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"Impact of bitter-tasting amino acids and peptides on mechanisms regulating gastric acid secretion and satiety"

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Don't be afraid to fail

be afraid not to try.

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List of abbreviations

cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
CaSR	calcium sensing receptor
CHRM3	muscarinic M3 receptor
CNS	central nervous system
DB	denatonium benzoate
ENaC	epithelial sodium channel
GDP	guanosine diphosphate
GLP-1	Glucagon-like peptide 1
GPCR	G-protein-coupled receptor
GTP	guanosine-5'-triphosphate
HED	homoeriodictyol
HCl	hydrochloric acid
H^+/K^+ ATPase	hydrogen /potassium adenosintriphosphatase
HRH2	histamine receptor H_2
IP1	myo-Inositol 1 phosphate
IP3	inositol 1,4,5-trisphosphate
PDE	phosphodiesterase
PKA	protein kinase A
PKC	protein kinase C
SDA	subdiaphragmatic vagal deafferentation
SSTR2	somatostatin receptor type 2
T2R	bitter taste receptor

I. Introduction

Overweight and obesity have been a growing problem all over the world for more than 40 years. In 2016, around 1.9 billion adults worldwide were overweight or obese, with 340 million children and adolescents afftected.[1] For the adult Austrian population, the Nutrition Report stated that 28.9 % were overweight and 12.1 % were obese.[2] For Austrian children aged 15 years, the "Obesity Update 2017" published by the Organization for Economic Co-operation and Development (OECD) reported an obesity prevalence of 12.5 %.[3]

Overweight and obesity are main risk factors for type II diabetes mellitus and are associated with various co-morbidities, like coronary heart diseases and some types of cancer.[1, 4] As a consequence, overweight and obesity as well as their co-morbidities are not only a burden to the individual person, but also to the health care systems due to high costs for treatments. One promising strategy to combat the growing incidence of overweight is to develop satiety-enhancing foods by optimizing their composition. So far, commercially available "*light*" products, which are mainly reduced in their carbohydrate and/or fat content, have not been proven effective for weight management.[5] In comparison to carbohydrates or fat, isocaloric amounts of proteins are known to induce a longer-lasting feeling of satiety.[6, 7] Considering the evidence that protein rich foods promote satiety in humans[8, 9], an optimization of proteins for enhancing satiety is a promising strategy for maintaining a healthy body weight. Thus, the question arises whether it is possible to identify an amino acid pattern with a maximum effect on satiety. Furthermore, since bitter taste has been shown to promote satiety in humans and most of the proteinogenic L-amino acids are bitter taste-active compounds, an effective and palatable bitter taste at the same time must also be addressed in studies.

1. The role of gastric acid secretion in the regulation of satiety

In the satiety cascade, it is distinguished between the terms “satiation”, which is the process that stops further eating, and the term “satiety”, which defines a feeling of fullness between meals.[10] In the following work, the term satiety is used as a preamble for both, satiation as well as satiety.

Satiety is regulated on a hormonal and neuronal level (**Figure 1**). On a hormonal level, the hunger hormone ghrelin is produced by oxyntic glands in the stomach, which is suggested to induce meal initiation.[10] In response to food intake, plasma glucose levels increase, leading to a release of the satiety hormone insulin from the pancreas, which stops further eating.[10, 11] Another satiety hormone, the Glucagon-like-peptide 1 (GLP-1) is released from the ileum and colon. GLP-1 has been shown to decrease energy intake and gastric emptying, as well as to promote gastric acid secretion.[10] GLP-1 is part of the ileal brake that controls the passage of the chyme from the stomach into the intestine for nutrient absorption.[10, 12]

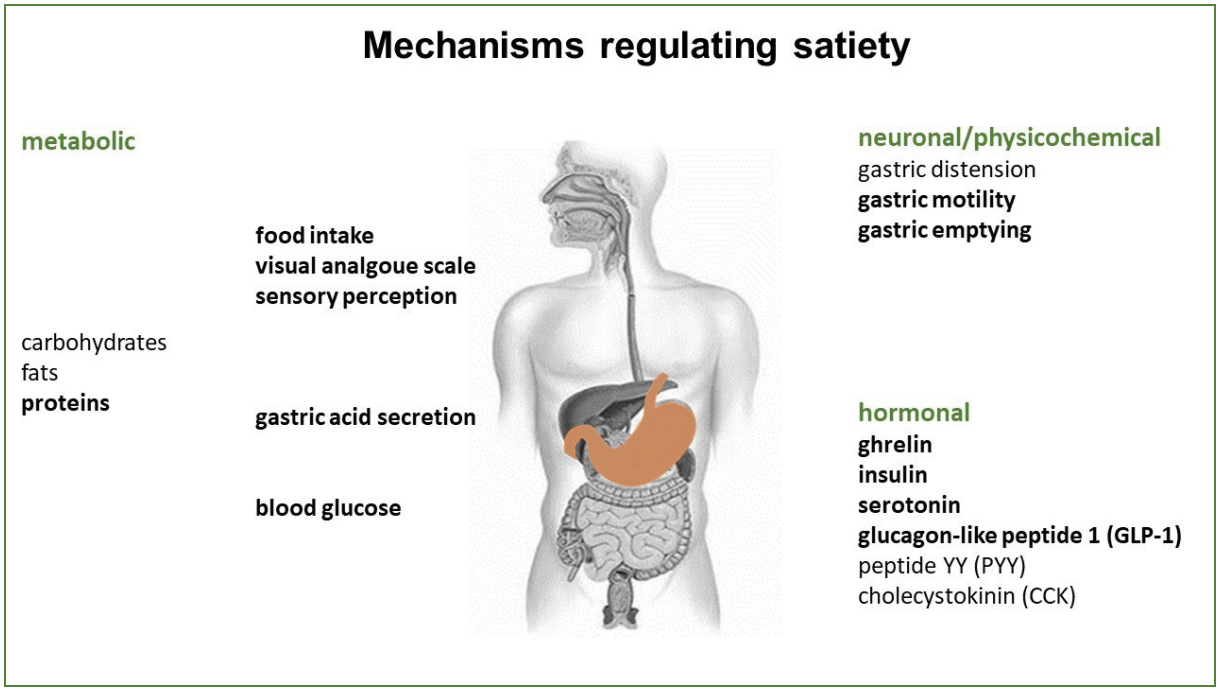

Figure 1: Schematic overview[3] of the mechanisms regulating satiety. The present thesis focused on parameters depicted in bold.

There is also evidence that peripheral serotonin increases satiety in healthy human subjects.[13, 14] Namkung and colleagues[15] postulated that modulating the serotonergic system might be a good strategy for anti-obesity treatments.[15] Although the exact mechanisms are not fully understood yet, it is suggested that peripheral serotonin influences blood glucose levels as well as glucose uptake by the liver and muscle tissues. Furthermore, pancreatic insulin secretion is influenced by serotonin. Of the total amount of the body’s serotonin, 90 % is produced by non-neuronal enterochromaffin cells in the gut. Among other factors, dietary protein intake promotes intestinal serotonin release which stimulates intestinal motility.[15-17]

On a neuronal level, hunger and satiety are controlled by neural signals from the periphery and the hypothalamus, specifically in the nucleus area.[10] Following food intake, the stomach initiates satiation via mechanoreceptors activating neurons that send tension signals to the brain. More specific, the *vagus nerve* mediates signals to the brain regarding emptiness or fullness of the stomach, and regulates gastric motility as well as gastric emptying. Gastric motility leads to relaxation of the proximal stomach, which serves as a food reservoir. The distal stomach grinds and mixes the chyme by peristaltic contractions. These peristaltic contractions also control closing and opening of the pylorus for gastric emptying [18], and promote a maximum output of gastric acid.[19] Gastric emptying limits the amount of the chyme that is released into the intestines. A delayed gastric emptying is known to result in a lasting gastric distension which has been shown to increase satiation[20].

During digestion, secretion of gastric acid is necessary to break down nutrients into absorbable molecules, which initiates the satiety cascade. Gastric acid is produced by parietal cells, located in the upper part of the stomach, the fundus region. The proton pump hydrogen /potassium adenosintriphosphatase (H^+/K^+ ATPase) is expressed in cytoplasmic tubulovesicles. After stimulation of the parietal cell, the H^+/K^+ ATPase delocalizes and integrates into microvilli of the apical membrane. This proton pump actively transports potassium ions (K^+) into the lumen in exchange to protons (H^+). Luminal K^+ for the proton pump is provided by K^+ channels. Furthermore, a chloride ion (Cl^-) influx is facilitated via passive diffusion through apical Cl^- channels, leading to the formation of HCl in the stomach lumen (**Figure 2**).

The stomach is part of the enteric nervous system. Here, acetylcholine from antral as well as oxyntic neurons stimulates gastric acid secretion directly via binding to the muscarinic M3 receptor (CHRM3), leading to activation of a Ca^{2+}-dependent protein kinase C (PKC) pathway.[21] Food-induced distension of the stomach activates the neural system as follows: whereas high distension promotes, low distension inhibits secretion of gastric acid. Cholinergic neurons act directly on parietal cells and regulate the release of histamine and somatostatin, which are paracrine modulators of gastric acid secretion.[22] Histamine is produced by enterochromaffin cells and stimulates gastric acid secretion via activation of a histamine receptor (HRH2) followed by a cAMP-mediated translocation of the H^+/K^+ ATPase, and activation of the protein kinase A (PKA).[21] An indirect stimulation by histamine occurs after its binding to H3 receptors, leading to inhibition of the somatostatin receptor.[23] Somatostatin is produced by oxyntic and D cells, and is known as the main inhibitor of gastric acid secretion through binding to its receptor (SSTR2) (**Figure 2**).

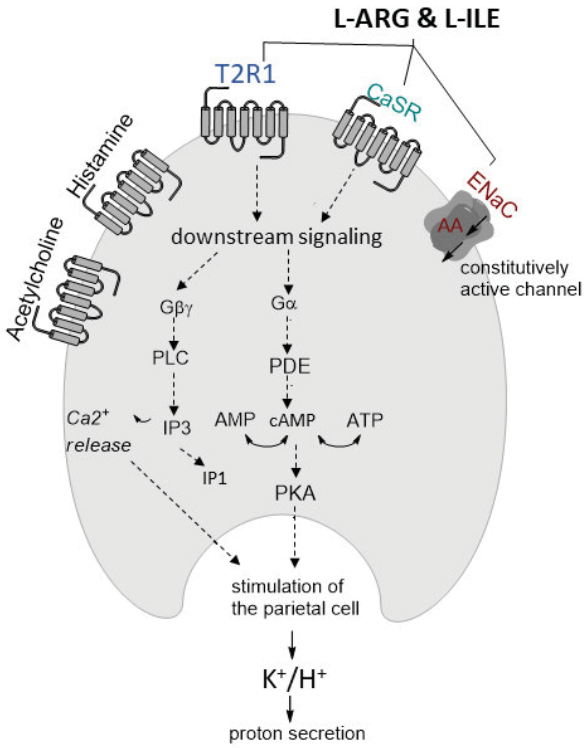

Figure 2: Schematic representation of HCl formation by parietal cells.[24, 25]

Receptors for histamine, acetylcholine and somatostatin belong to the group of G-protein coupled receptors (GPCRs). After binding of a ligand to a GPCR, guanosine diphosphate (GDP) is transformed into guanosine-5'-triphosphate (GTP), and the subunits of this heterodimeric receptor (Gα and Gβγ) are separated. The subunit Gα stimulates the adenylyl cyclase which leads to a cyclic adenosine monophosphate (cAMP)-mediated activation of PKA I and II.[26] The subunits Gβγ activate the phospholipid dependent pathway, leading to activation of PKC and intracellular Ca^{2+} release from the endoplasmic reticulum. The isoforms of PKC, PKC α and PKC ε have been identified as the active isoenzymes stimulating gastric acid secretion.[27]

In the stomach, the breakdown of proteins by gastric acid plays a pivotal role in protein digestion. The low gastric pH activates the cleavage of pepsinogen into its active form pepsin, which specifically cleaves peptide bonds. Secretion of gastric acid and the degradation of proteins into absorbable peptides and amino acids activate the signaling cascade. Proteins are known (i) to be the strongest stimulators of gastric acid secretion among dietary macronutrients, and (ii) to induce a stronger feeling of satiety in comparison to carbohydrates or fat after dietary intake in isocaloric amounts.[6, 7]

2. Influence of proteins, peptides and free amino acids on mechanisms regulating satiety

2.1 Proteins and amino acids and their influence on hormonal and metabolic parameters of satiety signaling

Proteins and amino acids are known to regulate outcome measures of satiety, resulting in a reduced energy intake between 12.5 % to 23 % depending on the served protein-rich test meal.[5-7] For example, in a human intervention study, application of a high-protein yoghurt induced a stronger feeling of satiety, detected by means of a visual analogue scale, in comparison to administration of crackers or chocolate served in isocaloric amounts of 160 kcal.[6] Another study confirmed those results: In comparison to meals high in fat or carbohydrates, a high protein meal reduced caloric energy intake by 18 % and 23 %, respectively, in healthy lean and obese men.[7] A decrease of energy intake has also been detected in another human intervention study conducted by Ballinger and colleagues[28]. In this study, a total of 10 g D- or L-phenylalanine was nasogastrically administered 20 minutes before a standardized test meal was served.[28] Whereas the L-phenylalanine preload decreased food intake to 1,089 ± 86 kcal in comparison to a water control which resulted in a mean intake of 1,587 ± 174 kcal, administration of D-phenylalanine had no effect.[28] A differential effect of individual amino acids on food intake is also indicated by results from another human intervention trial reported by Butler and colleagues[29]. In this study, an encapsulated amino acid mixture containing 3 g L-phenylalanine, 2 g L-valine, 2 g L-methionine, and 1 g L-tryptophan administrated 30 min before a test meal decreased the mean total food intake by 10 %.[29] These studies clearly demonstrate that proteins as well as free L-amino acids are able to promote satiety as demonstrated by an increased subjective feeling of hunger and a decrease in food intake.

Also, a metabolic regulation of the satiety cascade induced by dietary proteins and amino acids has been demonstrated, such as a regulation of blood glucose and ghrelin concentrations, and gastrointestinal satiety hormones. For example, a mean decrease of blood glucose concentrations by 0.6 ± 0.2 mmol/L after administration of 20 - 40 g whey protein in 300 mL tap water as a pre-load to a test meal was detected in healthy subjects by Akhavan and colleagues[8]. The authors of this study suggested that a whey protein preload could be a tool for blood glucose control in healthy as well as in insulin-resistant subjects.[8] In a study with type 2 diabetes patients, administration of 18 g whey protein stimulated insulin release, whereas blood glucose response decreased, indicating an improved glycemic control.[30] In another study, it was reported that cod protein has the highest potential to improve insulin sensitivity in comparison to protein sources from beef, pork, milk in insulin-resistant men and women.[31] Since cod protein contains relatively

high arginine concentrations compared to other dietary proteins, authors of this human intervention trial hypothesized a satiating role for arginine.[31] Moreover, an improvement of insulin sensitivity has been detected in a long-term intervention study with diabetic subjects after treatment with L-arginine. Here, an oral application of 3 g of L-arginine, three times per day for one month, improved insulin sensitivity by 34 %.[32] Increased insulin sensitivity has also been demonstrated in healthy Wistar rats after treatment with 20 g L-arginine/kg diet.[33] Another amino acid that has been shown to be metabolically active is L-isoleucine.[34] Oral application of this amino acid in a dosage of 1 mmol/kg body weight decreased the glucose response by 61 % in comparison to 25 g glucose alone in healthy volunteers.[34]

A satiating effect induced by dietary proteins is also indicated by their effect on plasma concentrations of the orexigenic hormone ghrelin. In healthy men, for example, the plasma ghrelin response induced by a 57 g protein-containing breakfast was 46 % higher than that induced by a 46 g carbohydrate-containing meal.[35] In addition, also glucose and insulin responses were improved by the protein rich meal.[35] Whether individual amino acids show differential effects on orexigenic hormones has still to be elucidated since literature evidence is scarce. In 2015, McGavigan and colleagues[36] published that an oral application of 0.07 g/kg L-cysteine decreased plasma acyl ghrelin concentrations by 270 pg/mL in comparison to the vehicle control that resulted in respective concentrations of 90 pg/mL in healthy subjects.[36]

A further hormone that is involved in the mechanisms regulating satiety, is GLP-1. An augmented release of GLP-1 by 29.0 % has been shown in Wistar rats receiving food enriched with 200 g sardine protein per kg diet for two months in comparison to casein.[37] An increase of plasma GLP-1 by 29 pmol/L has also been detected in healthy human subjects who ingested a high-protein breakfast (60 % w/w protein) in comparison to a high-carbohydrate (60 % w/w carbohydrate) or high-fat (60 % w/w fat) test meal.[38] After the high-carbohydrate breakfast, GLP-1 concentrations increased by 21 pmol/L, whereas a GLP-1 increase by 17 pmol/L was analyzed after the high-fat meal.[38] An influence on plasma GLP-1 concentrations was also detected for free amino acids, which was demonstrated for example by Alamshah and colleagues[39]. In this study, C57BL/6 mice received 16-24 mmol L-arginine per kg body weight by oral gavage twice daily.[39] The authors suggested that the satiating effect is directly mediated by the brain, since involvement of the vagus nerve was excluded by using rats that underwent subdiaphragmatic vagal deafferentation (SDA) surgery.[39] The SDA method eliminates all vagal afferents from the upper gut and the liver, but keeps the efferent vagal neurons intact.[40] In another study in mice, oral application of 1 g L-arginine per kg body weight increased GLP-1 by 29.1 ± 3.8 pM/L in comparison to basal concentrations of 8.80 ± 1.3 pM/L.[41] Moreover, in a human intervention study, administration of a test solution containing 1.56 g L-tryptophan induced an increase by 432 % of plasma GLP-1 concentrations in obese subjects and in lean subjects by 394 % in comparison to the tap water con-

trol.[42] This study further supports the hypothesis that proteins and free amino acids regulate mechanisms of satiety on a hormonal level by an increase of the satiety hormone GLP-1. With this evidence, an impact of dietary protein and amino acids on plasma concentrations of the anorexigenic hormone GLP-1 has been demonstrated. However, no structural requirement for amino acids or proteins to modulate the peripheral GLP-1 response has yet been identified.

Another parameter which has been shown to be regulated in the satiety cascade is the hormone serotonin, which also has an anorexigenic effect. Up to now, there is no clear evidence for a regulation of peripheral serotonin levels after protein intake, however it was shown that a modulation of energy intake was induced after oral application of serotonin via a sublingual spray for five times a day over two months to overweight women. In comparison to the baseline control, the daily energy intake decreased by 206 kcal and the BMI was lowered by -1.04 kg/m² after the intervention.[43]

Since proteins as well as amino acids are hypothesized to regulate satiety by promoting insulin release, a link to peripheral serotonin can be assumed. Moreover, the amino acid tryptophan is the substrate for serotonin biosynthesis, and has been shown to reduce energy intake by 227 kcal in healthy men, when 2 g – 3 g L-tryptophan were applied encapsulated 45 min prior to an *ad libitum* lunch.[44] L-arginine is another amino acid that is suggested to stimulate central serotonin release, which has been demonstrated in rats after intracerebral injection of 100 µM L-arginine.[45] In rats, also a diet rich in L-leucine (up to 37.5 g/kg chow) reduced energy intake, although, at the same time, decreased plasma serotonin levels in comparison to control. The authors of this study hypothesized that L-leucine competes with L-tryptophan for the same transport systems into enterochromaffin cells, which might have led to a decreased serotonin synthesis.[46]

A pivotal part of satiety regulation is neuronally mediated. One nerve that is critically involved in the regulation of food intake, is the vagus nerve that innervates the gastrointestinal tract and sends signals to the brainstem. Proteins as well as free amino acids are known to induce satiety via neuronal signaling. In 1984, Li and Anderson investigated the role of the vagus nerve on the regulation of protein and carbohydrate intake in rats. For this purpose, one group of rats was vagotomized and received a meal containing either 50 % casein or 83.5 % cornstarch. The loss of the functional vagus nerves reduced the intake of the protein-rich meal, but not that of the meal high in carbohydrates.[47] In an animal study with rats, Darcel and colleagues[48] showed that intestinal perfusion of an 8 % solution containing protein hydrolysates increased vagal afferent fibers and inhibited gastric motility.[48] In a review by Fromentin and colleagues,[49] the authors stated that a high-protein diet may inhibit the activation of neurons in the nucleus accumbens, leading to a reduced in energy intake.[49]

There is not only evidence for proteins, but also for free amino acids to neuronally mediate satiety signals. Tomé and colleagues[50] summarized that there are amino acids with an excitatory or an inhibitory effect on vagal afferent fibers. The amino acids L-alanine, L-arginine, L-leucine, L-lysine, L-serine, L-tryptophan, L-valine, and monosodium glutamate were classified as excitatory amino acids, whereas L-cysteine, L-glycine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, and L-threonine were classified as inhibitory amino acids.[50] Moreover, in a rat study, L-leucine (1.31 g/100 g) and L-valine (0.96 g/100 g) were demonstrated as pivotal amino acids for insulin secretion mediated via the vagus nerve.[51] Furthermore, in 2013, Jordi and colleagues[52] published that intragastrically administered L-amino acids in a dosage of 6.7 mmol/kg decreased food intake and delayed gastric emptying mediated via the vagus nerve in rats.[52] In another study, a delay of gastric emptying by 48.17 % has been detected when 1 g/kg L-tryptophan was orally administered to rats in comparison to a water control (100 %) after 120 min. Area under the curve-values were calculated by $^{13}CO_2$ % values versus time in minutes.[53] These evidences suggest that dietary amino acid pattern might have an impact on vagal neuron activity and subsequent regulation of mechanisms involved in food intake.

Meyer-Gerspach and colleagues[42] reproduced that intragastric infusions of 1.56 g L-tryptophan applied to diabetic patients decreased gastric emptying, which was detected by means of a 50 mg ^{13}C sodium acetate breath test over time (15 min to 240 min).[42] In healthy humans, a delay of gastric emptying after intragastric administration of 100 mM L-arginine has also been demonstrated when gastric emptying was determined at the time when intragastric titration was no longer possible due to inadequate intraluminal volume.[54]

The previous paragraph clearly demonstrates that protein and free amino acids are able to interact with different mechanisms involved in the regulation of satiety. Proteins and free amino acids promote secretion of gastric acid and modulate blood glucose concentrations. Moreover, a regulation on a hormonal as well as a neuronal level after protein and amino acid administration has been reported. However, if there is a possible structure-activity relationship of amino acids and parameters regulating satiety, is still an open question.

2.2 Proteins and free amino acids regulate the secretion of gastric acid

In 1960, Saint-Hilaire and colleagues[55] demonstrated that proteins are more potent stimulants of gastric acid than carbohydrates and fats.[55] The strengths of gastric acid secretion induced by proteins depends on the protein source.[56] In a human intervention study by McArthur and colleagues, a test meal with soy protein stimulated gastric acid secretion less potentially than beef protein after administration of equal amounts of 26 g.[56] This study indicates a pivotal role of the amino acid pattern. Also free amino acids have been shown to stimulate gastric acid secretion.[54, 57, 58] A human intervention study in infants showed that intravenous application of an amino acids

mixture (0.15 mg x kg KG^{-1} x h^{-1}) stimulated gastric acid secretion.[57] However, Taylor and colleagues[54] demonstrated that of the tested free L-amino acids, only the aromatic amino acids L-phenylalanine and L-tryptophan, applied in an amount of 100 mM and 50 mM, respectively, stimulated gastric acid secretion after intragastric application of 600 mL of the test solutions.[54] This stimulating effect on gastric acid secretion induced by L-tryptophan and L-phenylalanine was hypothesized to be mediated by an increase of serum gastrin levels.[54] Later, McArthur and colleagues[59] showed that also an intravenous infusion of free amino acids in an amount up to 12.5 mmol/h, stimulated gastric acid secretion. Recently, Kitay and colleagues[60], showed that exposure of *ex vivo* parietal cells from rats to 10 mM L-arginine stimulated gastric acid secretion via a direct activation of H^+, K^+ ATPase.[60] The effect of L-arginine and possibly other amino acids might be concentration dependent since intravenous infusion of 600 mg x kg KG^{-1} x h^{-1} L-arginine in male subjects inhibited gastric acid secretion.[61] This finding provides evidence that not only the application form, but also the concentration and the structure of L-amino acids are crucial for a stimulating or inhibiting effect on gastric acid secretion.

Since L-isomers of amino acids are known as the physiologically active form in humans, the majority of studies investigating the impact on gastric acid secretion were conducted with L-amino acids. However, food processing like fermentation, the ripening process or the use of acidic or alkaline conditions often result in isomerization of L- into D-amino acids. For example, soy protein was reported to contain 27.7 % D-aspartic acid, 19.7 % D-phenylalanine and 9.9 % D-alanine.[62] Another example would be zein, the corn protein, which was found to consist of 40.2 % D-aspartic acid, 31.3 % D-phenylalanine and 17.6 % D-alanine.[62] Furthermore, the microbiota, the submandibular glands and oral epithelial cells are suggested as sources for D-amino acids.[63] So far, it is not clear, in which amounts D-amino acids are ingested with the daily diet, as D-amino acids can be converted by oxidases and transaminases into L-amino acids.[64] However, an earlier study from 1976 investigated the influence of 50 mM L- and D-amino acids on gastric acid secretion in dogs by means of intragastric titration. In this study, L-histidine and glycine were identified as the strongest stimulators among the amino acids tested, although a functional role of D-amino acids on gastric acid secretion could not be excluded.[58]

The inconsistent effects of free amino acids on gastric acid secretion might be explained by different concentrations and possibly structural differences of amino acids, application routes and detection methods applied. Moreover, the different test models (cell culture, animals or humans) used might have contributed to differences in effect sizes. Also species differences might play a role since intragastric application of L-histidine and glycine to dogs stimulated gastric acid secretion[58], whereas intravenous administration of histidine to humans had no effect on gastric acid secretion, and only a slight stimulation was detected when glycine was administered[59] Overall, the cited literature demonstrates that proteins and free amino acids promote satiety via a regula-

tion of gastric acid secretion. However, since effects of single amino acids are not conclusive, further research is necessary.

2.3 Physicochemical properties of proteins and amino acids influence satiety

Satiety induced by proteins is also modulated by their physicochemical properties. For example, a low pH in the stomach lumen modulates protein folding and, therefore, its structure. Already in 1968, an influence of the gastric pH on the digestibility of proteins was suggested by Lennard-Jones and colleagues[65], who concluded that more gastric acid is needed for the digestion of proteins compared to that of carbohydrate and fats, since, among other factors, proteins have a higher buffer capacity.[65]

There is evidence that protein-rich meals regulate the fasted gastric pH in a range of 1.9 to 6.7.[66] In a study conducted by Dekker and colleagues[66], it was investigated whether the gastric pH profile influences protein digestion. For this purpose, the authors used a pH of 1.9 as a reference in comparison to an *in vivo* dynamic profile, at which the pH was gradually increased up to 6 over time. Using the dynamic pH model, a lower protein degradation was detected. This lower protein digestibility is thought to be associated with a decreased activity of the proteolytic enzyme pepsin, which demonstrates a pH optimum between 1.5 and 2.5.[66] In addition, in this study, a formation of a protein gel structure after heat treatment was investigated. Since the formation of a dense biopolymer structure reduces a proteins' digestibility, it was assumed that, in this study, the cleavage sites were not pepsin accessible.[66] Moreover, in 2014, Zhang and Vardhanabhuti[67] investigated whether a pH-dependent formation of heated whey protein aggregates show a differential impact on food intake *in vitro*.[67] In this study, it was found that an amount of 3 % to 9 % aggregated whey protein is optimal for a fast degradation rate and that the degradation rate increases with increasing protein concentrations. Furthermore, heat-induced whey protein aggregates formed by a pH above the isoelectric point were digested faster. The authors hypothesized that the pH influences the folding and aggregation of proteins, leading to more accessible peptide bonds for enzymatic cleavage.[67] Recently, a focus was also set on amino acid release during the gastric phase of protein digestion.[68] In this study, an *in vitro* digestion was conducted by simulating different phases of digestion starting from simulation of saliva to simulation of intestinal juice, containing pancreatin and bile. Furthermore, amino acid release in pigs was analyzed in samples of gastric and intestinal liquids and solid samples after administration of three meals containing 33 % of skimmed milk powder. In this study, it has been detected that the release of essential compared to non-essential amino acids was highest in the gastric phase *in vitro* as well as in *vivo*. The ratio between amino acids present in the stomach of pigs in comparison to the amino acids content in the original sample of skimmed milk powder was shown to be 67:33%.[68] The authors of this study proposed a need for further mechanistical studies on protein digestion and amino acid

release in the stomach.[68] Currently, a minor role has been postulated for amino acid release in the stomach, whereas a major role for protein digestion is attributed to the intestines.[68]
To summarize, these studies show that the satiating effects of protein and amino acids are influenced by the gastric pH.

In addition, there is growing evidence that a satiating effect of proteins and amino acids is also mediated via chemoreceptors in the gastrointestinal tract. For example, a mediation of CCK-release via the GPCR GPR93 chemoreceptor was demonstrated in the entero-endocrine cell model STC-1 after exposure to 6.25 mg/mL of a peptone solution.[69] In another study, conducted by Nakajima and colleagues,[70] a CCK release mediated via the calcium sensing receptor (CaSR), another GPCR, was also detected in STC-1 cells after treatment with protein hydrolysates in a concentration of 5 mg/mL.[70] The CaSR has been demonstrated to be targeted by L-amino acids in mechanisms regulating gastric acid secretion. For example, an exposure of 30 mM L-phenylalanine and L-tryptophan to *ex vivo* parietal cells of rats regulated the gastric pH by 0.083 ± 0.005 and 0.082 ± 0.013 Δ pH units/min, respectively, whereas D-phenylalanine had no effect.[71]

In sensory studies, L-amino acids have been predominately described as bitter-tasting, whereas D-amino acids have been revealed as sweet-tasting.[72, 73] In addition, there is increasing evidence that demonstrates a pivotal role for bitter taste and bitter taste receptors (T2R) as targets for proteins and amino acids regulating mechanisms of satiety. Recently, Zhang and colleagues[74] demonstrated that beef protein hydrolysates block T2R4 activation in transfected HEK-293 cells in dependence of their hydrolyzation grade. The peptide fraction with the least bitter taste, for example, was identified to block T2R4 activation.[74] The authors suggested that this effect was due to a low L-tryptophan content, since this amino acid has previously been shown to activate T2R4.[75] In a study conducted by Kohl and colleagues[75] it has been demonstrated that 10 mM L-tryptophan activated T2R4, whereas 53.3 mM L-phenylalanine targeted T2R1 in transfected HEK-293 cells.[75] Moreover, D-tryptophan, in a concentration range from 10 mM to 21.7 mM, activated T2R4 and T2R39. The authors of this study hypothesized that the bitter off-taste of D-tryptophan and D-phenylalanine mediates T2R activation in HEK-293 cells.[75] At almost the same time, Bassoli and colleagues also tested tryptophan and phenylalanine in both isomeric forms on T2R activation in transfected HEK-293 cells.[76] These authors detected that 30 mM L-tryptophan activated T2R4/39/43/49, whereas 100 mM L-phenylalanine activated T2R1/4/8/39 by means of intracellular calcium mobilization. However, according to the results published by Kohl and colleagues[75] 30 mM D-tryptophan was identified to target T2R4/39, whereas 100 mM D-phenylalanine activated T2R1 and T2R39.[75, 76] These results clearly demonstrate that L-amino acids target T2Rs. Whether this effect is correlated to the bitter taste of amino acids still needed to be elucidated and was part of the overall research hypothesis addressed by this PhD thesis.

Bitter taste has also been demonstrated to promote satiety mediated by a stimulation of gastric acid secretion.[24, 77, 78] A stimulating effect on gastric acid secretion induced by the bitter-tasting herbal substance *Gentiana Lutea L.* has been published by the European Medicines Agency.[77] Furthermore, in 2012, our working group identified that the stimulating effect of beer on mechanism of gastric acid secretion in the parietal cell model HGT-1 is mediated by bitter beer acids α, β and iso-α-acids.[78] This stimulating effect was hypothesized to be mediated via acetylcholine receptor, since an upregulation of this receptor on a mRNA level was detected.[78] In another study, the bitter-tasting wine constituents procyanidin B2 and catechin were found to stimulate mechanisms of gastric acid secretion in HGT-1 cells.[79] Furthermore, bitter tastants have also been demonstrated to promote satiety in healthy humans.[80] Andreozzi and colleagues[80] demonstrated that an encapsulated bolus administration of 18 mg of the bitter-tasting quinine resulted in a total energy intake from an *ad libitum* meal of 514 ± 248 kcal, which was significantly lower in comparison to the energy intake after administration of a placebo control (596 ± 286 kcal).[80] Also, a satiating effect induced by a bitter-tasting aqueous extract of *Gentiana lutea root,* which was microencapsulated and introduced into a vanilla pudding, was detected in a randomized human intervention trial. Application of this bitter extract promoted satiation by inducing a reduction of mean energy intake of 30 %.[81]

Since satiety is also promoted by a delay in gastric emptying, Janssen and colleagues[82] investigated a mixture of bitter-tasting compounds in mice. Compared to a water control, this bitter solution delayed gastric emptying more potently which was detected by means of a ^{13}C octanoic breath test.[82] In addition, a decreased food intake by 49 % four hours post load pointed to an increased satiety.[82] This effect was reproduced by another study in mice, in which a delayed gastric emptying was also detected after intragastric application of the bitter-tasting denatonium benzoate (DB) in a dosage of 60 µmol/kg.[83] Moreover, in this publication, 1 µmol/kg of DB was intragastrically applied to humans where DB induced an increase of the satiation scores, which were assessed by a visual analogue scale.[83] However, recent evidence demonstrates that the bitter-tasting compounds quinine was not detected as being effective for increasing satiety in humans, when applied alone or in a combination with sweet- and umami-tasting compounds. Since identified effects on satiety have a small effect size so far, authors of the study suggest that applications routes via naso-duodenal-ileal catheter or intraduodenal probably mask a small effect.[84, 85] Furthermore, by using these application forms, the gastric and oral perception is not involved, and it is not clear so far whether it needs to be activated by bitter-tasting compounds for influencing satiety.[84]

To summarize, there is evidence for proteins and amino acids acting on neuronal, hormonal and metabolic mechanisms regulating satiety, although no structural requirements are known yet and

systematic studies elucidating the impact of amino acids on mechanisms of gastric acid secretion as key mechanisms of protein digestion and satiation are lacking.

3. Hypothesis

Proteins have been shown to induce a stronger feeling of satiety in comparison to isocaloric amounts of carbohydrates and fats. For the digestion of proteins, secretion of gastric acid is mandatory for acidic and enzymatic hydrolysis of proteins into peptides and free amino acids. In sensory studies, L-configurated amino acids have been described as predominantly bitter-tasting, whereas respective amino acids in their D-configuration are described to be sweet-tasting.[72, 86] Moreover, bitter-tasting amino acids have been shown to activate T2Rs by means of Ca^{2+} mobilization in transiently transfected HEK-293 cells. A treatment of HEK-293 cells with L-phenylalanine, i.e. activated these T2Rs 1/4/9/39, whereas L-tryptophan was demonstrated to target the T2Rs 4/39/43/49.[75, 76]

Since several bitter-tasting compounds have been demonstrated to modulate proton secretion in the parietal cell model HGT-1, and the strength of proton secretion correlated with described bitter taste intensities[78], this native cell model has been validated as a suitable tool for the identification of bitter-masking and bitter modulating compounds (**Figure 3**).[87] Furthermore, HGT-1 cells express all relevant proteins for gastric acid secretion and for T2R signaling.[24, 78] T2Rs in humans belong to the group of GPCRs. The α subunit of gustducin initiates the cAMP dependent pathway after dissociation, whereas $G\beta\gamma$ induces the inositol 1,4,5-trisphosphate (IP3) signaling cascade.[88] An increase of cAMP concentrations activates PKA and IP3 promotes intracellular Ca^{2+} release from the endoplasmic reticulum. Phosphorylation activity of PKA as well as increased intracellular Ca^{2+} concentrations stimulate the secretion of protons via H^+,K^+-ATPase. IP3 will be further degraded to the more stable myo-Inositol 1 phosphate (IP1). (**Figure 3**).[89]

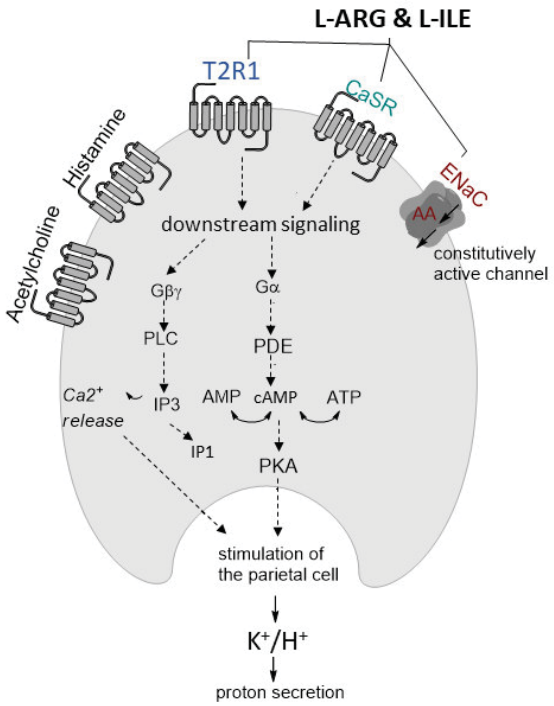

Figure 3: Schematic postulated mechanism of proton secretion in HGT-1 cells.[25]

Gastric acid secretion is a digestion signal, which initiates satiation after protein intake, and bitter taste has been shown to increase satiety in humans by decreasing food intake[80]. This provides a possible link between bitter-tasting amino acids, gastric acid secretion and satiety (**Figure 4**).

Hence, based on previous studies demonstrating that most of the free L-amino acids are bitter-tasting, and that bitter taste promotes satiety, in the here presented thesis, it was hypothesized that amino acids (1) influence mechanisms of gastric acid secretion by targeting T2Rs, which (2) promotes satiety via gastric motility (*ex vivo*) and regulates neuronal and metabolic parameters of satiety in healthy humans (*in vivo*) (**Figure 4**).

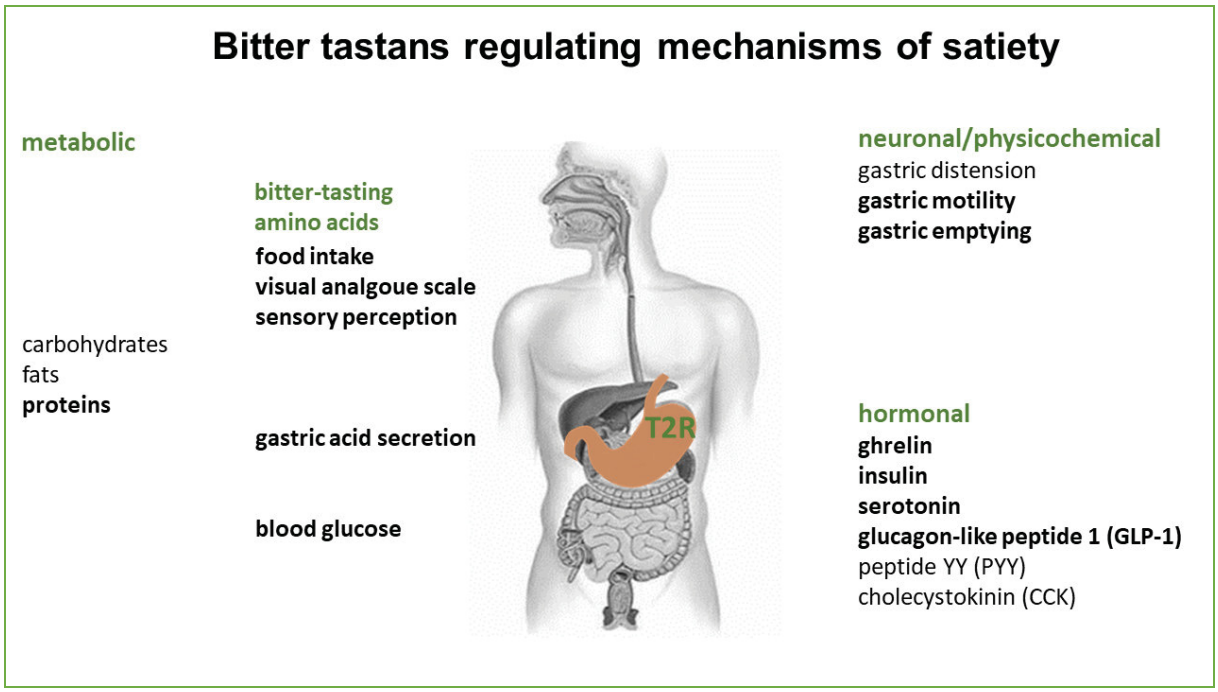

Figure 4: In the present PhD thesis, a role of T2Rs targeted by bitter-tasting amino acids in mechanisms regulating satiety were hypothesized.

Since previous studies demonstrated a regulation of gastric acid secretion by the aromatic amino acids L-phenylalanine and L-tryptophan via activation of the CaSR, an involvement of this receptor in proton secretion was also investigated. In addition, a regulation of the epithelial sodium channel (ENaC) in HGT-1 cells was also studied, since L-arginine enhances salt taste via ENaC activation[90] (**Figure 3**).

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II. Part I

Establishment and application of methods for the characterization of the effect of bitter-tasting compounds on mechanisms of gastric acid secretion and satiety.

1. Objectives

In Part I of this PhD thesis, the initial focus was to establish methods that enable to investigate gastric mechanisms regulating satiety. For this purpose, the parietal cell line HGT-1 was applied as a screening model for investigating bitter-tasting compounds on mechanisms regulating gastric acid secretion. For elucidating the role of T2R signaling on proton secretion in HGT-1 cells, a CRISPR/Cas9 approach was established to specifically knock-out *TAS2R43*. By means of organ tissue bath experiments, gastric motility in human stomach biopsies was analyzed for investigating neuronal parameters of satiety. In a human intervention study, the experimental protocols for investigating the influence of proteins and amino acids on food intake, blood glucose concentrations and satiety hormones in healthy subjects were developed.

2. Results

(1) Caffeine induces gastric acid secretion via bitter taste signaling in gastric parietal cells.

KI. Liszt, JP. Ley, B. Lieder, M. Behrens, **V. Stoeger**, A. Reiner, CM. Hochkogler, E. Köck, A. Marchiori, J. Hans, S. Widder, GE. Krammer, GJ. Sanger, MM. Somoza, W. Meyerhof and V. Somoza, DOI: 10.1073/pnas.1703728114 PNAS July 25, 2017 vol. 114 no. 30, pages: E6260-E6269

In this study, the bitter-tasting compound caffeine was analyzed for its impact on mechanisms of gastric acid secretion in HGT-1 cells as well as in a human intervention study. Expression of T2Rs and their signaling subunits gustducin as well as transducin, in HGT-1 cells have been detected. T2R43 and T2R10 have been identified as targets in the mechanism of gastric acid secretion. For this study, homoeriodictyol (HED) was used as a bitter-masking compound.

I assisted in establishing a stable T2R43 knock out in HGT-1 cells by means of CRISPR Cas9. Furthermore, I investigated the effect of HED on gastric motility during a short-term research visit in the laboratory of Professor Gareth Sanger at the Queen Mary University (London) and learned to apply the organ tissue bath method with human stomach specimen. I wrote the methods and results part for the organ bath in this manuscript.

(2) Appetite-inducing effects of homoeriodictyol: Two randomized, crossover interventions.

CM. Hochkogler, KI. Liszt, B. Lieder, **V. Stoeger**, A. Stuebler, M. Pignitter, H., Joachim, S. Widder, JP.Ley, GE. Krammer, V. Somoza, Mol Nutr Food Res. 2017 Dec;61(12), pages: 1-9. DOI: 10.1002/mnfr.201700459.

The flavanone HED was orally administered to healthy probands in two different human intervention studies. Satiety hormones as well as a hunger hormone have been detected in blood samples.

Within this study, I was introduced in designing and conducting a human intervention study.

(3) Human sweet receptor TAS1R3 is functional in human gastric parietal tumor cells (HGT-1) and modulates cyclamate and acesulfame K-induced mechanisms of gastric acid secretion.

M. Zopun, K. I. Liszt, **V. Stoeger**, M. Behrens, U. Redel, J. P. Ley, J. Hans and V. Somoza, Journal of Agricultural and Food Chemistry 2018 66 (19), 4842-4852, DOI: 10.1021/acs.jafc.8b00658

Effects of Cyclamate and Acesulfame K were diminished on proton secretion, after knock-down of T1R3. In HGT-1 cells a modulating effect on proton secretion was influenced by sweet taste receptor T1R3.

Within this study, I established the experimental protocol for the siRNA method together with M. Zopun.

(4) Impact of free N^{ε}–Carboxymethyllysine, its precursor glyoxal and AGE-modified BSA on serotonin release from human gastric tumor cells (HGT-1).

AK. Holik, **V. Stoeger,** K. Hölz, MM. Somoza, V. Somoza, Food Funct. 2018 Jul 1; 9(7): 3906–3915. DOI: 10.1039/c8fo01045e

In this *in vitro* study, I established an experimental protocol for studying the effect of post-translationally modified lysine (N^{ε}–Carboxymethyllysine) on proton secretion in human parietal cells.

(5) Serotonin biosynthesis and release from human gastric tumor cells (HGT-1) and its functional role in arginine-induced proton secretion.

AK Holik; **V Stoeger**; B Lieder; A Reiner; M Zopun; JK Hoi; N Kretschy; G Sanger; M Somoza; M Pignitter, V Somoza, *The FASEB Journal in revision*

The amino acid L-arginine has been detected to stimulate release of serotonin as well as proton secretion in HGT-1 cells. An involvement of the serotonin channel 5-HTR_3 is suggested.

For investigating this effect, I performed proton secretion assays and microarray experiments with L-arginine.

2.1 "Caffeine induces gastric acid secretion via bitter taste signaling in gastric parietal cells."

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Caffeine induces gastric acid secretion via bitter taste signaling in gastric parietal cells

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Edited by Robert J. Lefkowitz, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC, and approved June 16, 2017 (received for review March 7, 2017)

Caffeine, generally known as a stimulant of gastric acid secretion (GAS), is a bitter-tasting compound that activates several taste type 2 bitter receptors (TAS2Rs). TAS2Rs are expressed in the mouth and in several extraoral sites, e.g., in the gastrointestinal tract, in which their functional role still needs to be clarified. We hypothesized that caffeine evokes effects on GAS by activation of oral and gastric TAS2Rs and demonstrate that caffeine, when administered encapsulated, stimulates GAS, whereas oral administration of a caffeine solution delays GAS in healthy human subjects. Correlation analysis of data obtained from ingestion of the caffeine solution revealed an association between the magnitude of the GAS response and the perceived bitterness, suggesting a functional role of oral TAS2Rs in GAS. Expression of TAS2Rs, including cognate TAS2Rs for caffeine, was shown in human gastric epithelial cells of the corpus/fundus and in HGT-1 cells, a model for the study of GAS. In HGT-1 cells, various bitter compounds as well as caffeine stimulated proton secretion, whereby the caffeine-evoked effect was (*i*) shown to depend on one of its cognate receptor, *TAS2R43,* and adenylyl cyclase; and (*ii*) reduced by homoeriodictyol (HED), a known inhibitor of caffeine's bitter taste. This inhibitory effect of HED on caffeine-induced GAS was verified in healthy human subjects. These findings (*i*) demonstrate that bitter taste receptors in the stomach and the oral cavity are involved in the regulation of GAS and (*ii*) suggest that bitter tastants and bitter-masking compounds could be potentially useful therapeutics to regulate gastric pH.

gastric acid secretion | caffeine | homoeriodictyol | bitter taste receptors | TAS2Rs

Caffeine, a bitter-tasting methylxanthine alkaloid present in coffee and tea beverages, is the world's most frequently consumed psychoactive drug that functions as a stimulant of the autonomic and central nervous system (CNS) (1). It is also an activator of gastric acid secretion (GAS) (2–5). Although part of caffeine's effect appears to be mediated by antagonizing adenosine receptors and inhibition of phosphodiesterases (PDEs) (1), the observation that several other bitter-tasting compounds, such as denatonium benzoate (6); hop-derived beer bitter acids; α-, β-, iso-α-acids (7); and catechin and procyanidin B2 (8) cause gastrin release (6) or GAS (7, 8) indicates that bitter substance-evoked chemosensory mechanisms may be involved. Chemosensation potentially plays a role at three sites to regulate GAS: (*i*) bitter substances could excite oral taste cells and mediate their effects through cephalic regulation of gut physiology (9) or (*ii*) a bitter compound could also act in the gut through induction of gastrin and/or histamine release from enteroendocrine cells and/or (*iii*) by modulating acid production in GAS-producing parietal cells (10).

Bitter tastants elicit bitterness through a family of oral taste type 2 bitter receptors (TAS2Rs) (11). Humans express approximately 25 TAS2 receptors, of which five TAS2Rs, TAS2Rs 7, 10, 14, 43, and 46, can be activated by caffeine (12). In addition to the mouth, TAS2Rs have also been identified in nongustatory tissues, including airway epithelia (13), brain (14), intestinal cells (15, 16), and the gastric epithelia of rats and mice (17, 18). Beyond their chemosensory function, extraoral TAS2Rs are involved in nonsensory processes to expel or neutralize toxins in the upper and lower airways as well as in the gastrointestinal tract (19). Furthermore, the TAS2R pathway in the gut is involved in the regulation of food intake, digestion, and satiation (15, 16, 20, 21). Whereas, in the stomach, the endocrine effect of bitter substances on ghrelin secretion has been well described (20), a bitter compound-mediated exocrine function on acid production in parietal cells had not yet been discovered to our knowledge. Parietal cells can be activated by histamine or acetylcholine binding to their cognate histamine H2 or acetylcholine M3 receptors (22). Activation of these receptors results, either by Gs- and adenylyl cyclase/cAMP- or by Gq- and phospholipase C (PLC)/IP_3/Ca^{2+}-dependent pathways, in the activation of the H^+,K^+-ATPase, which pumps protons into the stomach lumen (22). In taste cells located on the tongue, the signaling cascade of TAS2Rs also includes a cAMP-dependent and a PLCβ2/IP3/Ca^{2+}-dependent pathway (23). Initiation of the latter major pathway leads to calcium release from intracellular compartments, which in turn activates transient receptor potential M5 ion channels. These channels mediate an influx of sodium ions and membrane depolarization (23), leading to ATP release and bitter perception. The α-subunit of gustducin has been described to stimulate

Significance

This study shows that caffeine's effect on gastric acid secretion (GAS) is more complex than has been previously thought. Oral and gastric bitter taste receptors are involved in the regulation of GAS in humans. This regulatory process can be modified by the bitter-masking compound homoeriodictyol. Practical applications of the results may include treatment of gastroesophageal reflux disease or peptic ulcer by manipulating gastric pH by means of bitter tastants and inhibitors.

Author contributions: K.I.L., J.P.L., B.L., M.B., V. Stöger, A.R., C.M.H., A.M., J.H., S.W., G.K., G.J.S., M.M.S., W.M., and V. Somoza designed research; K.I.L., B.L., M.B., V. Stöger, A.R., C.M.H., E.K., A.M., and G.J.S. performed research; J.P.L., J.H., S.W., G.K., and V. Somoza contributed new reagents/analytic tools; K.I.L., J.P.L., B.L., M.B., V. Stöger, A.R., C.M.H., E.K., A.M., J.H., G.J.S., M.M.S., W.M., and V. Somoza analyzed data; and K.I.L., B.L., M.M.S., W.M., and V. Somoza wrote the paper.

Conflict of interest statement: J.H., J.P.L., S.W., and G.K. are employees of Symrise, Holzminden, Germany.

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PDEs, resulting in low cAMP levels and PKA activities, which keep the IP3 type 3 receptor hypophosphorylated and sensitized (24). Therefore, the tonic activity of α-gustducin regulates taste cell responsivity. Transducin, a similar G protein also present in taste cells, can replace the function of α-gustducin (25, 26). TAS2R-expressing cells in the gastrointestinal tract have been reported to coexpress the downstream taste signaling components, suggesting that similar signal transduction pathways could also mediate gastrointestinal physiology (27). However, the detailed signal transduction pathways in extraoral chemosensitive cells are yet unknown.

This study investigated whether gastric and oral TAS2Rs contribute to the regulation of caffeine-induced mechanisms of GAS in humans. To study this hypothesis, the effect of caffeine on GAS was investigated in a human intervention trial, taking into account taste receptor activation in the mouth and the stomach. The underlying gastric mechanisms were studied by TAS2R expression analysis and by means of the validated HGT-1 cell culture model, which maintains the relevant characteristics of human parietal cells (28, 29).

Results

Oral Bitter Perception Reduces GAS in Human Subjects. Real-time gastric pH measurements were performed after caffeine administration in human subjects by means of Heidelberg pH diagnostic capsules (29–32). Heidelberg pH capsules are used to determine gastric acid secretory ability under conditions simulating the ingestion of food or beverages by means of radiotelemetry. For the measurements, overnight-fasted subjects swallow the pH capsule, followed by a saturated sodium bicarbonate solution. Ingestion of the bicarbonate solution triggers an increase in stomach pH and a subsequent attempt by the parietal cells to reestablish acidity. The impact of foods or beverages on the reacidification time can be analyzed by administration of the test material before or after the pH challenge. In this study, subjects swallowed a caffeine solution with or without a bitter-masking compound, homoeriodictyol (HED) (33, 34) 5 min after or 25 min before the bicarbonate challenge. Reacidification time was measured for three distinct delivery protocols (1–3), each of which assesses different sites of TAS2R activation (Fig. 1*A*). The subjects underwent the following interventions in 11 consecutive study site visits (Fig. 1*B*): For the first 8 visits, test compounds were administered 5 min after the bicarbonate solution. In delivery protocol (1), subjects drank 125 mL water, a caffeine solution (37.5, 75, or 150 mg caffeine in 125 mL water) with or without 30 mg HED, or an HED solution (30 mg HED in 125 mL water), thereby stimulating oral and gastric TAS2Rs (Fig. 1*B*). For delivery protocols 2 and 3, a dose of 150 mg caffeine was administered along with 125 mL water, either encapsulated to selectively stimulate gastric TAS2Rs, or as a sip-and-spit solution to activate only oral TAS2Rs, respectively (Fig. 1*B*).

During the final three visits, subjects were asked to drink 125 mL water or 150 mg caffeine with or without 30 mg HED in 125 mL water (delivery protocol 2) 25 min before the bicarbonate challenge to evaluate the effect of administration time. The intervention time of 25 min was chosen according to previous publications that demonstrated that caffeine starts to stimulate gastric acid after 30 min (2, 5).

Drinking the volume water control solution 5 min after the bicarbonate challenge resulted in a mean reacidification time of 23 ± 1 min (individual representative gastrogram shown in Fig. 1*C*). Oral application of caffeine by sip-and-spit or drinking led to prolongations ($P < 0.05$) of reacidification time by delta reacidification time values (reacidification time$_{\text{test compound}}$ − reacidification time$_{\text{water}}$) of 20 ± 6 min and 8 ± 2 min, respectively, compared with administration of a volume water control solution, indicating a delay of GAS (Fig. 1*D*). Stimulation of gastric sites only by encapsulated caffeine resulted in a shorter delta reacidification time of 5 ± 3 min relative to sip-and-spit administration ($P < 0.05$; Fig. 1 *C* and *D*). Individual gastrograms were quantified by determining the slope after the onset of reacidification. A higher slope indicates that, when reacidification has started, the gastric pH returns to its initial pH faster. The slope of the gastrogram (relative to water control) obtained after administration of encapsulated caffeine was higher (0.20 ± 0.16 pH units per min) compared with the slope calculated after drinking (−0.20 ± 0.10 pH units per min) and sip-and-spit intervention (−0.39 ± 0.05 pH units per min), whereby stimulation of oral receptors occurred (Fig. 1*E*). To extend the time period over which the effect of caffeine on GAS could be measured, we repeated the experiments with encapsulated caffeine administered with 125 mL water 25 min before the alkaline challenge. This intervention allows gastric pH changes to be recorded over a time period of 25 min to approximately 85 min after caffeine administration and revealed a stimulation of GAS, indicated by a reduced delta reacidification time of −23 ± 4 min by caffeine (Fig. 2*B*) compared with control treatment (empty capsule plus 125 mL water reacidification time, 41 ± 4 min; $P < 0.01$).

HED Reduces the Caffeine-Evoked Effects on GAS in Human Subjects. To determine if TAS2R bitter-taste receptors mediate the effect of caffeine on GAS, 125 mL water containing 150 mg caffeine and/or 30 mg of the bitter-masking compound HED (33, 34) were swallowed 5 min after the alkaline challenge (delivery protocol 1). Administration of HED alone resulted in a reacidification time of 21 ± 2 min, comparable to that of water (24 ± 1 min) as volume control.

Unexpectedly, concomitant administration of HED and caffeine resulted in accelerated gastric emptying in 4 of 10 subjects, as indicated by passing of the Heidelberg capsule into the duodenum before complete reacidification. The same effect was observed in 2 of 10 subjects after drinking a solution of 30 mg HED dissolved in 125 mL water. When HED and caffeine were administered encapsulated (delivery protocol 2), reacidification times could be analyzed in only six subjects, as four subjects demonstrated accelerated gastric emptying as seen after oral and gastric delivery (protocol 1). These results raised the question whether the bitter-masking compound HED promotes gastric motility by stimulating gastric relaxation. Experiments using strips of dissections of human stomach biopsy specimens revealed that treatment with 1 mM HED in an organ bath induced a maximum relaxation after 40 min, with mean tension values of 45.4 ± 6.7%, compared with water control values of 107 ± 5.7% (Fig. S1 *A* and *B*).

In those subjects who were subjected to delivery protocol 1 and did not respond with accelerated gastric emptying, HED largely reversed the effects of caffeine on reacidifcation time: whereas drinking of the caffeine solution 5 min after alkaline challenge resulted in a delta reacidification time of 8 ± 2 min, concomitant caffeine and HED administration revealed a mean value of 1 ± 1 min (Fig. 2 *A* and *B*), but showed no effect on the slope of the gastrogram (Fig. 2*C*). In contrast, gastric administration of encapsulated caffeine 25 min before alkaline challenge (delivery protocol 2) induced GAS compared with administration of water, resulting in a delta reacidification time of −23 ± 4 min (Fig. 2 *D* and *E*). Although the reversing effect of HED on the caffeine-mediated reacidification shown in Fig. 2*D* did not reach statistical significance in terms of reacidification time ($P = 0.087$; Fig. 2*E*), concomitant application of HED and caffeine reduced the slope of the gastrogram compared with caffeine administration, with mean respective values of 0.18 ± 0.13 pH units per min and 0.64 ± 0.26 pH units per min ($P < 0.05$; Fig. 2*F*).

The potent attenuation of caffeine's effects on GAS by the bitter-masking agent HED suggests that TAS2Rs are critically involved in caffeine's action in the mouth and the stomach.

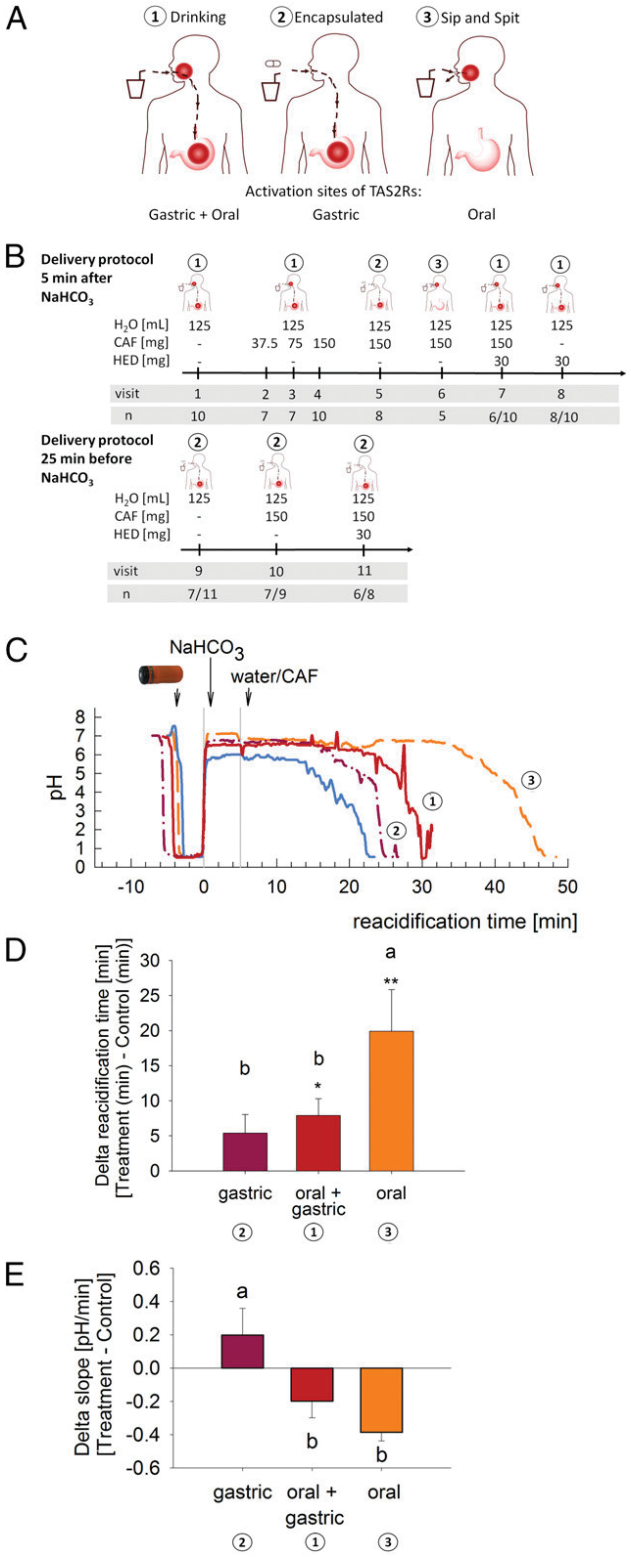

Fig. 1. Results of the gastric pH measurements demonstrate that the effect of caffeine (CAF) on reacidification time is influenced by the type of administration. (*A*) Overview of the different administration types in the human intervention trial. (*B*) Overview of the study procedure. (*C*) Gastrograms of different Heidelberg capsule measurements from one test subject combined in one graphic show that 150 mg caffeine diluted or administered with 125 mL water (blue line) administered by sip and spit (*3*) prolongs the reacidification time (i.e., time until the original pH is reached again) more than administration via drinking (*2*) or in encapsulated form (*1*). (*D*) Delta reacidification time of gastrograms show that sip-and-spit administration resulted in the highest prolongation of reacidification time compared with gastric and gastric plus oral administration. (*E*) Delta slope of the gastrograms indicate that encapsulated administration (gastric delivery) strongly stimulate GAS when reacidification has started. Data are displayed as mean ± SEM, n = 5–10; one-way ANOVA with Holm–Šídák post hoc test; significant ($P < 0.05$) differences are indicated by distinct letters [*$P < 0.05$, significant vs. water control (basal = 0) tested with paired Student t test].

Sensory Evaluation. To verify that the subjects were capable of sensing caffeine bitterness, the bitter recognition threshold of the same subjects who underwent the gastric pH measurements was determined by means of a threshold test, which yielded a result of 117 ± 44 mg/L for caffeine. In addition, the subjects rated the bitterness of 1,200 mg/L caffeine in the absence or presence of 240 mg/L HED in a blinded duo sensory test and confirmed the bitter-masking effect of HED reported by Ley et al. (33): Whereas the mean bitterness rating (±SD) for the caffeine solution was 7.5 ± 1.7, ratings for caffeine plus HED revealed mean values of 5.8 ± 1.9, corresponding to a −20 ± 8% reduction of caffeine-mediated bitterness by HED (Fig. S2*A*). The subjects' caffeine bitterness scores correlated with reacidification time (correlation coefficient, 0.66; $P = 0.03$; $n = 10$; Fig. S2 *B* and *C*) after caffeine administration by drinking (delivery protocol 1, 5 min after alkaline challenge), as well as with reacidification time after caffeine plus HED administered by drinking (correlation coefficient, 0.89; $P < 0.05$; $n = 6$; 5 min after alkaline challenge). No statistically significant correlation between bitter intensity rating and reacidification time was calculated after administration of encapsulated caffeine (delivery protocol 1; $P > 0.05$).

TAS2R Expression in HGT-1 Cells and Human Gastric Tissue. The mRNA expression of 25 human TAS2Rs in the HGT-1 cell line was investigated by quantitative RT-PCR (RT-qPCR) studies. The genes for the five TAS2Rs known to be activated by caffeine, TAS2Rs 7, 10, 14, 43, and 46 (12), as well as several other TAS2R genes, are expressed at similar or even higher levels than the M3 acetylcholine receptor CHRM3 gene, a major regulator of GAS (Table 1). Although *TAS2R5* and *TAS2R14* are the most highly expressed TAS2Rs, *TAS2R8*, *45*, and *60* mRNAs were not found in HGT-1 cells. HGT-1 cells also express mRNAs for TAS2R downstream signaling proteins PLCβ2, transducin (GNAT2), and α-gustducin (GNAT3) (11, 23) (Table 1). Like the parietal cell line HGT-1, the human gastric epithelium contains transcripts for the five cognate caffeine bitter receptors TAS2R7, TAS2R10, TAS2R14, TAS2R43 and TAS2R46 at levels similar to those of the M3 acetylcholine receptor, with ratios relative to that receptor of 0.76 ± 0.039, 0.97 ± 0.190, 1.16 ± 0.025, 0.62 ± 0.017, and 0.83 ± 0.071, respectively.

The presence of the broadly tuned, caffeine-sensitive TAS2R10 receptor (12) in the gastric epithelium was confirmed by immunohistochemical staining of stomach surgical specimens from the antrum and fundus/corpus region. The specificity of the TAS2R10 antibody was verified in transiently transfected HEK-293T cells (Fig. S3). In gastric mucosa, cell types were identified by H&E staining (Figs. S4 and S5). Parietal cells are localized in the glands of gastric fundus and body, and are scattered in the middle and, to a lesser extent, in the bottom part of the mucosa (Fig. S4). They are characterized by broad pink cytoplasms. Chief cells stain with basophilic cytoplasm and are mainly located in the bottom parts of the mucosa (Fig. 3*A* and Fig. S4). Localization of TAS2R10 staining was confined to parietal cells and to gastric chief cells in the fundus/corpus, showing strong cytoplasmic granular reactivity (Fig. 3 *A*, *a* and *b*). Staining of glandular cells in the gastric antrum was faint, consisting of very weak cytoplasmic and focal intermediate membranous reaction (Fig. 3 *A*, *e* and *f*). In contrast, mucus-producing foveolar cells in the fundus/corpus (Fig. 3 *A*, *a* and *b*) and antrum (Fig. 3 *A*, *e* and *f*) did not show

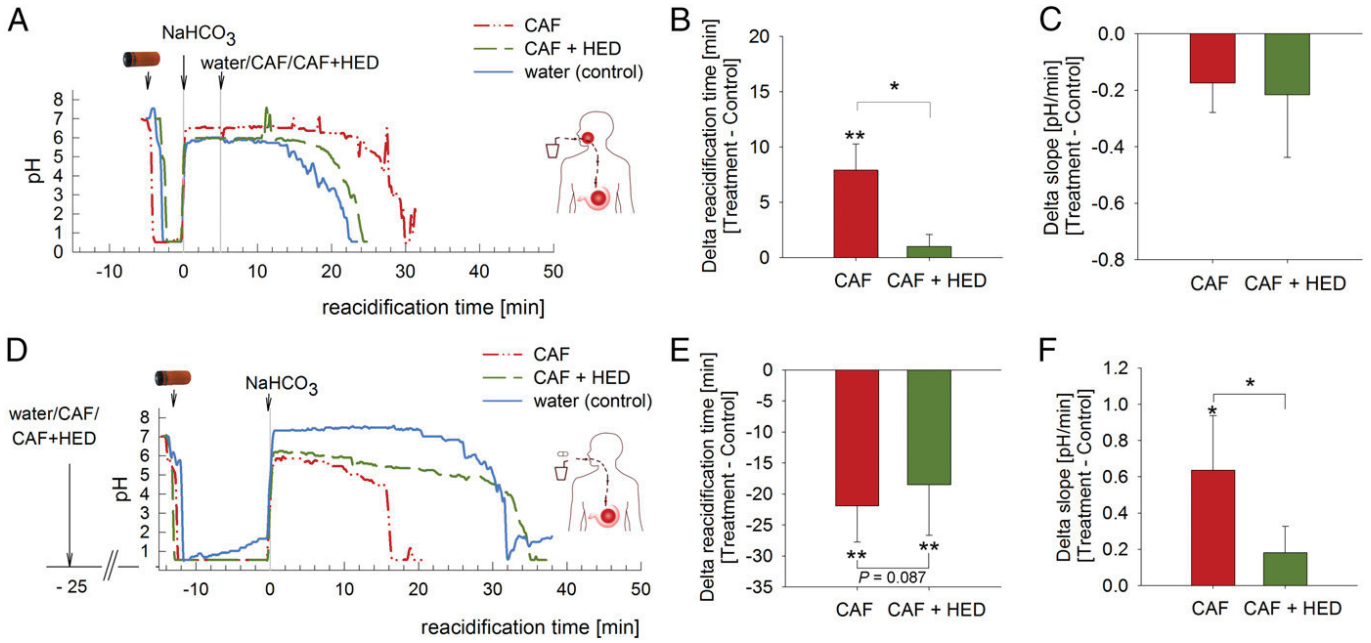

Fig. 2. Addition of HED reduces the caffeine-evoked effects on reacidification time or the slope in gastric pH measurements via administration by drinking 150 mg caffeine (CAF) with or without 30 mg HED dissolved in 125 mL water (*A*–*C*) or by encapsulated test compounds in combination with 125 mL water 25 min before alkaline challenge (*D*–*F*). (*A* and *D*) Gastrograms of Heidelberg capsule measurements according to the three different delivery protocols from one test subject are presented in one graphic. (*B* and *E*) Delta reacidification time of gastric pH measurements in subjects after consumption of CAF or CAF plus HED (basal = 0). (*C* and *F*) Delta slope of gastric pH measurements in subjects after consumption of CAF or CAF plus HED (basal = 0). Data are displayed as mean ± SEM: (*B* and *C*) CAF, $n = 10$; CAF plus HED, $n = 6$; (*E* and *F*) CAF, $n = 7$; CAF plus HED, $n = 6$ (*$P < 0.05$ and **$P < 0.01$ indicate significant differences by Student's *t* test).

expression of TAS2R10. Blocking experiments showed a clear staining reduction (Fig. 3 *A*, *c*, *d*, *g*, and *h*), supporting the epitope specificity of the antiserum. Like TAS2R10, the downstream signaling molecule transducin was localized in parietal and chief cells of the corpus/fundus, indicating that TAS2R10 and transducin are coexpressed in a substantial fraction of these cells. In addition, transducin immunoreactivity is present in the membranes of foveolar cells in gastric fundus/corpus (Fig. 3 *A*, *i* and *j*), but not in the antrum (Fig. 3 *A*, *m* and *n*). TAS2R10 and transducin expression was also detected in almost all HGT-1 cells (Fig. 3*B*), indicating that both proteins are coexpressed. Together, our data show that the mRNAs for caffeine's cognate TAS2Rs and at least one bitter receptor polypeptide are present in the human stomach and HGT-1 cells.

Effect of Bitter and Bitter-Masking Compounds on Proton Secretion in HGT-1 Cells. Following our hypothesis that bitter compounds induce mechanisms of GAS via TAS2Rs, various bitter compounds, such as theobromine, tannic acid, yohimbine, denatonium benzoate, sodium benzoate, and aristolochic acid were tested and verified for their stimulating effects on proton secretion in HGT-1 cells (Fig. 4*A*). The concentrations of the tested compounds were chosen based on preliminary experiments to elicit the strongest effect on proton secretion without impairing cellular viability [>90% compared with nontreated controls (100%)]. The responses were similar in magnitude, or even more pronounced, compared with those elicited by histamine, a major activator of proton secretion in parietal cells (10). These responses to bitter compounds indicate that several TAS2Rs could be activated in HGT-1 cells. Treatment of HGT-1 cells with 0.3–3,000 μM caffeine increased proton secretion, with 3,000 μM caffeine showing the highest effect (Fig. S6 *A* and *B*). The bitter-masking compounds HED and eriodictyol (ED), which have been described to reduce the bitter taste of caffeine in human sensory panels (33, 34), also reduced the caffeine-evoked proton secretion in HGT-1 cells (Fig. 4*B* and Fig. S6*C*).

Antagonistic or Agonistic Effect of HED and ED on TAS2Rs-Induced Ca^{2+} Mobilization in HEK-293T Cells. To identify the TAS2Rs that are targeted by HED and its structural analog ED, Ca^{2+}-mobilization in the presence of these compounds by transiently transfected HEK-293T cells was analyzed with or without costimulation with specific agonists of TAS2Rs (12). HED and ED were identified as agonists for TAS2R14 and as antagonists for TAS2Rs 43, 20, and 50 (Table S1). HED is also an antagonist for TAS2R31 (Table S1). As TAS2R43 can be activated by caffeine (12), the effect of caffeine and HED was further investigated in HEK-293T cells transiently transfected with TAS2R43. TAS2R43 in these cells was then activated by aristolochic acid or caffeine for the performance of calcium imaging experiments in the presence of increasing concentrations (0.03–30 μM) of HED and ED. Both compounds reduced TAS2R43 responses to aristolochic acid or caffeine (Fig. 4*C*).

Caffeine-Induced Proton Secretion Is Reduced in TAS2R43 KO HGT-1 Cells. To determine whether TAS2R43 is involved in mechanisms of caffeine-induced GAS, a homozygous 13-bp deletion in the TAS2R43 gene (Fig. S7) of HGT-1 cells was induced by using a CRISPR-Cas9 CD4-vector (i.e., TAS2R43-KO). As negative control (NC), HGT-1 cells were treated in parallel with the same vector containing a nontargeting scrambled guide RNA (gRNA). Off-target effects of the transfected gRNA were excluded by a whole-genome sequencing analysis. The stimulating effect of caffeine and the TAS2R43 agonist aristolochic acid on proton secretion in HGT-1 cells was substantially reduced in TAS2R43-KO cells compared with NC cells (Fig. 4*D*). These data demonstrate that TAS2R43 is involved in caffeine's action on proton secretion in HGT-1 cells.

Considering that caffeine's effect on proton secretion in HGT-1 cells is sensitive to TAS2R43 and HED, and that TAS2R43 is blocked by HED in *TAS2R43*-transfected HEK-293T cells, the data strongly suggest that caffeine mediates its effect through at least one TAS2Rs (TAS2R43) if not more.

Table 1. mRNA expression of TAS2Rs in HGT-1 cells normalized to the expression of the acetylcholine receptor (*CHRM3*)

	HGT-1	
Receptor/gene	Mean	SEM
CHRM3	1.00	0.035
TAS2R1	0.20	0.050
TAS2R3	9.87	0.848
TAS2R4	5.66	0.765
TAS2R5	12.08	0.822
TAS2R7	0.32	0.073
TAS2R8	No specific product	
*TAS2R9**	0.12	0.019
TAS2R10	0.97	0.100
TAS2R13	1.69	0.144
TAS2R14	12.39	1.347
TAS2R16	0.71	0.239
TAS2R19	4.40	0.678
TAS2R20	9.09	1.139
TAS2R30	8.02	0.717
TAS2R31	4.00	1.767
TAS2R38	0.14	0.045
TAS2R39	3.64	0.807
TAS2R40	0.51	0.052
TAS2R41	0.66	0.143
TAS2R42	2.24	0.444
TAS2R43	6.47	0.316
TAS2R45	Not detected	
TAS2R46	2.59	0.421
TAS2R50	2.91	0.290
TAS2R60	No specific product	
PLCB2	2.47	0.110
GNAT2	7.16	0.557
GNAT3	0.04	0.014

Data are shown as mean ± SEM; n = 3–4 biological replicates, tr = 3 technical replicates. The mRNA of *TAS2Rs* is similarly or even more highly expressed compared with the mRNA of *CHRM3* in HGT-1 cells.
*In one of three replicates, no product was detected.

Effect of Pharmacological Blockers on Caffeine-Induced Proton Secretion. Caffeine-evoked proton secretion was reduced by neither the PLCβ2 inhibitor U73122 nor the IP3 inhibitor neomycin (Fig. 4*E*). In contrast, it was reduced by the adenylyl cyclase inhibitor NKY80 (−22.5 ± 7.4%; $P < 0.01$), suggesting that cAMP but not Ca^{2+} signaling is involved in TAS2R-mediated regulation of acid secretion in HGT-1 cells.

Effect of Caffeine and HED on cAMP Levels in HGT-1 Cells. To confirm that adenylyl cyclase mediates proton secretion in HGT-1 cells via caffeine-dependent TAS2R stimulation, intracellular cAMP levels were determined in response to treatment with caffeine and HED (Fig. 4*F*). Treatment of HGT-1 cells for 10 min with 3.0 mM caffeine increased cAMP levels by 12 ± 4.6% ($P < 0.05$) in comparison with the treatment with DMEM (control, 100 ± 2.0%). However, coapplication of caffeine and HED (83.3 ± 2.7%; $P < 0.01$) and HED alone (84.9 ± 5.1%; $P < 0.05$) reduced cAMP levels in HGT-1 cells. Treatment with forskolin, a stimulator of adenylyl cyclase, increased cAMP levels to 131 ± 10.3% ($P < 0.05$) in HGT-1 cells in comparison with treatment with the solvent control ethanol (i.e., EtOH). These observations confirm that caffeine activates TAS2Rs signaling through changes in cAMP levels.

Discussion

Acid secretion in the stomach is a fundamental process that is finely regulated at different levels. Initial activation of GAS is regulated by the CNS when food is smelled and tasted (10, 35). When food enters the stomach, mechanical or chemical receptors (*i*) initiate GAS via activation of afferent/efferent fibers connected to the CNS, (*ii*) stimulate the gastrin-producing G cells or the histamine-producing enterochromaffin-like cells, or (*iii*) directly stimulate the HCl-producing parietal cells. Caffeine stimulates GAS, and, so far, it has been assumed that it acts via inhibition of PDE or by antagonizing adenosine receptors in gastric parietal cells (1). As caffeine activates five of the 25 human TAS2Rs (12), we hypothesized that its bitterness also contributes to its stimulating effect on GAS via activation of TAS2Rs.

First, we found that oral consumption of caffeine delayed GAS in healthy subjects, whereas caffeine that was administered encapsulated, being released in the stomach, accelerated this process compared with oral administration. The delay induced by oral caffeine presentation might be explained by findings reported by McMullen et al. (36). They demonstrated that caffeine in a coffee drink accelerated the heart rate without increasing the vascular tonus in comparison with caffeine administered encapsulated concomitantly to a decaffeinated coffee drink. The increase in heart rate was likely induced by vagal withdrawal instead of sympathetic activation. This finding is important, considering that the cephalic-phase response during digestion is thought to activate the vagus nerve to enhance digestion (36). The study of McMullen et al. (36) along with the present results suggest that orally sensed caffeine elicits vagal withdrawal that would reduce rather than enhance the digestive capacity, for example by delaying GAS. An explanation why the delaying effect of caffeine has not been discussed earlier might be that most of the previous studies investigating the effect of caffeine on GAS used gavages to bypass oral cavity receptors (2, 3, 5) and therefore did not take into account an inhibitory effect of caffeine on GAS by oral perception. Furthermore, we demonstrated that TAS2R bitter receptors in the stomach are involved in the caffeine-induced secretion of gastric acid. This conclusion is based on the observation that the bitter-masking compound HED similarly reduced caffeine's bitterness as well its effects on GAS evoked by combined oral/gastric or gastric-only caffeine application. The finding that concomitant oral ingestion of caffeine and HED accelerated passing of the Heidelberg capsule into the duodenum in 4 of 10 subjects compared with caffeine administration indicates that HED might induce gastric emptying. This hypothesis has been verified by measuring the effect of HED on gastric motility in strips of stomach dissections in an organ bath. Avau et al. (37) demonstrated that bitter compounds such as denatonium benzoate increased contractility in gastric strips of mice and caused an impairment of gastric relaxation after intragastric infusion. Whether a bitter-masking compound has opposite effects by causing gastric relaxation is an open question. The data presented here did not show any effects of HED on gastric secretion in humans, nor on proton secretion in HGT-1 cells at the concentrations tested. However, HED induced gastric relaxation, indicating physiological targets other than GAS.

To confirm that caffeine induces GAS via gastric TAS2Rs, we demonstrated that the mRNA of 22 of 25 *TAS2Rs* as well as transducin is present in HGT-1 cells, and that the five TAS2Rs that can be activated by caffeine are present in the stomach mucosa. These findings were corroborated by immunohistochemical detection of TAS2R10 and transducin in different gastric cell types for which useful antisera were available. So far, to our knowledge, only one validated antibody against human TAS2R38 (38) is known. Neither caffeine nor HED bind to TAS2R38. An antibody against TAS2R43 (OSR00171W; Thermo Fisher Scientific) was tested. Unfortunately, during validation in transfected HEK-239T cells, this antibody turned out to be unspecific. Nevertheless, we could demonstrate data for the validation of an antibody targeting TAS2R10 (Fig. S3) and show the expression of TAS2R10 on a protein level in gastric mucosa and HGT-1 cells.

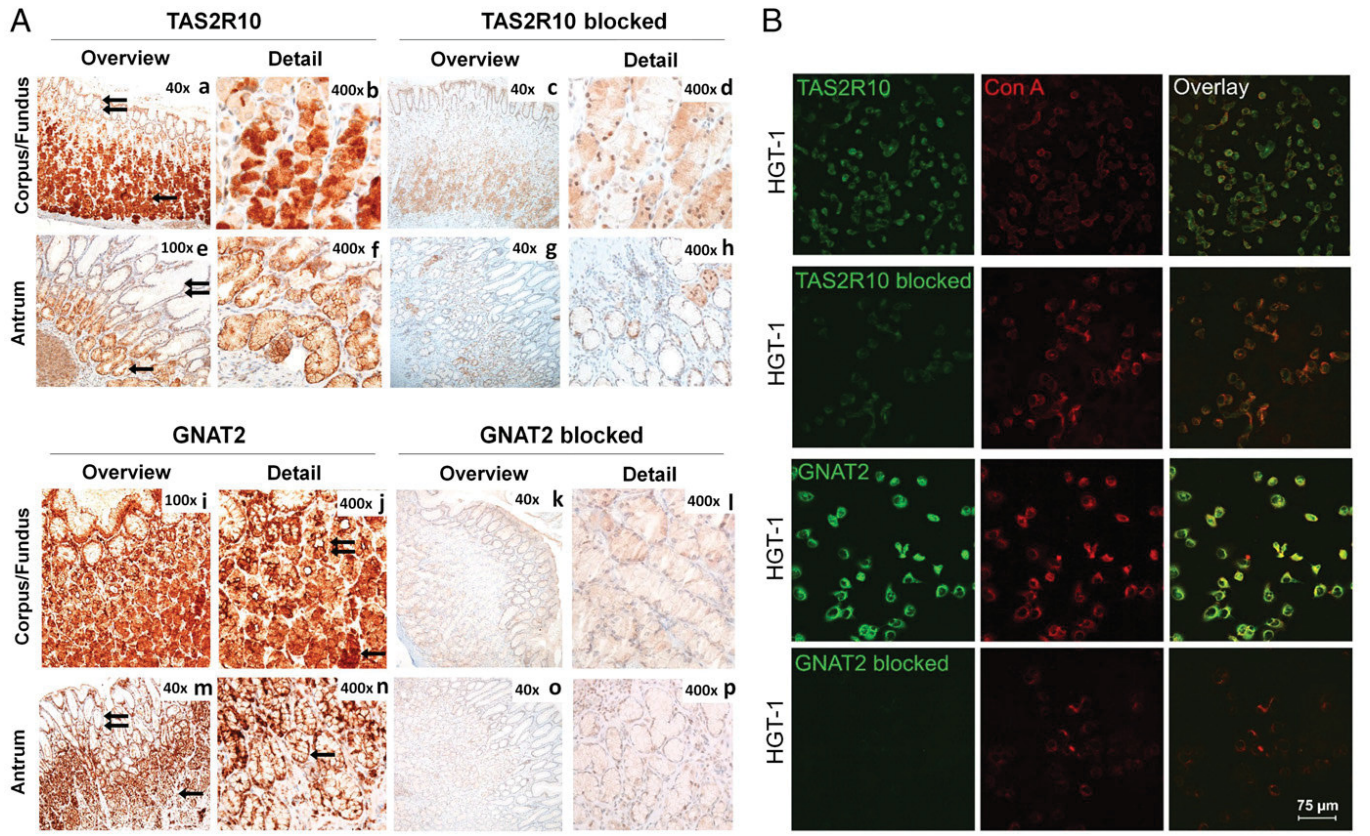

Fig. 3. (*A*, *a*–*p*) Immunochemical localization of TAS2R10 and GNAT2 in (*A*) gastric tissue and (*B*) HGT-1 cells with and without preincubation with a blocking peptide. (*a*) In the gastric corpus/fundus, cytoplasmic reactivity of TAS2R10 in parietal and chief cells (one arrow) was detected whereas foveolar cells were negative (two arrows). Detail (*b*) shows parietal and chief cells. In the gastric antrum (*e* and *f*), very faint cytoplasmic and focal membranous reactivity of TAS2R10 in glandular cells was detected (one arrow). Foveolar cells are negative (two arrows). (*f*) Detail showing glandular cells. GNAT2 was localized in the gastric fundus (*i* and *j*) parietal and chief cells (one arrow, *j*). Foveolar cells demonstrate membranous staining (two arrows, *j*). (*m* and *n*) In gastric antrum, membranous reactivity of GNAT2 in glandular cells (one arrow, *m* and *n*) was detected whereas foveolar cells were negative (two arrows, *m*). (*c*, *d*, *g*, *h*, *k*, *l*, *o*, and *p*) Corresponding negative controls. (*B*) Staining of HGT-1 cells with TAS2R10 and GNAT2 antisera (green) with and without specific blocking peptide and cell-surface labeling with con A (red).

As TAS2R10 was highly expressed in parietal cells, as detected by immunohistological staining, we focused on the cellular mechanisms in HGT-1 cells, which exhibit the characteristics of parietal cells (28, 29). Nevertheless, we cannot exclude that other cell types or gastrointestinal hormones may have contributed to the detected effects in the human intervention study. We demonstrated here that the bitter-masking agent HED reduced the stimulatory effect of caffeine on proton secretion in healthy subjects and in HGT-1 cells. As TAS2R43 is the only one of the five TAS2Rs that can be activated by caffeine and antagonized by HED, we also performed a CRISPR-Cas9 approach to knock out *TAS2R43* in HGT-1 cells. In these TAS2R43-KO cells, the effect of caffeine on proton secretion was reduced in comparison with control cells. To further confirm our hypothesis that TAS2Rs are involved in mechanisms regulating GAS, *TAS2R43* was transiently transfected into HEK-293T cells, which do not normally express any TAS2Rs. In this cell model, we demonstrated that HED antagonized caffeine-stimulated responses in *TAS2R43*-transfected cells. These results strongly indicate that TAS2R43 is involved in the proton secretory effect of caffeine. Nevertheless, involvement of other TAS2Rs or signaling pathways, such as adenosine receptors, or PDE inhibition cannot be excluded.

HED is also an agonist for TAS2R14, which is highly expressed in HGT-1 cells. The interaction of agonistic and antagonistic effects on TAS2Rs and the further activation of downstream signaling pathways seem highly complex, and not every TAS2R might be connected to the same downstream signaling cascade. One downstream signal for the induction of proton secretion is cAMP. Here, we show that caffeine increased cAMP levels in HGT-1 cells, an effect that was inhibited by HED. HED itself reduced the cAMP level in HGT-1 cells, but did not affect proton secretion in HGT-1 cells. Therefore, it remains unclear whether or which signaling pathways are affected by HED. Caffeine signaling via cAMP is supported by the fact that the adenylyl cyclase inhibitor NKY80 reduced the caffeine-evoked stimulation of proton secretion. Caffeine-induced activation of PLCβ2 and IP3 signaling can be excluded by the failure of the specific inhibitors U73122 (PLCβ2) and neomycin (IP3) to reduce proton secretion. Experiments in which caffeine and HED were tested in combination with the inhibitors showed no difference in proton secretion in comparison with caffeine and HED tested alone. That leaves the question of how caffeine can stimulate GAS via TAS2Rs. So far, only for sweet and glutamate taste receptors (TAS1R1/3) has an increase of cAMP levels via activation of adenylyl cyclase been demonstrated (39). The signaling cascade of bitter taste receptors has been proposed to reduce cAMP levels by activation of PDE via α-gustducin or transducin (23, 26, 40).

Based on our results, we hypothesize that bitter perception of caffeine in the mouth generates a signal of aversion, which leads, via vagal withdrawal, to inhibition of GAS (41). However, when bitter compounds reach the stomach, increased GAS could aid degradation or removal of the potential toxins. This hypothesis is

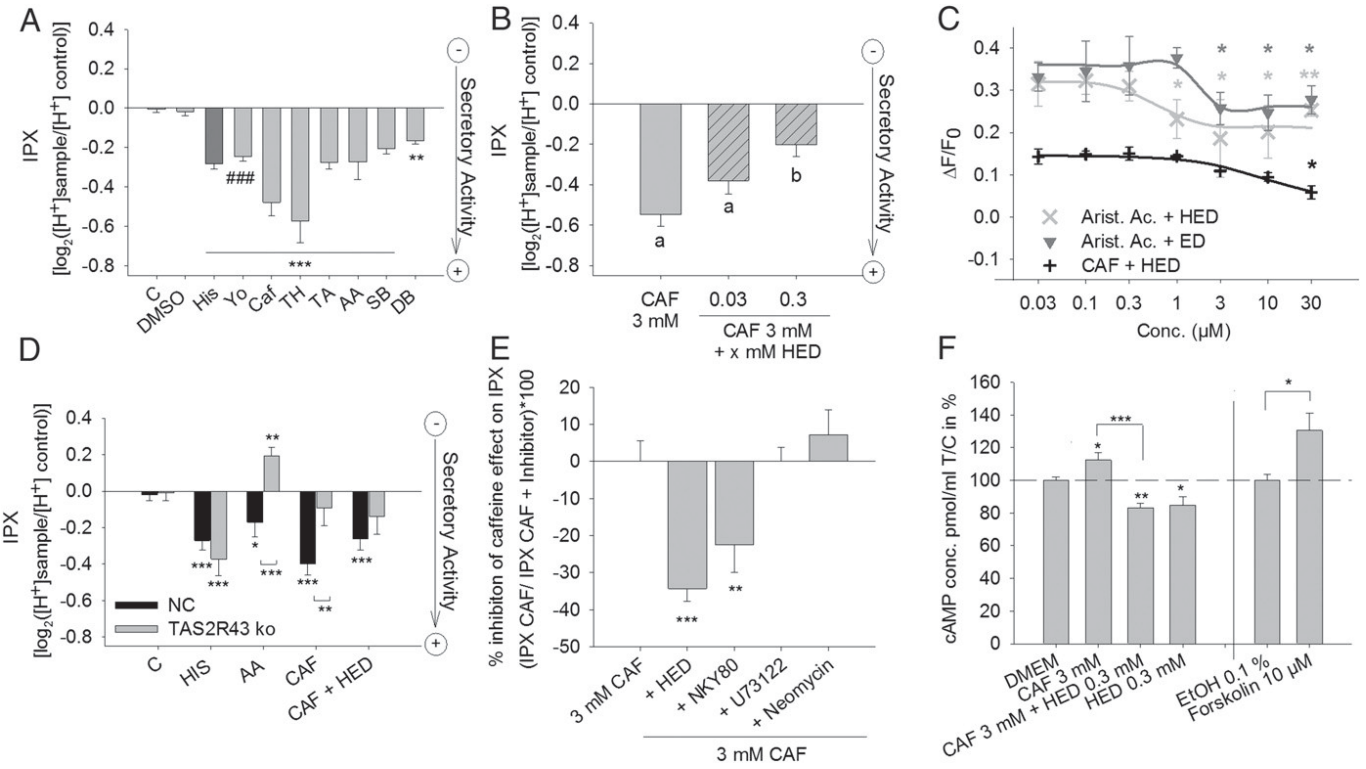

Fig. 4. Bitter tastants increase proton secretion in human gastric cells. Studies were performed with cultured HGT-1 cells loaded with the pH-sensitive fluorescent dye SNARF-1-AM and treated with test compounds for 10 min (*A*, *B*, and *D*). Results are presented as the IPX. A lower IPX value indicates increased proton secretion. Data displayed as mean IPX ± SEM. (*A*) IPX of HGT-1 cells after treatment with histamine (HIS; 1 mM), yohimbine (YO; 30 μM), denatonium benzoate (DB; 30 μM), caffeine (CAF; 3.0 mM), theobromine (TH; 0.3 mM), tannic acid (TA; 3 μM), aristolochic acid (AA; 0.3 μM), and sodium benzoate (SB; 3.0 mM) in comparison with untreated cells (i.e., control; marked as "C") or 0.1% DMSO-treated cells [solvent control for yohimbine; $n = 3$–16; six technical replicates (*tr*)]. (*B*) Coadministration of HED reduces the stimulating effect of caffeine on proton secretion ($n = 4$–37; $tr = 6$). (*C*) Inhibition curves of TAS2R43 assessed through calcium imaging experiments in transfected HEK-293T cells. Cells were costimulated with 0.03 μM aristolochic acid (Arist. Ac.) or caffeine 1 mM and increasing concentrations of the inhibitors HED or ED. Caffeine and aristolochic response amplitudes ($\Delta F/F_0$) were 0.14 and 0.39, respectively. Concentrations were chosen based on preliminary experiments to elicit the strongest effect. (*D*) IPX of HGT-1 cells transfected with nontargeting gRNA (NC) or HGT-1 cells with KO of TAS2R43 by CRISPR-Cas9 deletion treated with histamine (HIS; 1 mM), aristolochic acid (AA; 0.3 μM), caffeine (CAF; 3.0 mM), or 3.0 mM caffeine and 0.3 mM HED ($n = 5$–6; $tr = 6$). (*E*) Percentage inhibition of 3 mM caffeine effect on IPX of HGT-1 cells in comparison after treatment with 3 mM caffeine in combination with 0.3 mM HED, 5 μM U73122, 100 μM neomycin, or 30 μM NKY80 ($n = 3$–6; $tr = 6$). (*F*) cAMP concentration in HGT-1 cells after 10 min treatment with 3 mM caffeine, 0.3 mM HED, or in combination in comparison with DMEM, EtOH 0.1%, or forskolin 10 μM ($n = 4$; $tr = 2$). (*A*–*E*) Data presented as mean ± SEM. (*C*) Data presented as mean ± SD. Statistics: (*A*, *B*, and *F*) one-way ANOVA with Holm–Šídák post hoc test (*F*) vs. DMEM and (*A* and *C*–*F*) Student's *t* test. Significant ($P < 0.05$) differences are indicated by letters or as follows: $^{###}P < 0.001$ vs. DMSO 0.1%; $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$.

supported by previous studies demonstrating that extraoral TAS2Rs are probably involved in defense mechanisms in other parts of the gastrointestinal tract and form a "chemofensor complex" (18, 19, 42). The differential effect of site-specific TAS2R activation on GAS we demonstrated has not been reported so far to our knowledge and warrants further investigations. The expression of TAS2Rs in murine goblet cells (18), a cell type that secretes mucus to protect the epithelium, and the fact that bitter substances increase anion transport and fluid secretion in human and rat colon tissue (42), indicate defense-related functions of bitter taste receptors. Furthermore, in intestinal cells, Jeon et al. (43) identified a TAS2R38-dependent activation of the ATP-binding cassette B1 (ABCB1) via phenylthiocarbamide (PTC). As ABCB1 is an efflux transporter located on the apical membrane of intestinal epithelial cells to limit absorption of toxic substrates contained in food, TAS2R signaling has been assumed to limit the absorption of potentially hazardous bitter-tasting substances in the intestine (43).

Our results clearly demonstrate that the route of application of caffeine determines its effects on GAS, and suggest that other bitter tastants and bitter-masking compounds are also potentially useful therapeutics to regulate gastric pH. Finally, our results support the pleiotropic functions of taste receptors far beyond their role in taste.

Materials and Methods

Chemicals. The sodium salt of HED (3′-methoxy-4′,5,7-trihydroxyflavanone) and ED was provided by Symrise. All other chemicals were obtained from Sigma-Aldrich unless stated otherwise.

Identification of the Influence of Bitter Taste on GAS in Vivo. The human intervention study was designed as a single-blinded, randomized, controlled, longitudinal trial and was performed in accordance with good clinical practice guidelines and the Declaration of Helsinki. The experimental protocol was reviewed by the ethics committee of the city of Vienna (registration no. EK 13–180–VK_NZ), and the study has been registered at ClinicalTrials.gov (ID code NCT02372188). The subjects provided written informed consent after they had been given a detailed oral and written description of the study. The 13 healthy test subjects had no gastrointestinal complaints, were nonsmokers, did not take antibiotics for 2 mo before the test, and were between 21 and 32 y of age, with a body mass index between 19 and 25 kg/m^2. *Helicobacter pylori* infection was excluded by an immunochromatographic rapid capillary blood test (Diagnostik Nord). Average habitual caffeine consumption was 125 mg/d and determined by a food frequency questionnaire of caffeine-containing food and beverages. The Heidelberg capsule measurements were carried out at the Department of Nutritional and Physiological

Chemistry, University of Vienna, Austria. At each visit, subjects were subjected to one treatment, meaning that subjects who took part in all administration types and treatments completed 11 visits (Fig. 1*B*). Before the intervention, the trial subjects had to fast from food and liquid for 10 h. For the noninvasive measurement of gastric pH, the Heidelberg Detection System (Heidelberg Medical) was applied as described before (29, 30). When the subject arrived in the morning, the pH capsule was prepared by activation for 5 min in a sterile 0.9 NaCl solution and, as indicated by the Heidelberg Detection System software, the capsule was calibrated at pH 1 and pH 7. After calibration, the capsule was swallowed by the subject. When a pH of approximately 1–2 was stable over a period of 3 min, a stable position of the capsule in the stomach was considered to have been achieved. During the measurement, the subjects had to lie down on their left side to make sure that the capsule remained in the stomach. Each trial started with the administration of 5 mL of a saturated sodium bicarbonate solution ($NaHCO_3$), triggering an increase in gastric pH to values between pH 6 and pH 7 and subsequently leading to the secretion of stomach acid by the parietal cells. At the first 8 visits, 125 mL water (control), 37.5/75/150 mg caffeine diluted in 125 mL water, or 150 mg caffeine encapsulated in a gelatin capsule (Coni-Snap size 1; Capsugel) with 125 mL water were administered 5 min after the alkaline challenge (Fig. 1*B*). A total of 150 mg caffeine in combination with 30 mg HED or 30 mg HED alone, diluted in 125 mL, were administered by drinking 5 min after the alkaline solution. At visits 9–11, three new subjects joined to replace dropouts. There, an empty gelatin capsule (Coni-Snap size 1; Capsugel), 150 mg caffeine encapsulated, or 150 mg caffeine encapsulated with 30 mg HED were administered with 125 mL water 25 min before the alkaline solution. For exclusive activation of TAS2Rs in the mouth (delivery protocol 3), the subjects swallowed 125 mL water and rinsed their mouth with 150 mg caffeine diluted in 125 mL water without swallowing the caffeine 5 min after swallowing the alkaline solution. Reacidification time (i.e., time until original pH is reached again after administration of the alkaline challenge) as well as the slope of the gastrogram were analyzed by using Heidelberg Detection System software and ImageJ software (National Institutes of Health). Delta reacidification time was calculated by substraction of the reacidification time of the water or empty capsule control from the reacidification time after the treatment. The slope was calculated between the point when pH decreases and the point at which the original pH is reached again.

Organ Bath of Human Stomach Biopsy Specimens. Human stomach was obtained at surgery for obesity at Homerton University Hospital (London, United Kingdom) after ethical approval (REC 15/LO/2127) and informed written consent. Mucosa-free tissue from the fundus region of human stomachs was dissected parallel to the circular muscle fibers into strips (5–8 × 15 mm). Strips were tied up and mounted in organ bath chambers that contained a Krebs solution at 37 °C aerated with 5% CO_2 in O_2. After 1 h equilibration, the nerves were excited by electrical field stimulation (EFS) at 200 mA for 0.5 ms; 5 Hz were given for 10 s every 1 min. When there was a stable response to EFS, a frequency response with 1, 2, 5, 10, 15, and 20 Hz was generated. After switching back to 5 Hz and reaching a stable signal, NaHED was applied in a concentration of 1 mM for at least 50 min. Double-distilled (dd)H_2O was used as a vehicle control. Isometric force transducers (calibrated at 2 g; AD Instruments) and the software AcqKnowledge (Biopac) detected changes in muscle changes. Data are presented as percent changes in baseline tension. The number of patients is given as an *n* value.

Sensory Study. Taste sessions were carried out in the morning hours, and the 13 untrained panel subjects were asked not to consume anything besides water 30 min before the sensory duo test. The bitter recognition threshold of the subjects was determined by using a standardized test system starting with water and followed by nine solutions with increasing concentrations of caffeine, from 25 to 225 mg/L. Furthermore, the subjects had to rank the bitterness of a caffeine solution (150 mg/125 mL) and a caffeine (150 mg/125 mL) plus HED (30 mg/125 mL) solution by sip-and-spit on a scale of 1 (nothing) to 10 (extremely strong). This dual test was repeated four times in randomized order and under colored light. Statistical significance was calculated by Student's *t* test (double-sided, paired).

HGT-1 Cell Culture. The human gastric tumor cell line HGT-1 was obtained from C. Laboisse (Laboratory of Pathological Anatomy, Nantes, France) and cultured under standard conditions as described previously (8). Cytotoxicity of the tested substances and treatment reagents was excluded by MTT test as described before (8), and cell viability was determined by trypan blue staining. Tested cells had at least 90% cell viability.

Immunohistochemical Staining of Gastric Tissues. Histological specimens were obtained from two patients from the Pathologisch-Bakteriologisches Institut, Sozialmedizinisches Zentrum Ost–Donauspital, Vienna, Austria. The gastric fundus was derived from a sleeve gastrectomy of a 42-y-old adipose but otherwise healthy patient. The gastric antrum was derived from a 71-y-old patient undergoing distal partial gastrectomy for a benign gastrointestinal stroma tumor. Immunohistochemistry was performed on 5-μm-thick formalin-fixed, paraffin-embedded whole tissue sections. Slides were processed in the fully automated staining instrument Benchmark ULTRA by using an ultraView Universal DAB Detection Kit (Ventana Medical Systems). The following primary antibodies were applied: TAS2R10 (OSR00158W; Thermo Scientific), 1:750 for 28 min at 37 °C after heat-mediated antigen retrieval using EDTA buffer, pH 8.0, at 95 °C for 36 min (CC1 buffer; Ventana Medical Systems) and GNAT2 (transducin α-2 chain; AP11077c; Abgent), 1:50 for 28 min at 37 °C after heat-mediated antigen retrieval using EDTA buffer, pH 8.0, at 95 °C for 64 min (CC1 buffer; Ventana Medical Systems) and amplification at 95 °C (Amplification Kit; Ventana Medical Systems). All counterstaining was performed with hematoxylin. Blocking experiments to control for unspecific staining were performed by using the TAS2R10 control peptide (GST00040P; Thermo Scientific) and GNAT2 antibody blocking peptides (BP11077c; Abgent). For the TAS2R10 taste receptor, the blocking experiment consisted of the control peptide, 1:200, incubated together with TAS2R10 antibody, 1:750, for 120 min at 4 °C, and thereafter, incubation of the slide at 37 °C for 28 min. The GNAT2 antibody blocking peptide, 1:10, was incubated together with GNAT2 antibody, 1:50, for 120 min at 4 °C, and, thereafter, incubation of the slide for 28 min at 37 °C. All other steps were performed similarly to the staining procedure as described earlier.

Immunocytochemical Staining of HGT-1 Cells and HEK-293T-Gα16gust44 Cells. Transiently transfected HEK-293T-Gα16gust44 cells (TAS2R10 or TAS2R16) were prepared as described previously (19), and HGT-1 cells were seeded on coverslips 24 h before the staining procedure. Cells were fixed and stained as described previously (18) by using anti-HSV (1:15,000; Novagen), anti-TAS2R10, and anti-GNAT2 antibodies (*Immunohistochemical Staining of Gastric Tissues*) for 1 h at room temperature. Specificity of labeling was ensured as described in *Immunohistochemical Staining of Gastric Tissues*, and detection was carried out as described previously (18). Preabsorption of the anti-TAS2R10 and anti-GNAT2 antibody was performed with the corresponding immunogenic peptide (*Immunohistochemical Staining of Gastric Tissues*; Fig. S3). The HSV epitope was detected with anti-mouse antibodies conjugated with Cy3 (1:2,000; Sigma), biotin-labeled concanavalin A (con A) with streptavidin Alexa Fluor 633 (1:1,000; Molecular Probes), and TAS2R10 or GNAT2 with Alexa Fluor 488 goat anti-rabbit IgG (1:1,000; Molecular Probes).

Intracellular pH Measurement in HGT-1 Cells Indicating Proton Secretion. Intracellular pH, as indicator for proton secretion in HGT-1 cells, was measured using the pH-sensitive fluorescence dye 1,5 carboxy-seminaphto-rhodafluor acetoxymethylester (SNARF-1-AM; Life Technologies) as described before (8, 29). The intracellular proton index (IPX) in the cells was calculated by $\log_2$ transformation of the ratio between treated and untreated (i.e., control) cells. The lower the IPX, the fewer protons are in the cell, indicating a higher secretory activity in HGT-1 cells.

mRNA Expression of Bitter Taste Receptors in HGT-1 Cells and Human Biopsies Using RT-qPCR. Total RNA was extracted from HGT-1 cells and two human biopsy specimens by using the peqGold Total RNA Kit (Peqlab). Quantity and quality were checked spectrophotometrically. Reverse transcription was carried out with 2 μg RNA and the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time PCR was performed with an Applied Biosystems StepOneplus Real Time PCR system and Fast SYBR Green Master Mix (Thermo Fisher Scientific). Primers were designed using the National Center for Biotechnology Information (NCBI) primer designing tool (using Primer 3 and BLAST; Table S2). Cycling conditions were 20 s/95 °C (activation), 3 s/95 °C (denaturation), 30 s/60 °C (annealing), and 15 s/67 °C (elongation with fluorescence measurement). The PCR products were verified by melting curve analysis, agarose gel electrophoresis, and sequence analysis (Eurofins Genomics). Sequences were checked by using the NCBI BLASTn tool. Primers showing no product in HGT-1 in at least one of the three replicates (*TAS2Rs 8*, *9*, *45*, and *60*) were tested with cDNA derived from a human tongue biopsy provided by J.-D. Raguse (Charité, Berlin, Germany). Whereas primers for *TAS2Rs 8*, *9*, and *60* could be verified, *TAS2R45* was not detected. For *TAS2R45*, high-frequency copy-number variants are known, and some people do not possess the tested variant of the mRNA for this gene (44). *TAS2R46* could not be detected in the second human biopsy sample. The open-source software LinRegPCR was used for quantitative PCR

data analysis. This software enables the calculation of the starting concentration (N_0) of each sample, expressed in arbitrary fluorescence units. The calculated starting concentrations of the *TAS2Rs* were compared with the starting concentrations of the acetylcholine receptor (*CHRM3*), with previously described primers (8), which is typically expressed in parietal cells on a functional level.

Generation of TAS2R43 Homozygous KO HGT-1 Cell Line Using CRISPR-Cas9. A total of 40,000 cells were seeded in a 24-well plate. After approximately 24 h, cells were transfected with 495 ng GeneArt CRISPR Nuclease (CD4 Vector; A21175; Invitrogen) containing the gRNA targeting TAS2R43 gene TTTTTTGGCAAATGAGGTAC (5′–3′) or, as a control, a scrambled gRNA GTGGACGGTCGTGCGCTGT (5′–3′) with no target by using the transfection reagent Viromer RED (Lipocalyx) according to the manufacturer's protocol. Transfection efficiency was approximately 25%, verified with a CD4 monoclonal antibody (07-0403; Invitrogen/Thermo Fisher Scientific) by using a Guava soft-flow cytometer (Millipore) on the basis that only positively transfected cells express a CD4 protein. Cells were transferred in a six-well plate to increase cell number. After 3 d, CD4-positive cells (i.e., positively transfected cells) were enriched by using the Dynabeads CD4 Positive Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Cells were analyzed with a Genomic Cleavage detection kit (Thermo Fisher Scientific) according to the manufacturer's protocol. For genomic cleavage detection, the following primers were used: forward primer AGACTGCCATTGGGTCAAAGA (5′–3′) and reverse primer GATGTTGTTGGGGCCTTTGC (5′–3′). The following temperature protocol was used: 95 °C/10 min, 40 cycles of 95 °C/30s, 58 °C/30 s, 72 °C/30 s, and finally 72 °C for 7 min. A genomic cleavage of approximately 22% was detected. Single cells were isolated by serial dilution of positively transfected cells into two 96-well plates and observed for colony forming for 2 wk. Total cells of 39 wells in which clearly only one colony formed were harvested by trypsin/EDTA and first transferred to a 48-well plate, and, after confluence, to a 12-well plate to increase cell number. From each clone, half of the cells were frozen and the other half were used to extract DNA with a PureLink Genomic DNA Mini Kit (Life Technologies) according to the manufacturer's protocol. Before Sanger sequencing by Eurofins Genomics, the DNA extracts of 20 clones were amplified with AmpliTaqGold 360 Mastermix (Thermo Fisher Scientific), and PCR was carried out as described earlier for the genomic cleavage detection. Of 20 clones, 15 showed no deletion, four a heterozygous deletion, and one a homozygous deletion. Deletion on an mRNA level was also analyzed by means of Sanger sequencing following total RNA isolation of the WT, cells transfected with the scrambled gRNA, and the TAS2R43-KO cells as described earlier (Fig. S7).

Exclusion of Off-Target Effects Using Whole-Genome Sequencing. The quality-checked DNA was fragmented with a Covaris ultrasonicator. The resulting DNA fragments were purified, end-blunted, A-tailed, and adaptor-ligated. The concentration of the libraries was quantified by Bioanalyzer and real-time PCR. Each library was sequenced with Illumina's X Ten system with paired-end 125-bp read length according to the manufacturer's instructions. Sequencing-derived raw image files were processed by Illumina's basecalling software to yield raw data files in Fastq format. Reads were mapped to the human reference genome (GRCh37/hg19) using Burroughs–Wheeler Aligner (v0.7.12). Duplicate reads were removed from mapped data by using Picard tools (v1.118). SNPs and small insertions and deletions (InDels) were identified by using the Genome Analysis Toolkit (GATK; v3.3.0). Recommended best practices for variant analysis were followed, including InDel realignment and base quality score recalibration. The genomic variations were detected by using GATK's HaplotypeCaller. Variant quality score recalibration in GATK was applied to obtain high-confidence variant calls. Copy number variants were called by using CNVnator (v0.2.7). Structural variants (SVs) were detected by using Breakdancer or CREST. The SV annotation was done with SnpEff (v4.0). The University of California, Santa Cruz, LiftOver tool was used to get the corresponding coordinates from genome build 38 to build 37. Filtering of shared variants was done with GATK's SelectVariants tool. Reads mapping to off-target regions were extracted with SAMtools (v0.1.19).

cAMP Measurements in HGT-1 Cells. cAMP in HGT-1 cells was measured with the ELISA kit from R&D Systems according to the protocol.

Calcium Imaging Experiments in HEK-293T Cells. Calcium imaging experiments using HEK-293T cells transiently expressing TAS2Rs and stably expressing the chimeric G protein subunit Gα16gust44 were essentially done as described previously (12). Aristolochic acid was dissolved in C1 buffer at 0.03 μM concentration and caffeine at 1 mM concentration. Cells were exclusively stimulated with agonists and increasing concentrations of the antagonists in the concentration range of 0.03–30 μM. For screening which TAS2Rs are activated or inhibited by HED or ED, they were applied alone as well as coapplied with suitable TAS2R agonists. Intracellular Ca^{2+} concentration increases were measured to monitor changes in TAS2Rs activation upon coapplication of the putative inhibitors. Potential antagonistic/blocking activity of the compounds was addressed comparing the signals elicited by cells exclusively stimulated with the cognate agonists with signals elicited from cells costimulated with HED or ED in two test concentrations (10 and 100 μM) and the agonists. Inhibition curves were calculated with SigmaPlot 11 software after correcting signal responses and normalization to background fluorescence.

Statistical Analysis. Data shown are representative of at least three biological replicates. All data are expressed as mean ± SEM unless stated otherwise. All data have been verified for normality distribution, and statistically significant differences were considered if the *P* value was less than 0.05, determined by one-way ANOVA with Dunn's or Holm–Šídák post hoc test using SigmaPlot 11.0 software. Correlation analysis according to Spearman was calculated by SigmaPlot 11.0 software.

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2.2 "Appetite-inducing effects of homoeriodictyol: Two randomized, crossover interventions."

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Appetite-Inducing Effects of Homoeriodictyol: Two Randomized, Cross-Over Interventions

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Scope: Anorexia of aging, characterized by a decrease in appetite and/or food intake, is a major risk factor of under-nutrition and adverse health outcomes in elderly people. Recent in vitro evidence suggests homoeriodictyol (HED), a naturally occurring, bitter-masking flavanone, as a promising agent to increase appetite and food intake.

Methods and results: In two cross-over intervention trials, 30 mg NaHED, either solely ($n = 10$, Study I) or in combination with a 75 g glucose load ($n = 17$, study II) were administered to healthy adult subjects. Ratings of hunger were assessed at fasting and either 30 min (Study I) or 120 min (Study II) post intervention. Ad libitum energy intake from a standardized breakfast and plasma changes in hunger-/satiety-associated hormones PYY, GLP-1, ghrelin and serotonin were determined after blood drawings. Effects were more pronounced when NaHED was administered in combination with 75 g glucose since ad libitum energy (+ 9.52 ± 4.60%) and protein (+ 7.08 ± 7.97%) intake as well as plasma ΔAUC ghrelin values increased in study II solely, whereas plasma serotonin concentrations decreased after both interventions.

Conclusions: NaHED demonstrated appetizing effects in healthy adults when administered with a glucose load. Long-term intervention studies are warranted to verify these effects in compromised subjects.

1. Introduction

Beside the worldwide growing problem of obesity, another phenomenon called 'anorexia of aging' coexists. This condition is characterized by a loss of appetite and a reduced food intake, promoting weight loss and adverse health outcomes in the elderly. A decrease in body weight from the age of 65 years is recognized as an independent predictor of morbidity and mortality.[1]

Although especially nursing home residents and hospitalized elderly are at risk, also about 30% of community-dwelling elderly people are suffering from a loss of appetite,[2] sometimes leading to symptoms of severe protein-energy malnutrition, which are associated with several co-morbidities like dementia, heart disease and diabetes.[3]

Regulation of energy intake is a controlled process, including the feeling of hunger, which triggers meal initiation, due to acute energy depletion, and satiation, which determines the size of a meal.[4] In contrast, appetite is excited by the accessibility of food and the pleasure of eating, comparable to food reward, which also determines the intake of food.[5]

From a physiological perspective, the central nervous system and the gastrointestinal tract play the pivotal role in the control of energy homeostasis by secreting hormones regulating the complex cascade of hunger and satiety.[6] Central nervous system serotonin is well-known to exert anorexigenic effects, and also peripheral serotonin (5-hydroxytryptamine) released by enterochromaffin cells is hypothesized to promote satiation and satiety.[7]

The most important and widely studied gastrointestinal hormones promoting satiation include glucagon-like-peptide-1 (GLP-1) and peptide YY ($PYY_{3–36}$), whereas the stomach-derived hormone ghrelin, discovered in 1999, is the only gastrointestinal hormone exhibiting orexigenic effects.[8] Two different forms of circulating ghrelin are known: acyl-ghrelin and the more abundant des-acyl ghrelin. The mechanism of action is mainly attributed to the acylated form, which exerts its effects by binding to the growth hormone secretagogue receptor (GHSR-1alpha).[9] However, there is evidence that also the non-acylated form of ghrelin plays a role in appetite stimulation, independent from binding to GHSR-1alpha.[10]

A loss of appetite is often accompanied by a change in plasma concentrations of both, orexigenic as well as anorexigenic hormones.[11] Interestingly, plasma levels of these

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hormones seem to be influenced by age, since elderly people demonstrate lower fasting plasma ghrelin concentrations in comparison to younger adults,[12] whereas the postprandial release of PYY after energy intake is enhanced compared to younger control group.[13]

A poor appetite and an unintentional loss of body weight might not only occur during aging, but also in other circumstances in life, including moderately vigorous exercises[14] and states of diseases, like cancer, where malnutrition is caused either by the therapeutic regimen, or the cancer itself,[15] or psychological disorders, like stress or depression,[16] as well as exposure to extreme environmental conditions, like high altitudes >3600 m.[17]

For any of these conditions characterized by an unintentional decrease in food intake and loss of body weight, dietary approaches to enhance nutrient intake by increasing appetite are promising, although not very well studied. Pharmacological interventions with medications, e.g., megestrole acetate[18] or cyproheptadine hydrochloride,[19] effectively increased appetite, but are also known for various adverse effects like irritability, hyperactivity and drowsiness. Naturally occurring food compounds which promote appetite in populations at risk to counteract unintentional weight loss without side effects, have yet not been identified since most research in this area is focused on compounds that reduce appetite. Polyphenols, in particular, have been widely studied for their effects on glucose metabolism, which plays a crucial role in the regulation of satiety.[20,21] The tasteless polyphenol Homoeriodictyol (3'-methoxy-4',5,7-trihydroxyflavanone, HED) belongs to the class of flavanones which have been described for their bitter-masking effects.[22] HED is isolated from the Herba Santa shrub (*Eriodictyon californicum*), a plant which mainly grows in the Sierra Nevada, California coastal ranges and the Southern Oregon mountains. In ancient times, alcoholic liquid extracts of this plant were used to mask the bitter taste of quinine, and as natural remedy for treatment of the common cold and asthma. More recently, a concentration of 100 mg L^{-1} HED was shown to reduce the bitterness of a 500 mg L^{-1} caffeine solution by 43% in a sensory study, without exhibiting any side tastes.[23] In addition to the bitter masking effect, in vitro experiments carried out in our working group have shown that NaHED, the more water soluble sodium salt of HED, may also have the potential to stimulate mechanisms regulating hunger as it affected glucose uptake and serotonin release in Caco-2 cells.[24] Another recent study demonstrated that bolus administration of 18 mg quinine hydrochloride, a bitter taste receptor agonist, reduced ad libitum calorie intake in healthy adults,[25] providing evidence that bitter taste receptors may be involved in the regulation of food intake. We, therefore, hypothesized that bitter-masking agents, like HED, might exert opposite effects. Following this hypothesis, we studied the effect of a bolus administration of 30 mg NaHED to healthy adult subjects, either without (*Study I*) or with (*Study II*) a caloric load on ad libitum energy intake from a standardized breakfast, on the subjective feelings of hunger, and on plasma concentrations of satiety-regulating hormones. The two study designs were chosen as it was not clear from the literature whether NaHED would have an impact on food intake and associated plasma hormones in the presence or absence of a standardized caloric load. The study design has been chosen according to recently published works revealing the impact of another aroma compound, nonivamide, on food intake and satiety-associated hormones in healthy adults.[26]

2. Experimental Section

2.1. Human Intervention Study I

For *Study I*, 10 healthy test subjects were recruited: all were non-smokers, and were between 20 and 35 years of age, with a body mass index between 19 and 25 kg/m^2. Each volunteer was fully informed about the test, gave written consent, and was treated following the ethical principles of the declaration of Helsinki. The experimental protocol was reviewed by the ethics committee of the city of Vienna (registration no. EK 13–180–VK_NZ). The study was part of the intervention *'Influence of a Bitter Compound and Bittermasking Compound on Gastric pH'* which has been registered at clinicaltrials.gov, registration number NCT02372188. Prior to the intervention, subjects had to fast from food and liquid for 10 h, except for 200 mL of tap water that were allowed during this time period. Subjective feelings of hunger were recorded by means of visual analog scales (VAS). The volunteers had to make a sign on a 100 mm long scale, ranging from 0 (not hungry) to 100 (very hungry). Either 30 mg HED sodium salt (NaHED, Symrise product, purity >95%, prepared according to[22]) dissolved in 125 mL tap water or 125 mL tap water as control were administered. Since NaHED is more soluble in aqueous solutions compared to HED, and no difference between NaHED and HED in their ability to increase glucose uptake in differentiated intestinal Caco-2 cells was detected in previous in vitro studies,[24] NaHED was used for the experiments.

After ~30 min blood was drawn and the volunteers received a standardized breakfast, which consisted of 4 rolls, 3 slices of dark bread, 100 g strawberry jam, 60 g honey, 4 slices of ham, 4 slices of cheese, 180 g berry yogurt, 80 g creamery butter, 20 g sugar and 40 g coffee creamer as well as 200 mL of tea or coffee and water ad libitum. Mean total energy content of the breakfast was around 12.1 MJ, with a mean total macronutrient content of 335 g carbohydrates, 126 g fats, and 79.2 g proteins. Volunteers consumed the breakfast until satiation. The remaining food was weighed, and ad libitum energy intake as well as macronutrient intake was calculated by means of the German Food code and nutrient data base 'Bundeslebensmittelschlüssel BLS II' according to Hochkogler et al.[26]

2.2. Human Intervention Study II

This blinded, randomized, controlled, cross-over trial was performed in accordance with good clinical practice guidelines and the Declaration of Helsinki and approved by the Ethics Committee of the University of Vienna (no. UNIVIE00097). The volunteers provided written informed consent after receiving a detailed oral and written description of the study.

In total, 27 metabolically healthy, normal-weight (BMI between 18.5 and 25.0 kg/m²) sensorially untrained volunteers

Table 1. Characteristics of the study participants of study II.

	Female ($n = 11$)	Male ($n = 6$)
Age [y]	26.1 ± 3.70	25.4 ± 5.68
BMI [kg/m^2]	21.4 ± 2.22	21.9 ± 2.36
Fasting glucose [mg/dL]	74.3 ± 9.69	76.9 ± 10.1
Creatinine [mg/dL]	0.79 ± 0.11	0.93 ± 0.14
SGOT [U/L]	22.5 ± 7.27	26.0 ± 4.90
SGPT [U/L]	23.0 ± 8.65	26.0 ± 9.54
ALP [U/L]	68.2 ± 24.7	80.4 ± 34.4
GGT [U/L]	13.1 ± 4.61	18.9 ± 7.97
Cholesterol [mg/dL]	153 ± 30.9	162 ± 35.5
LDL [mg/dL]	77.3 ± 28.4	93.0 ± 27.5
HDL [mg/dL]	65.1 ± 5.58	52.6 ± 11.0
Chol/HDL	2.37 ± 0.49	3.17 ± 0.98
TG [mg/dL]	55.0 ± 23.2	82.0 ± 51.5

between 20 and 45 years, without reported tobacco consumption, alcohol or drug abuse were recruited via notices at blackboards at the University and via social media in March 2015. All subjects underwent a medical screening (hemogram, transaminases aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and gamma glutamyl transpeptidase as well as the blood lipids triglycerides, total, HDL and LDL cholesterol, creatinine, thyroid-stimulating hormone, and fasting and 1 and 2 h blood glucose) prior to their enrollment, in order to assess their eligibility. Thus, in total three venous blood samples were drawn. An oral glucose tolerance test (oGTT) was performed to ascertain the absence of metabolic diseases. Moreover, body weight was assessed by a digital scale in light clothes to the nearest of 0.1 kg. Body height was determined by a stadiometer, and blood pressure was measured on the upper left arm. The medical screening revealed 24 volunteers being eligible for the study. Due to personal reasons and some irregularities in blood drawings, seven participants dropped out. Plasma analyses for the medical screening were carried out by a commercial blood laboratory (Blutlabor Dr. Greiner, Wagramerstraße 122, 1220 Vienna, Austria). Baseline characteristics of the volunteers are shown in **Table 1**.

The study protocol included two visits, each separated by 7 days. Participants were asked to come to the study center at 8 a.m. after an overnight fast. After randomization, volunteers received either a glucose solution solely or a glucose solution supplemented with 30 mg NaHED. For the glucose+NaHED intervention, 75 g of glucose were dissolved in water, corresponding to a performance of a standard oral glucose tolerance test (oGTT). Since NaHED has a slight yellow color, bottles were wrapped in dark paper for blinding. To assess the subjects' feeling of hunger, participants were asked to mark their momentary hunger in a visual analog scale, as described above, before and 120 min after administration of the glucose solution. Two hours after administration of the glucose solution, a standardized breakfast as described above was served to evaluate the short-term effect of NaHED on food intake.

2.3. Blood Sample Collection and Quantitation of Satiety Hormones

In *Study I*, venous blood samples were drawn at fasting and about 30 min after administration of NaHED in EDTA coated tubes (Sarstedt, Numbrecht, Germany) for the determination of ghrelin, GLP-1, PYY and serotonin. In *Study II*, venous blood samples were drawn by means of a venous catheter at fasting and 15, 30, 60, 90, 120 min after consumption of the glucose solution in either EDTA coated tubes for determination of ghrelin, GLP-1, PYY and serotonin, or EDTA- and fluoride coated tubes for determination of glucose concentrations. Samples were centrifuged (1800 xg at 4 °C for 15 min) immediately for the preparation of plasma. Another centrifugation step (7000 × *g* at 4°C for 1 min) was necessary for those plasma samples, which were used for determination of serotonin concentrations in order to secure platelet-poor plasma.

Blood samples for the ghrelin assay were treated with AEBSF (4-(2-aminoethyl) benzene sulfonyl fluoride, Merck Millipore, Darmstadt, Germany) prior centrifugation. For stabilization, plasma samples were acidified with hydrochloric acid to a final concentration of 0.05 mol/L.

For determination of PYY concentrations, AEBSF as well as a dipeptidyl peptidase protease inhibitor (DPP IV, Merck Millipore, Darmstadt, Germany) were added to the whole blood sample prior to centrifugation.

A commercially available, colorimetric assay (Cayman Europe, Tallinn, Estonia) was used for the determination of plasma glucose concentrations. Total ghrelin, PYY and GLP-1 concentrations were quantified by means of sandwich ELISAs purchased from Merck Millipore, Darmstadt, Germany. Serotonin concentrations were assessed by a competitive ELISA (DLD Diagnostika, Hamburg, Germany).

All assays were carried out following the instructions written in the manufacturer's protocols.

2.4. Quantitation of NaHED in Plasma by LC-MS

In seven volunteers participating in *Study I*, NaHED plasma concentrations were quantified by means of LC-MS 30 min after administration of 30 mg NaHED dissolved in 125 mL tap water.

Quantitation of HED was performed by using eriodictyol (Carl Roth, HPLC purity >95%, Karlsruhe, Germany) which was added 400 μL of each plasma sample in an amount of 1.44 μg. Protein precipitation was induced by 400 μL formic acid (10%, v/v). The supernatant was loaded on a C18 SPE column (StrataX 30 mg, Phenomenex, Aschaffenburg, Germany), which had been preconditioned with 1 mL methanol and 1 mL water. After a washing step with 1 mL aqueous methanol (5%, v/v), HED was eluted twice with 500 μL methanol acidified with 2% formic acid. The eluted fraction was dried with nitrogen and reconstituted in 250 μL methanol. Prior to LC-MS analysis, the samples were passed through a 0.2 μm polyvinylidene fluoride membrane filter, and injected on a C18 column (Symmetry, 250 × 4.6 mm, 5 μm, Waters, Vienna, Austria) as part of an LC-MS system (LCMS-2020, Shimadzu, Korneuburg, Austria). The samples were chromatographically separated at 25°C using a flow rate of 1 mL/min, and a gradient starting with 30% methanol/70%

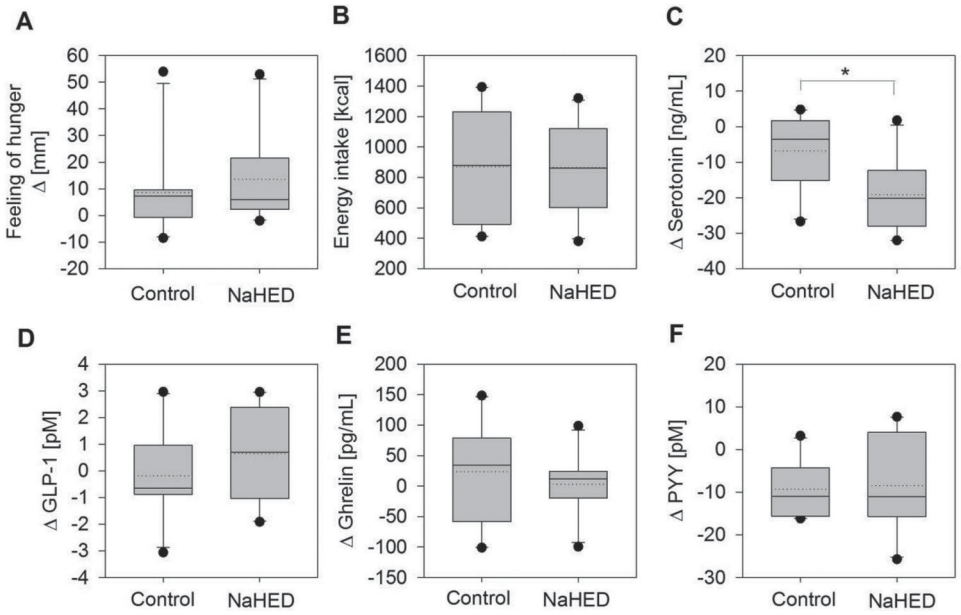

Figure 1. Administration of NaHED alone did not change energy intake but reduced plasma serotonin concentrations. Results of study I presenting the mean changes after administration of 30 mg homoeriodyctiol sodium salt (NaHED) after a time period of ~30 min of (A) feeling of hunger, (B) energy intake, blood plasma (C) serotonin, (D) GLP-1, (E) ghrelin and (F) PYY. Data presented as box plot, means are shown as dotted lines, $n = 10$. Statistic: Student's *t*-test. $^{*}p < 0.05$.

water and ending with 80% methanol/20% water after 30 min. The mobile phases were acidified with 0.1% (v/v) formic acid. The interface, desolvation line and heat block temperature of the mass spectrometer were set to 350, 100, and 100°C, respectively. The nebulizing and drying nitrogen gas flow were adjusted to 1.5 and 10 L/min, respectively. HED and eriodictyol were monitored in the SIM mode as [M-H]- ions at m/z 301 and 287, respectively. The recovery was calculated to be 97.4 ± 17.3%. The LOD was determined by a signal-to-noise ratio of 3 to be 0.36 ± 0.02 nM.

2.5. Statistics

Statistical analyses were performed using SigmaPlot 11.0 (Systat Software GmbH). Statistically significant differences were determined on the basis of a power of 80% and a niveau of 5%. Normality of the data was assessed by performing the Shapiro-Wilk test.

Differences between the two treatments (control versus NaHED) for the mean delta values (tx - t0) were calculated by a two-way repeated measures ANOVA for time points and treatment. A Student-Newman-Keuls test was performed as a post hoc test.

Total area under curve (AUC) and delta AUC (ΔAUC) were calculated over time for plasma hormones, and tested within and between the groups for statistical significance ($p < 0.05$) by conducting a one-sample/two-sample Student's *t*-test or, in case of non-normally distributed data, the Wilcoxon Signed rank test was performed.

Pearson correlation analyses were performed to determine the correlation between ad libitum energy intake from breakfast and the change of satiety hormone concentrations after administration of glucose solution calculated as ΔAUC.

3. Results

The effects of a bolus administration of NaHED on food intake and hormones involved in mechanisms regulating satiety were investigated in two separate studies. In study I, we examined whether administration of NaHED alone unfolds appetite-enhancing effects, whereas in study II, we investigated the effects in the presence of a standardized caloric load by conducting an oral glucose tolerance test.

3.1. Study I

In study I, in which NaHED was administered without a caloric load, no effect of 30 mg NaHED on the feeling of hunger, food intake or the plasma levels of PYY, GLP-1 or ghrelin have been detected in ten healthy subjects (**Figure 1**A, B, D, E and F).

However, administration of 30 mg NaHED reduced plasma serotonin concentrations by 19.2 ± 3.34 ng/mL, corresponding to a mean decrease of 38.2 ± 13.1 % ($p < 0.001$). Comparison of the delta serotonin levels after administration of NaHED versus water revealed a significant reduction ($p < 0.05$) as well (Figure 1C). In contrast, administration of 125 mL water as control did not significantly reduce absolute serotonin levels (- 6.81 ± 3.36 ng/mL, $p > 0.05$).

3.2. Plasma Concentrations of HED

Administration of 30 mg NaHED dissolved in 125 mL tap water resulted in a mean plasma concentration of 38.9 ± 22.1 nM HED,

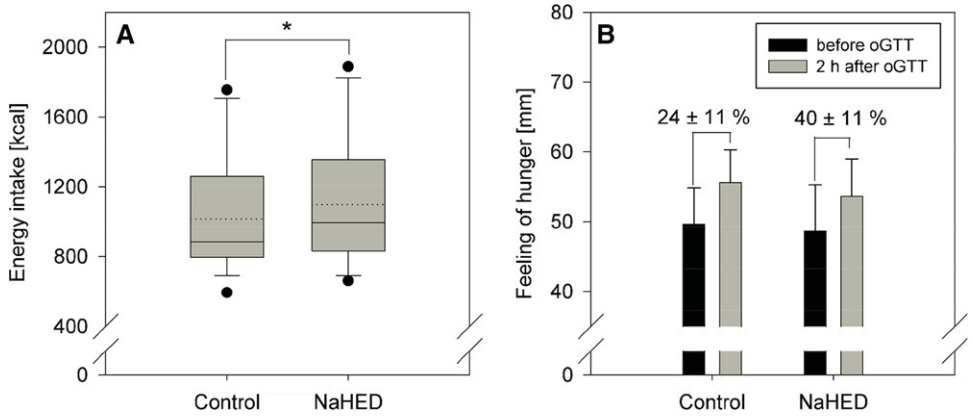

Figure 2. Administration of a glucose solution supplemented with NaHED enhanced food intake. Total ad libitum energy intake from a standardized breakfast (A) served 2 h after administration of glucose solution solely (Control) and supplemented with 30 mg NaHED and mean values in the feeling of hunger (B) determined by a 100 mm visual analogue scale (VAS) before and 2 h after oGTT ($n = 17$). Statistical difference ($p < 0.05$) between the two treatments was assessed by a one-sample student's t-test. Means are shown as dotted lines. Values for hunger feeling are shown as mean ± SEM.

Table 2. Mean macronutrient intake of healthy volunteers from a standardized breakfast 2 h after administration of 75 g glucose without (Control) or with supplementation of 30 mg NaHED.

	Control ($n = 17$)	NaHED ($n = 17$)	% change	*p*-value
Fat [g]	46.3 ± 5.04	46.9 ± 4.92	2.87 ± 5.80	0.15
Proteins [g]	37.8 ± 3.62	39.9 ± 3.79	7.08 ± 7.97	0.03
Carbohydrates [g]	104 ± 11.7	113 ± 5.56	7.55 ± 5.98	0.07

Values are shown as mean ± SD. Statistical difference ($p < 0.05$) between control and NaHED treatment was determined by means of a paired Student's *t*-test.

which was determined 30 min post load by means of LC-MS in blood plasma in 7 healthy subjects.

3.3. Study II

3.3.1. Ad libitum Energy Intake and Subjective Hunger Feeling

Ad libitum energy intake from the standardized breakfast (**Figure 2**A) consumed 2 h after the performance of the oGTT increased by 9.52 ± 4.6% from 1016 ± 83.7 kcal to 1099 ± 90.6 kcal ($p < 0.05$) after oral administration of 30 mg NaHED in comparison to the intervention with glucose solely (control). This increase was largely attributed to protein intake since it increased by 7.08 ± 7.98 % (**Table 2**). Fat and carbohydrate intake remained unchanged between the treatments.

Subjective hunger feeling was assessed at fasting and 2 h after administration of the glucose solution by means of a visual analog scale ranging from 0 to 100 mm. However, hunger feeling did not change significantly (Figure 2B).

3.4. Changes in Plasma Glucose Concentrations

Glucose concentrations were assessed only in *Study II* in order to investigate the effects of consumption of 75 glucose with or without NaHED. Administration of the glucose solution supplemented with NaHED enhanced glucose levels at t_{90} by 7.63 ± 3.77 % ($p < 0.05$), delta values changed from −2.67 ± 4.06 mg/dL to 6.51 ± 6.07 mg/dL in comparison to the fasting levels. Neither calculated AUC nor delta AUC differed between the treatments (data not shown).

3.5. Changes in Plasma Ghrelin Concentrations

Results of the changes of the plasma ghrelin levels after administration of the glucose solution in comparison to the fasting concentrations are presented in **Figure 3**. The two-way ANOVA revealed a less pronounced decrease of plasma ghrelin after consumption of 30 mg of NaHED at the time points t_{30}, t_{60}, t_{90} and t_{120} compared to glucose solution solely. Calculated ΔAUC values showed a statistical trend ($p = 0.09$) to be higher after administration of 30 mg NaHED when added to the glucose solution (control −30,143 ± 4587, NaHED −20,219 ± 3185). Mean delta values of plasma ghrelin concentrations were positively correlated with ad libitum energy intake from breakfast ($r = 0.42$, $p < 0.01$).

3.6. Changes in Plasma PYY and GLP-1 Concentrations

Postprandial plasma PYY and GLP-1 responses after administration of the glucose solution w/o NaHED did not differ between the treatments at any of the time points (**Figure 4**A and B).

3.7. Changes in Plasma Serotonin Concentrations

Plasma serotonin release was decreased 30, 90, and 120 min after administration of the glucose solution supplemented with NaHED in comparison to glucose solution solely. Delta AUC values were reduced from 219 ± 139 for the control treatment to −186 ± 78.4 after NaHED intervention (**Figure 5**A). A negative correlation was calculated (Figure 5B) between ad libitum energy

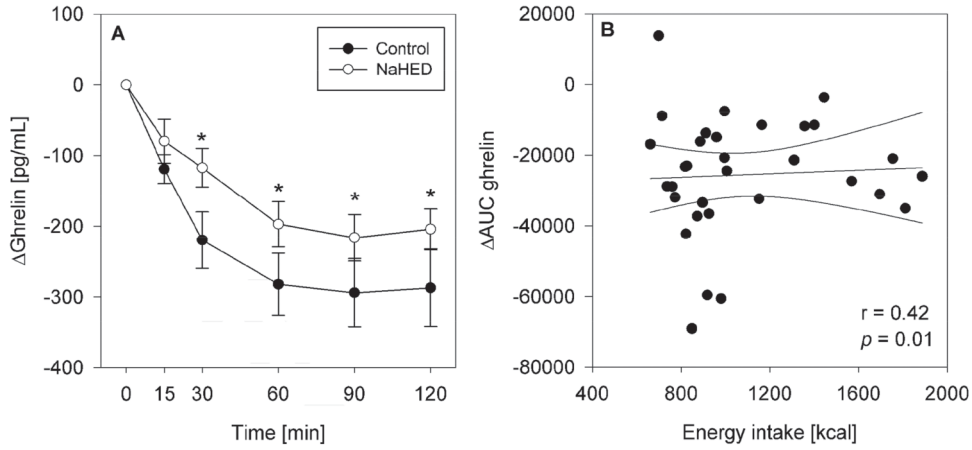

Figure 3. Administration of a glucose solution supplemented with NaHED increased plasma concentration of ghrelin. Mean changes in plasma ghrelin concentrations (A) 15, 30, 60, 90, and 120 min after administration of 75 g glucose without (Control) and with supplementation of 30 mg NaHED (NaHED) ($n = 17$) and correlation between delta AUC ghrelin and total energy intake (B). Values are shown as mean ± SEM. Statistical difference ($p < 0.05$) was determined by two-way repeated measures ANOVA for time and treatment. Pearson correlation coefficient was computed from the delta AUC for plasma ghrelin concentrations and between total energy intake ($n = 17$). Correlation were considered significant at $p < 0.05$.

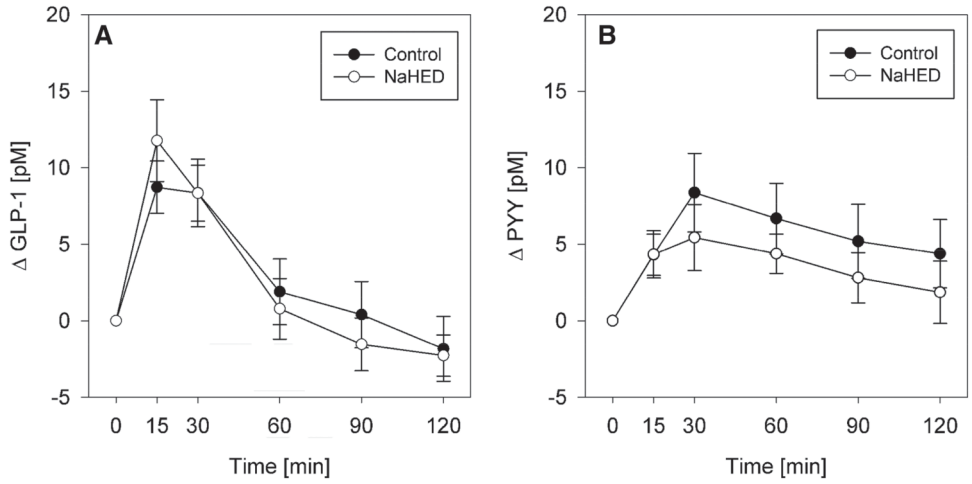

Figure 4. Administration of a glucose solution supplemented with NaHED did not change plasma concentrations of GLP-1 and PYY. Mean changes in plasma GLP (A) and PYY (B) concentrations 15, 30, 60, 90, and 120 min after administration of 75 g glucose without (Control) and with supplementation of 30 mg NaHED (NaHED) ($n = 17$). Statistical difference ($p < 0.05$) was determined by two-way repeated measures ANOVA for time and treatment.

intake from the standardized breakfast and delta AUC values of plasma serotonin levels ($r = -0.37$, $p = 0.04$).

4. Discussion

In two human intervention studies, we studied the short-term effects of a bolus administration of 30 mg NaHED when administered alone or concomitantly with 75 g glucose on ad libitum food intake and satiety-associated plasma hormones. The main results of this study suggest that bolus administration of the flavanone HED enhances appetite, as indicated by an increased ad libitum energy intake from breakfast which was accompanied by elevated plasma ghrelin when consumed in combination with a caloric load. Moreover, plasma serotonin concentrations decreased after HED administration, independent of the caloric load.

Ghrelin is mainly produced in the oxyntic glands of the gastric fundus.[9] It became popular for its orexigenic effects since its plasma concentrations were shown to decrease postprandially and to increase with a rising feeling of hunger.[27] Recent evidence suggests that the two forms in which ghrelin occurs in the plasma show similar effects, as acyl-ghrelin and non-acylated ghrelin exerted similar changes in response to meals in fasted subjects.[28] Here, we investigated the changes in plasma concentrations of total ghrelin. In *Study I*, administration of 30 mg NaHED without a caloric load had no effect on ghrelin release. This finding is in accordance with another study conducted in healthy volunteers, showing that filling of the stomach due to the consumption of 225 mL of water did not change ghrelin concentrations.[29] However, a less pronounced postprandial decrease in plasma ghrelin concentrations (calculated delta AUC for the control treatment −30,143 ± 4587, for NaHED treatment −20,219 ± 3185, corresponding to an increase by 8.82 ± 14.1 %) was determined after

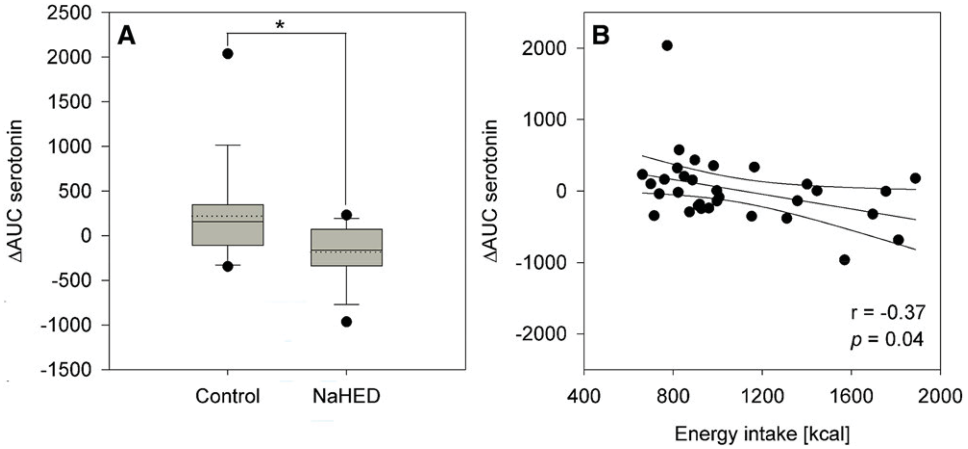

Figure 5. Administration of a glucose solution supplemented with NaHED decreased ΔAUC of plasma serotonin. Calculated delta AUC values for plasma serotonin concentrations (A) and correlation between delta AUC serotonin and total energy intake (B). To determine the difference between delta AUC the Wilcoxon signed rank test was performed. Statistical significant difference ($p < 0.01$) is indicated as "**". Means are shown as dotted lines. Pearson correlation coefficient was computed from the delta AUC for plasma serotonin concentrations and between total energy intake ($n = 16$). Correlation were considered significant at $p < 0.05$.

concomitant administration of 30 mg NaHED and 75 glucose, indicating an orexigenic effect of HED.

The performance of an oral glucose tolerance test has been evaluated as a suitable tool to investigate the ghrelin secretion in humans.[26,29] Our data indicate that HED exerts its appetizing effects only when it is administered in combination with a caloric load. We also hypothesize that increased ad libitum energy intake from the standardized breakfast might at least partly be attributed to changes of plasma ghrelin, since an inverse correlation for both outcome measures was calculated.

The enteroendocrine anorexigenic hormones $PYY_{3\text{-}36}$ and GLP-1 are both secreted by intestinal L-cells. Plasma concentrations of $PYY_{3\text{-}36}$ as well as GLP-1 did not change after administration of NaHED, neither in combination with water nor with glucose load. However, postprandial release of GLP-1 was assessed after the consumption of glucose solution, reaching peak plasma concentrations at the time point t_{15}, whereas PYY reached its peak concentrations after 30 min, similar to data as reported earlier.[30]

In vitro experiments revealed that incubation with NaHED in different concentrations increased the sodium-coupled glucose transporter SGLT-1-mediated glucose uptake in fully differentiated human intestinal Caco-2 cells.[24] Since the here presented data show that postprandial plasma glucose levels were increased by 7.63 ± 3.77 % after administration of NaHED 90 min after the glucose load, our results point to a potential impact on glucose metabolism, which has to be verified in future studies.

Besides the well-known satiating effects of central serotonin in the brain, mainly mediated by the receptors 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C}, the knowledge about the involvement of peripheral serotonin in mechanisms regulating satiety is sparse.[31] The majority (about 95%) of total body's serotonin is stored in enterochromaffin cells in the intestines, and is released after food intake to affect intestinal motility.[32] In the last years, evidence emerged that peripheral serotonin also plays a role in energy balance regulation, although it is discussed controversially whether peripheral plasma exhibits orexigenic or anorexigenic effects.[7,33] However, it has been shown that peripherally injected serotonin induces hypophagia in rats.[34]

We demonstrate here that a bolus administration of 30 mg NaHED dissolved in water decreases plasma serotonin levels by 39.6 ± 17.4 %. When consumed in combination with a glucose load, a decrease in the delta AUC values of plasma serotonin by 381 ± 143 % in comparison to control treatment was detected. We hypothesize that not only central but also peripheral serotonin might induce satiation signals, since an inverse correlation was calculated for plasma serotonin and energy intake from the standardized breakfast.

To our knowledge, the here presented study shows for the first time that a bolus administration of a polyphenol in an amount of 30 mg exerts appetizing effects, as shown by an increase of the ad libitum energy intake from a standardized breakfast by 9.52 ± 4.6 % when administered concomitantly with a caloric load. Since the subjects' feeling of hunger, determined by a VAS, did not differ between the two treatments, we hypothesize that enhanced appetite contributed to the observed increase in food intake. Appetizing agents may be used as a treatment for underweight in older adults, since about 25–30% of the elderly population is suffering from underweight, a condition which is called 'anorexia of aging'.[35] Although it is still unclear if the decreased energy intake is only a consequence or the cause of this problem, in a recent study, it has been shown that an increase of 137 kcal of the daily energy consumption for a period of two months increased body weight by 1.6% in institutionalized older adults.[36] Thus, the observed mean increase of the energy intake of 83.2 ± 36.0 kcal from the standardized breakfast may contribute to weight gain in the long term.

Nutrient recommendations for elderly people (> 65 years) regarding the daily protein intake have been revised recently since a decreased protein intake is associated with a higher risk for frailty and sarcopenia.[37] Accordingly, the older population should consume 1.0 to 1.2 g proteins per kg of body weight daily, instead of 0.8 g proteins per kg of body weight recommended for adults.[37] In this study, administration of a glucose solution supplemented

with 30 mg NaHED has been shown to enhance mainly the protein intake from a standardized breakfast, which might make HED a promising natural compound to counteract protein malnutrition. Moreover, protein hydrolysates are often used in elderly nutrition in order to meet nutrient recommendations, to support good health, and to prevent age-related muscle mass loss. Furthermore, in comparison to proteins, protein hydrolysates are more easily digested and absorbed faster,[38] whereas their dietary intake is largely limited due to its bitter-tasting attributes.[28] In this context, it is conceivable that HED may not only act as an appetizing agent, but might also be beneficial as an additive in bitter-tasting proteins[39] since it has been shown to have bitter-masking properties, at least in aqueous solutions.[22]

Our studies were conducted as 'proof-of-concept' trials in order to provide initial information about the effects of a bolus administration of 30 mg NaHED on appetite and energy intake in healthy adults (age between 20 and 45 years). Studies with compromised elderly subjects are warranted to confirm the observed appetizing effect of HED when administered as bolus dose. Furthermore, long-term human intervention studies with vulnerable groups are essential in order to elucidate whether the observed short-term effects sustain and contribute to weight gain. Moreover, the relevance of lemon fruits might be further investigated in depth, since they contain the flavonoid glycoside eriocitrin which can be metabolized into HED through Phase II enzyme activity.[40]

5. Conclusion

Polyphenols have been widely studied for their anti-obesity mode of action. Here, we provide original evidence that phenolic compounds may act also as appetite-inducing agents: When ingested concomitantly with a caloric load of 75 g glucose, bolus administration of NaHED enhanced food intake from a standardized breakfast, increased plasma total ghrelin concentrations and increased peripheral serotonin levels in normal weight subjects. Bitter masking compounds like HED are commercially added to pharmaceuticals or non-caloric, bitter-tasting foods like tea, but not used in high-caloric foods and beverages, like, e.g. soft drinks, since these products do not warrant bitter masking. That said, the use of HED as an appetizing compound would be limited to targeted applications. However, it has to be elucidated in long-term intervention studies whether HED is a promising botanical that may help to combat underweight in the elderly population.

Abbreviations

5-HT, serotonin; GLP-1, glucagon-like-peptide1; NaHED, homoeriodyctiol sodium salt; oGTT, oral glucose tolerance test; PYY, peptide YY (3-36); VAS, visual analog scale

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Conflict of interest

The authors J. Hans, S. Widder, J.P. Ley, and G.E. Krammer are employees at Symrise AG, Holzminden, Germany which, holds IP on homoeriodictyol, its derivatives and its use.

Keywords

appetizers, food intake, homoeriodictyol, peripheral serotonin, plasma ghrelin

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2.3 "Human sweet receptor TAS1R3 is functional in human gastric parietal tumor cells (HGT-1) and modulates cyclamate and acesulfame K-induced mechanisms of gastric acid secretion."

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Human Sweet Receptor T1R3 is Functional in Human Gastric Parietal Tumor Cells (HGT-1) and Modulates Cyclamate and Acesulfame K-Induced Mechanisms of Gastric Acid Secretion

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Supporting Information

ABSTRACT: The noncaloric sweeteners (NCSs) cyclamate (Cycl) and acesulfame K (AceK) are widely added to foods and beverages. Little is known about their impact on gastric acid secretion (GAS), which is stimulated by dietary protein and bitter-tasting compounds. Since Cycl and AceK have a bitter off taste in addition to their sweet taste, we hypothesized they modulate mechanisms of GAS in human gastric parietal cells (HGT-1). HGT-1 cells were exposed to sweet tastants (50 mM of glucose, D-threonine, Cycl, or AceK) and analyzed for their intracellular pH index (IPX), as an indicator of proton secretion by means of a pH-sensitive dye, and for mRNA levels of GAS-associated genes by RT-qPCR. Since the NCSs act via the sweet taste-sensing receptor T1R2/T1R3, mRNA expression of the corresponding genes was analyzed in addition to immunocytochemical localization of the T1R2 and T1R3 receptor proteins. Exposure of HGT-1 cells to AceK or D-threonine increased the IPX to 0.60 ± 0.05 and 0.80 ± 0.04 ($P \leq 0.05$), respectively, thereby indicating a reduced secretion of protons, whereas Cycl demonstrated the opposite effect with IPX values of −0.69 ± 0.08 ($P \leq 0.05$) compared to controls (IPX = 0). Cotreatment with the T1R3-inhibitor lactisole as well as a *TAS1R3* siRNA knock-down approach reduced the impact of Cycl, AceK, and D-thr on proton release ($P \leq 0.05$), whereas cotreatment with 10 mM glucose enhanced the NCS-induced effect ($P \leq 0.05$). Overall, we demonstrated Cycl and AceK as modulators of proton secretion in HGT-1 cells and identified T1R3 as a key element in this response.

KEYWORDS: *cyclamate, acesulfame K, gastric acid secretion, sweet taste receptor, K_{ATP}-channels, HGT-1 cells*

INTRODUCTION

Noncaloric sweeteners (NCSs) are widely used as sugar substitutes to increase the sweetness of foods and beverages without contributing to their caloric load. NCSs are known agonists of the human sweet taste receptor (T1R2/T1R3), which consists of the subunits T1R2 and T1R3 that respond to a variety of compounds, including natural sugars, sweet-tasting D-amino acids, and artificial sweeteners, such as cyclamate (Cycl) and acesulfame K (AceK).[1,2] Cycl and AceK are classified as high-intensity sweeteners since their sweet taste is 30 and 200 times more intense than that of sucrose, respectively, whereas glucose evokes a 30% less intense sweet sensation compared to sucrose (see review by Edwards et al., 2016).[3] Lactisole, a sweet taste receptor antagonist, inhibits sweet taste by acting on T1R3.[4] Previous studies have been aimed at identifying the yet not fully elucidated binding sites for sweet tastants on the sweet taste receptor. On one hand, it has been reported that Cycl and AceK, e.g., act on the heteromeric T1R2/T1R3.[2,5] On the other hand, it has been hypothesized that the homodimeric T1R3 receptor may respond to sweet-tasting compounds in various cell lines[6−8] and in humans.[9]

Apart from their molecular mechanism of action, the health effects of NCSs beyond their impact on sweet taste perception are also still controversial.[10] One concern is that regular intake of NCSs may stimulate food intake through their impact on the release of orexigenic and anorexigenic peptides in the gastrointestinal tract,[11] thereby contributing to the global increase of obesity and associated degenerative diseases.[10]

From a physiological perspective, the gastrointestinal tract and the central nervous system play a pivotal role in the control of energy homeostasis, not only by secreting hormones regulating the complex cascade of hunger and satiety[12] but also by regulating gastric motility and gastric acid secretion (GAS).[13] Hypersecretion of gastric acid may cause gastric discomfort accompanied by symptoms like epigastric pain, reflux, or heartburn[16] and may result in accelerated gastric emptying which counteracts satiation due to reduced gastric distention.[17] Satiating foods,

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in particular dietary proteins and amino acids,[14] but also endogenous satiating compounds, such as the sugar acid 2-buten-4-olide,[15] have been shown to suppress GAS via reduction of activity of the vagus nerve and gastric-related hypothalamic neurons. However, the effects of NCSs on mechanisms regulating gastric acid release still have to be elucidated.

One cell model to study the mechanisms of GAS *in vitro* is the HGT-1 cell line, established from the primary tumor of a 60-year-old patient[18] and known to express the principal transport proteins facilitating GAS.[19] Recently, HGT-1 cells have been verified as a suitable model for the identification of bitter and bitter-modulating compounds,[20] showing a high correlation between the proton secretory potential of a bitter compound and its sensory bitter perception,[20] whereas the associations between sweet-tasting substances and proton release have not been studied. Since some NCSs show a bitter off taste, we hypothesized that NCSs modulate mechanisms of GAS in HGT-1 cells and aimed at elucidating whether the sweet taste sensing receptor proteins (T1R2/T1R3) are involved in this response. Following this hypothesis, we investigated the proton secretory potentials of NCS Cycl, AceK, and the sweet-tasting amino acid D-thr in HGT-1 cells and studied the impact of the T1R3 antagonist lactisole and a *TAS1R3*-siRNA knock down. Moreover, involvement of cAMP and glucose in the regulation of NCS-stimulated proton secretion as well as the regulation of gastric acid associated genes, the H^+/K^+-ATPase alpha-subunit (*ATP4A*), the histamine H2 receptor (*HRH2*), the somatostatin receptor (*SSTR2*), the acetylcholine receptor M3 (*CHRM3*), and the identified bitter taste receptors of impact *TAS2R1*, *TAS2R38*, *TAS2R31*, and *TAS2R43*[21] were also studied.

MATERIALS AND METHODS

Chemicals. All the chemicals, including sodium cyclamate, acesulfame potassium, D-threonine, and histamine, were purchased from Sigma-Aldrich (Austria), unless stated otherwise. GIV3727 and lactisole (99%) were provided by Symrise (Germany). Substances insoluble in water were dissolved in 0.1% (v/v) DMSO.

Cell Culture. The human gastric cells HGT-1 (passage number 56) were obtained from Dr. C. Laboisse (Laboratory of Pathological Anatomy, Nantes, France) and were cultured in Dulbecco's Modified Eagle Medium (DMEM), containing 4 g/L of glucose, supplemented with 10% fetal bovine serum, 2% L-glutamine, and 1% penicillin/streptomycin and cultivated applying standard conditions of 37 °C temperature in a humidified atmosphere of 95% air with 5% CO_2, as described previously.[22]

Cell Viability. Analysis of cytotoxic effects of the selected compounds was performed by staining the cells with 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reagent, as described earlier.[22] Briefly, cells were seeded in a 96-well culture plate 24 h before use. Cells were treated with the test compounds dissolved in DMEM for up to 60 min. Absorbance was measured at 550 nm and the reference wavelength at 690 nm by means of an Infinite 200 Pro Plate Reader (Infinite M200 Plate Reader, Tecan, Switzerland). As a consequence, the cell viability was determined in relation to the medium-only treated control cells (untreated controls = 100%). Only the treatments exceeding 90% viability in comparison to untreated control were used.

Intracellular pH Measurement in HGT-1 Cells. The intracellular pH in HGT-1 cells was quantitated as proxy for proton secretion using the fluorescent pH indicator 1,5-carboxy-seminaphtorhodafluor acetoxymethyl ester SNARF-1-AM (SNARF, Invitrogen, Austria).[20−26] This assay in HGT-1 cells has been described previously.[21−24] Briefly, 1×10^5 HGT-1 cells were seeded in a black 96-well plate. After 24 h, cells were washed once with Krebs−Ringer-HEPES−buffer (KRHB; 10 mM HEPES, 4.7 mM KCl, 130 mM NaCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 1.2 mM KH_2PO_4, adjusted to a pH of 7.4 with 5 M KOH) and stained with 3 μM SNARF-1 in KRHB for 35 min under standard conditions. Later cells were treated for 10 min with test substances dissolved in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g L^{-1} of glucose. For the experiments investigating the effect of glucose, substances were dissolved in non-nutritive DMEM. Histamine (1 mM) was used as a positive control. Control cells were treated with DMEM only. Additionally, DMEM containing 0.1% DMSO was used as solvent control. Fluorescence was measured at 580 and 640 nm emission after excitation at 488 nm by means of an Infinite 200 Pro plate reader. After analyzing the intracellular pH values using a calibration curve, the concentration of intracellular H^+ was calculated. The ratio between treated samples and control cells (DMEM only or DMEM containing 0.1% DMSO) was calculated. After $\log_2$ conversion, the intracellular proton index (IPX) was calculated. The lower the IPX values, the more H^+ ions have been secreted by the HGT-1 cells.[20−24]

RNA Isolation, cDNA Synthesis, and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). HGT-1 cells, at a density of 7×10^5, were seeded in 6-well culture plates 24 h before the experiment. Cells were washed once with phosphate-buffered saline (PBS) and treated with 50 mM Cycl, AceK, D-thr, glucose, and 10 μM forskolin or 0.1% DMSO (control) for 10, 20, or 60 min under the standard conditions described above. After washing with ice-cold PBS on ice, cells were lysed, and total RNA content was collected using the peqGOLD Total RNA Isolation Kit (Peqlab) following the manufacturer's protocol. RNA quantity and quality were spectrophotometrically analyzed at 260 and 280 nm, followed by calculating the ratio of the two wavelengths using the NanoQuant Plate by means of the Infinite 200 PRO Plate Reader. Isolated RNA samples showed a ratio ranging between 1.8 and 2.2. A total of 2 μg of RNA was used for transcription into cDNA by applying the High Capacity cDNA Kit (Applied Biosystems, Austria) according to the manufacturer's protocol. Real-time PCR was performed with 100 ng of cDNA amplified with Fast SYBR Green Master Mix (Applied Biosystems, Austria). The sequences of the reverse and forward primers are shown in Table 1. Primer pairs for human *TAS1R1* (Assay ID: qHsaCID0013443), human *TAS1R2* (Assay ID: qHsaCID0016106), and human *TAS1R3* (qHsaCED0002321) were purchased from BIO-RAD (Austria). The remaining sequences were designed with NCBI Primer Blast, and the following oligonucleotide primers for human *SUR1*, *Kv11.1*, and *SLC2A1* (encoding GLUT1) were purchased from Sigma: for human *SUR1*, 5′-GCTGTCCAAAGGCACCTACT-3′ (forward) and 5′-TGAATGTCCTTCCGCACCTG-3′ (reverse); for human *Kv11.1*, 5′-CACCTTCCTGGACACCATCA-3′ (forward) and 5′-AAGCCGTCGTTGCAGTAGAT-3′ (reverse); for human *SLC2A1*, 5′-ATTGGCTCCGGTATCGTCAAC-3′ (forward) and 5′-GCTCAGATAGGACATCCAGGGTA-3′ (reverse). Analyses were carried out in triplicate by means of the StepOnePlus Real-Time PCR System (Applied Biosystems, Austria). The measured mRNAs (N_0 values) and efficiencies were calculated for each reaction setup with LinRegPCR v.12.8. Results of target genes were normalized to the geometric mean of two internal standard genes TATA-box binding protein (*TBT*) and peptidylprolyl isomerase A (*PPIA*).[22] The effects of treatments on gene expression were analyzed in comparison to non-treated control cells. Cycling conditions in the system were: 20 s/93 °C (activation), 3 s/95 °C (denaturation), 30 s/60 °C (annealing), and 15 s/72 °C (elongation).

Immunocytochemical Staining of HEK-293T-Gα16gust44 and HGT-1 Cells. Transient transfection of HEK-293T-Gα16gust44 cells was conducted as described before.[27] Briefly, HEK-293T-Gα16gust44 cells were seeded onto glass coverslips in 24-well plates 24 h before transiently transfecting the cells by using Lipofectamine 2000 (Invitrogen, Germany) with cDNA coding for human sweet receptor subunits *TAS1R2* and *TAS1R3*. HGT-1 cells were seeded onto coverslips 24 h before the immunocytochemical detection. Cells were fixed with methanol:acetone (1:1, v/v) and stained as described previously,[27] using antibodies against the C-terminal epitope tags (anti-FLAG antiserum for T1R2 (1:2000) and anti-HSV antiserum for T1R3 (1:15000) (Novagen, Germany)), goat anti-T1R2 (1:100), and rabbit anti-T1R3 (1:100) (Santa Cruz Biotechnology, USA). Detection was performed as reported previously,[27] and preabsorption of the anti-T1R2 antibody with the specific blocking peptide (1:100)

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Table 1. Sequences of All Primer Pairs Used in the RT-qPCR Experiments[21,23,24a]

target	amplicon size	5′ → 3′ sequence	target	amplicon size	5′ → 3′ sequence
PPIA	144	F:CCACCAGATCATTCCTTCTGTAGC	*TAS2R1*	172	F:AAATGGCTCCGCTGGATCTC
		R:CTGCAATCCAGCTAGGCATGG			R:GTGGCAAGCCAAAGTTCCAA
TBT	130	F:CCCGAAACGCCGAATATAATCC	*TAS2R38*	216	F:CCCAGCCTGGAGGCCCACATT
		R:GACTGTTCTTCACTCTTGGCTC			R:TCACAGCTCTCCTCAACTTGGCA
ATP4A	176	F:CGGCCAGGAGTGGACATTCG	*TAS2R31*	218	F:TTGAGGAGTGCAGTGTACCTTTC
		R:ACACGATGGCGATCACCAGG			R:ACGGCACATAACAAGAGGAAAA
CHRM3	117	F:AGCAGCAGTGACAGTTGGAAC	*TAS2R43*	148	F:ATATCTGGGCAGTGATCAACC
		R:CTTGAGCACGATGGAGTAGATGG			R:CCCAACAACATCACCAGAATGAC
HRH2	154	F:TGGGAGCAGAGAAGAAGCAACC			
		R:GATGAGGATGAGGACCGCAAGG			
SSTR2	189	F:TCCTCCGCTATGCCAAGATGAAG			
		R:AGATGCTGGTGAACTGATTGATGC			

[a]F: Forward, R: Reverse.

(Santa Cruz Biotechnology) was carried out. The FLAG and HSV epitopes were detected by means of IgG-conjugated antimouse and antigoat antibodies (1:2000) (Molecular Probes, USA), respectively, biotin-conjugated concanavalin A (1:2000) with Streptavidin Alexa Fluor 633 (1:1000) (Molecular Probes), T1R2 with Alexa Flour 488 donkey antigoat IgG (1:2000) (Molecular Probes), and T1R3 with Alexa Fluor 488 goat antirabbit IgG (1:2000) (Molecular Probes). To visualize the nuclei, cells were stained with DAPI (4′,6-diamidino-2-phenylindole). Finally, immunofluorescent pictures were taken by confocal laser-scanning microscopy (Leica TCS-SP8, Germany).

Small Interfering RNA (siRNA) Knockdown of *TAS1R3* Gene Expression in HGT-1 Cells. *TAS1R3* expression levels were reduced in HGT-1 cells by treatment with siRNA. Gene-specific siRNA targeting of *TAS1R3* (5′-GCCUGAAGAUCCGCUGGCA-3′) was purchased from Sigma (Austria). 3×10^4 cells were seeded 24 h prior to transfection with the HiPerFect Transfection Reagent (Qiagen, Austria) using mock transfection or a transfection reagent containing either 1−10 nM *TAS1R3* siRNA (toxicity was excluded by MTT) or All-stars Negative Control siRNA (Qiagen, Austria), according to the manufacturer's protocol (Qiagen). Transfections were performed at 37 °C, 5% CO_2, in a humidified atmosphere. After 48 h transient transfection of *TAS1R3* siRNA, the knockdown efficiencies were assessed by means of RT-qPCR (Supporting Information Figure 1).

cAMP Measurement. Cells were seeded in 96-well plates at a density of 1×10^5 per well under standard conditions 24 h before the experiment. Prior to performing the experiment, the culture medium was discarded, washed once with PBS, and treated with test substances dissolved in KRHB or KRHB containing 0.1% DMSO under standard conditions at 37 °C in a humidified atmosphere of 95% air with 5% CO_2 for 10 min. Cyclic AMP levels were determined by means of a cAMP assay kit, cAMP-Glo (Promega, Germany). The assay was performed according to the manufacturer's instructions. Cyclic AMP values were calculated from the external standard curve.

Statistical Analysis. Sigma Plot software 11.0 (Sytat Software) was used for all statistical analysis. Data are shown as the mean ± standard error of the mean (SEM). A minimum of three biological and two technical replicates were conducted in all cell culture experiments. The statistical analyses are stated in the legend of the corresponding table or figure, applying a one-way ANOVA Holm-Sidak post hoc test for comparison between control and various concentrations of the tested substance, and two-way ANOVA for comparison between control and time-dependent treatments. Treated overcontrol values are demonstrated as T/C (C = 100%). P-values ≤0.05 were considered as statistically significant.

RESULTS

Effects of Cycl, AceK, and D-Thr on H^+ Release in HGT-1 Cells. Treatment of HGT-1 cells with Cycl, AceK, or D-thr in a concentration of 50 mM for 10 min resulted in a stimulation of proton secretion, as shown by reduced IPX values of −0.69 ± 0.08 (Cycl) or an inhibition as shown by increased IPX values 0.60 ± 0.05 (AceK) and 0.80 ± 0.04 (D-thr) in relation to nontreated control cells (0.02 ± 0.08) ($P \leq 0.05$, Figure 1).

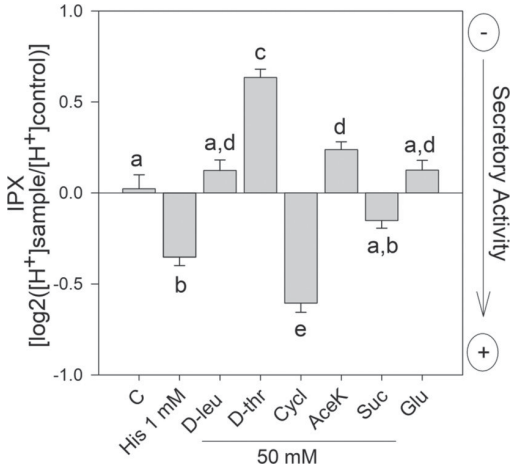

Figure 1. IPX after treatment of the cells with positive control histamine (His, 1 mM), D-leucine (D-leu, 50 mM), D-threonine (D-thr, 50 mM), cyclamate (Cycl, 50 mM), acesulfame K (AceK, 50 mM), sucrose (Suc, 50 mM), or glucose (Glu, 50 mM) for 10 min. Results are displayed as IPX and illustrated in comparison to untreated cells (Control, C) as the mean ± SEM, n = 3−4; tr = 5−6. (Statistics: one-way ANOVA Holm-Sidak post hoc test; significance differences are marked with the letters, $P \leq 0.05$).

Treatment with 50 mM D-leucine (D-leu), sucrose (Suc), and glucose did not affect the intracellular pH ($P > 0.05$, Figure 1). Concentration-dependent treatments revealed the highest concentration (50 mM) of test compounds to elicit the highest impact on proton release in HGT-1 cells (Figure 2).

Impact of Glucose, Cycl, AceK, D-Thr, and Forskolin on mRNA Expression of Targeted Genes Involved in Mechanisms Regulating Proton Secretion in HGT-1 Cells. The cDNA transcripts generated from HGT-1 RNA by gene-specific oligonucleotide primers for *TAS1R1*, *TAS1R2*, and *TAS1R3*, as well as the primers mentioned in Table 1, were amplified by means of RT-qPCR.

Glucose had no impact on the targeted genes of interest ($P > 0.05$, Table 2). Apart from *SSTR2*, *ATP4A*, and *TAS1R1* ($P > 0.05$), Cycl induced the mRNA expression of *TAS1R3* and *HRH2* at all three treatment times, whereas *CHRM3* mRNA was reduced after 10 min of Cycl exposure ($P \leq 0.05$, Table 2). Cycl and AceK are known to have a bitter aftertaste in addition to their

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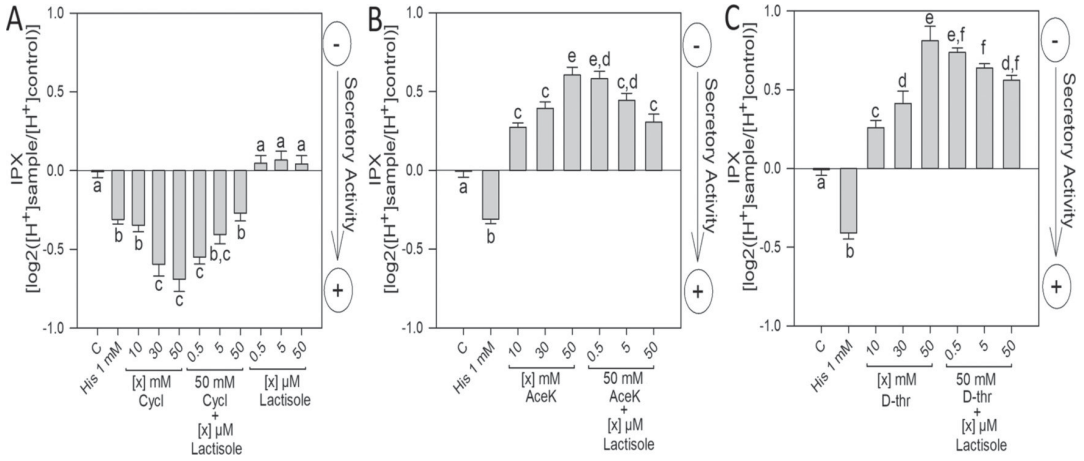

Figure 2. IPX of HGT-1 cells after treatment with (A) cyclamate (Cycl, 50 mM), in the presence or absence of lactisole, or lactisole alone, (B) acesulfame K (AceK, 50 mM), in the presence or absence of lactisole, and (C) D-threonine (D-thr, 50 mM), in the presence or absence of lactisole for 10 min. Findings are depicted as IPX and demonstrated in comparison to the untreated cells (Control, C) as the mean ± SEM, n = 3−4; tr = 5−6. (Statistics: one-way ANOVA Holm-Sidak post hoc test; significant differences are shown with the letters, $P \leq 0.05$).

sweet-tasting character. The identified bitter taste responsive receptors for Cycl are T2R1 and T2R38,[28] while those are T2R31 and T2R43 for AceK.[29] We, therefore, studied the potential involvement of these respective T2Rs in proton release. Cycl down-regulated mRNA expression of the bitter receptors *TAS2R1* and *TAS2R38* after 20 and 60 min exposure, respectively ($P \leq 0.05$, Table 2). Treatment of the cells with AceK also regulated *ATP4A*, *HRH2*, *SSTR2*, *TAS1R1*, *TAS1R3*, *TAS2R31*, and *TAS2R43* expression ($P \leq 0.05$, Table 2). Apart from *ATP4A*, D-thr also demonstrated an impact on the targeted genes at various times of treatment ($P \leq 0.05$, Table 2). Likewise, except for *SSTR2*, forskolin caused changes of the mRNA expression level of the genes shown in Table 2. Notably, forskolin (10 μM), dissolved in 0.1% DMSO (v/v), did not show a regulative effect when compared to cells treated with the solvent control, consisting of cell culture media with 0.1% DMSO (v/v) only (data not shown, $P > 0.05$). HGT-1 gene expression of *TAS1R2* was below the limit of detection, with ΔcT_{TAS1R2} values above 35 (RT-qPCR amplification, running 40 cycles; N_o values of *TAS1R2* calculated from LinRegPCR v.12.8 were normalized over the N_o values of *PPIA*; $3.6 \times 10^{-3} \pm 5.1 \times 10^{-4}$ (as the mean ± SEM, n = 3; tr = 3)).

Immunocytochemical Detection of the T1R2 and T1R3 Receptor Proteins in HGT-1 Cells. To test the specificity of the antibodies directed against T1R2 or T1R3 receptor proteins, HEK 293T-Gα16gust44 cells were transiently transfected with cDNA of *TAS1R2* and *TAS1R3* (Figure 3). Antibodies (FLAG-tag, green) selective for the T1R2 receptor protein (red) and (HSV-tag, green) the T1R3 receptor protein (red) were used to stain transfected HEK 293T-Gα16gust44 cells (Figure 3). Both T1R2 and T1R3 receptor proteins were detected by using the anti-FLAG and anti-HSV antisera indicating the specificity of the antisera (Figure 3). Subsequently, HGT-1 cells were analyzed with the validated antiseras against T1R2 and T1R3 receptor protein (green) and concanavalin A (ConA, red) directed toward cell surface glycoproteins. Signals gained for T1R2 were relatively weak in comparison to that for T1R3 (Figure 3, green). In addition, preincubation with the specific T1R2/T1R3 antisera reduced the staining signals (Figure 3).

Impact of Lactisole on Cycl- and AceK-Induced Proton Release in HGT-1 Cells. IPX values were analyzed after treating the cells with Cycl, AceK, or D-thr in the presence or absence of T1R3 antagonist lactisole.

Coincubation of 50 mM Cycl, AceK, D-thr, and 50 μM lactisole for 10 min most effectively reduced the effects of Cycl, AceK, and D-thr, revealing IPX values of −0.27 ± 0.05; −0.69 ± 0.08, 0.30 ± 0.05; 0.60 ± 0.05, and 0.56 ± 0.06; 0.80 ± 0.04, respectively, in relation to control cells (0.02 ± 0.08) ($P \leq 0.05$, Figure 2). Treatment of the HGT-1 cells with lactisole alone showed no significant effect on proton release ($P > 0.05$, Figure 2A).

Impact of Cycl and AceK on Proton Release in *TAS1R3* Knock-Down HGT-1 Cells. In order to clarify the involvement of T1R3 in Cycl- and AceK-induced changes in proton secretion, the mRNA expression of *TAS1R3* was knocked down by treatment of the cells with *TAS1R3* siRNA (10 nM). Mean knock-down efficiency, assessed by means of RT-qPCR, was 51.0 ± 3.1% (Supporting Information, Figure 1). Moreover, the effects of Cycl and AceK on proton release were reduced by 39.0 ± 5.1% and 51 ± 3.8% in *TAS1R3* knocked-down cells compared to mock transfected, nontargeted control cells ($P \leq 0.05$, Figure 4).

Role of cAMP in Cycl, AceK, and D-Thr-Induced Changes in Proton Release in HGT-1 Cells. In order to determine the role of T1R3 downstream signaling on Cycl-, AceK-, and D-thr-induced changes in proton secretion, and to elucidate whether these effects are mediated via elevated $[cAMP]_i$, the cAMP activator forskolin and its inhibitor NKY80 were used. Treatment of HGT-1 cells with 10 μM forskolin for different time points from 10 to 55 min resulted in a stimulation of proton secretion ($P \leq 0.05$), which was decreased by 3 μM NKY80 as indicated by $AUC_{10-55\ min}$ values (n = 4; tr = 5 statistics: one-way ANOVA Holm-Sidak post hoc test, $P \leq 0.05$, data not shown). HGT-1 exposure to NKY80 and 0.1% DMSO had no effect on proton release in HGT-1 cells ($P > 0.05$, data not shown).

Treatment of the cells with 50 mM Cycl, AceK, or D-thr in the presence of 3 μM NKY80 reduced the effect of Cycl and AceK ($P \leq 0.05$, Figure 5), whereas for D-thr, the level of a statistical significant difference was not reached ($P = 0.127$, Figure 5).

Impact of Cycl, AceK, Glucose, and Forskolin on the Intracellular cAMP Concentration ($[cAMP]_i$) in HGT-1 Cells. Apart from glucose, Cycl, AceK, and forskolin induced an increase in $[cAMP]_i$, which was reduced by treatment of the cells

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Table 2. RT-qPCR Results of HGT-1 Cells Treated with 50 mM Glucose, Cyclamate, Acesulfame K, D-Threonine, or 10 μM Forskolin[a]

target	treatment	10 min	20 min	60 min
ATP4A	glucose	1.12 ± 0.07	1.20 ± 0.08	0.97 ± 0.09
	DMSO	1.00 ± 0.07	0.90 ± 0.04	0.96 ± 0.08
	cyclamate	1.00 ± 0.04	1.06 ± 0.05	1.06 ± 0.09
	acesulfame K	**0.80 ± 0.04***	**1.31 ± 0.08***	0.88 ± 0.07
	D-threonine	1.00 ± 0.08	1.10 ± 0.05	0.93 ± 0.03
	forskolin	0.96 ± 0.08	**0.75 ± 0.02***	1.04 ± 0.05
HRH2	glucose	0.88 ± 0.10	0.96 ± 0.06	0.89 ± 0.03
	DMSO	0.87 ± 0.08	0.84 ± 0.04	0.97 ± 0.05
	cyclamate	**1.11 ± 0.03***	**1.14 ± 0.08***	**1.20 ± 0.10***
	acesulfame K	1.07 ± 0.10	**1.21 ± 0.05***	1.15 ± 0.11
	D-threonine	**1.24 ± 0.07***	**1.24 ± 0.05***	1.08 ± 0.08
	forskolin	0.97 ± 0.04	**0.80 ± 0.03***	**1.10 ± 0.05***
CHRM3	glucose	1.11 ± 0.06	0.93 ± 0.09	0.86 ± 0.04
	DMSO	1.10 ± 0.06	1.01 ± 0.05	0.90 ± 0.06
	cyclamate	**0.73 ± 0.09***	1.01 ± 0.23	0.96 ± 0.23
	acesulfame K	**0.65 ± 0.07***	**1.77 ± 0.06***	0.87 ± 0.09
	D-threonine	**0.70 ± 0.05***	1.22 ± 0.20	1.30 ± 0.36
	forskolin	**1.51 ± 0.08***	1.10 ± 0.10	**0.64 ± 0.14***
SSTR2	glucose	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.01
	DMSO	1.03 ± 0.02	1.05 ± 0.02	0.94 ± 0.02
	cyclamate	0.98 ± 0.04	1.01 ± 0.05	0.98 ± 0.02
	acesulfame K	0.92 ± 0.03	**0.88 ± 0.04***	1.03 ± 0.04
	D-threonine	**0.81 ± 0.03***	0.96 ± 0.03	1.02 ± 0.01
	forskolin	1.02 ± 0.03	1.02 ± 0.02	1.02 ± 0.04
TAS1R1	glucose	0.89 ± 0.07	1.11 ± 0.10	1.10 ± 0.11
	cyclamate	1.16 ± 0.06	0.90 ± 0.03	0.98 ± 0.07
	acesulfame K	1.13 ± 0.06	**0.71 ± 0.14***	1.12 ± 0.12
	D-threonine	1.12 ± 0.06	**0.70 ± 0.13***	0.98 ± 0.07
TAS1R3	glucose	1.15 ± 0.10	1.19 ± 0.06	1.19 ± 0.22
	cyclamate	**1.34 ± 0.15***	**1.45 ± 0.16***	**1.51 ± 0.20***
	acesulfame K	**0.74 ± 0.08***	**1.34 ± 0.08***	**1.52 ± 0.18***
	D-threonine	1.05 ± 0.06	**1.35 ± 0.10**	1.40 ± 0.25
TAS2R1	cyclamate	0.87 ± 0.20	**0.65 ± 0.11***	0.67 ± 0.23
TAS2R38	cyclamate	0.99 ± 0.19	0.55 ± 0.18	**0.36 ± 0.07***
TAS2R31	acesulfame K	0.96 ± 0.06	0.88 ± 0.10	0.96 ± 0.04
TAS2R43	acesulfame K	1.07 ± 0.10	1.03 ± 0.06	**1.32 ± 0.06***

[a]Data are indicated as average ± SEM in comparison to untreated control (set to 1), and forskolin results were analyzed over DMSO (0.1%, v/v) treatment. Calculations were made from at least three biological replicates. Statistics; two-way ANOVA Holm-Sidak post hoc test; significant difference between treatments over control are depicted as *; $P \leq 0.05$.

with the adenylate cyclase inhibitor NKY80. In addition, lactisole attenuated the impact of Cycl and AceK on $[cAMP]_i$ ($P \leq 0.05$, Table 3), whereas neither lactisole nor NKY80 demonstrated a significant effect on $[cAMP]_i$ ($P > 0.05$, Table 3).

Impact of Glucose on Cycl- and AceK-Induced Proton Release in HGT-1 Cells. In order to further elucidate the mechanisms of T1R2/T1R3, we studied caloric sweetener glucose in NCS-induced proton release. Cells treated with 50 mM Cycl and 5 or 10 mM glucose for 10 min showed IPX values of −0.24 ± 0.07, −0.35 ± 0.06, and −0.46 ± 0.04, respectively, in relation to the control cells (0.05 ± 0.05) ($P \leq 0.05$, Figure 6A). Cotreatment with 50 mM AceK and 5 or 10 mM glucose resulted in IPX values of 0.52 ± 0.13, 0.70 ± 0.19, and 0.84 ± 0.08, respectively, in relation to the control cells (0.09 ± 0.21) ($P \leq 0.05$, Figure 6B). The effect of 10 mM glucose coincubated with 50 mM Cycl or AceK was significantly reduced by addition of 30 μM glibenclamide, a K_{ATP} inhibitor, resulting in IPX values of −0.18 ± 0.06; −0.46 ± 0.04, and 0.51 ± 0.09; 0.84 ± 0.08, respectively, compared to nontreated control cells (0.05 ± 0.05 and 0.09 ± 0.21, respectively) ($P \leq 0.05$, Figure 6A, 6B). Cotreatment of 1 mM histamine and with 10 mM glucose resulted in an IPX value of −0.80 ± 0.05 in relation to the control cells (−0.05 ± 0.03) ($P \leq 0.05$, Figure 6C). Glucose did not change the histamine-induced proton secretion ($P > 0.05$, Figure 6C). Histamine-induced proton release was suppressed by glibenclamide with IPX values of −0.80 ± 0.05 and −0.36 ± 0.06 in relation to the control cells (−0.05 ± 0.03) ($P \leq 0.05$, Figure 6C). Additionally, 30 μM glibenclamide alone inhibited the H^+ release, with an IPX value of 0.18 ± 0.04 in relation to the control cells (−0.05 ± 0.03) ($P \leq 0.05$, Figure 6).

Impact of Glucose, Cycl, and AceK on mRNA Expression of *SUR1*, *Kv11.1*, and *SLC2A1* in HGT-1 Cells. After exposing the cells to 50 mM glucose, Cycl, or AceK for 10, 20, and 60 min, mRNA expression profiles of *SUR1*, *Kv11.1*, and *SLC2A1* were measured by means of RT-qPCR.

Glucose did not show any effect on the expression level of all targeted genes ($P > 0.05$, Table 4), except for *SLC2A1* at the time points of 10 and 20 min ($P \leq 0.05$, Table 4). Cycl revealed a

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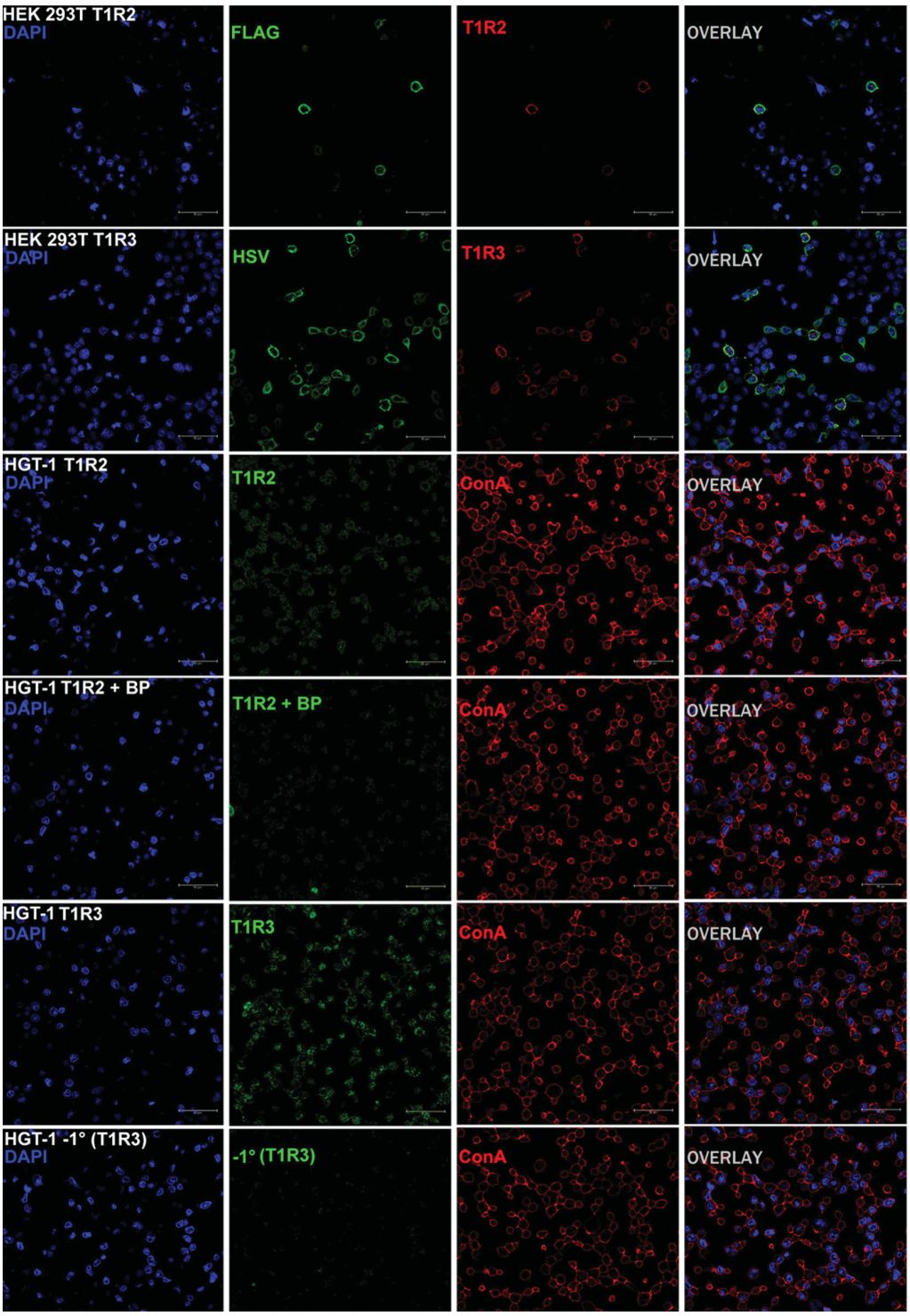

Figure 3. Specific immunostaining of the transiently transfected HEK 293T-Gα16gust44 cells expressing human T1R2 and T1R3 is exhibited by the epitope (green) tagged specific anti-T1R2 and -T1R3 antibodies (red). Nuclei of the cells are visualized with DAPI (blue). In HGT-1 cells, T1R2 and T1R3 receptor proteins are visualized by the anti-TAS1R2 and -TAS1R3 antiserums (green). Anti-T1R2 antibody preincubated with the specific blocking peptide (T1R2+BP). As a negative control for the anti-T1R3 staining, −1° (T1R3) represents the treatment of HGT-1 cells without anti-T1R3 antibody. Cell surface labeling is demonstrated with concanavalin A (ConA, red). Scale bar: 50 μm.

regulatory influence on the mRNA expression level of *SUR1*, *Kv11.1*, and *SLC2A1* at the time points of 10, 10, and 60 min, respectively ($P \leq 0.05$, Table 4). AceK most effectively regulated *SUR1*, *Kv11.1*, and *SLC2A1* ($P \leq 0.05$, Table 4).

DISCUSSION

The secretion of gastric acid is essential for protein digestion and absorption of calcium, iron, and vitamin B12, preventing bacterial overgrowth and enteric infections, and helping to reduce or

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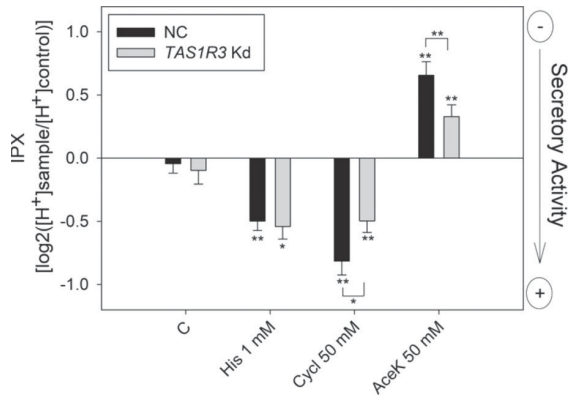

Figure 4. Effect of Cycl and AceK on H^+ release in *TAS1R3* knockdown cells (*TAS1R3* kd). *TAS1R3* was knocked down 51.0 ± 3.1% by using siRNA (10 nM). IPX of HGT-1 cells transfected with nontargeting negative control siRNA (NC) or HGT-1 cells with knockdown of *TAS1R3* by siRNA, treated with histamine (His, 1 mM), cyclamate (Cycl, 50 mM), or acesulfame K (AceK, 50 mM) for 10 min. Results are calculated in comparison to nontargeting negative control siRNA (C, NC) as the mean ± SEM, $n = 3$; tr = 5−6. (Statistics: Student's t test; significant differences are expressed with **; $P \leq 0.001$,*; $P \leq 0.05$).

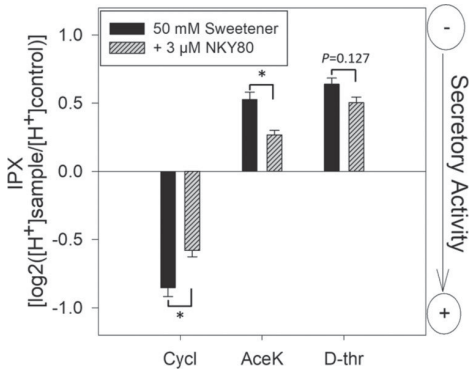

Figure 5. IPX of HGT-1 cells after coincubation of cyclamate (Cycl, 50 mM), acesulfame K (AceK, 50 mM), D-threonine (D-thr, 50 mM), and NKY80 for 10 min. Findings are exhibited as IPX and demonstrated in comparison to untreated cells (control cells, set to 0) or 0.1% DMSO-treated control cells (solvent control for NKY80, set to 0) as the mean ± SEM, $n = 3$−4; tr = 5−6. (Statistics: one-way ANOVA Holm-Sidak post hoc test; significant differences are expressed with *, $P \leq 0.05$).

eliminate the allergenicity of foods and is among the key factors regulating food intake. The control of acid secretion is accomplished by a complex interaction of neurocrine, paracrine, and hormonal pathways.[30] One of our recent studies has demonstrated that activation of oral and gastric bitter receptors by caffeine stimulates the secretion of gastric acid in healthy subjects and that this effect is reduced by the bitter masking compound homoeriodictyol. In vitro studies using a CRISPR-Cas9 approach in HGT-1 cells demonstrated a key role for the bitter receptor *TAS2R43* targeted by caffeine and homoreiodictyol, respectively.[21] Apart from its reducing effects on caffeine-stimulated GAS, we also showed that the bitter-masking/bitter receptor (T2R43) inhibiting compound homoeriodictyol increased food intake in healthy subjects when administered as a bolus of 30 mg.[31]

The impact of NCS on food intake and mechanisms regulating satiety is still controversial. In addition, how NCS affects GAS is unknown. Here, we demonstrate that Cycl and AceK are modulators of GAS in human parietal cells (HGT-1 cells).

Table 3. cAMP Formation after Treatment with Cyclamate, Acesulfame K, Glucose, or Forskolin in the Presence and Absence of NKY80 or Lactisole for 10 min[a]

		NKY80	Lactisole
		3 µM	50 µM
control	100 ± 2.4[a]		94.3 ± 6.4[a]
cyclamate 50 mM	181 ± 9.6[b]	128 ± 5.4[c]	151 ± 3.3[d]
acesulfame K 50 mM	195 ± 8.7[b]	113 ± 2.8[c]	154 ± 6.7[d]
glucose 50 mM	110 ± 8.1[a]	105 ± 3.2[a]	107 ± 6.1[a]
0.1% DMSO control	100 ± 3.5[a]	87.2 ± 5.6[a]	
forskolin 10 µM	266 ± 38[b]	136 ± 8.2[c]	

[a]Data are displayed as [%T/C], average ± SEM in relation to control (set to 100%, marked with "a") or control containing 0.1% DMSO for forskolin and NKY80 (set to 100%), $n = 3$; tr = 2. Statistics: one-way ANOVA followed by Holm−Sidak post hoc test was employed, and significant differences between treatment over control are calculated and compared to their respective measurement and marked with letters; $P \leq 0.05$.

HGT-1 cells have been established as an in vitro model for studying mechanisms of GAS.[18−24,32,33] These cells express all functional proteins responsible for proton secretion[19] which is analyzed by quantitating the intracellular concentration of protons using a fluorescent dye that shows a characteristic pH-dependent fluorescence.

In this mechanistic study, treatment of HGT-1 cells with 50 mM Cycl increased proton secretion, whereas the same concentrations of AceK and D-thr showed a reducing effect compared to untreated control cells. At low concentrations (≤1 mM), neither NCS evoked proton release in HGT-1 cells (data not shown). RT-qPCR analyses demonstrated a regulation of mRNA expression of prosecretory gene encoding for the H^+/K^+-ATPase (*ATP4A*), the histamine (*HRH2*), and the acetlycholine (*CHRM3*) receptor and the antisecretory somatostatin receptor (*SSTR2*) for Cycl, AceK, and D-Thr, whereas treatment of HGT-1 cells with glucose had no effect, either on the mRNA expression of the selected genes or on proton secretion. Coincubations of Cycl and AceK with lactisole, a known antagonist for T1R3,[4] diminished the effect of both NCS compounds. To clarify whether the T1R2/T1R3 is involved in this response, mRNA expression of *TAS1R2* and *TAS1R3* as elements of the heterodimer sweet taste receptor[2] were analyzed by RT-qPCR. Whereas the mRNA level of *TAS1R2* was below the limit of quantification, Cycl and AceK regulated the mRNA expression level of *TAS1R3*. Overall, a down-regulation or an up-regulation of a prosecretory gene does not necessarily result in a decreased proton release or vice versa, although it provides some evidence that these proteins play a role in the cellular response. To follow up on this hypothesis, protein expression of T1R2 and T1R3 in HGT-1 cells was analyzed by immunocytochemistry, using commercially available antibodies which were validated in transfected HEK-293T cells. Staining signals for T1R3 dominated over those for T1R2. Here, we hypothesize that the structure of the T1R2/T1R3 heterodimer in parietal cells is different in the tongue taste cells[2] and in HGT-1 cells, although data accurately characterizing the protein structure are lacking. Thus, we cannot exclude that the receptor in HGT-1 cells is not a heterodimer but rather a T1R3 homodimer. This might be an actual possibility in view of the fact that such homodimers have been found and recognize sugars, as demonstrated for insulating secreting-pancreatic β-cells,[6] adipocytes 3T3-L1 cells,[7] and GLP-1-secreting Hutu-80 cells.[8] On the other hand, another possibility is that HGT-1

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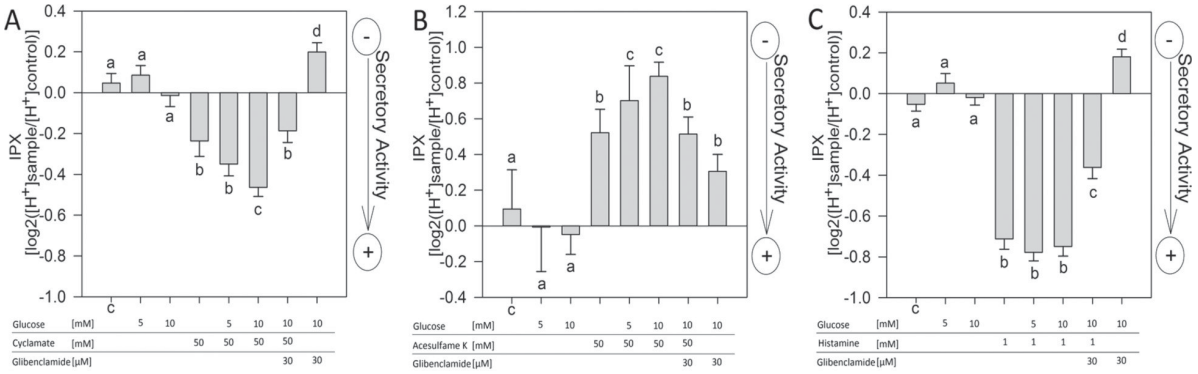

Figure 6. Enhancing effect of 5 and 10 mM glucose on 50 mM cyclamate-, acesulfame K-, or 1 mM histamine-induced H^+ secretion. IPX of HGT-1 cells treated with (A) cyclamate, (B) acesulfame K, or (C) histamine in the presence or absence of glibenclamide for 10 min. Data are presented as IPX and expressed treated over untreated control cells as the mean ± SEM, $n = 3$; tr = 5−6. (Statistics: one-way ANOVA Holm-Sidak post hoc test; significant differences are shown with the letters, $P \leq 0.05$).

Table 4. RT-qPCR Results of HGT-1 Cells Treated with 50 mM Glucose, Cyclamate, or Acesulfame K[a]

target	treatment	10 min	20 min	60 min
SUR1	glucose	1.03 ± 0.10	0.90 ± 0.08	0.84 ± 0.08
	cyclamate	**0.65 ± 0.09***	0.96 ± 0.18	1.18 ± 0.21
	acesulfame K	**1.68 ± 0.38***	**1.46 ± 0.27***	**1.31 ± 0.23***
Kv11.1	glucose	1.00 ± 0.13	1.12 ± 0.12	1.15 ± 0.22
	cyclamate	**1.29 ± 0.19***	0.96 ± 0.12	**1.61 ± 0.22***
	acesulfame K	**1.60 ± 0.27***	**1.70 ± 0.20***	1.34 ± 0.25
SLC2A1	glucose	**1.20 ± 0.10***	**1.21 ± 0.13***	1.00 ± 0.11
	cyclamate	0.98 ± 0.14	1.18 ± 0.16	**1.53 ± 0.15***
	acesulfame K	**1.35 ± 0.18***	**1.30 ± 0.12***	**1.54 ± 0.20***

[a]Data are indicated as average ± SEM in comparison to untreated control (set to 1). Calculations were made from at least three biological replicates. Statistics: two-way ANOVA Holm−Sidak post hoc test; significant difference between treatments over control are depicted as *; $P \leq 0.05$.

cells express both hetero- and homodimers, with a prevalence of homodimers. Future studies are warranted to elucidate the protein structure of the T1R2/T1R3 receptor in HGT-1 cells and in gastric tissues. However, our hypothesis is confirmed by results from the *TAS1R3* siRNA knockdown approach: in HGT-1 cells showing a 51% lower *TAS1R3* mRNA level compared to nontransfected cells, the effect of Cycl and AceK on proton secretion was significantly reduced. This suggests the involvement of T1R3 in NCS-induced proton release, although it also indicates additional cell surface proteins are involved in this response. This hypothesis is supported by our data showing opposite effects of Cycl and AceK on proton secretion. Cycl, reported to also target the bitter receptors T2R1 and T2R38,[28] stimulates the secretion of protons, whereas AceK, an agonist of T2R31 and T2R43,[29] shows a reducing effect compared to control cells. We demonstrated involvement of T2R43 by using the T2R43 antagonist 4-(2,2,3-trimethylcyclopentyl) butanoic acid (GIV3727)[34] which decreased the AceK-evoked response (Figure S2). A sensory study by Świąder et al.[35] demonstrated that the sweet taste intensity of Cycl and AceK in water solutions increased with higher concentrations in comparison to equi-sweet concentrations of sucrose, whereas at lower concentrations, up to about 0.5 ppm, AceK demonstrated a less intense bitter off-taste compared to Cycl. The human T1R2/T1R3 heterodimer possess various ligand-binding sites for sweeteners. AceK, e.g., binds to the amino-terminal domain of T1R2,[5] whereas Cycl acts on the transmembrane helical domain of T1R3 as an agonist.[36] There is, however, evidence demonstrating that a T1R3 homodimer is functional in response to AceK in Hutu-80 cells.[8] Nevertheless, we do not exclude the potential involvement of T1R2. Future studies are needed to discriminate between the efficacy of T1R and T2R by means of T1R and T2R targeted knock-down approaches. The outcome from a sensory study by Galindo-Cuspinera et al.[37] suggests that the transmembrane helical domain of the T1R3 section of the sweet taste receptor also functions as an allosteric binding site for saccharin for which lactisole is a known antagonist. The authors also demonstrated that saccharin at high concentrations (>25 mM) elicits a bitter taste character and suppresses the sweet response, whereas Cycl at high concentration exhibits a bitter and sweet character. Another explanation for the opposite effects of Cycl and Acek apart from the involvement of additional cell surface receptors and concentration-dependent responses might be antagonistic effects: A recent publication by Behrens et al.[38] demonstrated antagonistic activities for Cycl and saccharin on each other's bitter responsive T2Rs. However, we can not explain the opposite effects by Cycl and AceK on proton secretion in HGT-1 cells yet, neither by our current nor by previous findings where the stimulating effect of the bitter tasting caffeine, targeting T2R7, T2R10, T2R14, T2R31, and T2R43[32] on proton secretion, was reduced by the T2R43 antagonist homoeriodictyol.[21] Although the 50 mM concentrations of Cycl and AceK applied to HGT-1 cells in this mechanistic work are comparatively high, it can not be excluded that in gastric cells the density of taste receptors is lower than that on the tongue, requiring higher concentrations of NCS to elicit a similar response. Future studies are needed to clarify whether antagonistic activities of Cycl and AceK on TAS2Rs, beyond their role as agonists for T2R1/T2R38[32] and

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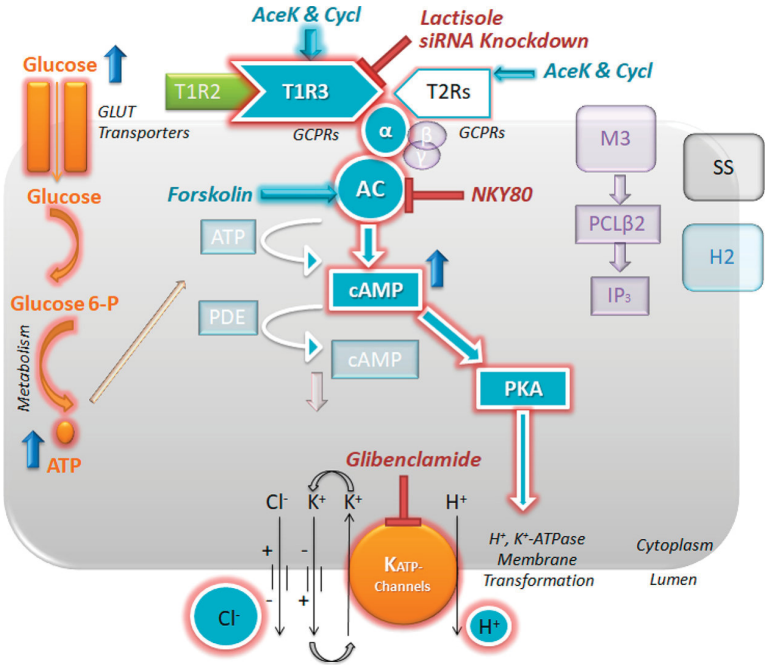

Figure 7. Illustrative proposed mechanism of sweetener-induced H^+ secretion in HGT-1 cells. The main T1R3 stimulatory pathway as well as T2Rs is elicited in dark blue and glowed with red. The glucose-dependent mechanism is demonstrated in orange. T1R3 agonists and AC activator forskolin are shown in blue. Inhibitors, as well as *TAS1R3* siRNA knockdown, are depicted in red. Activation of T1R3 is followed by induction of the secondary signaling cascade through cAMP. cAMP activates protein kinase A (PKA) which subsequently stimulates the H^+, K^+-ATPase that is accompanied by H^+ Cl^- secretion. Other signaling mechanisms, which might presumably be activated during H^+ secretion induced by NCS, are illustrated in the background, such as histaminic (light blue, H2), cholinergic (purple, M3), and somatostatin (black, SS) receptors.

T2R31/T2R43,[33] respectively, might help to explain the contrary effects of these NCSs on proton secretion. In addition, involvement of other cell-surface proteins cannot be excluded. Therefore, the role of T1R3 was further elucidated by analyzing NCS-associated downstream signaling pathways.[6−8] First, the impact of Cycl and AceK on intracellular cAMP was studied by using the adenylate cyclase activator forskolin[39] and its inhibitor NKY80.[40] Increased cAMP concentrations analyzed after exposure of HGT-1 cells to forskolin and either of the NCSs tested were reduced by NKY80 cotreatment. In addition, glucose did not evoke a response. Involvement of T1R3 was verified by lactisole, which also lowered the cAMP increase by Cycl and AceK. Although the opposite response of HGT-1 cells to Cycl and AceK on proton secretion is not reflected by comparable effects of these compounds on cAMP, our results indicate a link between T1R3 activation and proton secretion. This hypothesis is supported by effects of NKY80, which reduced the Cycl and AceK-evoked response on proton secretion.

T1R3-expressing cells have been shown to also express glucose transporters (GLUTs) and K_{ATP}-sensitive K^+ channels (K_{ATP}) which are considered cellular "glucose sensors".[41] Conducting RT-qPCR experiments, we observed a regulation of the mRNA expression level of these genes by Cycl and AceK in HGT-1 cells. Since Sidani et al.[42] demonstrated that glibenclamide inhibits H^+, K^+-ATPase-mediated and histamine-induced acid secretion in isolated gastric glands from wild-type mouse, we also tested the impact of the K_{ATP} inhibitor glibenclamide[41] which markedly diminished the effect of Cycl and AceK on proton secretion in HGT-1 cells. Since the transport of K^+ is directly linked to H^+ exchange, these results support our hypothesis of Cycl and AceK as modulators of GAS. Assuming that the cations gained from cyclamate (Na^+) and acesulfame (K^+) would also contribute to this opposite effect on proton release, the cells treated with 50 mM NaCl and KCl showed no effect on Cycl- and AceK-induced proton secretion (data not shown). To elucidate whether Cycl and AceK not only act through binding to the sweet taste receptor but also via activation of GLUTs, we studied glucose in coincubation experiments as suggested by Yee et al.[41] who described that a mixture of NCSs and a caloric sweet tastant (glucose) augment the cellular NCS response. This hypothesis is supported by our data demonstrating that glucose did enhance the impact of Cycl and AceK on proton secretion, while not showing an effect when cells where treated with glucose solely. Consistent with our results, Li et al.[43] demonstrated that a much higher concentration of glucose (>50 mM) is required to stimulate the canonical sweet taste receptor in taste buds. Moreover, it has been hypothesized that the sweet taste receptor in β-cells is not identical to the canonical sweet taste receptor expressed in the taste buds of the tongue, as a relatively high concentration of NCS was required to stimulate the sweet taste receptor in β-cells.[44] In addition, the heterodimeric sweet taste receptor is known to detect the sweet taste of caloric sugars, for example sucrose at the EC_{50} = 100 mM,[43] whereas it senses the sweet taste of Cycl at EC_{50} = 2.2 mM and AceK at EC_{50} = 120 μM.[45] Since the 10 min 50 mM glucose exposure in this work also did not alter intracellular cAMP concentrations nor mRNA expression of *TAS1R3*, future experiments will be needed to elucidate the different pathway kinetics of NCS and glucose. Although glucose is a known agonist of the sweet taste receptor[2] and a substrate for ATP formation upon cellular uptake by GLUTs,[46] these cellular responses might follow different kinetics as compared to those of NCS, which are many times sweeter than glucose and may have, therefore, significantly different T1R3 binding kinetics than glucose. Glucose is assumed to bind to the amino-terminal

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domain of both subunits, T1R2 and T1R3, with distinct binding affinities.[47] Cycl has been shown to bind to the transmembrane domain of T1R3,[36] while the amino-terminal domain of T1R2 is the binding site for AceK.[5] Therefore, we hypothesize that the enhancing effect of glucose on the cellular response to Cycl and AceK predominantly depends on an initial T1R3 activation by the NCS compounds. However, we cannot exclude an enhancing effect on cAMP concentrations via metabolic transformations of glucose through gylcolysis, the Krebs cycle, and oxidative phosphorylation reactions, finally increasing ATP as the primary substrate for the adenylate cyclase to form cAMP[48] (Figure 7).

The contrary effects of Cycl and AceK on proton secretion in HGT-1 cells might also be explained by different kinetics. In our mechanistic studies, which were intended to prove the hypothesis that NCSs have an impact on mechanisms regulating GAS via sweet taste receptor, both NCS compounds were used at equal concentrations, although AceK is about 7 times sweeter than Cycl (in relation to sucrose),[3] which are higher than those commonly added to foods and beverages. The European Union (EU) allows maximum doses for Cycl in various foods from 250 mg/L to 2500 mg/kg, with typical carbonated soft drinks containing up to 1.4 mM. For AceK, the doses range from 25 mg/L to 2500 mg/kg.[49]

In summary, our study demonstrates that (i) T1R3 is a major functional part of the T1R2/T1R3 receptor and that (ii) Cycl and AceK are T1R3-dependent modulators of proton secretion in HGT-1 cells. However, future studies are needed to identify their efficacy of Cycl and AceK on GAS in vivo when applied in food-representative concentrations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b00658.

Results demonstrating the *TAS1R3* siRNA knockdown efficiencies determined by means of RT-qPCR and the effect of GIV3727 and AceK-induced proton release (PDF)

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■ ABBREVIATIONS USED

Cycl, sodium cyclamate; AceK, acesulfame potassium; D-thr, D-threonine; NKY80, 2-amino-7-(2-furanyl)-7,8-dihydro-5(6*H*)-quinazolinone; SNARF, 1,5-carboxy-seminaphtorhodafluor acetoxymethyl ester; IPX, intracellular proton index; HGT-1, human gastric parietal tumor cells; NCS, noncaloric sweeteners; GAS, gastric acid secretion; AC, adenylate cyclase

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2.4 "Impact of free Nε–Carboxymethyllysine, its precursor glyoxal and AGE-modified BSA on serotonin release from human gastric tumour cells (HGT-1)."

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Impact of free N^{ε}-carboxymethyllysine, its precursor glyoxal and AGE-modified BSA on serotonin release from human parietal cells in culture

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Advanced glycation end products (AGEs) are frequently encountered in a western diet, in addition to their formation *in vivo*. *N*-Epsilon-carboxymethyllysine (CML), one of the chemically diverse compounds formed in the reaction between reducing carbohydrates and amines, is often used as a marker of advanced glycation, and has been shown to stimulate serotonin release from cells representing the central (SH-SY5Y cells) and the peripheral (Caco-2 cells) serotonin system *in vitro*. Here, we investigated the effect of glyoxal, free CML, and protein-linked AGE-BSA on serotonin release from human gastric tumour cells, which originate from an adenocarcinoma of the stomach and have recently been shown to be capable of serotonin synthesis and release. Microarray experiments showed both CML and glyoxal to alter genes associated with serotonin receptors. Furthermore, treatment with glyoxal resulted in a small change in RAGE expression while CML did not alter its expression. On a functional level, treatment with 500 μM CML increased extracellular serotonin content by 341 ± 241%, while treatment with 1 mg mL^{-1} AGE-BSA led to a reduction by 49 ± 11% compared to non-treated cells. The CML-induced serotonin release was reduced by the HTR3 antagonist granisetron. Incubation with the RAGE antagonist FPS-ZM1 abolished the effect of AGE-BSA on serotonin release, while no impact on CML-induced serotonin release was observed. Furthermore, treatment with 5 mM CML stimulated proton secretion as a functional outcome measure, assessed using a pH sensitive dye. Taken together, these results indicate a likely HTR3-mediated, RAGE-independent effect of free CML on serotonin release and a RAGE-dependent mechanism for the protein linked AGE-BSA.

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Introduction

Advanced glycation end products (AGEs) are formed by thermal processing of foods at high temperatures. In addition to the formation of AGEs, the high thermal heat treatment encountered in many typical western diets, may alter other food properties such as energy density. A study by Birlouez-Aragon *et al.*,[1] testing a standard diet high in AGEs and a steamed diet low in AGEs, reported the standard diet to have a higher energy density compared to the steamed diet. In addition, consumption of the standard diet high in AGEs resulted in a higher total energy intake compared to the low AGE meals.[1] By regular ingestion as part of a typical western diet, AGEs partially enter systemic circulation.[1–4] N^{ε}-Carboxymethyllysine (CML) is a stable and well-characterized AGE which is often used as a marker for AGEs in foods[5–7] and has been detected in human plasma, centring at a concentration around 3 μM.[8] In addition to CML, one of its precursors, glyoxal, has been identified in human plasma at concentrations around 0.3 μM.[9] Both forms, protein-bound and free CML, have been investigated in several studies, the latter to lesser extent. A study on HEK-293 cells indicated protein-linked CML and free CML to induce p38 MAPK activation. This study used HEK-293 cells only expressing the extracellular domain of RAGE, and thus lacking the ability to intracellularly transduce RAGE-mediated signals. Zill *et al.*[10] suggested a RAGE-mediated pathway for both protein-bound and free CML. A RAGE-depended pathway has also been described by Schmid *et al.*[11] in experiments on the expression of heat shock proteins in which Caco-2 cells were incubated with casein-linked or free CML. Furthermore, free CML is known to stimulate serotonin

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release from cells representing central and peripheral serotonin.[12,13] Serotonin is an important neurotransmitter in the regulation of feeding and satiety.[14] The activation of several serotonin receptors has been described to increase or decrease food intake, for some of which alterations in gene expression have been shown after CML treatment.[13] Several amino acids also influence satiety; a study on adult male Sprague-Dawley rats on an amino acid supplemented diet indicated CML's precursor amino acid, lysine, increases satiety.[15]

While the effects of AGEs have been studied in several cell lines and their accumulation in diverse tissues *in vivo* have been assessed, less information is available on the potential effects on the stomach. In a study by Morioka *et al.*,[16] positive Glycer-AGE immunostaining was observed in the stomachs of healthy rats. Argirova and colleagues[17] assessed the effects of water-soluble Maillard reaction products (MRPs), derived from different model systems, on the mechanical activity of the gastric smooth muscle from rats. Several of the compounds applied in this work led to contractions, among which the reaction products formed between glucose and arginine produced the strongest effect. Furthermore, the authors reported the precursor carbohydrates and amino acids not to have an impact on the mechanical gastric smooth muscle activity. AGEs have also been reported to interfere with the expression of gastric smooth muscle contractile markers involving RAGE and NF-κB in streptozotocin-induced diabetic rats.[18] In a study by Wang *et al.*[19] gastric muscle tissue from patients undergoing gastric surgery was collected and the expression of contractile proteins correlated with the CML levels of individuals in a diabetic and a control group. Decreased expression of contractile proteins and phosphorylation levels of proteins involved in the contractile regulation were found in the diabetic group. In addition, this group showed increased CML concentrations in the gastric muscle layer. Fujiwara *et al.*[20] have shown dietary uptake of compounds possessing a catechol group to enhance the formation of CML. In their experiments, CML was shown to accumulate at the surface area of the gastric epithelial cells in the stomach of streptozotocin-induced diabetic mice which received 500 mg kg^{-1} day^{-1} epicatechin.

The human gastric tumour cell line (HGT-1), derived from a poorly differentiated adenocarcinoma of the stomach, was introduced by Laboisse and co-workers in 1982.[21] This cell line has since been shown to possess the transporters required in gastric acid release[22] and consequently been used in the determination of a test compound's effect on proton secretion using the fluorescent properties of a pH sensitive dye.[23,24] As little information is available on the effects of dietary derived AGEs on the stomach and HGT-1 cells have been shown to release serotonin upon stimulation[25] (unpublished data), we used this cell line in assessing the effects of free CML, its precursor glyoxal and protein-linked AGE-BSA on serotonin release. Further, we investigated a potential HTR3 link as serotonin has been suggested to alter gastric acid release *via* HTR3 receptors.[26]

Materials and methods

Materials

Synthetic CML, free from proteins and hydrochloride, was obtained from Iris Biotech (Markredwitz, Germany). AGE-BSA and FPS-ZM1 were ordered from Merck Millipore. Glyoxal and all other reagents required were ordered from Sigma-Aldrich (Austria).

Cell culture

HGT-1 cells were received from C. Laboisse (Laboratory of Pathological Anatomy, France) and cultured in Dulbecco's modified Eagle medium (DMEM). The medium was supplemented with 10% fetal bovine serum, 4 mM L-glutamine and 1% penicillin/streptomycin. Cells were maintained at 37 °C in a humidified incubator at 5% CO_2 and sub-cultured at 90% confluence.

Viability assay

Negative effects of any of the treatments on metabolic activity was excluded by MTT-assay (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide). Briefly, HGT-1 cells were seeded in 96-well plates at a density of 100 000 cells per well and left to settle overnight. Next, the medium was aspirated and test compounds added dissolved in either Krebs-Ringer HEPES buffer or serum-free medium. After incubation for 20 to 90 min, the compounds were removed and MTT working solution (diluted in serum-free medium to a final concentration of 1 mg mL^{-1}) added. After formation of purple formazan crystals (approx. 15 min), the MTT-working solution was replaced by DMSO and the absorbance recorded at 570 nm.

Gene expression

DNA microarrays. DNA microarrays were prepared using a maskless synthesis approach as described previously.[27] Two microarrays were synthesized at once as described by Sack *et al.*[28] HGT-1 cells were seeded in 6-well plates prior to incubation with either CML (500 μM, 5 mM) or glyoxal (0.3 μM, 3 μM) for 90 min in serum-free medium.[12] RNA was isolated using the RNeasy Mini Kit (Qiagen). To verify no DNA co-purification occurred and to check the integrity of the isolated RNA, agarose gel electrophoresis was carried out in addition to determining $A_{260/230}$ and $A_{260/280}$ ratios photometrically. Using the SuperScript III Kit (Invitrogen) and random nonamer primers carrying a Cy3-tag (Tebu Bio), 10 μg RNA were reverse transcribed[29] and cleaned up using the QIAquick PCR purification kit (Qiagen). The hybridization solution consisted of the generated Cy3-tagged cDNA, synthetic quality controls (25mer, 2× 60mer), BSA, herring sperm DNA and MES buffer. After allowing the DNA to hybridize for 20 h at 42 °C under constant rotation in the dark, the microarrays were washed. Afterwards, the microarrays were scanned using an Axon GenePix 4400A device (Molecular Devices). The scanned images were analyzed using NimbleScan 2.1 (NimbleGen) and normalized using robust multichip analysis. After extracting normalized intensities, fold-changes of CML/glyoxal-treatment

to the untreated control were calculated and genes reaching either 1.2 up- or down-regulation input into the DAVID pathway analysis software (http://david.abcc.ncifcrf.gov), which was used for identifying potential candidates[30] for further analysis.

qPCR

HGT-1 cells were seeded into 24-well plates at a density of 1.5×10^5 cells per well 24 h prior incubation with CML (500 μM) or glyoxal (3 μM) for 15–90 min. RNA was isolated using the PeqGold Total RNA kit (Peqlab), a spin-column based kit which employs DNA-removing columns in the first isolation step. RNA isolation was followed by reverse transcription using the high capacity cDNA kit (Life Technology). SYBR green master mix (Life Technology) was used in the qPCR reaction on a StepOnePlus device (Applied biosystems). The sequences of all primers used are shown in Table 1.[13,31] Data analysis was carried out using LinReg v2013.0 [32,33] and normalized to the expression of TBP (TATA-box binding protein) and PPIA (peptidylprolyl isomerase A).

Serotonin release

The serotonin concentration after stimulation with CML (5–5000 μM), glyoxal (0.03–30 μM), lysine (5–5000 μM), BSA (0.5–1 mg mL^{-1}) or AGE-BSA (0.5–1 mg mL^{-1}) was determined by ELISA (serotonin high sensitive, DLD Diagnostika) as described previously[34] with the following modification: the incubation time was extended from 5 min to 20 min.

CML concentration

The CML concentration of AGE-BSA and BSA was determined after hydrolysis as described by Teerlink *et al.*[8] Briefly, the protein (0.2–4 mg) was dissolved in water and sodium borohydride (100 mM in 200 mM sodium borate buffer pH 9.2) added. The mixture was incubated in a Pyrex glass reaction tube with a Teflon-lined screw cap for 2 h at room temperature before TFA (200 g L^{-1}) was added and the protein pelleted by centrifugation at 2000*g* for 10 min. The supernatant was removed prior to addition of another aliquot of TFA (100 g L^{-1}) and a further centrifugation step. After the removal of all TFA, HCl (6 M) and internal standard (^{13}C-CML, kindly provided by Dr John Baynes) were added. After heating the mixture to 110 °C for 20 h, the acid was evaporated and the solid material dissolved in LC-MS/MS starting eluent. The starting conditions were 90% acetonitrile and 10% of a 6 mM ammonium formate buffer (pH 2.2). Gradient component A was acetonitrile supplemented with 6 mM ammonium formate and gradient component B was aqueous 6 mM ammonium formate buffer (pH 2.2). The following gradient was set: 0–2 min 90% B, 3–5 min 87% B, 9–15 min 30% B, 20–30 min 90% B. The flow rate was set to 0.4 mL min^{-1}. The analysis was carried out on a Shimadzu 8040 LC-M/MS using a Luna 3 μm HILIC 200 A column (100 × 3 mm, Phenomenex) equipped with a HILIC 4 × 2.00 mm SecurityGuard pre-column. A total of 10 μL sample and standards was injected and a valve used for only allowing effluent between 9 and 15 min of the run time to enter the MS. The MS interface settings were as follows: nebulizing gas flow 3 L min^{-1}, DL temperature 170 °C, heat block temperature 350 °C, drying gas flow 13 L min^{-1}. Lysine, CML and ^{13}C-CML were detected in positive MRM mode. Table 2 shows the corresponding settings. The transition from 205 *m*/*z* to 83 *m*/*z* was used in the quantification of CML. The calibration range was 100–10 000 ng mL^{-1} for CML and 250–10 000 ng mL^{-1} for lysine.

Proton secretion

HGT-1 cells were seeded in a 96-well plate (FluoroNunc™ F96 MicroWell™, VWR) at a density of 100 000 cells per well. After allowing the cells to settle for 24 h, the cells were washed with Krebs-HEPES buffer (10 mM HEPES, 11.7 mM D-glucose, 4.7 mM KCl, 130 mM NaCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 1.2 mM KH_2PO_4, adjusted to pH 7.4 with 5 M KOH). Then, the cells were stained with the pH-sensitive fluorescent dye 1,5-carboxy-seminaphtorhodafluor acetoxymethylester (3 μM, SNARF-1-AM, Life Technologies) for 30 min before washing twice and adding test compound (100 μL, 500 μM CML, 5 mM CML) or standard (pH calibration pH 7.2–8.2, potassium, buffer calibration solution: 20 mM HEPES, 18 mM D-glucose, 110 mM KCl, 20 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$). The fluorescence was measured using an Infinite 200 Pro Plate reader (Tecan) with the excitation wavelength set to 488 nm and the emission recorded at 580 nm and 640 nm for 30 min. The intracellular proton index (IPX) was calculated using the pH calibration generated and the emission ratio and performing a $\log_2$ transformation.

Table 2 MRM settings used for the quantification of CML in AGE-BSA and BSA

Analyte	Precursor (*m*/*z*)	Product (*m*/*z*)	Dwell time (ms)	Q1 pre bias (V)	CE	Q3 pre bias (V)
CML	205	84	400	−12	−19	−13
CML	205	130	200	−16	−14	−20
Lys	147	84	100	−12	−10	−13
^{13}C-CML	207	130	100	−16	−14	−20

Table 1 Sequences of the primers used in qPCR

Target	Forward primer	Reverse primer	Product length (bp)
TBP	CCCGAAACGCCGAATATAATCC	GACTGTTCTTCACTCTTGGCTC	130
PPIA	CCACCAGATCATTCCTTCTGTAGC	CTGCAATCCAGCTAGGCATGG	144
RAGE	ACTACCGAGTCCGTGTCTACC	GGAACACCAGCCGTGAGTT	79

Statistical analysis

All data are displayed as average with standard deviation as stated in the corresponding table or figure legend. Data shown as fold-change (denominated T/C in the figures) were calculated in relation to the control which was set to 1 or 100%. For statistical analysis SigmaPlot 11 was used.

Results

Cell viability

None of the treatments applied had any negative effects on cellular viability as determined by MTT-assay (data not shown).

Gene expression

In the microarray experiments, HGT-1 cells were treated with CML (500 μM, 5 mM) or glyoxal (0.3 μM, 3 μM) for 90 min. The scatterplots of $\log_2$ intensities of treatment *vs.* control (Fig. 1) show a stronger impact on gene regulation by treatment with CML (1A, B) compared to glyoxal treatment (1C, D) as illustrated by a wider distribution of probes. A solid diagonal line represents a fold-change of 1, *i.e.* no difference between control and treatment. The scatterplots suggest treatment with 5 mM CML to have the biggest impact of the conditions tested. In the next step, probes reaching a fold-change of 1.2 up- or down-regulation were further assessed using the free online tool DAVID. The first cluster obtained after DAVID analysis is shown in Table 3 for CML (A: 500 μM, B: 5 mM) and in Table 4 for glyoxal (A: 0.3 μM, B: 3 μM). All four clusters were found to contain serotonin receptors, reaching similar enrichment scores each.

The mRNA expression of RAGE was analysed by qPCR after incubation with 500 μM CML or 3 μM glyoxal for 15, 30, 60 or 90 min (Table 5). Treatment with glyoxal resulted in alterations in RAGE expression at 30 min (0.73 ± 0.15, $p < 0.05$). The other incubations did not yield any changes in RAGE expression compared to the control.

Serotonin release

Exposure of HGT-1 cells to 500 μM or 5 mM CML increased the extracellular serotonin concentration to $341 \pm 241\%$ ($p < 0.05$, Fig. 3A) and $1247 \pm 438\%$ ($p < 0.05$, Fig. 2A), respectively, while glyoxal did not induce changes in the serotonin levels at any of the concentrations tested (Fig. 2B). The effect of 500 μM CML was not changed by co-incubation with the RAGE antagonist FPS-ZM1 ($100.5 \pm 39\%$, CML set to 100%, $n = 7$). Co-incubation with the HTR3 inhibitor granisetron decreased the CML-induced serotonin release by 36% (Fig. 3). Incubation with AGE-BSA at concentrations of 0.5 or 1 mg mL^{-1} decreased the serotonin concentration in the extracellular supernatant to $60 \pm 23\%$ ($p < 0.05$, Fig. 2D) and 49 ± 11 ($p < 0.05$, Fig. 2D), respectively. These effects were abolished in a co-incubation

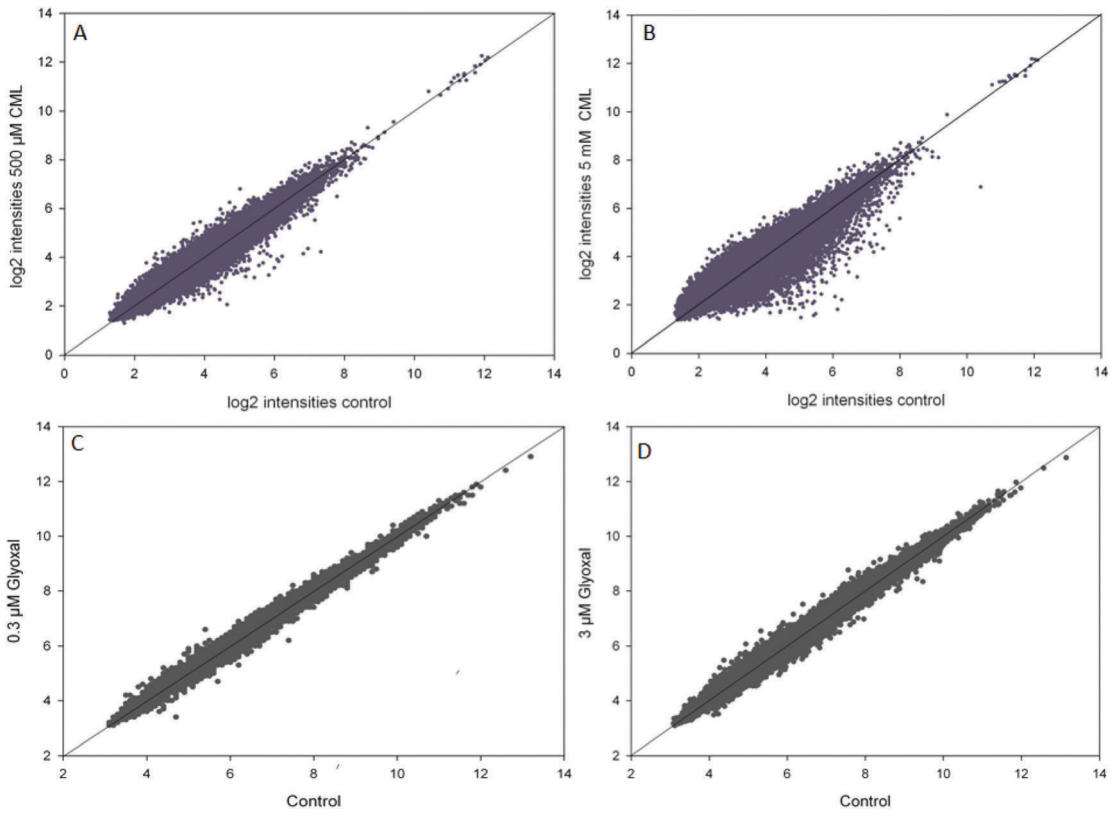

Fig. 1 Scatterplots of $\log_2$ transformed intensities of microarray data for control and treatment (A, B: CML; C, D: glyoxal), data are shown as average of at least three independent biological replicates.

Table 3 Microarray data after treatment with 500 μM CML (A) or 5 mM CML (B), showing the cluster with the highest enrichment score after DAVID pathway analysis. Probes reaching a fold-change below 0.8 or above 1.2 in all three biological replicates were used in the analysis

	p-Value	Benjamini
A: 500 μM CML		
Serotonin receptor signalling pathway	1.7×10^{-8}	8.0×10^{-5}
G-protein coupled serotonin receptor activity	4.4×10^{-6}	6.5×10^{-3}
Serotonin binding	7.1×10^{-6}	5.3×10^{-3}
5-Hydroxytryptamine receptor family	4.6×10^{-5}	1.1×10^{-1}
Neurotransmitter receptor activity	8.5×10^{-5}	2.5×10^{-2}
Behaviour	6.1×10^{-4}	3.6×10^{-2}
Regulation of behaviour	8.0×10^{-4}	5.3×10^{-1}
Release of sequestered calcium ion into cytosol	1.5×10^{-3}	5.1×10^{-1}
Serotonergic synapse	1.1×10^{-2}	2.1×10^{-1}
Vasoconstriction	2.0×10^{-2}	8.6×10^{-1}
Adenylate cyclase-inhibiting G-protein coupled receptor signalling pathway	3.6×10^{-2}	9.2×10^{-1}
Positive regulation of phosphatidylinositol biosynthetic process	5.0×10^{-2}	9.5×10^{-1}
B: 5 mM CML		
SM01381	1.1×10^{-10}	4.3×10^{-8}
Neuroactive ligand–receptor interaction	1.4×10^{-9}	3.8×10^{-7}
Receptor	5.2×10^{-8}	1.3×10^{-5}
Lipid moiety-binding region: S-palmitoyl cysteine	1.3×10^{-4}	1.1×10^{-1}
G-protein coupled receptor	4.3×10^{-4}	1.9×10^{-2}
Transducer	2.0×10^{-3}	6.3×10^{-2}
G-protein coupled receptor, rhodopsin-like	4.9×10^{-3}	9.6×10^{-1}
GPCR, rhodopsin-like, 7TM	8.3×10^{-3}	9.7×10^{-1}
G-protein coupled receptor signaling pathway	2.9×10^{-1}	1.0
G-protein coupled receptor activity	7.4×10^{-1}	1.0

Table 4 Microarray data after treatment with 0.3 μM glyoxal (A) or 3.0 μM glyoxal (B), showing the cluster with the highest enrichment score after DAVID pathway analysis. Probes reaching a fold-change below 0.8 or above 1.2 in all three biological replicates were used in the analysis

	p-Value	Benjamini
A: 0.3 μM glyoxal		
Neuroactive ligand–receptor interaction	1.4×10^{-11}	1.3×10^{-9}
SM01381	1.7×10^{-8}	1.1×10^{-6}
Receptor	8.2×10^{-8}	1.5×10^{-5}
G-protein coupled receptor	7.9×10^{-7}	7.3×10^{-5}
Transducer	1.9×10^{-6}	1.2×10^{-4}
Topological domain: extracellular	3.6×10^{-6}	1.2×10^{-3}
G protein-coupled receptor, rhodopson-like	8.3×10^{-5}	2.2×10^{-2}
GPCR, rhodopsin-like, 7TM	1.0×10^{-4}	1.4×10^{-2}
Transmembrane helix	2.0×10^{-4}	9.4×10^{-3}
Transmembrane	2.2×10^{-4}	8.1×10^{-3}
Topological domain: cytoplasmic	2.7×10^{-4}	4.5×10^{-2}
Integral component of plasma membrane	3.8×10^{-4}	5.8×10^{-2}
Glycosylation site: N-linked	1.1×10^{-3}	9.0×10^{-2}
Cell membrane	1.3×10^{-3}	3.0×10^{-2}
Transmembrane region	2.0×10^{-3}	1.3×10^{-1}
Plasma membrane	5.6×10^{-3}	3.6×10^{-1}
Glycoprotein	5.9×10^{-3}	9.6×10^{-2}
Membrane	6.0×10^{-3}	8.9×10^{-2}
Disulfide bond	8.2×10^{-3}	1.1×10^{-1}
Disulfide bond	9.1×10^{-3}	4.0×10^{-1}
Integral component of membrane	7.8×10^{-2}	8.4×10^{-1}
G-protein coupled receptor activity	1.8×10^{-1}	9.7×10^{-1}
B: 3.0 μM glyoxal		
G-protein coupled serotonin receptor activity	4.6×10^{-7}	1.6×10^{-4}
Serotonin receptor signaling pathway	9.6×10^{-6}	5.9×10^{-3}
Serotonergic synapse	1.0×10^{-4}	8.2×10^{-3}

Table 5 RAGE mRNA expression assessed by qPCR after treatment with glyoxal (3 μM) or CML (500 μM) for 15–90 min, data are shown as average and standard deviation of at least three independent biological replicates, statistics: two-way ANOVA *vs.* control, * = $p < 0.05$

	90 min	60 min	30 min	15 min
Control	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.08	1.00 ± 0.07
CML	1.01 ± 0.09	0.97 ± 0.02	0.93 ± 0.14	0.79 ± 0.08
Glyoxal	1.01 ± 0.05	0.82 ± 0.13	0.73 ± 0.15*	1.02 ± 0.24

experiment with FPS-ZM1. The co-incubation resulted in values of 79 ± 28% and 108 ± 60% for 0.5 and 1 mg mL^{-1} AGE-BSA. Unmodified BSA used at the same concentration did not alter serotonin release compared to the control ($p > 0.05$, data not shown). Similarly, FPS-ZM1 did not show any effects by itself ($p > 0.05$, data not shown). The treatment with 5, 50, 500 or 5000 μM lysine also did not change the extracellular serotonin concentration, leading to values of 148 ± 108%, 149 ± 86%, 108 ± 22% and 98 ± 22%, respectively (Fig. 2C).

Proton secretion

Incubation of HGT-1 cells with 500 μM CML did not induce any changes in proton secretion as calculated using the intracellular proton index (IPX), leading to an IPX value of −0.1203 ± 0.0382 (SEM) *vs.* control cells receiving buffer only. However, incubation with 5 mM CML resulted in a slight stimulation of proton secretion (Fig. 4), giving an IPX value of −0.1554 ±

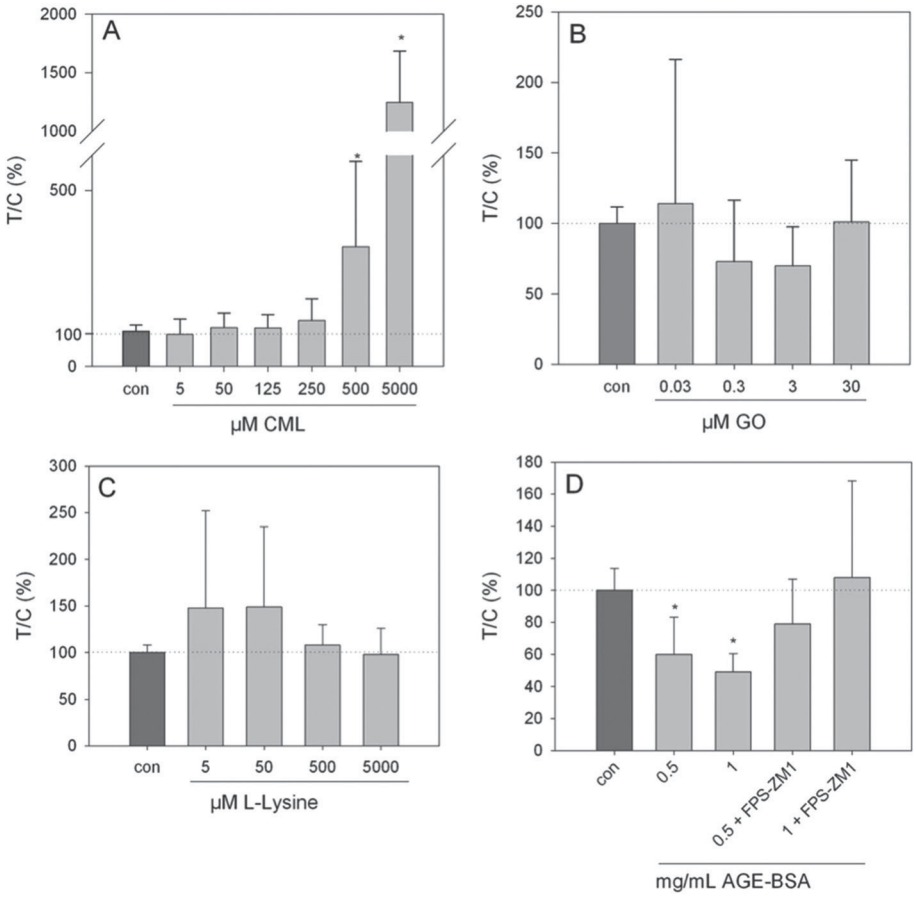

Fig. 2 Serotonin release from HGT-1 cells after 20 min treatment with CML (A, 5 µM–5 mM), glyoxal (B, 0.03–30 µM), lysine (C, 5 µM–5 mM) or AGE-BSA (D, 0.5–1 mg mL^{-1}) determined by ELISA, data are shown as average with corresponding standard deviation from at least three independent biological replicates, statistics: Kruskal–Wallis one-way analysis of variance on ranks, multiple comparisons *vs.* control (Dunn's method).

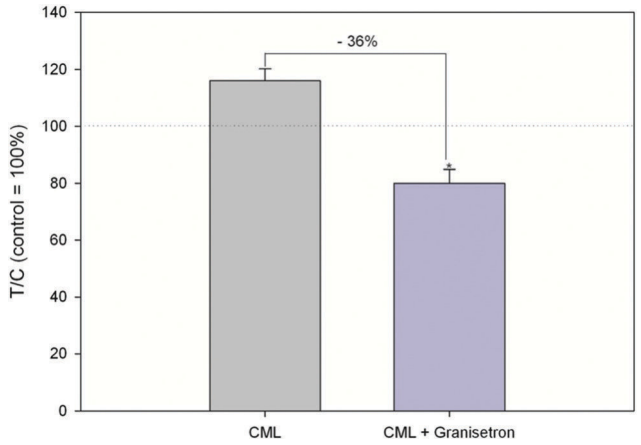

Fig. 3 Serotonin release after treatment with 500 µM CML or co-incubation of HTR3 antagonist granisetron (10 µM) and 500 µM CML, data are shown as average with corresponding standard deviation and calculated in relation to CML treatment (set to 100%), Statistics: Mann–Whitney rank sum test CML *vs.* control (p = 0.002; not depicted in graph), t-test CML *vs.* granisetron (* = $p < 0.001$).

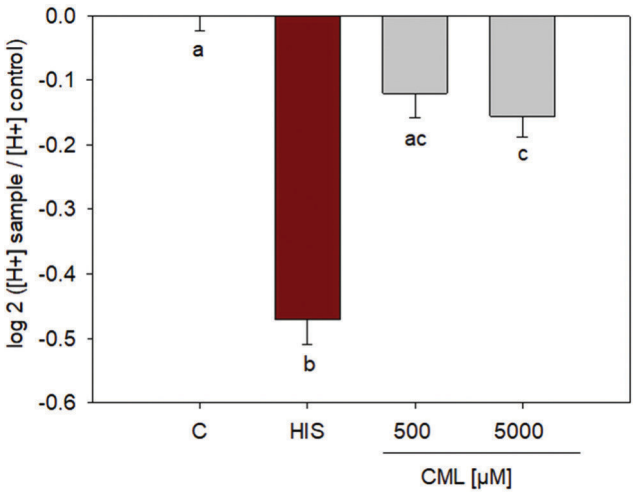

Fig. 4 Proton secretory activity after treatment of HGT-1 cells with 500 µM or 5 mM CML; n = 3, data are shown as average with corresponding standard error of the mean, one-way ANOVA followed by Holm-Sidak *post hoc* test.

0.0323 ($p < 0.05$, one-way ANOVA followed by Holm-Sidak *post hoc* test).

CML concentration

The analysis of AGE-BSA and BSA by LC-MS/MS showed CML to be present in both modified and unmodified protein. A CML concentration of 10.2 ± 0.65 mmol mol^{-1} lysine ($n = 3$) was found in the AGE-BSA tested and a concentration of 0.15 ± 0.08 mmol mol^{-1} lysine ($n = 3$) was detected in the BSA sample.

Discussion

Advanced glycation end products are ingested on a regular basis as part of a western diet. The advanced glycation end product CML and its precursor glyoxal are detectable in human plasma,[8,9] both in increased concentrations in individuals suffering from diabetes.[9,35] CML is predominantly found in a protein-linked state in plasma, but free CML has also been detected in concentrations up to 100 nM.[36] As such, most *in vitro* studies into the effects of CML are carried out using either modified BSA or casein. However, recently, a conversion of free CML to protein-linked CML *in vivo* has been suggested in a study on the long-term exposure of rats on a high fat diet to large quantities of free CML.[37] In addition, several studies showed comparable effects of free and protein-linked CML in different cell lines.[10,11] As CML formation in the stomach has been reported to increase in the presence of catechols,[20] and several Maillard reaction products have been indicated to exert influence on the mechanical activity of the gastric smooth muscle,[17] we assessed the effect of CML on human parietal cells in culture, deriving from an adenocarcinoma of the stomach (HGT-1 cells). Furthermore, we have recently shown this cell line to be capable of serotonin biosynthesis and release upon stimulation.[25] In our previous work on human SH-SY5Y cells, used as a model system of central serotonin, we could show treatment with 500 μM CML to increase serotonin release.[13] Similarly, we have reported treatment with 500 μM CML to increase serotonin release from Caco-2 cells,[12] indicating CML to influence serotonin release from peripheral sources, thus leading us to test whether CML also influenced gene expression of serotonin related genes and serotonin release from HGT-1 cells, representing cells from another peripheral site. First, we analysed the genome-wide impact of free CML and its precursor glyoxal on the gene expression of HGT-1 cells by microarray. Cluster analysis revealed both CML and glyoxal to influence the expression of serotonin receptors. As RAGE has been described to be overexpressed in gastric cancer cells compared to adjacent non-cancerous tissue,[38] we analysed RAGE gene expression by qPCR. This experiment showed RAGE to be expressed by HGT-1 cells and glyoxal to alter its gene expression. CML, however, had no impact on RAGE gene expression. Next, we assessed the impact of glyoxal and CML on extracellular serotonin levels. This showed CML to have a strong impact on extracellular serotonin levels, while glyoxal showed no effect. However, the potential degradation of serotonin was not assessed, and whether the enzyme required for serotonin degradation, MAO-A, is expressed in HGT-1 cells is unknown. As a result, the total serotonin concentration may be underestimated, and it is unknown whether glyoxal or CML have any impact on a potential serotonin degradation, which may also result in an increased or decreased serotonin release compared to untreated control cells.

In order to test whether the increase in serotonin release after treatment with CML is related to RAGE, we carried out co-incubation experiments with the RAGE antagonist FPS-ZM1. This, however, unlike our experiments on Caco-2 cells,[12] had no impact. As we used a longer incubation time compared to our Caco-2 study, and RAGE protein expression seems likely from our qPCR results, we next set out to elucidate whether a RAGE-mediated mechanism could be shown using a commercially available RAGE ligand, AGE-BSA, thus demonstrating that the cell system used in this study is capable of showing RAGE-related mechanisms. Unexpectedly, this experiment revealed the opposite effect on serotonin release compared to free CML. The treatment with AGE-BSA decreased serotonin release from HGT-1 cells and was reversible by co-incubation with the RAGE antagonist, indicating a RAGE-mediated pathway.

In order to exclude the impact of AGE-BSA to be caused by the protein itself regardless of the degree of modification, we analysed native BSA. BSA did not to have any influence on serotonin release, indicating, like the experiment using the RAGE antagonist, the effect of AGE-BSA is caused by the protein modification. Next, we determined the CML concentration in the modified and unmodified BSA. This showed, as expected, that the modified protein contained significantly higher amounts of CML. However, the highest concentration of free CML used, 5 mM, roughly corresponds to 1 mg mL^{-1} which is identical to the highest concentration of AGE-BSA used, thus making the CML concentration in the AGE-BSA incubation significantly lower than used in the incubations with free CML. However, a number of other modifications may be present in the AGE-BSA, which may also explain the opposite effect compared to the incubation with free CML. To test if the CML-induced serotonin release was caused by the amino acid itself rather than the lysine-modification, we incubated the cells with L-lysine in the same concentration range as CML. However, treatment with lysine could not induce the effects observed after incubation with CML. Recently, the amino acid L-arginine has been described to induce serotonin release from HGT-1 cells in a likely HTR3-mediated manner[25] (unpublished data). In an attempt to clarify whether, similarly to L-Arg, the CML-induced serotonin release is HTR3-related, we carried out co-incubation experiments with the HTR3 inhibitor granisetron. In this case, the effect of 500 μM CML on serotonin release were counteracted, indicating a HTR3-related pathway. This pathway may be further analysed to elucidate the differences between AGE-BSA and free CML in terms of serotonin release. A study by Collison *et al.*[39] described a link

between RAGE activation and Ca^{2+} mobilization. Furthermore, a link between AGE, RAGE and Ca^{2+} homeostasis of the myocardium has been shown. This study by Petrova *et al.*[40] showed myocardial overexpression of RAGE to reduce the systolic and diastolic Ca^{2+} concentration. Calcium ions and their role in neurotransmitter release are well described (see review by Augustine[41]) and differences in Ca^{2+} mobilization after treatment with free CML or AGE-BSA may be involved in the serotonin release induced by these compounds. Furthermore, HTR3 receptors are ion channels[42] and, thus, form an exception compared to the G-protein coupled HTR receptors. In this study, we only investigated the involvement of HTR3, thus it cannot be excluded that other HTRs may also be involved and account for the discrepancy in observed serotonin release. SERT activity and potential 5-HT degradation were not analysed and should also be taken into consideration in further experiments as the 5-HT degradation product 5-hydroxyindole acetic acid has been reported to be biologically active. For instance, it has been demonstrated to decrease glucose uptake in a study on isolated rat soleus muscle.[43] Differences in the intracellular signalling cascade utilizing mediators such as Ca^{2+}, cAMP or pERK/ERK will also have to be addressed in future studies. However, we consider the effect of CML on HTR3-mediated pathways as physiologically relevant, in particular because an HTR3-related link between serotonin and gastric acid release has been suggested by Lai *et al.*[26] In addition, the unmodified amino acid L-Lys has been reported to delay gastric emptying in a dose dependent manner in both rats and humans.[44] As a result, we assessed the impact of CML on proton secretion. This showed the highest concentration tested, 5 mM to have a small, yet significant, stimulating effect, suggesting the influence of CML on factors involved in proton secretion to be further examined in future studies.

In conclusion, our current work shows AGE-BSA to reduce serotonin release in a likely RAGE-dependent manner. Furthermore, we show a likely HTR3-mediated, RAGE-independent impact of free CML on serotonin release in HGT-1 cells, in addition to showing an effect of free CML on cellular proton secretion.

Abbreviations

AGE	Advanced glycation endproduct
BSA	Bovine serum albumin
CML	N^{ε}-Carboxymethyllysine
MRP	Maillard reaction product
RAGE	Receptor for advanced glycation end products
T/C	Treated over control

Conflicts of interest

There are no conflicts to declare.

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2.5 "Serotonin biosynthesis and release from human gastric tumour cells (HGT-1) and its functional role in arginine-induced proton secretion."

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Serotonin biosynthesis and release from human gastric tumour cells (HGT-1) and its functional role in arginine-induced proton secretion

Short title: Serotonin biosynthesis by HGT-1 cells

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Abbreviations:

5HT serotonin

AADC aromatic amino acid decarboxylase

HGT-1 human gastric cancer cell line

KRHB Krebs-Ringer-HEPES buffer

PCPA p-chlorophenylalanine

SERT serotonin transporter

TPH tryptophan hydroxylase

Abstract

Among mammals, most of the body's serotonin is located in the gastrointestinal tract where it has been shown interact with many pathways. However, little information is available on serotonin and its potential function in the stomach. We investigated whether HGT-1 cells, derived from an adenocarcinoma of the stomach, are capable of serotonin synthesis and consequent release. Thus, HGT-1 cells and human stomach specimen were immunostained to indicate the presence of the neurotransmitter. Incubation with the tryptophan hydroxylase inhibitor p-chlorophenylalanine reduced the fluorescence signal intensity obtained from HGT-1 cells by 27%. Treatment with 30 mM L-Arg increased extracellular serotonin to 147 ± 18 % compared to control cells. Granisetron, a 5-HT_3 receptor antagonist, reduced the L-Arg induced serotonin release and proton secretion. Gastric antral mucosa specimen stained positive for serotonin and treatment of human stomach strips with 30 mM L-Arg resulted in an increase in muscle tension to 149.9 ± 55.29 % (control set to 100%) *ex vivo*. Taken together, the *in vitro* data gathered from HGT-1 cells suggests L-Arg to impact serotonin release and proton secretion in a likely HTR3-related mechanism. The *ex vivo* data showed serotonin to be present in the human stomach and L-Arg to influence gastric motility.

Keywords: Human gastric tumour cells, serotonin release, immunofluorescence, proton secretion

Introduction

Within the central nervous system (CNS) the monoamine serotonin (5HT), synthesised from the essential amino acid L-tryptophan, is an important neurotransmitter associated with a manifold of different disorders such as schizophrenia (1) ,depression (2) in the regulation of satiety (3, 4). Serotonin is also synthesised in large amounts outside the CNS, especially within the enterochromaffin cells in the mucosa of the gastrointestinal (GI) tract (5).. Choi et al. (6) carried out investigations into the distribution of different cell lineages on three healthy human donor stomachs. Their analysis revealed distinct patterns of enteroendocrine cells, showing serotonin-positive cells in the proximal stomach and in the antrum. In addition, the presence of the serotonin transporter (SERT) in the antrum was demonstrated. The release of serotonin from the upper GI tract has long been known to play a key role in the mechanism of certain forms of nausea and vomiting. Clinically, serotonin may also be of importance in a number of gastrointestinal diseases. In a study on constipation-predominant IBS patients, diarrhea-predominant IBS patients and healthy controls, diarrhea-predominant IBS patients were shown to have increased platelet-depleted plasma serotonin concentrations under fed and fasting conditions (7). Consequently, serotonin receptor antagonists have been suggested in the treatment of IBS. The serotonin type 3 receptor antagonist alosetron has been described to relieve abdominal discomfort and pain in a multicentre, randomized, double-blind, placebo-controlled study on women suffering from diarrhea-predominant IBS (8). Serotonin is thought to mostly act locally, stimulating and sensitising abdominal vagal nerve terminals which project to the brainstem; in some circumstances plasma concentrations of serotonin may also be increased, sufficient to pass through the blood-brain-barrier (9). While serotonin has been studied in the context of gastrointestinal diseases, less information is available on the influence of peripheral serotonin, released postprandially from the enterochromaffin cells, on gastrointestinal mechanisms regulating satiety. Two studies carried out in the early eighties suggest peripheral administration of serotonin to induce hypophagia (10, 11). Recently, peripheral serotonin has been indicated to influence glucose and lipid metabolism (12). A role for 5-HT released from endocrine cells by the presence of acid within the lumen (13) and entering the blood circulation has also been to an ability to inhibit gastric acid secretion (14). It is important to understand how serotonin is released from endocrine cells in response to stimulation with different compounds. For this purpose, several cell line models have been established. These include neuroblastoma cells (SH-SY5Y) (15) and enterochromaffin cells (QGP-1) (16), but also the human colon carcinoma cell line Caco-2

has been reported to synthesize and degrade serotonin (17). Thus, we aimed at investigating the capacity of a stomach-derived cell line model, human gastric tumour cells (HGT-1) to synthesise and release serotonin after nutrient- stimulation and to investigate serotonin's functional role for gastric acid secretion.

The human gastric cell line HGT-1 was established by Laboisse and co-workers (18) from a poorly differentiated adenocarcinoma on the posterior wall of the body of a patient's stomach in 1982. HGT-1 cells have been shown to express histamine H_2 receptors, which have been demonstrated to mediate adenylate cyclase activation and cAMP production (18). Furthermore, this cell line has been described to express acetylcholine receptors, the H^+/K^+ ATPase proton pump, an omeprazol-binding site possessing K^+ channel (19), angiotensin 1-converting enzyme (20), and transporters required for gastric acid secretion (21). HGT-1 cells have consequently been established as a valuable cell model for assessing the effects of different components, e.g. compounds encountered in coffee beverages, on proton secretion by measuring the intracellular pH value (22, 23). Here, we demonstrate this cell model's capacity of synthesizing serotonin and its subsequent secretion in response to exogenous stimuli by incubating with L-Arg, an amino acid which as recently reported to influence food intake (24), delay and inhibit gastric emptying and enhance gastric adaptive relaxation (25). As 5-HT3 receptors have been suggested to be involved in gastric acid release (26) and HGT-1 cells are capable of proton secretion, we carried out co-incubation experiments with the 5-HT_3 inhibitor granisetron (27).

Materials and Methods

Materials

All reagents were obtained from Sigma-Aldrich (Austria) unless stated otherwise. Anti-5HT and Anti-TPH2 antibodies were purchased from Merck-Millipore (Austria).

Cell culture

The human gastric tumour cell line (HGT-1, Dr. C. Laboisse, Nantes, France) was cultivated using Dulbecco's modified Eagle medium (DMEM) containing 4.5 g L^{-1} glucose. The medium was supplemented with 4 mM L-glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin (100 units penicillin, 171 µM streptomycin). Cells were maintained in

a humidified incubator at 5% CO_2 and sub-cultured at 90% confluence, using cells between passage 60 and 80 in experiments. Caco-2 cells were obtained from ATCC (American Type Culture Collection) and maintained in DMEM containing 4.5 g L^{-1} glucose, 10% fetal bovine serum and 1% penicillin/streptomycin. QGP-1 cells were cultivated in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Cell Viability Assay

HGT-1 cells were seeded in 96-well plates at a density of $1x10^5$ cells per well and left to settle for 24 h. Next, the cells were incubated with Krebs-Ringer-HEPES buffer (KRHB, supplemented with 0.1% ascorbic acid, pH 7.4) or serum-free DMEM for 5 min. Then the incubation solution was replaced with MTT working solution (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide, 100 µL, 1 mg mL^{-1}) and incubated for 15 min at 37°C in a humidified incubator. Finally, the MTT working solution was removed, 150 µL DMSO added and the absorbance read at 570 nm.

qPCR

HGT-1 cells were seeded in 24-well plates at a density of $1.5x10^5$ cells per well. The cells were treated with L-Arg for 3 h prior to RNA isolation using the PeqLab total RNA kit (Peqlab, VWR, Austria). Then, the isolated RNA was reversely transcribed with the high capacity cDNA kit (Applied Biosystems, Thermo Fisher Scientific). The synthesized cDNA was added to a qPCR mix containing fast SYBR green master mix (Applied Biosystems, Thermo Fisher Scientific) and the amplification assessed on a StepOnePlus device (Applied Biosystems, Thermo Fisher Scientific). Tata-box binding protein (TBP), glyeraldehyde 3-phosphate dehydrogenase (GADPH) and peptidylprolyl isomerise A (PPIA) were used as endogenous controls. The sequences of all primers are provided in Table S1. Primers have either been designed using PrimerBlast or have been taken from PrimerBank. LinReg v2013.0 was used in the data analysis.

DNA Microarrays

Microarray analysis was carried out as described previously (28), using a reaction chamber allowing for the simultaneous synthesis of two microarrays (29). HGT-1 cells were seeded in

6-well plates and left to settle for 24 h. Thereafter, cells were incubated with 50 mM L-Arg for 3 h prior to RNA isolation (RNeasy Mini Kit, Qiagen). Microarray labelling, hybridization and analysis were carried out as described previously (15), however an Axon GenePix 4400A was used for scanning the microarrays.

TPH2 expression

TPH2 expression was determined by Western Blot. Briefly, HGT-1 cells were seeded in 6-well plates, left to grow for 24h, washed with ice-cold PBS and lysed with RIPA buffer supplemented with protease inhibitor cocktail, PMSF and, sodium vanadate. The lysate was passed through a 22-gauge needle and centrifuged at full speed and 4°C for 15 min. Next, the protein concentration in the supernatant was assessed by Bradford assay. A total of 20 µg protein or 3 µL pre-stained protein standard (New England Biolabs) were loaded per lane of a 16% polyacrylamide SDS-gel. After electrophoresis, the proteins were transferred onto an activated (2 min methanol, 5 min blotting buffer) PVDF membrane. The membrane was blocked using 5% BSA dissolved in TBS-T for 1 h at room temperature. Then, primary antibody (rabbit anti-TPH2, 1:200, Merck Millipore or rabbit anti-β-actin, 1:2000, Santa Cruz) diluted in blocking solution was added and incubated over night at 4°C. After washing four times with TBS-T, secondary antibody (goat anti-rabbit, 1:2000, CST cell signalling) diluted in blocking solution was added and incubated for 1 h at room temperature. Afterwards, four washing steps with TBS-T were carried out prior to pipetting 500 µL Signal Fire Chemiluminescence reagent (New England Biolabs) onto the membrane. Finally, after 5 min incubation at room temperature an image was recorded using a Fusion FX7 device (Vilber). Image analysis was carried out using ImageJ.

Aromatic Amino Acid Decarboxylase (AADC) activity

The method used in the assessment of the enzymatic activity of the aromatic amino acid decarboxylase was modified from Vieira-Coelho et al. (17) Briefly, HGT-1 cells grown to confluence in a T175 flask were washed with HBSS and detached from the flask by scraping after addition of 1 mL HBSS. The cells were then subjected to three freeze-thaw cycles in liquid nitrogen, homogenised using a Potter-Elvehjem device, passed through a small-bore needle, and centrifuged at 4°C (full speed, 15 min). A total of 100 µL of the supernatant was added to incubation medium (100 µL) and incubated for 15 min at room temperature. The incubation medium consisted of: tolcapone (4 µM), paragyline (300 µM), pyridoxal

phosphate (240 µM), sodium borate (110 µM), Na_2HPO_4 (150 µM), KH_2PO_4 (350 µM) and HBSS. Next, 200 µL 5-HTP (0 – 9 mM) and ascorbic acid, giving a final concentration of 0.1%, were added. After 15 min incubation in the dark, 60 µL 6 M hydrochloric acid were added and centrifuged at 4°C for 15 min. The supernatant was passed through a 0.2 µm filter prior to injecting 50 µL onto the HPLC column. The analytical method used is described in the LC-MS/MS section of the supplemental information. The total protein content of the cell lysate was determined by Bradford assay.

Serotonin staining

Immunofluorescence (HGT-1 cells)

For serotonin staining, HGT-1 cells were seeded onto glass coverslips located in a 24-well plate. p-chlorophenylalanine (100 µM) (30) was added to one part of the cell suspension during seeding. After two days, the cells were washed once with ice-cold PBS and fixed with a mixture of acetone and methanol (50:50 % v/v) for 2 min at -20°C. The fixed cells were stained using a primary anti-serotonin antibody. The detailed procedure is described in the supplemental information.

Immunohistochemistry (HGT-1 cells, tissue)

Histological specimens were obtained from two patients from the Pathologisch-Bakteriologisches Institut, Sozialmedizinisches Zentrum Ost – Donauspital, Vienna, Austria after signing a written informed consent that covers all diagnostic procedures on the extracted tissue. One specimen derived from a 79-year old female patient undergoing distal partial gastrectomy for benign peptic ulcer with perforation. The other specimen derived from a 47-year old male patient with proximal high-grade adenocarcinoma. Immunohistochemistry was performed on 5 µm thick formalin-fixed, paraffin-embedded whole tissue sections. Slides were processed in the fully automated staining instrument Benchmark ULTRA using ultraView Universal DAB Detection Kit (Ventana Medical Systems). The primary antibody M0758 (clone 5HT-H209, Agilent DAKO) was applied at 1:5 for 32 minutes at 37°C. No pretreatment was performed. All counterstaining was performed with hematoxylin. Blocking experiments in order to control for unspecific staining were performed using normal mouse IgG1 serum replacing the primary antibody. All other steps were performed similar to the staining procedure as described above.

Serotonin release

Serotonin ELISA

Serotonin release was carried out as described previously (31). A more detailed procedure is given in the supplemental information.

Gastric motility

Stomach specimens from bariatric surgery were used for this study. Tissue collection was approved by the local ethical committee in the UK (REC 15/LO/2127) and patients gave written informed consent. The proximal region of the stomach was separated in the mucosa and muscularis mucosa by blunt dissection. Afterwards, 5-8 x 15 mm muscle strips were mounted in 10 mL organ bath chambers equipped with isometric force transducers (MLT0201, AD Instruments, Chalgrove, UK) and filled with Krebs' solution (gassed with 95 % O_2, 5 % CO_2), maintained at 37^0C. After 1 h of tissue equilibration under 2 g tension and changing bath solution every 15 minutes, nerves were excited by electrical field stimulation (EFS). This was given every 10 s for 1 min at 5 Hz, 0.5 ms bipolar pulse duration and 200 mA with a stimulator (STG2008, Scientifica, Uckfield, UK). When the responses to EFS, usually consisting of cholinergically-mediated muscle contraction (32), were continuously consistent a frequency response curve was constructed by application of EFS at 1,2,5,10,15 and 20 Hz, followed by changing bath solutions and switching back to 5 Hz. Strips were then treated with 30 mM L-Arginine for 15 minutes (afterwards precipitation in the Krebs' solution occurred). Changes in baseline tension were determined before and 5 min after application on the top of the peak for at least 3 EFS-induced responses. Data acquisition was done by Biopac Inc. CA, USA.

Proton secretory activity

Proton secretory activity and corresponding data analysis were determined as described previously (22, 23). HGT-1 cells were treated with 0.1 – 10 µM serotonin, 30 mM L-arginine. Furthermore, co-incubation experiments with 5-HT_3 receptor inhibitor granisetron (10 µM) or SERT inhibitor fluoxetine were carried out.

LC-MS/MS

Briefly, tryptophan, 5-hydroxytryptophan and serotonin in the cellular supernatants collected were analysed in a single run by LC-MS/MS in positive MRM mode. The detailed method is described in the supplemental information.

Statistical analysis

SigmaPlot 11 was used for statistical analysis. Data are presented as average ± SEM or standard deviation. The number of biological replicates is stated in legend of the corresponding figure or table. Treatments are shown in relation to control cells set to 1 or 100%, labelled as treated over control in the figures. Image J was used for reading out the fluorescence of single cells. Proton secretion data was subjected to Nalimov's outlier test prior to data analysis.

Results

Cell viability

Cell viability was assessed using the MTT assay. None of the treatments led to a significant decrease in metabolically active HGT-1 cells compared to the corresponding control cells. ($p > 0.05$, data not shown).

Serotonin release

The serotonin-sensitive ELISA detected serotonin in the supernatants collected from cells treated with Krebs-Ringer HEPES buffer (+0.1% ascorbic acid). Compared to Caco-2 cells ($3.74 \times 10^{-5} \pm 8.27 \times 10^{-6}$ pg serotonin corrected for cell number and incubation volume) and QGP-1 cells ($9.56 \times 10^{-6} \pm 4.81 \times 10^{-6}$ pg serotonin corrected for cell number and incubation volume), the highest serotonin concentration was detected in HGT-1 cells ($2.76 \times 10^{-4} \pm 1.49 \times 10^{-6}$ pg serotonin corrected for cell number and incubation volume).

Serotonin biosynthesis

The presence of the two enzymes required in the biosynthesis of serotonin was analyzed. The expression of TPH2 was determined by Western blotting. This yielded a single band at approx. 61 kDa on average, fitting the expected molecular weight of approx. 56 kDa (Figure 3A-B). β-Actin was used as a loading control. Likewise, this showed one band (Figure 3A).

The expression of AADC was tested by the capability of cellular lysate to react 5-HTP provided in excess to serotonin. This experiment showed a rise in the determined serotonin with increasing 5-HTP concentrations (Figure 3C). From this graph, a Lineweaver-Burk plot was derived which led to a value of 0.28 mM for K_m and 85 nmol mg protein^{-1} h^{-1} for v_{max}.

Effects of L-Arg on gene expression, serotonin release, proton release and gastric motility

Gene expression

First, the effects of 50 mM L-Arg on the gene expression of HGT-1 cells were assessed in a genome wide screening using customized cDNA microarrays. The scatter plot shown in Figure S2 shows a broad distribution of probes, suggesting a strong impact of L-Arg treatment on gene expression. Pathway analysis using the freeware DAVID, suggested the expression of serotonin receptors to be altered (Table 1A, B). As a result, we first assessed the expression of *HTR1A, 2A, 1B, 3A, 3B, 3C, 3D, 3D, 4* and *7*, in addition to the genes encoding the enzymes required in serotonin synthesis and the serotonin reuptake transporter *SLC6A4*. This showed *SLC6A4, TPH1, TPH2, HTR3C, HTR3D* and *HTR7* to be consistently expressed over 4-5 biological replicates. However, *HTR1B, HTR3B* and *HTR3E* were only detectable in some of the samples. *HTR1A, HTR2A, HTR3A* and *HTR4* were detected in none of the samples tested. Treatment of HGT-1 cells with L-Arg for 3 h regulated the gene expression of *SLC6A4, TPH2, HTR3C* and *HTR7* (Table 2).

Serotonin staining

Figure 1 shows a typical result of HGT-1 cells stained using an anti-serotonin antibody. Figure S1 shows an additional HGT-1 staining experiment using another antibody.
In a second set of experiments, HGT-1 cells were seeded in either DMEM or DMEM containing 100 µM PCPA. In both cases, positive serotonin staining was observed (Figure 1D-E). However, cells that received PCPA at seeding, exhibited less pronounced serotonin staining (Figure 1E). After image analysis with Image J, a fold-change of 0.73 was calculated, indicating a reduction in serotonin staining by 27% in the PCPA treated group. In the absence of a blocking peptide, the primary anti-5HT antibody was pre-incubated with pure serotonin for 30 min prior to the incubation. In this experiment, control cells receiving the pre-blocked version of the antibody showed a reduction in serotonin staining by 23%,

suggesting that part of the antibody was prevented from binding to cellular serotonin by the pre-incubation. Figure 2 shows characteristic staining for serotonin in human benign and carcinoma stomach samples with positivity in scattered single cells.

Serotonin release

Figure S3A shows a chromatogram of the supernatant collected from HGT-1 cells. Both L-Trp and 5-HT were detected in the supernatant. However, the 5-HTP concentration was too low to be detected by LC-MS/MS. The incubation with 30 mM L-Arg increased the serotonin concentration in the cellular supernatant to 147 ± 18 % compared to cells treated with KRHB only (+0.1% ascorbic acid; Figure S3B). This result was verified by LC-MS/MS where an increase to 162 ± 48 % was detected (Figure S3C). No significant difference between the neutral pH control and the control adjusted to pH 9.5 was found (data not shown in figure). Similarly, no difference between L-Arg simply added to KRHB and the pH adjusted L-Arg solution was detected (data not shown in figure).

In the experiment using 10 µM granisetron, the L-Arg induced serotonin release was prevented by the 5-HT_3 antagonist, reducing the increase to 124 ± 23% observed after L-Arg treatment to 95 ± 16%, compared to the control set to 100%. Granisetron alone did not impact the serotonin release compared to control cells treated with KRHB only (Figure 4).

Proton secretion

After a 10 min incubation time with 0.1 µM serotonin, HGT-1 cells showed an inhibition of the proton secretion in comparison to the untreated control analysed by means of intracellular proton index [IPX]: 0.21 ± 0.03 vs. control> -0.00 ± 0.02) (Figure 5A). Statistics were done for 3-6 biological and 6 technical replicates with Kruskal-Wallis one Way ANOVA on Ranks with Holm-Sidak post hoc test vs. non-treated control, $p < 0.05$. Incubation with 10 µM of the 5-HT_3receptor antagonist granisetron reduced the L-Arg induced proton secretion from IPX -3.48 ± 0.13 to -3.00 ± 0.53
($p < .0.05$, Figure 5B), while incubation with SERT inhibitor fluoxetine showed no impact (data not shown).

Gastric motility

After 15 minutes treatment of the stomach strips with 30 mM L-Arg, traces showed no consistent changes in responses to EFS (data not shown) but an increase of the baseline gastric muscle tension up to 150 ± 55 % in comparison to the 100 % vehicle control (Figure 5C).

Discussion

The indole amine serotonin has been described to exert a wide variety of actions in the human body in health and disease, reaching from its role in gastrointestinal function and the regulation of nausea, vomiting and satiety to several brain disorders such as Schizophrenia. Several cell lines have been shown to release serotonin upon stimulation, among which enterochromaffin QGP-1 cells and enterocyte-like Caco-2 cells represent cell models of peripheral serotonin release. As several reports indicate the presence of serotonin in the stomach, we investigated the capacity of a cell line originating from an adenocarcinoma of the stomach to synthesize and consequently release serotonin into the cellular supernatant upon stimulation. Moreover, a functional role of serotonin on mechanisms regulating gastric acid secretion was elucidated.

First, we analyzed the expression of the enzymes involved in the biosynthesis of serotonin on the functional level. In an attempt to assess the activity of TPH in HGT-1 cells, we could not see a robust increase in 5-HTP with increasing substrate concentrations of L-Trp. In Caco-2 cells, a similar challenge was described by Nakamura (30) and co-workers, who opted to use living cells rather than a cell-free enzyme assay and were successfully showing *de novo* synthesis of serotonin by using the TPH inhibitor p-chlorophenylalanine. In order to still show the presence of TPH on the protein level, we carried out Western blot experiments using an antibody directed against TPH2. This revealed a single band with the expected molecular weight of the targeted protein. The isoform TPH2 has been reported to be primarily expressed in the brain, while TPH1 has been shown to be abundantly expressed in the periphery. However, TPH2 expression was shown in the intestine, although to a weaker extent than TPH1 and more restricted in its distribution (30). Similar to the study by Vieira-Coe et al. (17), reporting the ability of Caco-2 cells to synthesize and degrade serotonin, we determined the activity of AADC based on their method. In this experiment, we showed an increase in the serotonin concentration with increasing substrate concentrations of 5-HTP, thereby indicating the presence of functional AADC. Taken together, the protein expression

of TPH and the presence of functional AADC suggest HGT-1 cells to be capable of synthesising serotonin. To conclusively show that serotonin is in fact present in HGT-1 cells, we performed immunostaining experiments, using two different antibodies directed against serotonin. In both cases, HGT-1 cells stained positive for serotonin. In an experiment using the TPH inhibitor PCPA, we observed a decrease in the signal intensity in cells being treated with the inhibitor compared to cells receiving culture medium only. Furthermore, human adenocarcinoma excised from the proximal stomach showed scattered single cell staining positive for serotonin. This pattern is similar to the expression pattern of serotonin-cells in healthy stomach tissue, in which only a low number of serotonin expressing cells was found compared to other cell types present (6). This finding is also concordant with the literature demonstrating only a low number of scattered carcinoma cells with serotonin expression. In addition, only a very low percentage of serotonin expression in gastric adenocarcinomas has been described (33). Regardless of the number of cells staining positive, this suggests serotonin to be present in proximal stomach samples of both healthy and diseased donors. Next, we compared the serotonin content of HGT-1 cells to cell lines which have been described to release serotonin upon stimulation in previous studies. In the cellular supernatants of the enterochromaffin cell line QGP-1 and differentiated Caco-2 cells, serving as cell model of enterocytes, a serotonin-sensitive ELISA detected smaller quantities than in the supernatant collected from HGT-1 cells. L-Tryptophan (L-Trp) has to be present in the cells in order to synthesise serotonin. However, the antibody used in the ELISA may cross-react with L-Trp when it is present in high concentrations. As the actual L-Trp level in the supernatant collected from HGT-1 cells was unknown, we analysed L-Trp, 5-HTP and 5-HT by LC-MS/MS to exclude the Trp level in the sample exceeds the level reported to cross-react by the ELISA manufacturer. This showed L-Trp to be present, but not in concentrations high enough to interfere with the ELISA. Interestingly, we detected both L-Trp and serotonin, but not 5-HTP. This fits with the conversion of L-Trp to 5-HTP being the rate limiting step, and hence formed 5-HTP being reacted to serotonin immediately.

In addition to showing the enzymes required in the biosynthesis of serotonin on a functional level, we assessed the mRNA expression of the cultured cells, including several serotonin receptors and the serotonin reuptake receptor SERT by qPCR. This demonstrated *TPH1, TPH2, SLC6A4, HTR3C/D* and *HTR7* to be expressed. However, we failed to detect the second enzyme required in the synthesis of serotonin, *AADC*. As we were able to show AADC activity, and AADC is a prerequisite in the biosynthesis of serotonin, it seems likely

that the AADC mRNA level was below the limit of detection of our qPCR method. In addition, mRNA expression of the receptor subtypes *HTR1A, HTR2A* and *HTR3A* was below the limit of the detection. However, genes encoding for serotonin receptors have been described in stomach tissue previously. A study by Van Lelyveld (34) reported very low gene expression levels of *HTR3A* and *HTR3B* in the stomach. Furthermore, the authors showed expression of *HTR3C, HTR3E* and *HTR4* in both antrum and fundus of healthy donor samples (34). Among the currently known seven serotonin receptor families, 5-HT_3 receptors are the only ones belonging to the family of cys-loop ligand-gated ion channels (35). Serotonin has been suggested to modulate the secretion of gastric acid *via* this subtype of serotonin receptors (26). Furthermore, a link between intragastric pH and serotonin release has been shown using perfused rat stomach (36). In this study, at an intragastric pH of 2, the basal serotonin release into the vasculature was reported to be ten times higher than that into the gastric lumen (36). This may be of particular interest in HGT-1 cells as they have previously been used to study the influence of a number of compounds on proton secretion (22). Hence, we tested the effect of serotonin on proton secretion from HGT-1 cells, showing incubation with 0.1 µM serotonin to significantly reduce proton secretion, thus suggesting serotonergic pathways may influence proton secretion in this cell line similarly as suggested by Lai et al. (26). The 5-HT_3 receptors are also of interest in the context of gastric emptying. An *in vivo* study on male Wistar rats carried out by Doihara et al. (37) reported a delay in gastric emptying by TRPA1 agonists involving a serotonergic pathway. In this study, a delay in gastric emptying was observed after administration of 1 mg x kg^{-1} serotonin. The effect of TRPA1 agonists on gastric emptying was abolished in experiments using either the TPH inhibitor PCPA or the 5-HT_3 receptor inhibitor granisetron. Additionally, serotonin has been suggested to play a role in emesis (38) (35).

As a HTR3-involving delay in gastric emptying after serotonin administration has been described previously(37), we assessed the effect of L-Arg, an amino acid recently shown to delay and inhibit gastric emptying in a study on male Sprague-Dawley rats (25) and to enhance gastric adaptive relaxation, on gastric motility on human stomach strips. This indicated L-Arg to increase baseline gastric muscle tension. Next, we tested the effect of L-Arg on serotonin release from HGT-1 cells. This showed, both by ELISA and LC-MS/MS, treatment with L-Arg to increase the serotonin content in the cellular supernatant collected. The increase could either be due to a rise in serotonin release or due to an impact on the serotonin transporter SERT. *SERT* expression has been reported in the stomach (39) and

could also be detected by qPCR in our study. Our microarray experiment revealed treatment with L-Arg to have a strong impact on gene expression of HGT-1 cells. Pathway analysis with the free online tool DAVID showed the cluster with the second highest enrichment score to consist predominantly of genes involved in serotonin release. As a result, we carried out qPCR experiments, showing L-Arg to alter the gene expression of *HTR3C*, *HTR7*, *SERT*, and *TPH2*. Interestingly, even though TPH2 has been described to be most abundant in the brain, we could detect this isoform by Western blot and treatment with L-Arg influenced only the expression of *TPH2*, not however, *TPH1*. As L-Arg influenced the expression of *HTR3* and the *HTR3C* subunit has previously been described in the modulation of gastric acid secretion (26), we carried experiments into a possible link between L-Arg induced serotonin release and the 5-HT_3 receptor by performing co-incubation experiments with the 5-HT_3 receptor antagonist granisetron. The increase in extra-cellular serotonin after treatment with L-Arg was prevented by co-incubation with granisetron, indicating a 5-HT_3 receptor-mediated pathway may be involved. A similar control of serotonin release by 5-HT_3 autoreceptors has been shown for enterochromaffin cells from isolated intestinal segments of guinea pigs (40). Moreover, a study showing delayed gastric emptying after serotonin administration, revealed this effect to be abolished by either inhibiting the 5-HT_3 receptor or TPH as well (37). In the present study, the effect of L-Arg on proton secretion was decreased by granisetron co-incubation, pointing to involvement of 5-HT_3 receptors not only in the regulation of serotonin release, but also of the proton secretion HGT-1 cells.

In order to test whether the results obtained from HGT-1 cells may point to a possible regulation *in vivo* and may thus be used as a screening system for identifying potentially interesting compounds, will need to be addressed in either human intervention studies or animal trials. In addition, the mechanism linking gastric acid and serotonin release may be analysed further in HTR3 knock-out models and by identifying cellular mediators involved such as Ca^{2+} mobilization, cAMP or ERK activation.

In conclusion, this study shows serotonin synthesis and release from HGT-1 cells as model system for peripheral serotonin in the stomach. Furthermore, experiments using the 5-HT_3 receptor antagonist granisetron suggest this serotonin autoreceptor to be involved in both proton and serotonin secretion, although, to elucidate the clinical impact serotonin may have after release from endocrine cells of the stomach, animal trials and human intervention studies need to be conducted.

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Declaration of Interests

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Author contributions

Author contributions: A.K.H., V. Stöger, B.L., M.Z., J.H., A.R., G.J.S., and V. Somoza designed research; A.K.H., V. Stöger, B.L., M.Z., J.H., A.R., performed research; G.J.S. and V. Somoza contributed new reagents/analytic tools; A.K.H., V. Stöger, B.L., M.Z., M.P., J.H., A.R., G.J.S., and V. Somoza analyzed data; and A.K.H., B.L., G.J.S., V.Stöger, and V. Somoza wrote the paper.

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Table 1: Clusters generated by DAVID functional annotation clustering software using the microarray data gathered after 3h incubation with L-Arg or DMEM only.

A:cluster 1, enrichment score: 3.41		
	p-value	Benjamini
disulfide bond	1.14E-05	3.75E-03
glycoprotein	4.90E-04	3.30E-02
glycosylation site: N-linked	1.00E-03	2.80E-01
signal peptide	1.90E-03	3.90E-01
signal	3.40E-02	5.20E-01

B: cluster 2, enrichment score: 3.14		
	p-value	Benjamini
serotonin receptor signaling pathway	2.30E-07	4.60E-04
G-protein coupled serotonin receptor activity	2.30E-06	1.30E-03
serotonin binding	5.00E-05	1.40E-02
serotonergic synapse	1.70E-04	1.30E-02
5-hydroxytryptamine receptor family	8.10E-04	4.90E-01
neurotransmitter receptor activity	2.70E-03	3.10E-01
release of sequestered calcium ion into cytosol	1.50E-02	8.90E-01
adenylate cyclase-inhibiting G-protein coupled receptor signaling pathway	2.40E-02	9.20E-01
vasoconstriction	5.80E-02	9.60E-01
dendrite	2.10E-01	9.30E-01

Table 2 qPCR data obtained after treating HGT-1 cells with 30 mM L-Arg for 3h. Data are shown as average ± SEM; n = 4-5, tr= 2-3; Statistics: Mann-Whitney Rank Sum Test

	control	**L-Arg**	**p-value**
TPH1	1.00 ± 0.04	1.50 ± 0.16	0.078
TPH2	1.00 ± 0.11	10.0 ± 2.0	<0.001
SLC6A4	1.00 ± 0.05	10.8 ± 1.2	<0.001
HTR7	1.00 ± 0.06	2.88 ± 0.57	0.034
HTR3D	1.00 ± 0.11	2.83 ± 0.75	0.138
HTR3C	1.00 ± 0.09	2.62 ± 0.45	<0.001

Figure legends

Figure 1 Immunofluorescence of HGT-1 cells. Overlay of nuclear staining and serotonin staining (A), serotonin staining only (B), nuclear staining only (C). A representative image is shown; at least three biological replicates and multiple technical replicates have been stained. Immunofluorescence of HGT-1 cells treated either with DMEM only (D) or tryptophanhydroxylase inhibitor PCPA (E). n=1, tr=2

Figure 2 Immunohistochemical staining for serotonin in human stomach samples. Gastric antral mucosa demonstrating cytoplasmic reactivity in scattered individual enterochromaffin-like cells within gastric glands, overview (A), detail (B).
Gastric adenocarcinoma with cytoplasmic reactivity in scattered individual carcinoma cells, overview (C), detail (D).

Figure 3 Western blot analysis of the protein expression of TPH2. (A) Three biological replicates have been tested for both TPH2 expression and the endogenous control □-actin. (B) Representative image of the full-length blot showing the protein ladder and the TPH2 band. (C) Graph of the serotonin formed normalized to protein concentration and incubation time *vs.* the substrate concentration. Data were collected from 3-4 biological replicates with 1-2 technical replicates.

Figure 4 Influence of 10 µM of the 5-HT_3 receptor antagonist granisetron on L-Arg induced serotonin release. Data are shown as average ± standard deviation calculated in relation to L-Arg (set to 100%). n = 3-4, tr = 2; Statistics: One-Way ANOVA *vs.* control followed by Holm-Sidak *post hoc* test.

Figure 5 (A) Impact of incubation with 0.1 – 1 µM serotonin on proton secretion in HGT-1 cells, n= 3-6, tr= 6; Statistics: one-way ANOVA on Ranks followed by Dunn's *post hoc* test *vs*. control, (B) Impact of 10 µM 5-HT_3 receptor antagonist granisetron on L-Arg induced proton secretion, n= 3, tr= 3-6, Statistics: one-way ANOVA followed by Holm-Sidak *post hoc* test, (C)

Impact of incubation with 30 mM L-Arg on gastric motility of human stomach strips. Representative traces for a vehicle control (H_2O) and a 30 mM L-Arg treatment

Figure 1

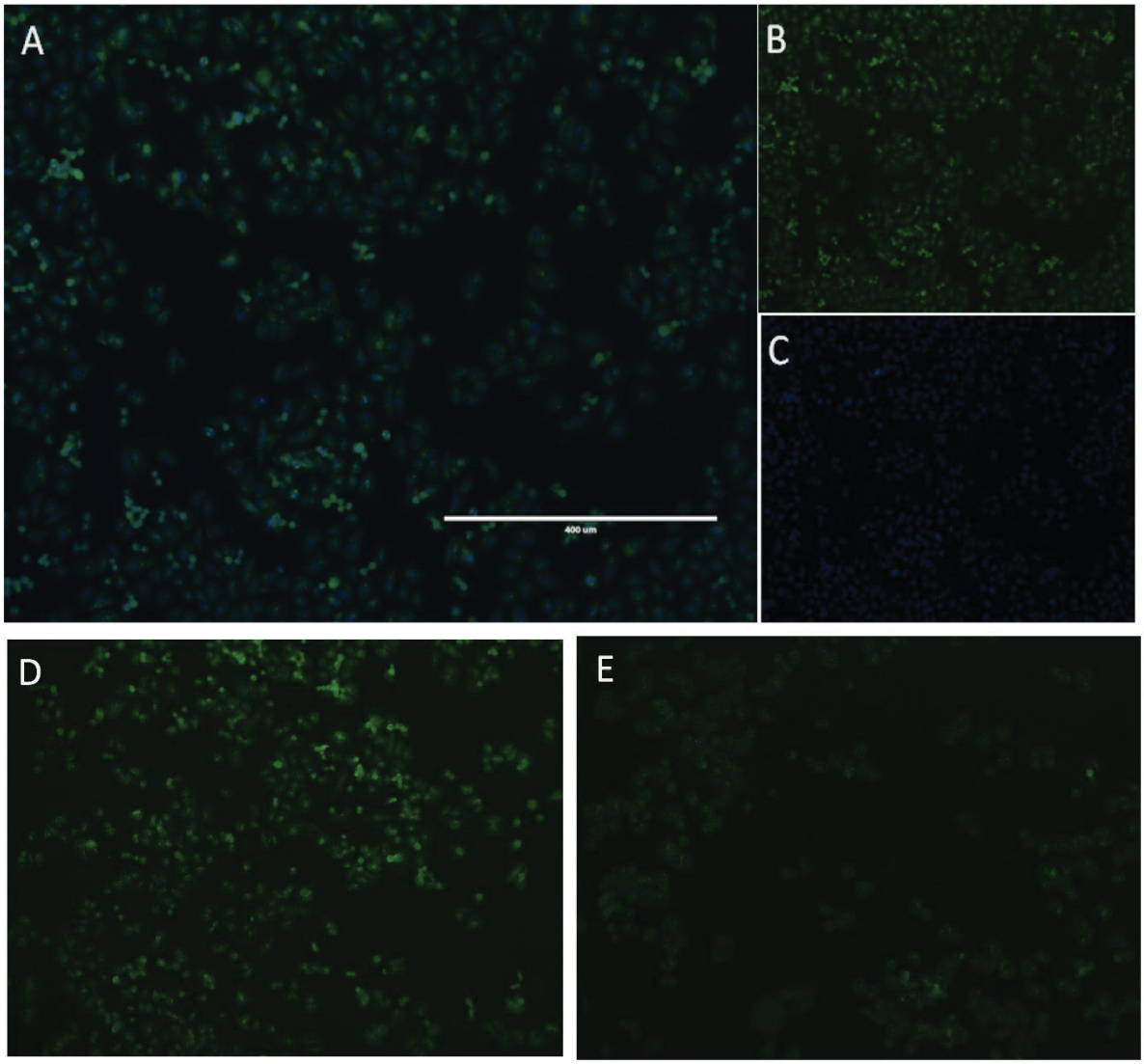


Figure 2

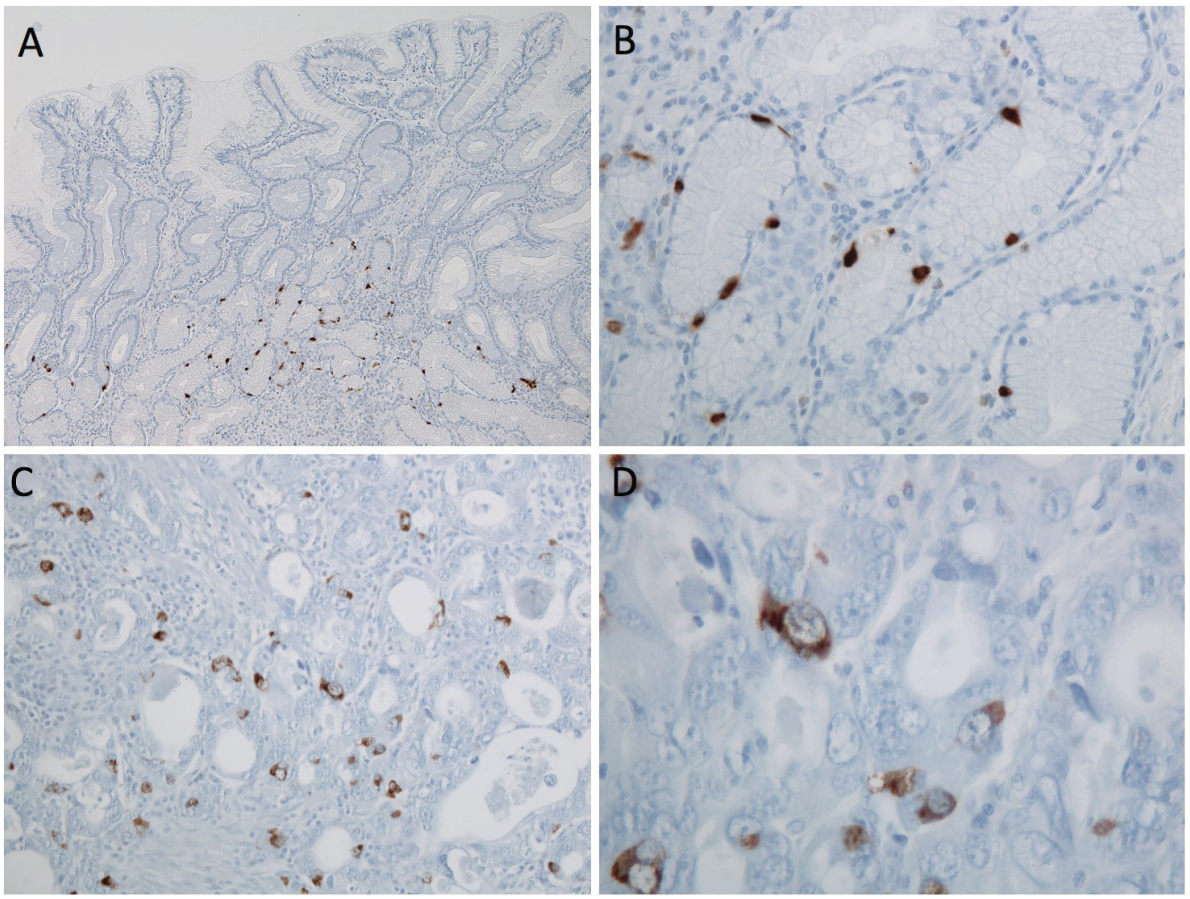


Figure 3

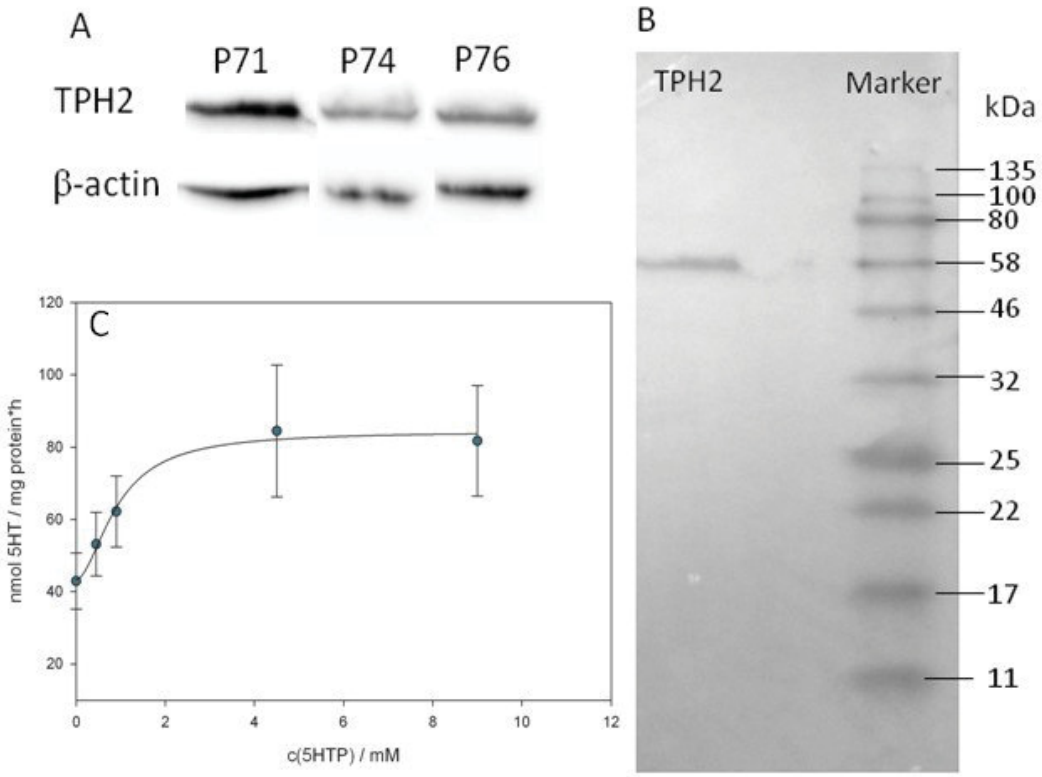

A
P71
P74
P76
TPH2
β-actin
B
TPH2
Marker
kDa
135
100
80
58
46
32
25
22
17
11
C
nmol 5HT / mg protein*h
c(5HTP) / mM
120
100
80
60
40
20
0
2
4
6
8
10
12

Figure 4

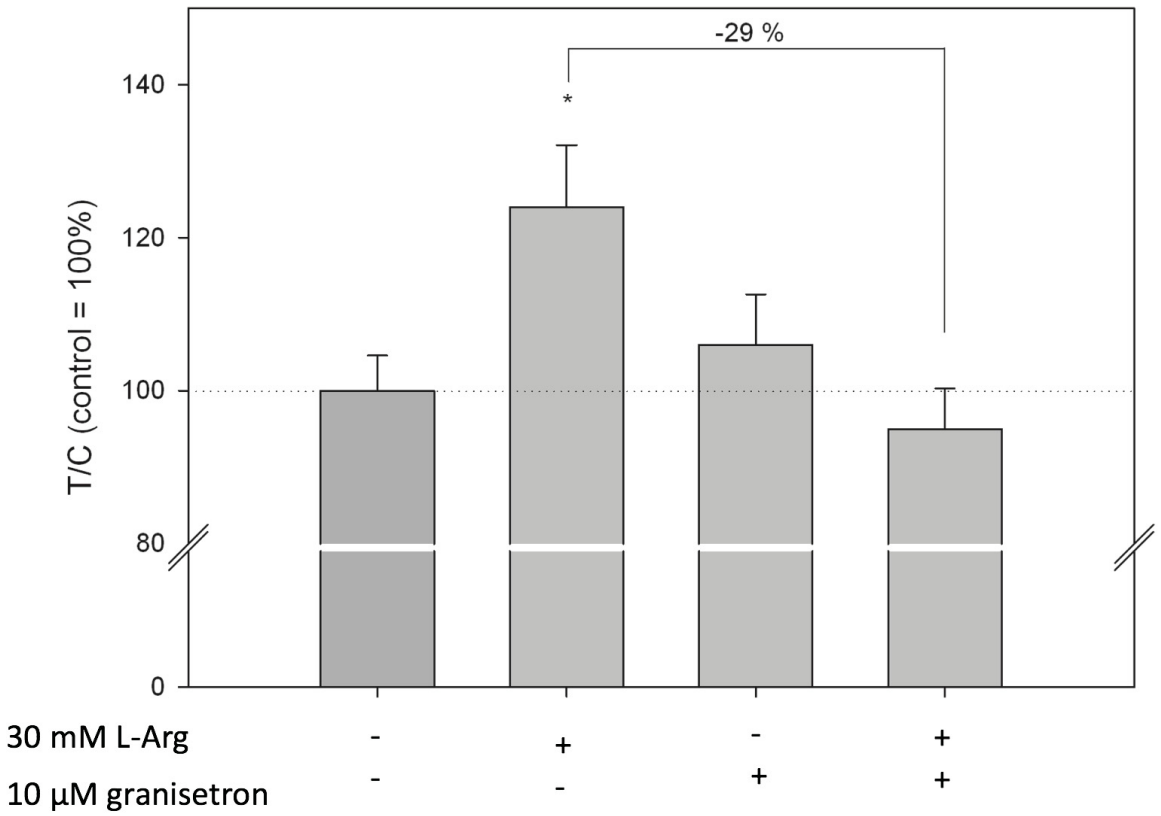
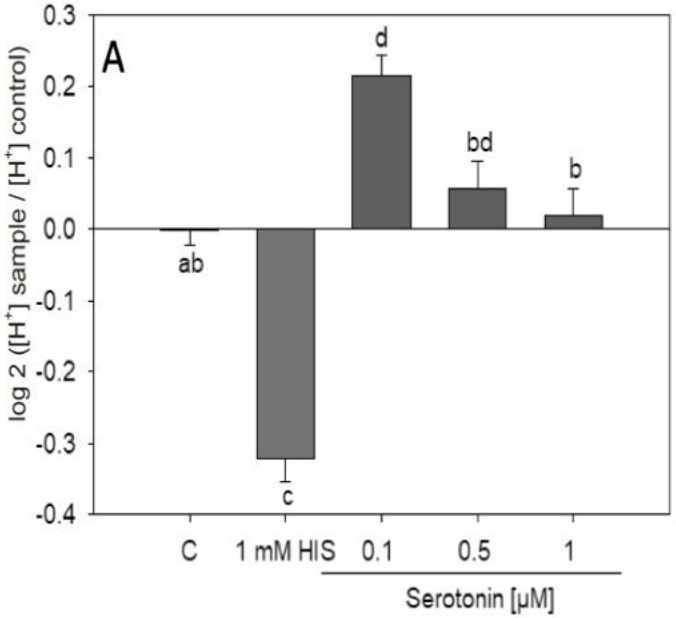
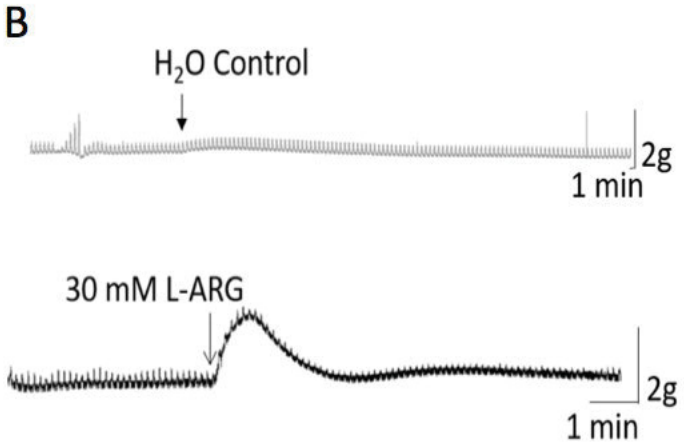


Figure 5


A
log 2 ([H+] sample / [H+] control)
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
ab
c
d
bd
b
C
1 mM HIS
0.1
0.5
1
Serotonin [µM]


B
H2O Control
2g
1 min
30 mM L-ARG
2g
1 min

112

Supplemental information

Methods and materials

Immunofluorescence (HGT-1 cells)

Next, the fixed cells were washed twice with PBS and blocked for 1 h with 5% FBS and 0.5% Triton X-100 in PBS (blocking solution) at room temperature and gentle agitation. Then, the solution was exchanged with primary antibody (1:100, anti-serotonin, raised in rabbit, Merck Millipore) diluted in blocking solution and incubated first at 37°C prior to incubation for 15 min at room temperature with gentle agitation. Thereafter, the cells were washed four times with PBS for five minutes each. Then, secondary antibody (1:1000, Alexa-488, goat anti-rabbit (Molecular Probes, Thermo Fisher Scientific), diluted with PBS containing 5% FBS and 0.2% Triton X-100) was added and incubated for 1 h at room temperature with gentle agitation. Afterwards, the cells were washed four times with PBS for 5 min each. During the last wash, 5 µL of DAPI stain (fixed cells nucBlue, Molecular Probes, Thermo Fisher Scientific) were added. Finally, the coverslip was briefly dipped into ddH_2O and mounted using Immu-Mount solution (Life Technologies, Thermo Scientific). Fluorescent images were taken using an EVOS FL microscope (Thermo Fisher Scientific) equipped with an Olympus 10x objective. For control purposes, images of untreated cells, cells treated with primary antibody only and cells treated with secondary antibody only were taken. For testing the specificity of the primary antibody, some coverslips were incubated with primary antibody, which was exposed to pure serotonin for 30 min prior to the experiment. Data analysis was carried out using Image J, assessing the fluorescence of at least 150 cells per image of two technical replicates.

Serotonin ELISA

Briefly, HGT-1 cells were seeded in 24-well plates at a density of $1.5x10^5$ cells per well. One day after seeding, the cells were washed with pre-warmed PBS and subsequently incubated with 200 µL KRHB (supplemented with 0.1% ascorbic acid, pH 7.4) or 30 mM L-Arg for 5 min in a humidified incubator at

37°C in the dark. Since L-Arg induced a change in the pH, both a pH control and a pH adjusted L-Arg solution were tested in addition. The incubation solutions having a pH of 9.5 were passed through a 0.22 µm filter prior to incubation to remove insoluble material. To assess whether 5-HT_3 receptors are involved, co-incubations with 10 µM 5-HT_3 antagonist granisetron were carried out. After incubation, the supernatant was collected, diluted 1:5 with KRHB and the serotonin concentration assessed using a serotonin-sensitive ELISA (DLD Diagnostika, Hamburg, Germany). Caco-2 cells were seeded in 12-well plates and differentiated for 21 days, within which period the culture medium was exchanged every two to three days. On day 21, the cells were washed with pre-warmed PBS, incubated for 5 min with 150 µL KRHB (+0.1% ascorbic acid), the supernatant collected and used in the ELISA without further dilution. Similarly, QGP-1 cells were seeded at a density of 250 000 cells per well in 24-well plates, settled for 72 h, and incubated with 250 µL KRHB (+0.1% ascorbic acid) and the collected supernatant centrifuged for 5 min at 1,000x g at 4°C and tested for its serotonin content without further dilution.

LC-MS/MS

The cells were seeded and incubated as described above. However, the collected supernatants were acidified with 0.1% formic acid, and passed through a 0.2 µm filter prior to LC-MS/MS analysis. A total of 50 µL was injected. The analysis was carried out on a Shimadzu LC-MS 8040 equipped with a Synergi 4 µm Fusion-RP 80 A column (150 x 2 mm, Phenomenex) and a security guard cartridge (Phenomenex). A binary gradient of water (+0.1% formic acid, A) and acetonitrile (+0.1% formic acid, B) was used in the chromatographic separation: 0-3 min 0% B, 3-10 min change to 90% B, 13-18 min 90% B, 18-23 min change to 0% B, 23-30 min 0% B. In order to protect the MS from contamination, the eluent was directed to the waste outlet from 0 to 2 min and 8.7 to 30 min, while everything eluting between 2 and 8.7 min was directed to the MS. The MRM settings chosen are shown in Table 2. The MS interface was set as follows: nebulizing gas flow 3 L min^{-1}, DL temperature 150°C, heat block temperature 350°C

and drying gas flow 17 L min^{-1}. The calibration range used for the quantification of serotonin in HGT-1 supernatants was 0.4 – 8.3 ng mL^{-1}.

Tables

Table S1 Sequence and product size of the primers used for qPCR experiments

Target	Forward primer	Reverse primer	Product size
TPH1	TAAGACCTGGGGAACCGTATT	TGGAAAAACCTGTACGCTCTTT	173
TPH2	ATCTCGGCGAAGAAGTTCTGA	CAGGGCACATCCTCTAGCTC	165
AADC	TGGGGACCACAACATGCTG	TCAGGGCAGATGAATGCACTG	121
HTR1A	TCATCGTGGCTCTTGTTCTG	CGGGGTTAAGCAGAGAGTTG	108
HTR1B	CTGGTGTGGGTCTTCTCCAT	AGAGGATGTGGTCGGTGTTC	109
HTR2A	GTTGCTTACTCGCCGATGATA	TGCCAAGATCACTTACACACAAA	144
HTR3A	GAAGCCAACCACCGTATCCAT	CCACATCCACGAACTCATTGAT	218
HTR3B	TCTCCCTACCTCTAAGTGCCA	CTCAATGGTCCCAGATGAGTTC	116
HTR3C	TTCCGGTCTCACTGCCTATATC	AAGGTGAAGGTACAGTTCTGTTG	129
HTR3D	CCCTACGTGGTAAACTTTCTGG	TGTGATGAAGTGCTAGTGGCT	179
HTR3E	AGACGCATCCCGGAACATC	GGCACGAGAAGGTTTATGACA	165
HTR7	CTCCATCACCTTACCTCCACTC	ATGCCACTGCGGTAGAGTAAAT	110
SERT	ACGGAGTTCTACAGAAGGTTGT	ATAGAGTGCCGTGTGTCATCT	118
TBP	CCCGAAACGCCGAATATAATCC	GACTGTTCTTCACTCTTGGCTC	130
GAPDH	AGGTCGGAGTCAACGGATTTG	GGGGTCATTGATGGCAACAATA	95
PPIA	CCACCAGATCATTCCTTCTGTAGC	CTGCAATCCAGCTAGGCATGG	144

Table S2 MRM settings for the simultaneous detection of L-Trp, 5-HTP and serotonin by LC-MS/MS

Target	Transition (m/z)	Dwell time (ms)	Q1 pre bias (V)	CE (V)	Q3 pre bias (V)
L-Trp	205 -> 146	400	-12	-19	-13
5-HTP	221 -> 204	400	-12	-13	-13
	221 -> 162	400	-12	-19	-13
5-HT	177 -> 160	400	-12	-13	-13
	177 -> 115	400	-12	-30	-13

Figures

Figure S1 (A) Serotonin staining of HGT-1 cells; (B) Serotonin staining of HGT-1 cells at higher magnification

Figure S2 Scatter plot of log2 transformed intensities of control and treatment with L-Arg for 3h. The diagonal line represents a fold change of 1, i.e. equal intensity in control and treatment.

Figure S3 Typical chromatogram of the cellular supernatant collected from HGT-1 cells, showing the traces of L-Trp, 5-HTP and 5-HT (A). In the absence of 5-HTP, its retention time is shown with an arrow. Serotonin in the cellular supernatant after treatment of HGT-1 cells with 30 mM L-Arg for 5 min analysed by ELISA (B, n=5) or LC-MS/MS (C, n=4). Data are shown in relation to the control (set to 100%). (D) Typical chromatogram obtained after injection of a mixed standard of 5-HT, 5-HTP and L-Trp. The chromatogram shows 5-HTP at a retention time of 5 min.

Figure S1

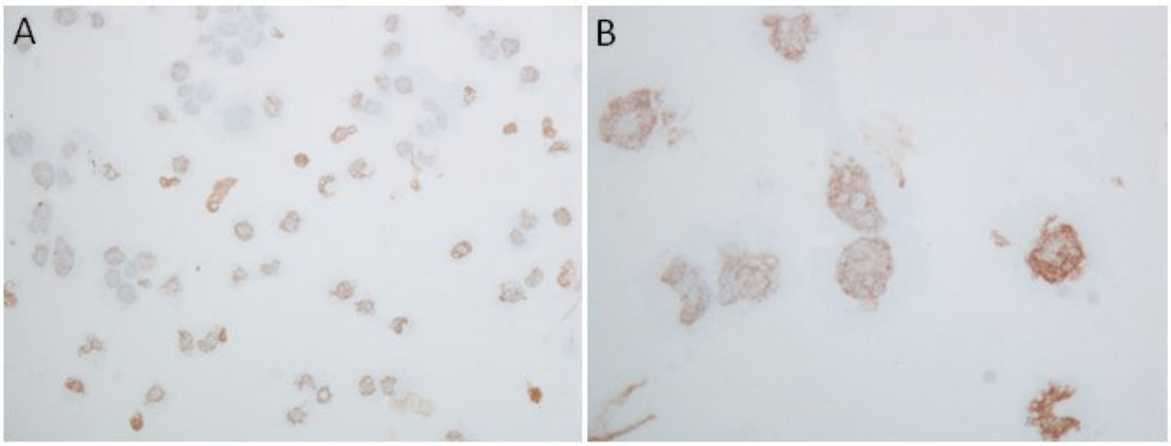


Figure S2

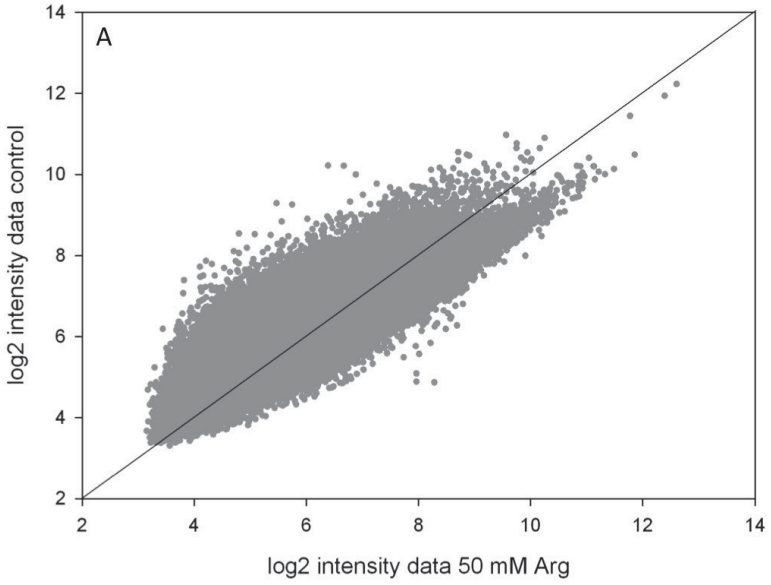
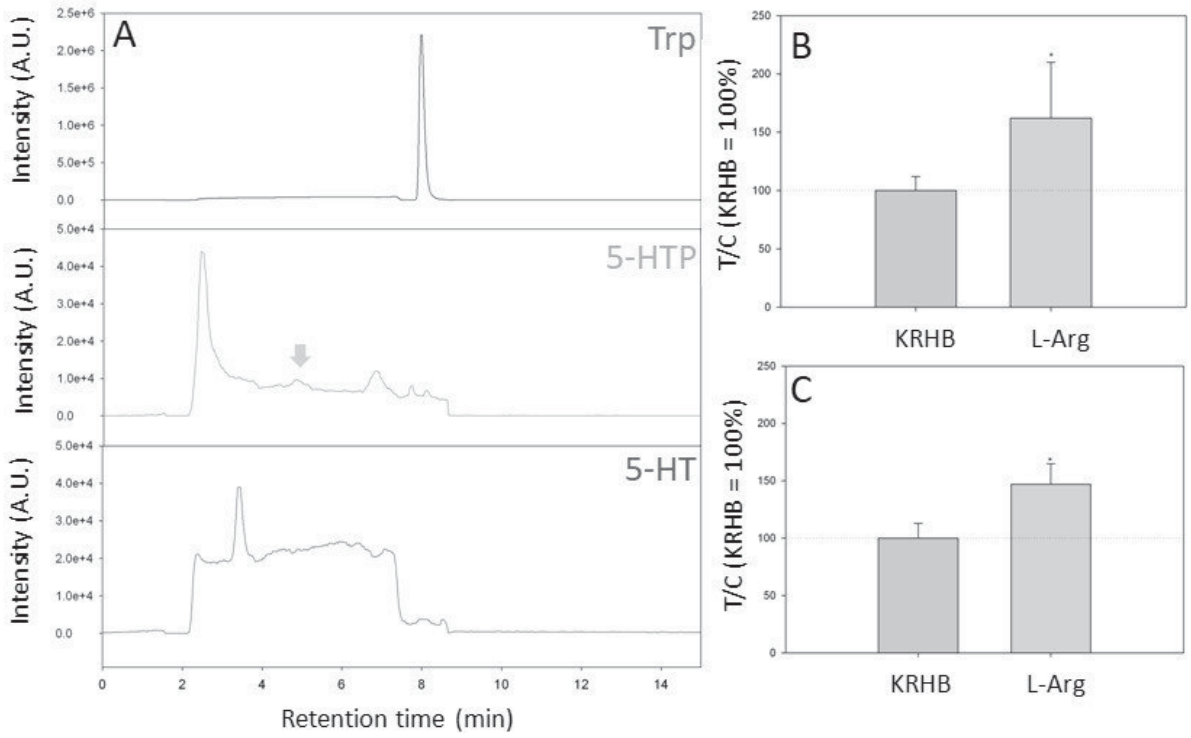

A
log2 intensity data control
log2 intensity data 50 mM Arg
14
12
10
8
6
4
2
2
4
6
8
10
12
14

Figure S3


A
Trp
5-HTP
5-HT
Intensity (A.U.)
Retention time (min)
B
C
T/C (KRHB = 100%)
KRHB
L-Arg

3. Conclusions and Perspectives

The aim of part I was to establish the methods that enabled the investigation of the hypothesis of this PhD thesis: *"Impact of bitter-tasting amino acids and peptides on mechanisms regulating gastric acid secretion and satiety"*. This hypothesis was generated since intake of dietary proteins was shown to reduce food intake and to regulate mechanism of satiety.[6, 7] In addition, most of the proteinogenic L-amino acids are bitter-taste active[73, 91], and bitter taste has been shown to induce satiety in humans[80, 81]. As T2Rs are expressed in the gastrointestinal tract[92] and bitter taste has been shown to modulate gastric acid secretion[78, 79], in this PhD thesis, the satiating potential of bitter-tasting amino acids and peptides was investigated.

For this purpose, in the publication *(1) Caffeine induces gastric acid secretion via bitter taste signaling in gastric parietal cells*, it has been demonstrated for the first time, that HGT-1 cells express T2Rs as well as the subunits of taste-specific GPCRs, gustducin and transducin. In this study, a knock-out of *TAS2R43* by means of CRISPR/Cas9 demonstrated that the bitter-tasting caffeine stimulates proton secretion mediated by T2R43, and that this effect was diminished after addition of the bitter masking compound HED. In addition, experiments with HED in organ tissue baths indicated that T2R signaling regulates mechanisms of gastric acid secretion also neuronally. On the other hand, the bitter masking compound HED was unraveled to promote appetite by regulating metabolic and hormonal parameters of satiety. After oral application of HED, an increase in food intake, associated with increased plasma concentrations of ghrelin, was detected. In addition, plasma levels of the satiety hormone serotonin were decreased after a 30 mg HED bolus administration to healthy subjects. The results of this human intervention study were published in (2) *Appetite-inducing effects of homoeriodictyol: Two randomized, crossover interventions*. These two studies demonstrate that T2Rs are involved in the mechanism of gastric acid secretion. Results from the application of the bitter-masking compound HED to healthy subjects also indicate a role of T2Rs in mechanisms regulating satiety. For investigating a mechanistical approach of satiety, HGT-1 cells were identified as a model for peripheral serotonin release. Treatment of these cells with L-arginine induced secretion of protons, and also increased the release of serotonin. By means of a genome-wide cDNA microarray, the gene encoded for serotonin receptor 5-HT_3 was identified to be regulated by HGT-1 cells treatment with L-arginine. In addition, experiments with L-arginine alone and in combination with a 5-HT_3 antagonist on proton secretion indicate a crucial role of this serotonin receptor in mechanisms regulating satiety. In publication *(5) Serotonin biosynthesis and release from human gastric tumour cells (HGT-1) and its functional role in arginine-induced proton secretion*, HGT-1 cells were further validated as a suitable model for investigating mechanisms of satiety.

In conclusion, methods established in part I enabled the investigation of the impact of bitter-tasting compounds on metabolic, hormonal and neuronal parameters of satiety. A knock out of

TAS2R43 by means of CRISPR/Cas9 supported the involvement of T2R43 in mechanisms of proton secretion in HGT-1 cells. In addition, experiments with HED tested on stomach specimen indicated a role for bitter tastants in the neuronal regulation of satiety. Furthermore, application of HED to healthy subjects showed that administration of this bitter-masking compound promotes appetite by increasing food intake and plasma hormones in healthy subjects.

III. Part II

Influence of bitter-tasting amino acids on mechanisms of gastric acid secretion and satiation *in vitro* and *in vivo*

1. Objectives

In part I of this thesis, methods that enable to investigate the influence of tastants on mechanism regulating satiety via proton secretion were established. In part II, the hypothesis was to elucidate whether amino acids differentially modulate mechanism of gastric acid secretion and whether taste qualities and or structural characteristics may explain these differences. Moreover, the question was addressed whether a stimulating effect on mechanisms of gastric acid secretion *in vitro* translates into improved satiety and reduced food intake in a human intervention trial.

Starting with the *in vitro* experiments, a screening of the effect of ten different taste-active amino acids in both enantiomeric forms on proton secretion in HGT-1 cells was carried out. Since literature data on taste qualities of amino acids is conflicting, a sensory study with 10 amino acids in their L- as well as D-configuration was conducted. By means of correlation analysis, structural key determinants of the selected amino acids that stimulated proton secretion were identified.

As a mechanistic approach, the expression of genes regulating gastric acid secretion was analyzed by means of RT-qPCR. Moreover, for elucidating a regulation of T2R mRNA levels, a cDNA microarray was performed. An influence of T2R signaling on proton secretion was investigated by use of T2R antagonists and a CRISPR/Cas9 knock-out experiment. GPCR down-streaming signals cAMP and IP1 were also investigated in HGT-1 cells.

To validate the regulation of metabolic, hormonal and neuronal parameters of satiety by a dietary protein and an amino acid, a proof of concept study with healthy male subjects was conducted. Since it has been previously demonstrated that the capsaicinoid nonivamide promoted satiety, the focus of the current human intervention study was to investigate whether 3.2 g of the bitter-tasting amino acid L-arginine alone and/or in combination with 2 g of a wheat protein hydrolysate are able to further enhance nonivamide-induced satiety. Thus, effects on food intake, gastric emptying, blood glucose, and satiety hormones like insulin, serotonin, and GLP-1 were analyzed.

2. Results

(1) Identification of bitter-taste intensity and molecular weight as amino acid determinants for the stimulating mechanisms of gastric acid secretion in human parietal cells in culture.

V. Stoeger, KI. Liszt, B. Lieder, M. Wendelin, M. Zopun, J. Hans, JP. Ley, GE. Krammer, V. Somoza. **J Agric Food Chem. 2018 Jul 5;66(26):6762-6771. DOI: 10.1021/acs.jafc.8b01802.**

In this publication, correlation analysis identified bitter taste intensities of L-amino acids and their molecular weight as key determinants for stimulating gastric acid in the parietal cell model HGT-1. L-arginine has been detected as the most stimulating amino acid on proton secretion. In addition, L-arginine and L-isoleucine have been identified as the only amino acids being intensely bitter-tasting in both enantiomeric forms.

I participated in the experimental design and carried out the sensory study. Furthermore, I analyzed the effect of 20 amino acids on proton secretion and selected candidates that were co-incubated with blockers for bitter and sweet taste receptors. In addition, I analyzed the expression of mRNA of genes regulating gastric acid secretion were analyzed by RT-qPCR. I did the statistical analysis of the data and wrote a draft for the manuscript.

Data of this publication were presented at the "*Annual Meeting of the American Chemical Society 2017*" in Washington DC in an oral presentation.

(2) The bitter-tasting amino acids L-arginine and L-isoleucine differentially regulate proton secretion via T2R1 signaling in human parietal cells in culture.

V. Stoeger, AK. Holik, K. Hölz, T. Dingjan, J. Hans, JP. Ley, GE. Krammer, M. Niv., MM. Somoza, V. Somoza, **J Agric Food Chem. 2019, *in review***

This manuscript shows a pivotal role for L-arginine, L-isoleucine in T2R signaling on gene expression, and proton secretion by using available T2R antagonists. Moreover, T2R1 expression on a mRNA and protein level has been identified to be functional to regulate proton secretion and to modulate the effect of L-arginine and L-isoleucine by means of a knock-out of *TAS2R1* in HGT-1 cells. Results suggest an involvement of the CaSR, but not that of ENaC.

In this *in vitro* study, I participated in the experimental design and investigated the effect of L-arginine and L-isoleucine on TAS2R expression by means of cDNA microarray. In addition, I analyzed the expression of *TAS2R1* on mRNA as well as on protein level and its functional role on proton secretion by means of CRISPR/Cas9. I assisted for the detection of the T2R1 by Western Blot. Finally, I analyzed the data and wrote a draft of the manuscript.

Data of this publication were presented at the "*Annual Meeting of the European Chemoreception Research Organization 2019*" in Trieste (Italy) in a poster presentation.

(3) Wheat protein hydrolysate fortified with L-arginine enhances satiation induced by the capsaicinoid nonivamide in moderately overweight male subjects.

V. Stoeger, B. Lieder, J. Riedel, K. Schweiger, JK. Hoi, V. Ruzsanyi, M. Klieber, P. Rust, J. Hans, JP. Ley, GE. Krammer, V. Somoza, **Mol Nutr Food Res, 2019, DOI:10.1002/mnfr.201900133**

An increase of the satiety enhancing effect of nonivamide in combination with a wheat protein hydrolysate alone and fortified with L-arginine has been investigated in healthy men.

I participated in the experimental design and wrote the application for the ethical approval. I planned, organized and conducted a medicinal screening of the subjects together with a medical doctor from an external blood laboratory. I prepared the study solutions containing the test compounds and the ^{13}sodium acetate for detecting gastric emptying and monitored the collection of blood and breath samples at defined time points. I introduced a master student to prepare an *ad libitum* standardized breakfast, to calculate energy intake with the nutritional software nut.s v1.32.50, to assess the subjective feeling of hunger by means of visual analogue scales and to detect plasma levels of glucose, insulin and GLP-1 by means of a colorimetric assays and ELISA. I assessed the levels of plasma ghrelin by means of ELISA and calculated $^{13}CO_2$ concentrations of each subject considering their body surface by means of the Dubois formula. I computed the statistical analysis and drafted the publication.

Data of this human intervention study were the basis for the patent application (PCT/EP/2019/051301) "Zubereitungen enthaltend argininreiche Proteine sowie Nonivamid", G. Krammer, J. Ley, J. Hans, **V. Stoeger**, V. Somoza

2.1 "Identification of bitter-taste Intensity and molecular weight as amino acid determinants for the stimulating mechanisms of gastric acid secretion in human parietal cells in culture."

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Identification of Bitter-Taste Intensity and Molecular Weight as Amino Acid Determinants for the Stimulating Mechanisms of Gastric Acid Secretion in Human Parietal Cells in Culture

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Supporting Information

ABSTRACT: Secretion of gastric acid, aimed at preventing bacterial growth and aiding the digestion of foods in the stomach, is chiefly stimulated by dietary intake of protein and amino acids (AAs). However, AAs' key structural determinants responsible for their effects on mechanisms regulating gastric acid secretion (GAS) have not been identified yet. In this study, AAs have been tested in the parietal cell model HGT-1 on GAS and on mRNA expression of genes regulating GAS. AAs' taste intensities from 0 (not bitter at all) to 10 (very bitter) were assessed in a sensory study, in which ARG (L: 6.42 ± 0.41; D: 4.62 ± 0.59) and ILE (L: 4.21 ± 0.43; D: 2.28 ± 0.33) were identified as bitter-tasting candidates in both isomeric forms. Pearson correlation showed that GAS in HGT-1 cells is directly associated with the bitter taste quality (r: −0.654) in combination with the molecular weight of L-AA (r: −0.685).

KEYWORDS: *parietal cells, gastric acid secretion, HGT-1, amino acids, bitter*

INTRODUCTION

The secretion of gastric acid (GAS) provides a chemical barrier against pathogens in the stomach, aids the absorption of iron, calcium, and vitamin B12, and the digestion of food.[1] Hence, food intake stimulates GAS, with protein-rich meals being most effective, and dietary fat delaying the acid secretory response.[2] Although proteins and proteinogenic canonical amino acids (AAs) have been widely studied for their impact on GAS and associated mechanisms regulating food digestion and food intake through modulation of anorexigenic and orexigenic pathways,[3−7] the question of whether structural requirements or taste qualities of AAs play a role in this gastric response has not been elucidated yet. In foods like beer, cheese, and ripened ham, as well as fruits and vegetables, free AA concentrations are lower than in proteins but may reach up to about 100 mg/100 g.[8,9] Moreover, amino acid controlled formula diets, e.g., those for infants, are limited in their protein contents and contain higher amounts of free amino acids.[10]

GAS by parietal cells in the fundus region of the stomach is mediated by the proton pump H^+/K^+-ATPase. Upon activation of prosecretory cell surface receptors, such as G-protein coupled receptors (GPCRs), the histamine H2 receptor (HRH2), or the acetylcholine receptor M3 (CHRM3) by histamine or acetylcholine, respectively, calcium is mobilized from the endoplasmic reticulum. Intracellular Ca^{2+} mobilization activates the cAMP-dependent kinase cascade, provoking a movement of the H^+/K^+-ATPase to the apical surface. This proton pump actively secretes H^+ ions in exchange for K^+ ions on the apical side of the cell, leading to gastric acidification in the presence of Cl^- ions. GAS is down-regulated by binding of somatostatin to its corresponding receptor (SSTR2).[11]

The secretion of gastric acid together with enzyme activity by pepsin, and peristaltic movements of the stomach facilitate protein degradation, resulting in degradation to smaller peptides and free AAs. The low pH evoked by GAS activates the gastric proteolytic enzyme pepsin which preferably cleaves protein structures next to hydrophobic and aromatic AAs like tryptophan and phenylalanine.[12] However, there is recent evidence for cleavage specificity of pepsin that shows a broader target range,[13] suggesting that pepsin cleaves not only peptide bonds but also nucleic acids. McArthur and colleagues[14] demonstrated that proteins stimulate GAS when applied in form of milk to healthy volunteers.[14] Intragastric titrations after application of milk revealed a mean maximum acid output of 115% in comparison to a H_2O control, which induced 40% mean maximum acid output.[14] Apart from protein-rich foods, there is also evidence that free AAs can induce GAS. In a human intervention study with healthy volunteers, Taylor and colleagues[3] identified the aromatic AAs L-TRP and L-PHE as the most potent stimulators of gastric acid secretion among 19 tested proteinogenic canonical AAs, applied via nasogastric tube in concentrations of 50−100 mM[3].

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Beside proteins and AAs, also bitter-tasting compounds have been identified as stimulators of GAS, for example, the hop-derived α-, β-, and iso-α-bitter acids in beer[15] or the bitter-tasting phenolic wine constituents catechin, syringic acid, and procyanidin B.[16] In healthy volunteers, coffee[17] and its main bitter tastant caffeine[18] stimulated GAS which was measured in real-time by a pH-sensitive Heidelberg capsule. There is emerging evidence for the presence and regulating function of extraoral bitter taste receptors in human tissues.[19,20] One of our recent studies revealed that caffeine evoked its stimulating effect on GAS via activation of the bitter taste receptors TAS2R10 and TAS2R43. The bitter-masking compound homoeriodictyol (HED)[21] was shown to reduce the caffeine-evoked mechanism of gastric acid secretion in parietal cells in culture, whereas it also ameliorated the caffeine-induced bitter perception in a nontrained sensory panel.[22] HED is a plant flavanoid extracted from *Eriodictyon californicum* and is known to decrease bitter taste by targeting TAS2R43.[22]

Since these previous studies demonstrated involvement of TAS2Rs in mechanisms regulating GAS, we hypothesized bitter taste-active AAs to be GAS stimulants. In general, L-forms of several AAs have mainly been considered as more bitter, whereas the D-forms have been evaluated mostly as sweet.[15,16] Since high molecular weight AAs have also been described as more bitter than low molecular weight AAs,[23−25] we also hypothesized the molecular weight playing a role in AAs' potential to stimulate GAS. However, no consistent sensory data on taste qualities of taste-active proteinogenic AAs are available in the literature. Although previous sensory studies have identified different outcome parameters like relative taste, taste threshold, or taste recognition level,[23,26,27] taste intensities have not been published so far. Moreover, most D-AAs have not been sensorial characterized, as they were not available as a free form at the time point when the sensory study was carried out.

Kawai and colleagues[25] described the taste intensities of AAs by means of a labeled magnitude scale and identified at which tested concentrations AAs are perceived as a certain taste quality, ranging from barely detectable to strongest imaginable. Results revealed that most of the AAs not only have one dominant overall taste quality but also show additional other taste qualities.[25] Although Kawai and colleagues[25] provided important data on the overall taste qualities of AAs, sensory studies using concentrations of AAs which can be linked to their functional impact on mechanisms regulating GAS are missing.

Apart from the taste quality, we also were interested whether a structural component of an AA might determine its impact on GAS. Generally, investigated proteinogenic AAs are characterized by a 2-aminocarboxylic moiety.[25] AAs greatly vary in their molecular weights and chemical characteristics.[23]

Moreover, AAs exist in L- and D-configurations. L-AAs are considered to be the physiologically dominant form in humans, as only this enantiomeric form is proteinogenic and can be found mostly in animal or plant derived food. The D-enantiomers are only generated by prokaryotes, e.g., in fermented food such as cheese since bacteria therein are able to transform D-AAs from L-AAs. In the here presented study, structural characteristics as well as the molecular weight and hydrophobicity of AAs were tested for their impact on mechanisms regulating GAS in HGT-1 (human gastric tumor cell line-1) cells, a well-established cell model for parietal cells which has been used to identify GAS modulating compounds in wine,[28] beer,[15] and coffee.[17,22,29] This parietal cell model expresses relevant genes that regulate GAS: histamine receptor 2 (*HRH2*), acetylcholine receptor (*CHRM3*), somatostatin receptor (*SSTR2*), and the H^+/K^+-proton pump (*ATP4A*)[30] as well as bitter taste receptors.[18] Moreover, inhibitors for sweet (lactisole)[31] and bitter taste receptors 4-(2,2,3-trimethylcyclopentyl)butanoic acid (TMPB)[32] and sodium salt of HED)[28] were used to elucidate if bitter (T2Rs) and/or sweet (T1R2/R3) signaling is involved in the mechanism of GAS on a functional level

MATERIALS AND METHODS

Chemicals. L- and D-amino acids (synthesis grade, purity ≥98.5), cell culture media Dulbecco's modified Eagle's medium (DMEM) and its supplements (L-glutamine, penicillin and streptomycin), as well as the primer oligonucleotides were purchased from Sigma-Aldrich, except D-forms of ALA, SER, VAL and ILE were obtained from Carbolution Chemicals GmbH (Germany) and fetal bovine serum (FBS) from Gibco (USA). Phosphate-buffered saline 1× (PBS) was bought from Biozym Biotech Trading GmbH (Austria). Cell viability was tested by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) obtained from Carl Roth GmbH + Co. KG (Austria). For the sensory study, L-ILE and L-ARG were purchased from Fagron GmbH & Co KG (Germany). The fluorescence dye 1,5 carboxy-seminaphto-rhodafluor acetoxymethylester (SNARF-1-AM) was obtained by Thermofisher Scientific (USA) and also RT-qPCR reagents. Lactisole, HED, and TMPB (purity each 99%) were provided by Symrise AG, (Germany). In cell culture studies, TMBP and HED were used as blockers for TAS2Rs in HGT-1 cells. In the context of sensory studies these compounds were defined as bitter taste maskers.

Cell Culture. The human gastric tumor cells (HGT-1),[2215,28,33] were cultivated with DMEM, supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin and streptomycin in a 37 °C humidified environment and 5% CO_2. Per well, 100 000 cells were seeded in either a transparent (for cytotoxicity assays) or a black 96-well plate (for the proton secretion assays), 1 day prior to the test. For detection of gene expression levels, 700 000 cells were seeded per well in 6-well plates.

Cell Viability. MTT[16] dye was used for determining metabolic activity of HGT-1 cells. HGT-1 cells were incubated with the tested substances for different time points (10 to 180 min). Afterward, substances were washed off with PBS solution and incubated with MTT in a concentration of 0.83 mg/mL for 30 min. Absorbance of the formazan salt, dissolved in dimethyl sulfoxide (DMSO), was measured at 570 nm, and the reference wavelength was 630 nm using a Tecan Infinite M200 Pro plate reader (Tecan, Switzerland).

Proton Secretion. Determination of proton secretion was carried out as described in HGT-1 cells previously.[15,22] HGT-1 cells represent a well-established model for identifying bitter compounds with a modulating effect on proton secretion,[28] via activation of extraoral bitter taste receptors.[18] In brief, cells were washed with Krebs−Ringer−HEPES buffer (KRHB) and stained with 3 μM intracellular pH indicator 1,5 carboxy-seminaphto-rhodafluor acetoxymethylester (SNARF-1-AM) for 30 min under standard conditions (37 °C, 5% CO_2). Subsequently, cells were washed again with KRHB and treated with AAs in L-and D- configurations in a concentration range from 5 to 50 mM for 10 min. These concentrations in a millimolar range have been chosen according to receptor binding studies in transfected HEK cells.[34,35] AAs were selected to consider aliphatic, aromatic, hydroxylated, and basic characteristics for the proton secretion assay. Threonine (THR), proline (PRO), serine (SER), alanine (ALA), arginine (ARG), phenylalanine (PHE), valine (VAL), leucine (LEU), isoleucine (ILE), and tryptophan (TRP) as well as 0.5 mM HED, 5 + 50 μM lactisole and 10−100 μM TMPB were diluted in DMEM. As positive control, 1 mM histamine was used. For each experiment, appropriate solvent controls have been used. Measurement was done using an Infinite 200 Pro plate reader

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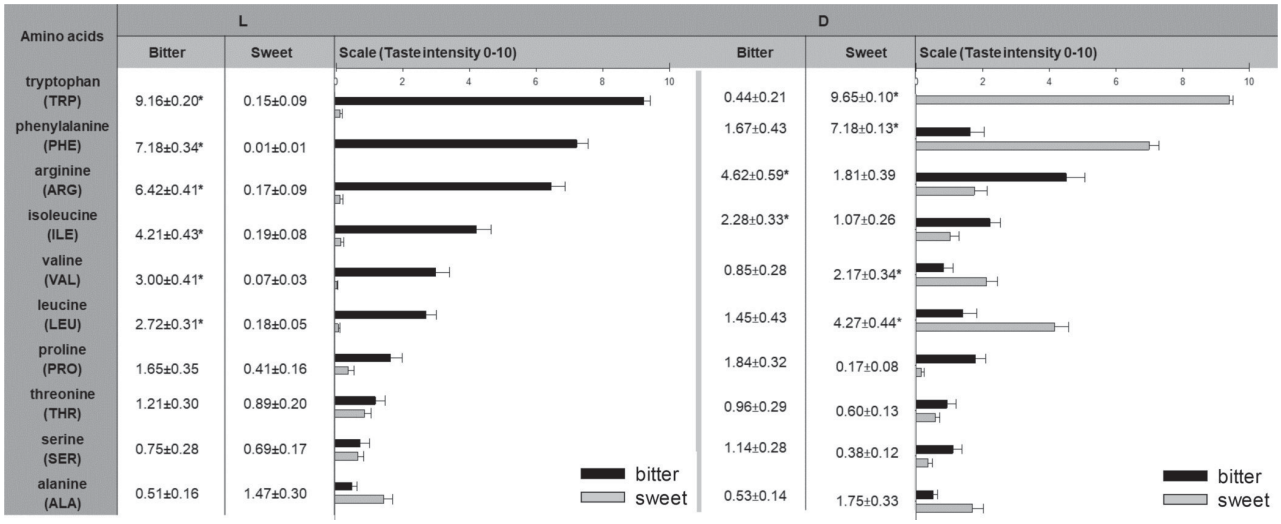

Figure 1. AAs are ranked from the most to the least bitter-tasting L-amino acid according to its assessed intensities from the sensory study. Bitter taste intensities are depicted as dark bars and sweet taste as light gray bars. L-TRP was the most bitter-tasting, and D-TRP the sweetest-tasting amino acid. Data are represented as the mean of the absolute intensities ± standard error of the mean (SEM) as well as in grouped bar charts for 3−4 technical replicates. In this sensory study, 19 panelists completed the study.

(Tecan, Switzerland). Excitation wavelength was 488 nm and the emission wavelengths were 580 and 640 nm. For calibration, intra- and extracellular pH was set by 2 μM nigericin in potassium buffer (20 mM NaCl, 110 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 18 mM D-glucose, and 20 mM HEPES) with a pH range from 6.8 to 8.2. The intracellular proton index (IPX) was calculated as the log2 data of the 580/640 ratio and compared to the untreated control. The lower the IPX, the more protons are secreted by the cell. The measurement of the intracellular proton concentration enables to test also acidic or basic food constituents, for example, ARG.

Sensory Study. A (quantitative) sensory profiling for the evaluation of bitter and sweet taste of L- and D-AAs by means of a nonstructured scale was carried out.[36,37] Test concentrations for the scale training were determined in preliminary experiments. A concentration of 0.03% caffeine was used as a bitter reference for 5 mM L-TRP, and 0.6% sucrose was used as a reference compound for sweet with an equal intensity for 1 mM D-TRP. A method-trained sensory panel of 6 male and 13 female assessors (mean age 31.5 ± 5.6 years) evaluated 10 AAs in a concentration of 25 mM. Pilot tests with 50 mM concentrated TRP revealed this concentration as too high for bitter and sweet intensity tests as the sensory perceptions were too intense. These selected amino acids are described in literature as taste-active AAs.[23,25,38] All assessors signed an informed consent on the first study day, and ethical principles for research involving subjects developed by the World Medical Association (WMA) in the Declaration of Helsinki were followed for the sensory tests.[39] Before each session, all panelists completed a scale (10 cm, nonstructured) training them by tasting the references for no taste (water), bitter (intense: 5 mM L-TRP, very intense: 25 mM L-TRP, and sweet (intense: 1 mM D-TRP, very intense: 25 mM D-TRP). In each session, five AAs were assessed without retasting, so that every AA was assessed four times by 19 panelist. Each assessor was asked to do a mouth rinse with a 20 mL of solution, which was spewed out afterward. The assessors received 20 mL of each sample in a randomized order. All solutions were prepared with nonsparkling mineral water ("Vittel"), which was also used as a control solution.[40] Between samples, the assessors rinsed their mouth with water.

mRNA Expression. After incubation of the cells with 50 mM L-ARG or L-ILE at the time points 15, 30, 60, and 180 min, cells were washed with ice-cold PBS. RNA isolation was carried out using the peqGold Total RNA Kit (Peqlab Biotechnology GmbH, Germany), according to the manufacturer's protocol. RNA concentration was determined spectrophotometrically at 260 and 280 nm. High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Austria) was used for the generation of cDNA. Quantitative PCR was carried out on a StepOneplus Real Time PCR system (Applied Biosystems, Thermo Fisher Scientific, Vienna, Austria) using the following temperature program: 20 s/95 °C for activation, 3 s/95 °C for denaturation, 30 s/65 °C for annealing, and 15 s/72 °C for elongation. RT-qPCR data was analyzed with the open access software LinRegPCR 11.0[41] to determine hypothetical mRNA starting concentrations, called "N_0". Primer sequences for the genes *HRH2*, *ATP4A*, *CHRM3*, and *SSTR2* and the housekeeping gene *PPIA* have previously been published.[15,16]

Statistical Analysis. Cell culture experiments were carried out with at least 3−4 biological replicates. Each biological replicate was carried out with 3−6 technical replicates, each referring to one well in a microtiter test plate or to a cell culture dish. The sensory study was carried out with 19 panelists who tested each AA four times. Results were analyzed with SigmaPlot 12.0 and are given as mean fold changes (in %) ± SEM. Outliers haven been detected by means of Nalimov's test. Significant differences were tested by one- or two-way ANOVA followed by Holm-Sidak method post hoc or by using Student's *t*-Test. By means of Shapiro Wilk, normality of the data has been tested. If there was no normality given, then significances have been detected by one-way ANOVA on Ranks. The level of significance was set at $p < 0.05$. The Pearson Product Moment method was applied to identify correlations between the structural characteristics (molecular weight, stereochemical configuration and taste quality) of the AAs and their impact on the IPX.

RESULTS

Cell Viability of HGT-1 Cells after Treatment with L- and D-AAs. Neither the tested L- or D-AAs nor their combination with HED, 4-(2,2,3-trimethylcyclopentyl)-butanoic acid (TMPB) or lactisole affected the cells' viability in the tested concentrations at any time point as assessed by means of MTT assays (data not shown).

Sensory Study. Figure 1 shows the bitter and sweet taste intensities evaluated by the panel for the tested AAs that are described as taste-active in literature.[23,25,38] L- and D-AA were tested in concentrations of 25 mM. TRP in the form of both isomers was used as a reference compound for the bitter and sweet scale in comparison to a test compound. The taste

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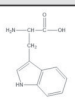
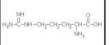
Table 1. AAs Sorted according to Their Bitter Taste Intensity of the L-Configuration[a]

amino acid	chemical structure	functional group	molecular weight [g/mol]	IPX [induced by 50 mM AA]		P – value (stereoisomerism)
				L	D	
TRP		aromatic	204.2	-0.66 ± 0.23	-0.04 ± 0.04	< 0.001
PHE*		aromatic	165.2	0.50 ± 0.05	1.58 ± 0.10	< 0.001
ARG*		guanidino	174.2	-2.71 ± 0.11	-2.37 ± 0.05	< 0.05
ILE*		aliphatic	131.2	0.58 ± 0.07	-0.40 ± 0.04	< 0.001
VAL*		aliphatic	117.2	0.99 ± 0.05	-0.44 ± 0.17	< 0.001
LEU*		aliphatic	131.2	1.38 ± 0.14	1.10 ± 0.13	< 0.05
PRO*		imino acid	115.1	0.44 ± 0.08	0.63 ± 0.09	p = 0.156
THR*		hydroxyl	119.1	1.43 ± 0.10	1.57 ± 0.05	p = 0.351
SER*		hydroxyl	105.1	1.40 ± 0.12	0.87 ± 0.10	< 0.01
ALA*		aliphatic	89.1	0.87 ± 0.10	-0.35 ± 0.08	< 0.001

[a]AAs were sorted according to their bitter taste intensity of the L-configuration and characterized by their chemical structure, functional group molecular weight, and the intracellular proton index (IPX), which is defined as the log2 data of the 580/640 ratio, indicates high proton secretion with low IPX values. The effect on the IPX in HGT-1 cells after 10 min incubation time of 50 mM AA, is given for the L- and D-forms. Significances of the tested AAs against media control are marked as bold and with an asterisk. Data are represented as mean fold change in percent ± SEM obtained from 3 to 4 biological replicates.

recovery rate of the panel was around 90%, meaning that the assessors detected the L- and D-TRP with the highest intensity of 10. Water has been used as reference. Figure 1 depicts L-TRP as most bitter, with a relative intensity of 9.16 ± 0.20. The second most bitter AA was L-PHE (7.18 ± 0.34), followed by L-ARG (6.42 ± 0.41) and L-ILE, the most bitter branched chained amino acid (BCAA) with an intensity of 4.21 ± 0.43. D-TRP was ranked as sweetest, with an intensity of 9.65 ± 0.10, followed by D-PHE with 7.18 ± 0.13 and D-LEU assessed with 4.27 ± 0.44. The BCAA D-VAL was still assessed as sweet with an intensity of 2.17 ± 0.34. Overall, the first six AAs listed in Figure 1 were assessed as being more bitter-tasting in their L- than compared to their D-form ($p < 0.05$). The other AAs tested (PRO, THR, SER, and ALA) were assessed as slightly bitter and sweet, without any significant difference between stereoisomers. Overall, statistical analyses by means of a two-way ANOVA revealed that the L-form of TRP, PHE, ARG, ILE, VAL, and LEU are more bitter taste-active AAs compared to their respective D-isomers and the other AAs ($p < 0.001$, except L-LEU p-value: 0.01). Also, for D-isomers, the sweetest taste-active AAs assessed by the sensory panel were TRP, PHE, VAL, and LEU ($p < 0.001$), although D-ARG ($p < 0.001$) and D-ILE ($p < 0.01$) were assessed as bitter-tasting.

Impact of L- and D-AAs on Proton Secretion in HGT-1 Cells. Table 1 represents the impact of the tested AAs on proton secretion in HGT-1 cells according to their revealed bitter taste intensity. By means of the proton secretory assay, AA were tested in their L- and D-configuration in a

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concentration of 50 mM, as it was shown for some AAs to activate T2Rs in a millimolar range.[34,35] All tested AAs except TRP, induced a significantly different effect on proton secretion in comparison to media controls ($p < 0.05$). For TRP, it has to be noted that the experimental protocol had to be adapted due to its strong autofluorescence: Whereas for all other AAs, fluorescence of the intracellular pH-indicator SNARF was measured after a 10 min exposure in the presence of the AA, TRP had to be removed by an extra washing step following the incubation. This additional step could have weakened the effect of TRP on proton secretion in HGT-1 cells. The additional washing step was verified by means of histamine of which the effect was decreased by 42.9 ± 0.07% (data not shown). Beside the AAs PRO and THR, all AA induced an enantiospecific effect (Table 1). Moreover, results for a two-way-ANOVA analysis depicted in Figure S1 showed that proton secretion was mostly stimulated by the L-ARG and L-TRP (IPX: −2.71 ± 0.11; −0.66 ± 0.23, $p < 0.05$), whereas L-THR and L-SER (IPX: 1.43 ± 0.10; 1.40 ± 0.12; $p < 0.05$) had the strongest inhibitory potential. In contrast to the L-isomers, the D-ARG and D-ILE equally with D-VAL and D-ALA (IPX: −2.37 ± 0.05, −0.40 ± 0.04, −0.44 ± 0.17, −0.35 ± 0.08) most effectively stimulated the proton secretion, whereas D-PHE (IPX: 1.58 ± 0.10) and D-THR (IPX: 1.57 ± 0.05) most effectively reduced the most proton secretion followed by D-LEU, D-PRO, and D-SER (IPX: 1.10 ± 0.13, 0.63 ± 0.09, $p < 0.05$, 0.87 ± 0.10, Table 1).

Correlation of Structural Characteristics and Taste Qualities of the Tested AAs with the Intracellular Proton Index. IPX induced by L-AAs in HGT-1 cells negatively correlated with their respective bitter-taste intensities (Figure 2A) (r: −0.654, $p < 0.05$) but not their sweet taste intensities. Furthermore, Pearson correlation analysis revealed a negative correlation of the IPX and the molecular weight of the tested L-AAs (r: −0.685, $p < 0.05$) (Figure 2B). There was also a correlation between the MW and bitter as well as sweet taste intensities of L- and D-AAs (data shown in supplemental Figure S3A,B). However, there was no correlation between the IPX induced by D-AAs and bitter intensities or sweet intensities ($p = 0.385$, Figure S4). A correlation between an AA's carbon as well as hydrogen content and bitter taste of all tested AAs was also detected (r: 0.559, $p < 0.01$; r: 0.543, $p < 0.01$). However, there was no statistically significant difference between L- and D-enantiomers (data not shown).

Co-Incubation with the Bitter Modulating Compound HED. Since Pearson correlation analysis revealed a significant association between bitter-tasting L-AAs and the IPX ($p < 0.05$, Figure 2), involvement of bitter-taste of AAs on the IPX in HGT-1 cells was studied in coincubation experiments using the bitter-masking compound HED.[21] HED was used in a micromolar range, as it has been shown in a sensory study that a maximum of 300 μM HED partially masked the bitterness of 1 mM caffeine up to 40%.[21] HED has been tested at one-hundredth of the AA concentration.[22] L-THR, the most inhibiting, and L-ARG, the most stimulating AAs of proton secretion, were used for these coincubation experiments. Moreover, L-ILE, which was bitter-tasting in both enantiomeric forms as well as the nonbitter but sweet taste-active AA L-SER were applied to HGT-1 cells. Incubation with HED did not have an impact on L-TRP-induced proton secretion (Figure S2). However, due to the autofluorescence signal of TRP, this effect was not further investigated. Addition

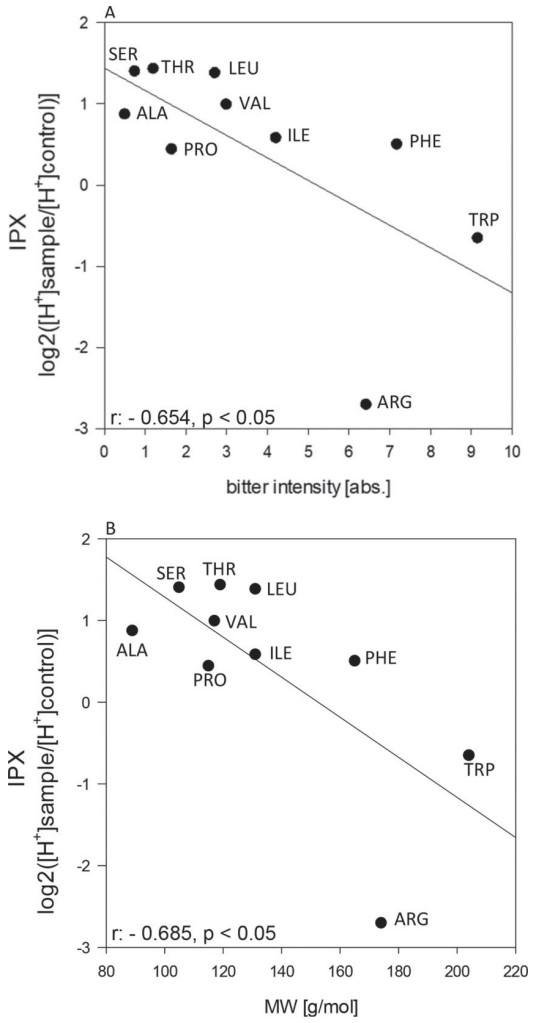

Figure 2. (A) Correlation of the bitter taste intensities and the IPX after incubation with 50 mM L-AAs in HGT-1 cells. Correlation was done with Pearson Product Moment method, r: −0.654, $p < 0.05$. (B) Correlation of the molecular weight and the IPX induced by L-AAs in HGT-1 cells. Correlation was done with Pearson Product Moment method (r: −0.685, $p < 0.05$).

of 0.1 mM HED to 10 mM L-THR treated HGT-1 cells increased the IPX up to 0.72 ± 0.11 in comparison to L-THR alone (IPX: 0.38 ± 0.06; $p < 0.05$). Addition of 0.1 mM HED to L-SER did not point to an involvement of bitter taste receptor signaling in the mechanism of proton secretion induced by L-SER ($P = 0.145$) (Figure 3A,B); however, this needs to be verified in future studies. A combination of 0.5 mM HED and 50 mM L-ARG increased the IPX (−1.98 ± 0.14; $p < 0.05$) in comparison to treatment with L-ARG alone (IPX: −2.68 ± 0.12). The same effect was observed when 50 mM L-ILE was coapplied with 0.5 mM HED, since the combination of HED and L-ILE increased the IPX up to 0.90 ± 0.18 ($p < 0.05$) compared to 50 mM L-ILE (IPX: 0.25 ± 0.07) (Figure 3C,D).

Time-Dependent mRNA Expression Induced by L-ARG and L-ILE on Genes Regulating GAS. *L-ARG. ATP4A* gene expression was downregulated after 30 min until 180 min of exposure of the HGT-1 cells to L-ARG (fold changes: 0.84 ± 0.08; 0.77 ± 0.08; 0.72 ± 0.14; $p < 0.05$) (Figure 3E). After

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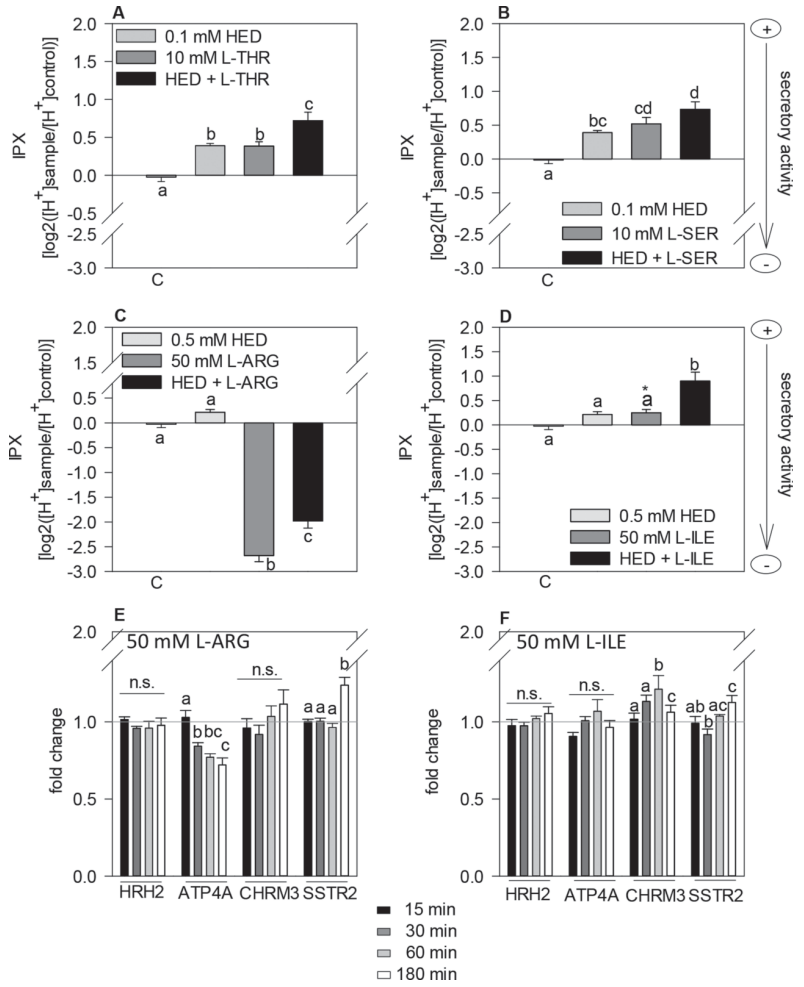

Figure 3. (A and B) Impact of bitter taste on proton secretion was tested with the bitter-masking compound HED. A coincubation with 0.1 mM HED, 10 mM L-THR, and 10 mM L-SER was done. Data are represented as mean fold change ± SEM 3−4 biological replicates. Results were tested by one-way ANOVA, $p < 0.05$. (C and D) Proton secretion was reduced when L-ARG in a concentration of 50 mM, was coincubated with 0.5 mM HED. The inhibiting effect of 50 mM L-ILE increased by addition of 0.5 mM HED. (E and F) Time-dependent mRNA expression in HGT-1 cells for 15/30/60 and 180 min after incubation with L-ILE and L-ARG is shown as mean fold changes ± SEM deviation. Effects were tested for 3−4 biological replicates with one-way ANOVA and Holm Sidak method post hoc. Overall significance level was $p < 0.05$.

180 min of treatment, *SSTR2* gene expression increased to 1.24 ± 0.15 in comparison to the untreated control cells set to 1 ($p < 0.05$).

L-ILE. mRNA expression of *CHRM3* in HGT-1 cells after 60 min exposure to 50 mM L-ILE increased up to a fold change of 1.21 ± 0.30 compared to untreated controls (= 1, $p < 0.05$) (Figure 3F), whereas *SSTR2* expression was down-regulated to 0.92 ± 0.12 after 15 min ($p < 0.05$). An *SSTR2* up-regulation in the presence of L-LEU was detected after 180 min (fold change: 1.13 ± 0.14; $p < 0.05$). HED had no effect on genes regulating GAS (data not shown).

Co-Incubation with the T2R Inhibitor TMPB and the T1R3 Inhibitor Lactisole. As there is evidence that (i) L-TRP targets T2R4[34,35] and (ii) TMPB is an inhibitor for T2R4,[32] both compounds were tested in coincubation experiments. The results validated that bitter-taste signaling via T2Rs contributes to proton secretion when AAs are applied to HGT-1 cells. Since Kohl and colleagues showed that 10 mM L-TRP activated T2R4,[34] the same concentrations were chosen for this experiment. After 10 min of exposure to TMPB (25 μM/100 μM) and 10 mM L-TRP, the stimulating effect of L-TRP on proton secretion was reduced (IPX: L-TRP: −0.14 ± 0.05; L-TRP + 25 μM TMPB: −0.03 ± 0.06; L-TRP + 100 μM TMPB: −0.03 ± 0.05; Figure 4A and 3B).

For analysis of the sweet signaling cascade, the sweet taste inhibitor lactisole[31] was used. D-PHE, which was assessed as a very sweet taste-active AA, and L-SER, which was neither bitter- nor sweet-tasting in both isomeric forms in the sensory evaluation, were analyzed for their impact on proton secretion. Figure 5 represents delta IPX values for HGT-1 cells exposed to either 50 mM L-PHE (A) or 50 mM L-SER (B). Delta IPX values of the coincubation of 5 μM lactisole and 50 mM L-PHE (ΔIPX: 0.03 ± 0.04) showed no change of the inhibitory effect of L-PHE alone. The same effect was detected when L-PHE was applied together with 50 μM lactisole (ΔIPX: 0.07 ± 0.05, $p > 0.05$). A comparison of the ΔIPX values between 5 and 50 μM of the inhibitor lactisole alone (ΔIPX 5 μM: 0.35 ± 0.03; 50 μM: 0.34 ± 0.03) and the coincubations with 50 mM D-PHE showed a statistical difference ($p < 0.001$, Figure 5A). For L-SER, a coincubation with lactisole in concentrations of 5 μM (ΔIPX: 0.15 ± 0.05) induced no change on the inhibiting effect of L-SER alone; on the contrary, 50 μM lactisole

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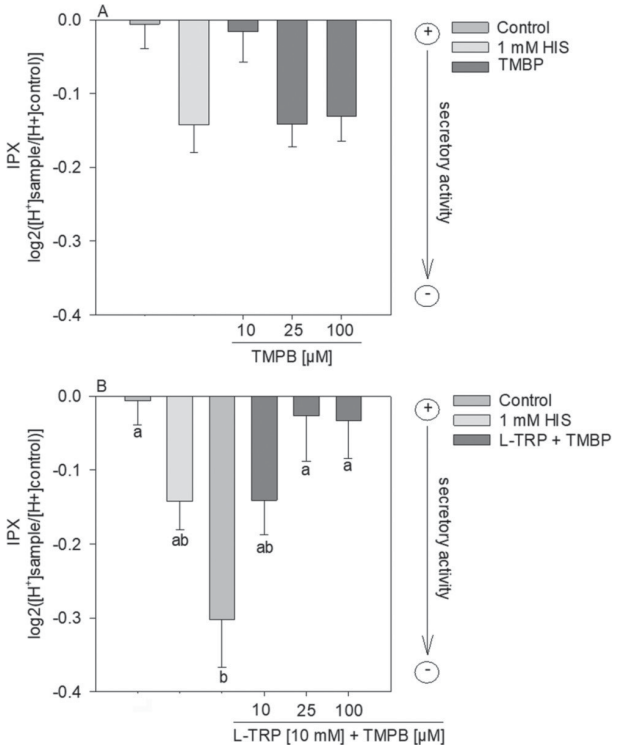

Figure 4. (A) Impact of different concentration of the T2R inhibitor TMBP on proton secretion in HGT-1 cells. (B) TMBP inhibited proton secretion induced by L-TRP in HGT-1 cells after 10 min incubation time. Data are represented as mean fold change ± (SEM) of 3−4 biological replicates. Results were tested by one-way ANOVA, $p < 0.05$.

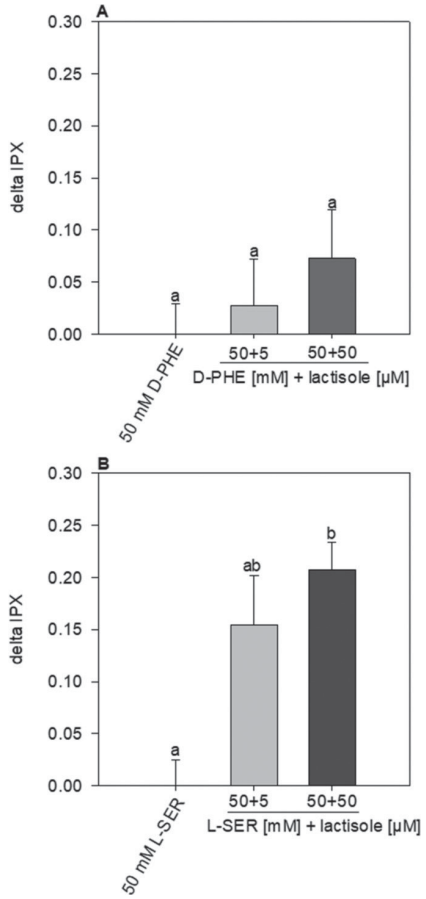

Figure 5. (A and B) Impact of 50 mM of the sweet-tasting amino acids D-PHE and L-SER in addition with the sweet taste receptor inhibitor lactisole (5 μM/50 μM) were tested on proton secretion. Graph depicts ΔIPX values referred to either D-PHE or L-SER alone.

inhibited proton secretion (ΔIPX: 0.21 ± 0.03) in comparison to the effect of L-SER alone ($p < 0.01$; Figure 5B).

DISCUSSION

The secretion of gastric acid helps to prevent bacterial overgrowth and enteric infections, is essential for the absorption of calcium, iron, and vitamin B12, and aids the digestion of dietary protein.[42] At low gastric pH, gastric pepsinogen released by the chief cells is activated to pepsin that enzymatically cleaves protein structures into peptides and some free AAs. Dietary intake of protein-rich foods and aromatic L-AAs have been demonstrated to stimulate GAS in healthy volunteers.[3,43] However, it has not been elucidated whether and which structural characteristics or taste qualities of dietary proteins and AAs are responsible for their stimulating effect on GAS.

Most of the proteinogenic AAs are described as bitter-tasting in their L-form, whereas their corresponding D-forms frequently taste sweet.[23,44] In a recent publication, we show that the bitter-tasting compound caffeine stimulates GAS in healthy volunteers by activation of a T2R bitter signaling cascade.[22,28] We here hypothesize that bitter-tasting AAs could be modulators of mechanisms of GAS demonstrated in human parietal cells (HGT-1). In accordance with our hypothesis a pronounced bitter-taste quality was identified as key determinant of AAs to stimulate proton secretion in parietal cells. In addition, we found that a high molecular weight of L-AAs is also favorable for increasing secretion of protons. To summarize, our data demonstrate that bitter-tasting and large AAs induce proton secretion in parietal cells in culture. This finding is in accordance with reports from the literature, showing that bitter taste-active L-AAs have a high molecular weight and that smaller AAs are sweet-tasting.[23−25]

As there is no homogeneous literature on bitter and sweet taste intensities for AAs, we started with a sensory study with method-trained panelist. In previous publications, taste qualities of some AAs have not been assessed in their free uncharged form because these AAs were only available as racemates, e.g., D-ARG and D-PRO.[26,38] Also procedures of the sensory tests applied were quite different regarding outcome measures, e.g., relative taste[45] or taste threshold level.[44] Here, we aimed at identifying taste intensities of D- and L-AAs. When TRP was administered, panelists validated that this AA was very bitter-tasting in its L-form and very sweet-tasting in its D-form in accordance with current literature.[23,24] An enantioselective taste perception was also determined in our sensory study for PHE, VAL, and LEU but not for ARG and ILE.

A correlation of proton secretion in HGT-1 cells and the bitter perception has been demonstrated as a reliable predictor for taste-active compounds.[28] Therefore, a taste-dependent, diverse impact of the AAs subjected to our sensory study on proton secretion in HGT-1 cells has been hypothesized. As a result, an enantioselective modulation on proton secretion in HGT-1 cells has been identified for TRP, PHE, ARG, ILE,

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VAL, LEU, SER, and ALA but not for PRO and THR. Among all tested AAs, both enantiomers of ARG were most stimulating on proton secretion in HGT-1 cells. This effect might be associated with the guanidino moiety of ARG, since a regulation on GAS by nitrogen oxide formed enzymatically from L-ARG by nitric oxide oxidases (NOS) was shown by Pique and colleagues[46] as well as Konturek and colleagues.[47] The authors of both of these studies hypothesized GAS was modulated by a mediating effect of NO on gastric mucosal vasodilation[46] and by a change of the release of the GAS hormones gastrin and somatostatin.[47] Furthermore, Sakai and colleagues found that the vasodilator ecabapide, directly stimulated the opening of Cl^- channels in rabbit gastric parietal cells.[48] One might speculate that also TRP regulates GAS because of its aromatic ring structure; however, these findings have to be verified in future studies.

L-THR is the most potently IPX-inducing L-AA, thereby demonstrating a strong diminishing effect on proton secretion in HGT-1 cells, which could be due to its OH group which possibly serves as a proton donor. In contrast to other AAs, D-THR modulated proton secretion to the same extent as its L-enantiomer, indicating characteristics other than only the isomeric form as determinants of GAS. This is supported by other results on the effects of L- and D-AAs on proton secretion in HGT-1 cells which revealed no homogeneous picture for the effect of L- versus D-AAs. In contrast to the effects on IPX, bitter-taste intensities of L-AAs correlated quite well with the IPX, whereas this was not the case for sweet taste intensities and D-AAs. The first outcome, demonstrating a correlation between a compound's bitter taste and its potential to induce proton secretion in HGT-1 cells, is in accordance with literature evidence on caffeine, beer, or wine constituents.[15,16,22] However, for the second outcome, no literature evidence for an enantioselective and bitter taste-active modulation on GAS induced by AAs is available.

Next, the question was addressed whether bitter or sweet taste of AAs is involved in the mechanism of proton secretion in HGT-1 cells. L-PHE, as the second bitter-tasting AA, did not show a stimulating effect on the IPX. Whether the aromatic ring of this AA plays a role in IPX modulation needs to be addressed in future studies. Therefore, L-ARG and L-ILE, two bitter taste-active AAs with contrary effects on proton secretion, were each coincubated with the known bitter-taste inhibitor HED. As a negative control, two nontaste active AAs (L-THR, L-SER) were also tested. L-ARG as well as L-ILE increased the IPX by 26.9 and 55.2%, respectively, when HED was added. A cotreatment of L-THR with HED increased the IPX by 52.8%, whereas the IPX effect induced by L-SER was not influenced by HED. An influence of the flavanone HED alone on the proton pump cannot be excluded as there is evidence for an inhibiting effect on H^+,K^+-ATPase by flavonoids.[49] This data indicates that bitter-taste receptors may regulate proton secretion in HGT-1 cells after treatment with L-ILE and L-ARG. However, whether HED not only masks the bitterness of caffeine[21] but also bitter-tasting AAs and peptides has to be tested in future sensory studies. Furthermore, PCR analysis of mRNA expression of genes regulating GAS in HGT-1 cells demonstrated that expression of *SSTR2* was upregulated by L-ARG and L-ILE, whereas only L-ARG downregulated expression of *ATP4A*. According to one of our previous studies,[17] these modifying effects on expression of genes regulating GAS indicate that secretion of protons is modulated by L-ILE and L-ARG.

Recently, we showed in a human intervention study that the stimulating effect of caffeine on GAS was reduced when coadministered with HED.[22] Moreover, in sensory studies with untrained and trained panelists, the bitter-taste of caffeine was masked by 20 ± 8% when assessed by untrained panelists and 43% assessed by trained panelists.[22,50] Furthermore, HED has been published to be an antagonist for the bitter-taste receptor T2R43 and an agonist for T2R39.[28] There is also evidence that TRP and PHE activate TAS2R 1/4/39, independent of their enantiomeric form in receptor-activating studies in HEK cells.[34,35] Thus, we examined TMPB[32] as an antagonist and L-TRP[34] as an agonist for T2R4. Since these results showed that TMPB partly prevented the stimulatory effect of L-TRP, we suggest T2R4 is involved in the mechanism of proton secretion. On the basis of data of proton secretion after application of L-AAs in combination with the bitter maskers HED[22] or TMPB,[32] it can be assumed that a bitter-masking effect in sensory studies is associated with a modulating effect on proton secretion by parietal cells.

To exclude involvement of the sweet taste of AAs, lactisole, targeting T1R3 as antagonist, was used.[21] When D-PHE, whose sweet intensity was rated very high, and L-SER, a nontaste active AA, were coincubated with lactisole, no influence on the AA-evoked effect on proton secretion was shown. These data indicate a negligible role of the sweet receptor activation. Thus, sweet taste and D-isomers of AAs might not be crucial for secretion of protons in HGT-1 cells.

Beside taste and isomerism, the molecular weight of the tested AAs was also identified as a determinant of proton secretion, shown by a correlation between the IPX induced only by L-configurated AAs and their molecular weight. This correlation indicates that a large molecular weight of L-AAs is preferable for a stimulating effect on proton secretion in HGT-1 cells. In the context of protein digestion, GAS is essential for protein degradation.[8] With these results, the present study significantly broadens the existing knowledge on AAs' structural requirements for stimulating GAS. However, whether HED masks the bitterness of not only caffeine[21] but also bitter-tasting AAs and peptides has to be tested in future sensory studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b01802.

Influence of L- and D-AAs on proton secretion in HGT-1 cells, influence of L-TRP in a combination with HED on proton secretion, correlation of the bitter intensities of L-amino acids with their molecular weight, correlation of the IPX induced by D-AAs in HGT-1 cells and their bitter intensity (PDF)

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The authors declare the following competing financial interest(s): M.W., J.H., J.P.L., and G.E.K. are employed by Symrise AG.

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■ ABBREVIATIONS

(AAs), amino acids; (GAS), gastric acid secretion; (GPCR), G-protein coupled receptor; (ATPase), H^+/K^+ adenosine triphosphatase; (HED), homoeriodictyol; (IPX), intracellular proton index; (T2R), bitter taste receptors

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2.2 "The bitter-tasting amino acids L-arginine and L-isoleucine differentially regulate proton secretion via T2R1 signaling in human parietal cells in culture."

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The Bitter-Tasting Amino Acids L-Arginine and L-Isoleucine Differentially Regulate Proton Secretion via T2R1 Signalling in Human Parietal Cells in Culture

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The Bitter-Tasting Amino Acids L-Arginine and L-Isoleucine Differentially Regulate Proton Secretion via T2R1 Signalling in Human Parietal Cells in Culture

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Abstract

This study aimed at identifying whether the bitter-tasting amino acids L-arginine (L-ARG) and L-isoleucine (L-ILE) differentially regulate mechanisms of gastric acid secretion (GAS) in human parietal cells (HGT-1 cells) via activation of bitter taste sensing receptors (T2Rs). In a first set of experiments, involvement of T2Rs in L-ARG and L-ILE-modulated proton secretion was demonstrated by co-treatment of HGT-1 cells with T2R antagonists. Subsequent whole genome screenings by means of cDNA arrays revealed T2R1 as a prominent target for both amino acids. Next, the functional role of T2R1 was verified by means of a *T2R1* CRISPR-Cas9 knock out approach. Here, the effect of L-ARG on proton secretion decreased by 65.7 ± 21.9 % and the effect of L-ILE increased by 93.2 ± 24.1 % in HGT-1 T2R1 ko vs. HGT-1 wt cells (p<0.05). Overall, our results indicate differential effects of L-ARG and L-ILE on proton secretion in HGT-1 cells and our molecular docking studies predict distinct binding for these amino acids in the binding site of T2R1. Further studies will elucidate whether the mechanism of differential effects involves structure-specific ligand-biased signalling of T2R1, or additional cellular targets.

Introduction

Dietary proteins and amino acids (AAs) have been shown to act on metabolic targets involved in mechanisms regulating satiety, energy expenditure, and body composition, although structure-based evidence is scarce.[1] In general, consumption of plant proteins, considered of lower nutritional quality than animal proteins, is increasing due to interest in ecological footprint reduction, although most plant proteins show taste deficits, e.g. bitter off-notes. On the other hand, bitter tasting food constituents, i.e. bitter tasting amino acids, have been demonstrated to stimulate mechanisms of gastric acid secretion in parietal cells[2] as an outcome measure of stimulated gastric digestion and satiation.

Parietal cells are the primary acid secretory cells of the stomach. The regulation of acid secretion by these cells is highly complex and not yet fully elucidated since many receptor-ligand interactions are required. The classical pathway of acid secretion involves the release of gastrin which is induced from antral G cells, causing a subsequent efflux of histamine from enterochromaffin-like cells. The released histamine binds to its cognate receptor on the basolateral membrane of the parietal cell, thereby inducing a cascade of events, including a rise in cytosolic Ca^{2+} and cAMP levels, which then trigger chloride and H^+ effluxes from the apical cell surface via a chloride channel and the H^+-K^+-ATPase.[3-7] Upon activation by stimulants like histamine, gastrin or acetylcholine, the H^+-K^+-ATPase, located on cytosolic tubular vesicular elements, traffics to the apical cell surface where the vesicles fuse with canaliculi along the apical membrane to secrete protons, which is essential for the formation HCl in the gastric lumen.[5-7] In one of our previous works, we demonstrated bitter receptors (T2Rs) are not only being localized on human parietal cells but also involved in the regulation of proton secretion.[8] Using caffeine to target the human bitter

receptors T2R7, 10, 14, 43 and 64,[9] a regulatory role for T2R10 and T2R43 has been shown by means of an siRNA and CRISPR-Cas9 approach, respectively.[8]

T2Rs of parietals cell in the stomach are potential targets for all bitter-tasting food constituents, including respective proteins and peptides for which gastric acid plays an essential role in the process of digestion into amino acids. Although promoting effects on gastric acid secretion by proteins have been widely studied,[10-12] the role of T2Rs as nutrient sensors in this process is largely unknown. Recently, we identified molecular weight and bitter taste intensity as key determinants responsible for the effects of a subset of proteinogenic L-amino acids on mechanisms regulating gastric acid secretion in the human parietal cell model HGT-1.[13] In that study, involvement of T2Rs was tested by means of the bitter masking compound homoeriodictyol,[14] which is known to target several T2Rs[8, 15] and to reduce the effect of the bitter tasting amino acids L-arginine (L-ARG) and L-isoleucine (L-ILE) on proton secretion.[13] However, whether L-ARG and L-ILE elicit their effects through agonistic or antagonistic activities on specific T2Rs, as demonstrated for sesquiterpenes and lactones by Brockhoff and colleagues[16] or whether other cellular targets are involved has not yet been elucidated. Differential effects of these amino acids seem likely due to the following reasons: 50 mM L-ARG was more effective than L-ILE in stimulating proton secretion in HGT-1 cells, and was also evaluated to be approximately 30% higher bitter intensity than L-ILE by a non-trained sensory panel.[13] These results, in addition to previous studies of our group[8, 17] demonstrate the HGT-1 cell line as a valuable model for the identification of bitter tasting and bitter taste modulating compounds.

T2Rs belong to the group of G-Protein Coupled Receptors (GPCRs). Upon activation, the heterodimeric complex is separated into two complexes: Gα and β1,γ13 gustducin. The first complex stimulates the enzyme phosphodiesterase which cleaves the phosphodiester bond of cyclic adenosine monophosphate (cAMP). The other complex

activates the phospholipase C β2 (PLCβ2), which generates the second messenger phosphatidylinositol-4,5-diphosphate (PIP2), resulting in the formation of inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG), and a Ca^{2+} release from the endoplasmic reticulum.[18] In oral-tissues, this Ca^{2+} release causes a depolarisation and, thereby, activation of taste buds via neurotransmitters. In gastrointestinal tissues, paracrine and endocrine secreting mechanisms are induced and an activation of proton pumps is hypothesized.[19] The human parietal cell line HGT-1 has been shown to express all of the known 25 T2Rs[8] and the functional proteins regulating the activity of the ATP-driven proton pump H^+,K^+-ATPase,[20, 21] and has been established for the identification of bitter tasting and bitter taste modulating compounds via modulation of proton secretion.[17]

Activation of T2Rs by amino acids in native cells has not been widely studied so far. Bassoli and colleagues[22] and Kohl et al.[23] demonstrated the bitter tasting L-tryptophan and L-phenylalanine to activate T2Rs in transfected HEK cells by means of Ca^{2+} mobilisation, whereas no activation was obtained for L-ARG.[23] The authors hypothesized that this difference was due to cytotoxic effects in the T2R transfected HEK-293 cell model, limiting the response to concentrations lower than those applied in sensory tests.[23] Bitter-tasting peptides and L-phenylalanine were previously shown to activate T2R1 receptor in transfected HEK-293 cells.[24] Furthermore, molecular docking predicted that these peptides bind within the same binding pocket at the T2R1 receptor, but in different orientations.[24]

Besides activation of T2Rs, amino acids target a variety of other receptors in the gastrointestinal tract, e.g. the GPCR GPRC6A, the Calcium Sensing Receptor (CASR), the metabotropic glutamate receptor (mGLUR) and the umami receptors T1R1 and T1R3.[25] For L-ARG, there is evidence to target the widely expressed receptors GPRC6A,[26, 27] CASR,[28] mGLUR,[29] and the umami taste receptors (T1R1/T1R3),[30]

whereas L-ILE has so far been associated with umami receptors solely.[31] Although both amino acids have been described for their bitter taste,[2, 32, 33] only for L-ARG is there evidence that its bitter taste is mediated via T2R38 activation. This has been shown in a sensory study investigating the bitterness perception of L-ARG and 6-n-propylthiouracil (PROP), with PROP perception primarily being controlled by polymorphisms in the *T2R38* gene whereas no specific T2R targeted by L-ARG could have been identified so far.[34-36]

Major dietary sources of L-ARG are seafoods, garlic and broccoli, whereas high amounts of L-ILE are found in cheese and ham.[37] Besides their role as essential amino acids for the synthesis of proteins, L-ARG has been shown to improve endothelial functions, wound healing, and gastrointestinal functions,[38] whereas L-ILE lowered blood glucose levels by decreasing liver glucose production.[39] Both amino acids were also demonstrated to differentially modulate proton secretion in HGT-1 cells,[13] although their mechanism of action herein is still unknown. Since there is evidence for bitter tasting peptides (e.g. Trp-Trp-Trp) and L-phenylalanine to activate T2R1 in transiently transfected HEK-293 cells,[23, 24] we hypothesized (i) that T2Rs are targeted by L-ARG and L-ILE in HGT-1 cells and (ii) that GPCR signalling is differentially regulated since there is evidence that GPCRs activate different transduction systems in a native environment.[40] In a first step, effects of commercially available T2R antagonists on proton secretion in HGT-1 cells were tested. Since the results indicated involvement of T2Rs, a whole genome screening of HGT-1 cells exposed to either L-ARG or L-ILE was conducted to elucidate mRNA differences for T2R associated genes and genes related to GPCR signalling pathways. Results indicated major involvement of one T2R, of which its functional impact on L-ARG and L-ILE-mediated proton secretion was demonstrated in a CRISP-Cas9 knock down experiment. Overall, these

149 results will help to understand the mechanism of action of bitter tasting food
150 constituents in parietal cells which are responsible for gastric acid secretion as key
151 determinant of protein digestion and gastric motility as important gastric satiety signal.
152
153
154 **Methods & Materials**
155 **Chemicals.** L-ARG and L-ILE (synthesis grade, purity ≥ 98.5 %), DMEM, L-glutamine,
156 penicillin, streptomycin, probenecid, benzamil hydrochloride hydrate, NPS2143
157 hydrochloride and primer oligonucleotides for T2R expression were purchased from
158 Sigma Aldrich (Austria). PBS (1×) was obtained from Biozym Biotech Trading GmbH
159 (Austria). Fetal bovine serum (FBS) was purchased from Gibco (USA). Cell viability
160 was tested by means of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
161 (MTT, Carl Roth GmbH & Co. KG in Austria). For detection of the proton secretory
162 activity, the fluorescence dye 1,5 carboxy-seminaphtorhodafluor acetoxymethylester
163 (SNARF-1-AM) was obtained from Thermo Fisher Scientific (USA). From this company
164 also, RT-qPCR reagents were obtained. Reagents for droplet digital-PCR (ddPCR)
165 were ordered from Bio-Rad (Austria). For *T2R1* knock out Opti-MEM I Reduced Serum
166 Medium, transfection reagent Lipofectamine™ CRISPRMAX™ Cas9 Transfection
167 Reagent, TrueCut™ Cas9 Protein v2 and GeneArt Genomic Cleavage Detection Kit
168 were obtained from Thermo Fisher Scientific (Austria). For Western Blot, RIPA buffer
169 supplemented with protease inhibitor cocktail, PMSF and, sodium vanadate (all
170 substances from Sigma Aldrich), T2R1 Polyclonal Antibody (1:300) from Invitrogen,
171 Signal Fire Chemiluminescence reagent (New England Biolabs). T2R inhibitors
172 4-(2,2,3-trimethylcyclopentyl)butanoic acid (TMBP) and 6-Methoxyflavaonone were
173 provided by Symrise AG.
174

Cell Culture. Human gastric tumour (HGT-1) cells provided by Dr. C. Laboisse, Nantes (France), were cultivated with DMEM containing 4 g/L glucose and supplemented with 10 % fetal bovine serum, 2 % L-glutamine and 1 % penicillin and streptomycin. Cells grew in a humid atmosphere with 5 % CO_2 in a 37 °C incubator.

Cell vitality. Cell viability was detected by staining with the yellow tetrazole MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent for 15 min after seeding in a 96-well plate 24 and incubated with test substances. The method was carried out as described previously.[2]

cDNA Microarrays. Gene expression analysis by means of microarray analysis were performed as described previously.[41, 42] HGT-1 cells in a concentration of 700,000 cells / 2.5 mL were grown for 24 h in 9.4 m² dishes and incubated with either 50 mM L-ARG or L-ILE for 3h. RNA purity was analyzed at A260/230 nm and A260/280 nm as well as by agarose gel electrophoresis. RNA was reverse transcribed according to Ouellet et al[43] by using SuperScript III (Invitrogen) and Cy3-labeled random nonamer primers (Tebu Bio). After purification of the samples (QIAquick purification columns, Qiagen), microarray hybridization was performed under constant rotation (42 °C for 20 h). The hybridization mix consisted of 3 µL herring sperm DNA (10 mg/mL), 15 µL acetylated BSA (10 mg/mL), 135 µL 2× MES buffer, 10 µL QC25 (Cy3-labeled, 100 nM), 10 µL ECO1BioA1 (Cy3-labeled, 100 nM), 10 µL ECO1BioD2 (Cy3-labeled, 100 nM) and the Cy3-labeled cDNA in 85 µL RNase-free water, with QC25, ECO1BioA1 and ECO1BioD2 serving as quality controls and reference sequences. Subsequent to the hybridization, microarrays were washed for 2 minutes in non-stringent wash buffer (SSPE; 0.9 M NaCl, 0.06 M phosphate, 6 mM EDTA, 0.01 % Tween20), for 1 minute in stringent wash buffer (100 mM MES, 0.1 M Na^+, 0.01 %

Tween20) and for about 5 seconds in final wash buffer (0.1× saline-sodium citrate buffer). After drying the microarrays in a microarray centrifuge, they were scanned at 2.5 µm resolution using a Axon GenePix 4400A microarray scanner (Molecular Devices). Data was extracted and intensities were determined by NimbleScan 2.1 (Roche NimbleGen).

RT-qPCR. In 9.4 m² dishes 700,000 cells were seeded and incubated with 50 mM L-ARG or 50 mM L-ILE for different time points (0/15/30/60/180 min). RNA isolation was done with PeqLab total RNA kit obtained from Peqlab (Austria). RNA integrity was analyzed by the A260/280 absorbance ratio using the nanoquant plate for the Infinite 200 PRO Plate Reader. All RNA samples had a ratio between 1.8 - 2.2. Reverse transcription was carried out using the High capacity RNA to cDNA Master Mix kit from Applied Biosystems (Germany). Primers for the internal control *peptidylprolyl isomerase A (PPIA)*, and *T2R1/4/20/39/43/50* were designed by using PrimerBlast and PCR products were verified by melting curve analysis, agarose gel electrophoresis, and sequence analysis (Eurofins Genomics). Sequences are provided in Table 1.[8] Predesigned primers were used for the *epithelium sodium channel* α (Quantitect primer assay, Qiagen, Germany). The open source software LinReg v2013.0 was used for data analysis.

Digital Droplet (dd)-PCR

HGT-1 cells were treated with 50 mM L-ARG or L-ILE in a time-dependent manner. RNA isolation and cDNA synthesis were carried out according to the same protocol as described for the RT-qPCR experiments. EvaGreen Supermix was used as reaction reagent for *calcium sensing receptor (CASR)* and *PPIA* expression. For analyzing *CASR* expression, non-diluted HGT-1 samples were used whereas for *PPIA* cDNA

analysed, samples were diluted 2.5 times. Droplet Generation Oil for Evagreen was used for droplet generation by pipetting 20 µL of the cDNA and 70 µL into the cartridge and put it into the droplet generator. Afterwards, droplets were transferred onto a 96-well microtiter plate (Eppendorf) and PCR was carried out at a temperature of 58.1 °C on a C1000 thermocycler. Data analysis was done by QuantaSoft.Ink software and droplet threshold was set at 30,000 droplets.

T2R1 protein expression

Western Blot analysis was performed as described previously.[44] HGT-1 cells were grown in 6-well plates for 24 hours. Afterwards, cells were washed with ice-cold PBS and lysed with RIPA buffer supplemented with a protease inhibitor cocktail, Phenylmethylsulfonyl fluoride (PMSF) and sodium vanadate. In the supernatant, protein concentration was determined by the Bradford assay. On a 16 % polyacrylamide SDS-gel, 20 µg of the protein or 3 µL prestained protein standard obtained from New England Biolabs were loaded and subjected to electrophoresis, Afterwards, the proteins were transferred onto an PVDF membrane which was activated for 2 min by methanol and for 5 min using the blotting buffer. After blocking the membrane for 1 h with 5 % BSA, the primary antibody (rabbit anti T2R1, 1:300, Thermo Fisher Scientific or rabbit anti-β-actin, 1:2000, Santa Cruz) was added over night at 4 °C. The secondary antibody was added after 4× of washing (goat anti-rabbit, 1:2000, CST cell signalling). Before Signal Fire Chemiluminescence reagent was pipetted onto the membrane, the membrane was washed four times. Chemiluminescence was detected by Fusion FX7 device (Vilber) and image analysis was carried out using ImageJ.[45]

ELISA

cAMP. Cells were seeded in cell culture dishes in a concentration of 2.5 million in 4 mL DMEM. Afterwards, cells were incubated with 50 mM L-ARG or 50 mM L-ILE for 10 min, subsequently washed 2x with cold (PBS) and lysed by the cell lysis buffer from the ELISA kit from R&D Systems. cAMP concentration was quantitated according to the supplier's protocol (R & D Systems). Absorbance was detected at 450 nm by the Infinite 200 PRO Plate Reader.

IP1. 24 h before assay, HGT-1 cells were seeded in a 24-well plate (400,000 cells / 500 µL DMEM) and incubated for 1 h with 50 mM L-ARG or 50 mM L-ILE solved in stimulation buffer provided by the IP1 ELISA kit from CISBIO. All other steps were carried out according to the suppliers' manual of CISBIO. Absorbance signal was detected at 450 nm and reference wavelength was set at 620 nm.

Proton secretory activity. Detection of proton secretion was performed as described previously.[2, 13] Briefly, this method is based on the quantification of intracellular protons. The lower the intracellular proton concentration, the higher the number of protons excreted.[46, 47] Experimentally, HGT-1 cells were seeded in a 96-well plate (FluoroNunc™ F96 MicroWell™, VWR) in a concentration of 100,000 cells / 200 µL DMEM 24 h before starting the assay. For the experiment, cells were stained for 30 min with 3 µM of the pH-sensitive SNARF-1-AM. Afterwards, cells were treated with L-ARG and L-ILE alone and in combination with inhibitors (TMBP, probenecid, 6-Methoxyflavanone, benzamil, NPS2143). Changes of the intracellular proton concentrations were detected at 580 nm and 640 nm. For detecting the intracellular pH, a calibration curve was generated by treating the cells with potassium buffer solutions in a pH range between 6.8 to 7.6. Intracellular H^+ concentrations were determined by calculating a linear calibration curve. Equilibration of the intra- and extracellular pH was achieved by means of a 2 µM Nigericin solution. Resulting data

were calculated as intracellular proton index (IPX), and expressed as ratio between media control and compound treated HGT-1 cells. Final data were $\log_2$ transformed.

Knock out. *T2R1* knock out was done by means of CRISPR/Cas9, starting with 15,000 HGT-1 cells grown in a 96-well plate for 24 h. A total of 50 ng gRNA of the non-targeting control and the gRNA of T2R1 (UUAUGGUCCCAUACUUCCUA) was allowed to form a complex with 250 ng of Cas9 Protein v2 and the Lipofectamine™ CRISPRMAX™ transfection reagent. After 48 h, a genomic cleavage detection was done according to the manufacturer's manual. As forward (5'-3') reverse primer (3'-5'), the following sequences ACAGGATAGACAGCAACGCGC and GGTGGTGAATGGCATTGACTTG were used, respectively. The cleavage efficiency was approximately 30 %.

Protein Modelling. Initial T2R1 models were built using the servers of GPCR-ModSim[48], I-TASSER[49] and Medeller.[50] The performance of the models was evaluated by enrichment with known actives and inactives sourced from BitterDB.[51] The best model was obtained by Induced fit docking (IFD; performed using Maestro[52]) (see 'Molecular docking' section below) performed using the GPCR-ModSim server model. An area under the curve (AUC) was generated by using SigmaPlot 12 software and Glide docking scores from known actives and inactives docked to the T2R1 model. The AUC generated from the enrichment was improved by IFD from 0.68 to 0.75. The extracellular loop 2 (ECL2) of this IFD-modified T2R1 model was sampled for 1000 conformations using Modeller[53], GalaxyLoop[54], ModLoop[55], and DaReUs-Loop.[56] Amino acid side chain conformations were predicted with SCWRL4.[57] Protein structures were prepared using the Protein Preparation Wizard available within Maestro[52] (assigned bond orders, added hydrogens, created disulfide bonds) and the protonation state of all side chains determined at pH 7 ± 2. Energy minimization of

hydrogen atoms only was carried out using default constraints of 0.3 Å RMSD in the OPLS3e force field. Binding sites were automatically identified using SiteMap.[58] A pairwise sequence alignment was done by using Jalview 2.11.0[59] between the model sequence and the template sequence (PDB: 2vt4).

Molecular docking. In total, 39 actives and 104 inactives for T2R1 were obtained from BitterDB.[51] Ligands were enumerated to 3-D conformers using LigPrep[60] at pH 7 ± 2. Receptor grid calculations were centered on the binding sites identified by SiteMap. Docking was performed using Glide Standard Precision (SP) mode.[58, 60-62] Ligands were docked flexibly, sampling nitrogen inversions and ring conformations, and a single pose generated per ligand.

Statistical Analysis. Data are presented as means ± standard error of the mean (SEM). All experiments were calculated from 2-4 biological replicates. Nalimov outlier test was performed prior ANOVA or Student's *t* test. Significant differences between untreated control and different treatments were determined using One- or Two-Way ANOVA with Holm-Sidak post hoc. Comparison of two groups (control/treatment) was performed with Student's *t* Test. Significances were set at a *p*-value of 0.05 and marked with letters or asterisks.

Results

Proton secretory activity (IPX). (**Table 2**) TMBP and 6-methoxyflavanone (MXO) were used as antagonists of T2R4/7/14/20/31/39/40/43. Combination of 25 µM TMBP and 50 mM L-ARG induced a mean ΔIPX of 0.51 ± 0.11 % compared to L-ARG treatment, whereas an opposite effect was detected for L-ILE and TMBP (ΔIPX -0.97 ± 0.08%, $p < 0.001$). A different regulation of proton secretion induced by L-ARG and L-ILE was also demonstrated in co-incubation experiments with 5 µM of the T2R antagonist MXO (L-ARG + MXO: ΔIPX + 0.51 ± 0.35 % vs. L-ILE + MXO: ΔIPX -0.93 ± 0.14 %; $p < 0.01$) (**Table 2**).

cDNA Microarray. A genome wide screening was performed after incubation of HGT-1 cells with 50 mM L-ARG or 50 mM L-ILE for 3 h. Δ $\log_2$ data (treatment - control) are depicted in **Figure 2** and demonstrate that cDNA expression was differentially regulated by the amino acids (One Way ANOVA on Ranks followed by Student-Newman-Keuls Method, post hoc, $p < 0.05$). Control was set to 0. A different regulation by L-ARG and L-ILE on known targets (GPCR6A, CASR, mGLUR) was not detected (**Figure S1**). In **Figure 2B**, data with a fold change between 0.5 – 2 were further analysed by means of DAVID pathway analysis (http://david.abcc.ncifcrf.gov), resulting in the displayed cluster with the highest annotation. (**C**) Pearson Product Moment Correlation analysis between fold changes (T/C) induced by L-ARG and L-ILE indicates a regulating role of T2R1 (r: 0.637; $p < 0.001$).

Western Blot & RT-qPCR. In HGT-1 cells the protein of T2R1 was detected (**Figure S6**). In **Figure 3**, the influence of L-ARG as well as L-ILE on *T2R1* mRNA expression in HGT-1 cells over time (L-ARG: 13.54 ± 0.57 to 8.54 ± 0.45, L-ILE: 6.44 ± 0.30 to 4.76 ± 0.58, $p < 0.05$) is depicted. HGT-1 cells showed a significantly

more pronounced regulation of *T2R1* mRNA expression after L-ARG exposure in comparison to treatment with L-ILE ($p < 0.001$). No regulation was seen for the *ENAC* α after incubation with 50 mM L-ARG or L-ILE over time. (**Figure S2**)

dd-PCR. mRNA expression of the *CASR* in HGT-1 cells was differentially regulated when comparing L-ARG and L-ILE treatment after 60 min and 180 min (fold change L-ARG: 1.02 ± 0.86, 0.95 ± 0.13, and L-ILE: 0.18 ± 0.04, 0.18 ± 0.03, $p < 0.01$) (**Figure S3**).

Impact of *T2R1* knock out on L-ARG and L-ILE-modulated proton secretion. The effect on proton secretion induced by L-ARG (IPX -71.7 ± 14.0 % compared to HGT-1 WT = 0 %) decreased by 65.7 ± 21.9 % after *T2R1* KO (**Figure 4A**). For L-ILE, in contrast, the *T2R1* KO in HGT-1 cells increased the effect of L-ILE on proton secretion by 93.2 ± 24.1 % in comparison to HGT-1 WT cells ($p < 0.05$) (**Figure 4B**).

In **Figure S4A**, glide docking of L-ARG to T2R1 predicted that $S^{6.60}$, A^{ECL2} and $C^{3.29}$ are interacting with the ligand via hydrogen bonds. For L-ILE there was one interaction suggested with $S^{6.60}$ (**Figure S4B**), which is also one of the predicted binding residues for docking of L-ARG to T2R1. A sequence alignment and the T2R1 model with known conserved motifs are depicted in **Figure S5**.

ELISA. Incubation with 50 mM L-ARG induced a stronger decrease of **cAMP** concentrations by -83.5 ± 9.07 % than the treatment of HGT-1 cells with 50 mM L-ILE by -33.48 ± 10.5 %. Effects induced by L-ARG and L-ILE were significant in comparison to untreated controls, which were set to 100 % ($p < 0.01$) (**Figure 5A**). **IP1** concentrations increased by 37.2 ± 21.02 % in HGT-1 cells after incubation with

379 50 mM L-ILE, whereas L-ARG induced a mean decrease by -3.88 ± 39.78 % ($p < 0.05$)

380 (**Figure 5B**).

Discussion

Bitter taste receptors (T2Rs) have been shown to be expressed in many different extra-oral tissues, like the airway, urethra or gastrointestinal tract,[19] and to regulate various metabolic pathways. In the gastro-intestinal tract, activation of gastric T2Rs by quinine increased plasma levels of the satiating hormone cholecystokinin, and reduced total energy intake from a subsequent meal,[63] Our group also showed a satiating potential of gastric T2R activation in healthy subjects who demonstrated an increased GAS after caffeine administration.[8] Moreover, in the gastric acid producing parietal cell line HGT-1, T2R10 and T2R43 signalling has been shown to be involved in caffeine-induced proton secretion.[8]

Dietary proteins are known for their satiating effects, which are mediated by a variety of gastrointestinal signals, including GAS. Plant proteins, in particular, as well as some protein hydrolysates and amino acids present a bitter off-taste, which limits consumer acceptance. We recently demonstrated the bitter taste of L-AAs to correlate with proton secretion in HGT-1 cells, although the underlying mechanisms still need to be elucidated.[2] As L-ARG and L-ILE were evaluated as bitter tasting by a sensory panel, but induced a contradictory effect on proton secretion in HGT-1 cells, these AAs were chosen as candidates for the here presented study.

The lack of complete correlation between activation of T2Rs in cell systems and sensory data has been addressed before, although no conclusive answer has been found yet. Brockhoff and colleagues[16] also reported inconsistent results for bitter-tasting and structurally related sesquiterpene lactones on T2R-dependent calcium signalling in T2R46 transiently transfected HEK-293 cells. The T2R signalling is complicated by the fact that the orthosteric binding site can be shared by agonists and antagonists.[64] Synergistic and antagonistic effects of compounds targeting multiple T2Rs[9] or even other cell surface receptors might contribute to cellular

responses which do not necessarily correlate with the bitterness perceived by sensory panels.

For the bitter tasting AAs L-ARG and L-ILE, there is evidence for an activation of T2R-linked GPCRs,[26, 65] although it was not known whether and which T2R is targeted. L-ARG has been shown to be involved in T2R signalling by enhancing the bitterness of PROP, which is a bitter substance commonly used for detection of *T2R38* polymorphisms.[36] For L-ILE, a distinct bitterness has been reported,[32, 33, 66] although the T2R responsible is unknown.[23]

In this study, we demonstrated involvement of T2Rs in L-ARG- and L-ILE-modulated proton secretion in HGT-1 cells by means of the T2R antagonists TMBP, MXO and probenecid.

TMPB binds at the orthosteric sites of T2R4/7/20/31/40/43 with high selectivity, known to target lysine in the sequence of the transmembrane helix 7 of T2R31/43.[67] The T2R antagonist MXO targets T2R14 and T2R39. Its efficacy depends on the C_6 methoxy group of the A-ring, and the size, electronegativity and the electron withdrawing effect of the B-ring.[68] The third T2R antagonist chosen is probenecid for which an allosteric inhibition of T2R16/38 and 43 has been reported,[69] indicating its binding to the receptor site being distinct from the orthosteric ligand binding site.[70] In order to test our hypothesis that T2Rs are involved in the modulating effect of L-ARG and L-ILE on proton secretion in HGT-1 cells, a range of T2R antagonists were tested in co-incubation experiments. These co-incubation experiments using TMBP or probenicid in combination with L-ARG or L-ILE revealed significant changes of the effect on proton secretion demonstrated for the individual AA, indicating involvement of the T2Rs targeted by the respective antagonist. Co-treatments of the cells with L-ARG or L-ILE and MXO revealed similar results, although the level of statistical significance was not met for L-ARG experiments ($p = 0.37$).

Based on these results, a cDNA microarray screening was performed to identify any of the 25 T2Rs representative GPCRs (see supplemental material) were regulated by L-ARG and L-ILE. Scatter plot analyses and David pathway analyses revealed L-ILE as the more potent AA to regulate overall mRNA levels and, specifically, *TAS2R* mRNA expression, with *T2R1* cDNA increasing most markedly. Next, involvement of T2R1 in the observed effects on proton secretion was studied. Since none of the commercially available T2R antagonists targets T2R1, a knock out approach by means of CRISPR/Cas9 was performed. Treatment of T2R1 knock out cells with L-ARG or L-ILE revealed significantly different effects on proton secretion compared to HGT-1 WT cells: the stimulating effect induced by L-ARG in HGT-1 WT cells[2] decreased in HGT-1 T2R1 KO cells, and the inhibiting effect on proton secretion by L-ILE[2] was significantly reduced in HGT-1 T2R1 KO cells. A different regulation on a functional level mediated by T2R1 was also published by Upadhyaya and colleagues,[24] who demonstrated that for bitter-tasting amino acids and peptides different EC_{50} concentrations are necessary to activate T2R1 in transfected HEK-293 cells.[24]

The presence of the T2R1 protein in HGT-1 cells was detected by western blot method. These results provide further evidence for a potential role of T2R1 in the L-ARG and L-ILE evoked effects mechanism on proton secretion in HGT-1 cells.

The hypothesis that T2R1 might be targeted by the amino acids L-ARG and L-ILE is supported by previously published results which demonstrated T2R1 activation by L-phenylalanine[22, 23] and by peptides containing L-ILE.[23] However, there is also evidence from transiently transfected HEK-293 cells, that L-ARG and L-ILE may not target T2Rs at physiologically relevant concentrations.[23] As discussed by Kohl and colleagues[23], the reason for these diverging results could be that it was not possible to use the same concentrations tested in sensory studies. Another hypothesis could also

459 be that the use of a heterologous cell model in which only one T2R is expressed vs. a
460 native cell model which does express all 25 T2Rs, is able to carry out T2R interactions.
461 Assuming that these molecules do interact with T2R1 directly, molecular docking
462 studies predict one shared binding interaction (S248$^{6.60}$) for L-ARG and L-ILE and the
463 T2R1 receptor model. Furthermore, L-ARG is suggested to also interact with C82$^{3.29}$
464 and A164 in the ECL2. The different binding interactions predicted for L-ARG and L-ILE
465 and the T2R1 model, might contribute to the different effects on downstream signalling
466 and eventually on proton secretion in HGT-1 cells. This outcome would go along with
467 results published by Upadhyaya and colleagues[24], who found that certain amino acids
468 and bitter-tasting peptides activate the T2R1 to a different extent, and their receptor
469 studies predict overlapping as well as different binding residues.[24] There is evidence
470 that antagonists for T2R bind to the same binding sites as agonists, therefore they are
471 called competitive antagonists and indicate a single binding pocket for T2R
472 activation.[71] In a recent publication on advanced glycation end-products (AGEs), AGEs
473 were shown to activate as well as inhibit T2R4 activity, associated with polar
474 interactions with N164, N165, and T166 present in ECL2.[72]
475 Apart from T2Rs, L-ARG and L-ILE might target other cell surface receptors that are
476 involved in proton secretion by HGT-1 cells. E.g., the stimulating effect induced by
477 L-ARG might be mediated by the formation of nitric oxide, which has recently been
478 demonstrated by Kitay and colleagues[73]. Authors of this study suggest that after
479 transportation of L-arginine into the parietal cell, L-arginine is converted into NO by
480 endothelial nitric oxide synthetase (eNOS), which subsequently results in a stimulation
481 of the proton pump mediated via soluble guanylyl cyclase (sGC) and cyclic guanosine
482 monophosphate (cGMP).[73]
483 Moreover, L-ARG has been described as a ligand for the CASR.[28] To elucidate a
484 possible involvement of this receptor, HGT-1 cells were treated with L-ARG or L-ILE,

and tested for their *CASR* mRNA expression by means of ddPCR (since qRT-PCR was not sensitive enough to quantify the very low expression levels). *CASR* mRNA levels were differentially regulated by L-ARG and L-ILE after 60 and 180 min treatments, indicating the CASR as a potential target for these AAs (**Figure S1C**).

Binding of agonists or antagonists to the CASR or to T2Rs results in changes of GPCR-mediated signalling pathways. Signal transduction is mediated via the second messengers cyclic adenosine monophosphate (cAMP) and/or inositol-1,4,5-triphosphate (IP3), which is degraded to IP1.[18, 74] In contrast to literature evidence,[27] L-ARG most potently decreased cAMP levels in HGT-1 cells in the current work, although it stimulated proton secretion at a higher level than L-ILE. These results indicate activation of the PDE pathway solely, because IP1, a stable downstream metabolite of the PLCβ2 pathway was, not affected.

Treatment of HGT-1 cells with L-ILE reduced cAMP concentrations less pronounced than L-ARG and increased IP1 levels compared to controls. The different GPCR signalling induced by L-ARG and L-ILE, might explain their divergent effects on proton secretion as cAMP was more potently regulated by L-ARG, whereas most pronounced changes in IP1 concentrations were induced by L-ILE. Moreover, the more pronounced effect of L-ARG on proton secretion might also be due to a stronger interaction via hydrogen bonds with the T2R1 receptor, which was predicted by molecular modeling. Furthermore, L-ARG had a stronger effect on T2R1 mRNA expression in HGT-1 cells than L-ILE. We suggest that the increase in T2R1 expression is involved in proton secretion mechanism.

As another cellular target of L-ARG, the epithelial sodium channel alpha (ENAC α), has been studied, based on evidence for L-ARG to enhance salt taste perception via ENACα activation.[75] However, we did not observe any changes in *ENAC* α mRNA

levels in HGT-1 cells after treatment with L-ARG or L-ILE (**Figure S2**). Investigation of the remaining β,γ,δ forms to fully exclude the involvement of this receptor in L-ARG-evoked effects is recommended for future studies.

As there is growing evidence for T2Rs to act as nutrient receptors and to regulate different metabolic pathways in the gastrointestinal tract, the focus of this *in vitro* study was to elucidate whether the bitter-tasting AAs L-ARG and L-ILE evoke divergent effects on proton secretion in HGT-1 cells. This question is of relevance, since sensory data evaluating bitter-tasting food constituents not always correlate closely with T2R activation in cell models. Elucidating the cellular mechanisms involved in T2R activation will help to understand these diverging effects of bitter tasting and bitter taste-modulating compounds.

The aim of the present *in vitro* study in HGT-1 cells was to elucidate why the bitter-tasting amino acids, L-ARG and L-ILE induce different effects on proton secretion. For this purpose, we started with co-incubation experiments with commercially available T2R antagonists for detecting if T2R are possibly involved in the mechanism of proton secretion. We observed a modulation of proton secretion, but could not identify which specific T2Rs are activated by L-ARG and L-ILE. Hence, cDNA expression of all 25 T2Rs after treatment with L-ARG and L-ILE was measured by means of the Microarray method. This experiment demonstrated that cDNA expression of T2R1 was strongly upregulated by L-ARG as well as L-ILE. We therefore validated this receptor by RT-qPCR. In addition, we quantitated the T2R1 protein, which indicated a potential functional role for it in proton secretion in HGT-1 cells. Since there is no specific T2R1 antagonist available, a T2R1 KO was induced in HGT-1 cells that changed the effect on proton secretion following L-ARG and L-ILE stimulation. However, since amino acids have many cellular targets, additional targets may be involved. Regulation of

mRNA expression of the *CASR* and the *ENAC* by the amino acids tested was studied, but did not reveal any changes. Additional factors of influence beyond T2R signalling cannot be excluded and have to be identified in future studies. Overall, we concluded that T2R1 plays an important role in the mechanism of L-ARG and L-ILE-modulated proton secretion in HGT-1 cells. The diverging effects of L-ARG and L-ILE on proton secretion could at least in part be explained by differentially regulated cAMP and IP1 levels. In addition, the deviation of the predicted T2R1 interaction residues for L-ARG and L-ILE could also play a role.

According to our hypothesis that T2R-mediated signalling is involved the modulating effect of L-ARG and L-ILE on proton secretion in HGT-1 cells, we demonstrated that T2R1 expression, as well as T2R1 signalling, are differentially regulated by solutions of 50 mM L-ARG and L-ILE which present a similar bitter taste intensity in human sensory studies. These findings were substantiated by diverging effects on second messengers of the GPCR cascade, resulting in a different regulation of proton secretion in HGT-1 cells after incubation with L-ARG and L-ILE. We also demonstrated that L-ARG and L-ILE modulate proton secretion in HGT-1 cells via T2R1 by CRISPR-Cas9 knock down approach. Molecular docking predicted different binding positions for L-ARG and L-ILE at the T2R1 and may explain differential downstream signalling, which warrants further study. The current results not only begin to shed light on the effects of nutritive amino acids, but also highlight the importance of further study of biased signalling[76, 77] in bitter taste receptors.

Abbreviations

Amino Acids (AAs), Gastric Acid Secretion (GAS), G-Protein Coupled Receptor (GPCR), Homoeriodictyol (HED), Intracellular Proton Index (IPX), bitter taste receptors (T2R)

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Conflict of interest statement

Joachim Hans, Jakob P. Ley and Gerhard E. Krammer are employed by Symrise AG.

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Table 1[8]

Primer	Forward	Reverse	Amplicon
hT2R1	AAATGGCTCCGCTGGATCTC	GTGGCAAGCCAAAGTTCCAA	172
CASR	GGAGCCACTCACCTTTGTG	GCACTGGCATCTGTCTCATCA	168
PPIA	CCACCAGATCATTCCTTCTGTAGC	CTGCAATCCAGCTAGGCA	144

Table 2

antagonist	targets	L-ARG		L-ILE		L-ARG vs. L-ILE
		Δ-IPX	H^+	Δ-IPX	H^+	p-value
50 µM NPS2143	CASR	0.16 ± 0.008	0:19	0.01 ± 0.007	0.87	0.198
5 µM benzamil	ENAC (α,β,γ,δ)	-0.78 ± 0.10*	↑	-0.71 ± 0.12*	↑	0.689
25 µM TMBP	T2R 4/7/20/31/40/43	0.51 ± 0.11*	↓	-0.97 ± 0.08*	↑	**< 0.001**
5 µM MXO	T2R14/39	0.51 ± 0.35	0:37	-0.93 ± 0.14*	↑	**< 0.01**
5 µM probenecid	T2R16/38/43	-1.07 ± 0.13*	↑	-0.87 ± 0.11*	↑	0.278

Table 2: Influence of different blockers co-incubated with L-ARG or L-ILE on proton secretion in HGT-1 cells. Δ effect: IPX-AA subtracted from IPX-antagonist + AA. Statistical differences were tested by t-Test, mean ±SEM, n = 3-4, p> 0.05. Differences to AA alone are marked with an asterisk (*).

Figure captions

Figure 1: Postulated mechanism on proton secretion in the parietal cell model HGT-1 after incubation with 50 mM L-ARG and L-ILE.

Figure 2: (**A**) Whole genome screening detected by means of microarray method, after 3h incubation time with 50 mM L-ARG and L-ILE in HGT-1 cells. cDNA expression of L-ARG vs. L-ILE is presented as $\Delta \log_2$ intensities (control subtracted from L-ILE and L-ARG), n = 2-3. (**B**) Functional Annotation Analysis of cDNA levels with a fold change between 0.5 and 2 was analysed with DAVID pathway analysis tool. (**C**) Pearson Product Moment Correlation indicates a prominent role for T2R1 on proton secretion in HGT-1 cells after treatment with L-ARG and L-ILE, n = 2-3,

Figure 3: *T2R1* gene expression by RT-qPCR after time-dependent incubation with L-ARG and L-ILE vs. medium-only treated HGT-1 cells (control). Statistical differences were tested by t-Test between L-ARG and L-ILE, control (a) is depicted as grey line = 1, n = 3-4, $p < 0.001$

Figure 4: Impact of L-ARG (**A**) and L-ILE (**B**) on proton secretion in HGT-1 WT and HGT-1 *T2R1* knock out cells; significances were tested by t-Test, $p < 0.05$, n = 3, Δ IPX was calculated as amino acid treated [%] over control [HGT-1 WT / HGT-1 knock out = 0 %].

Figure 5: Influence of L-ARG and L-ILE on GPCR signalling molecules (**A**) cAMP and (**B**) IP1 in HGT-1 cells. One Way ANOVA followed by Holm-Sidak post hoc, $p < 0.05$, n = 3. Different letters indicate significance, a = control, which was set to 100 %. Δ-values were calculated as treatment - control

Figure 1

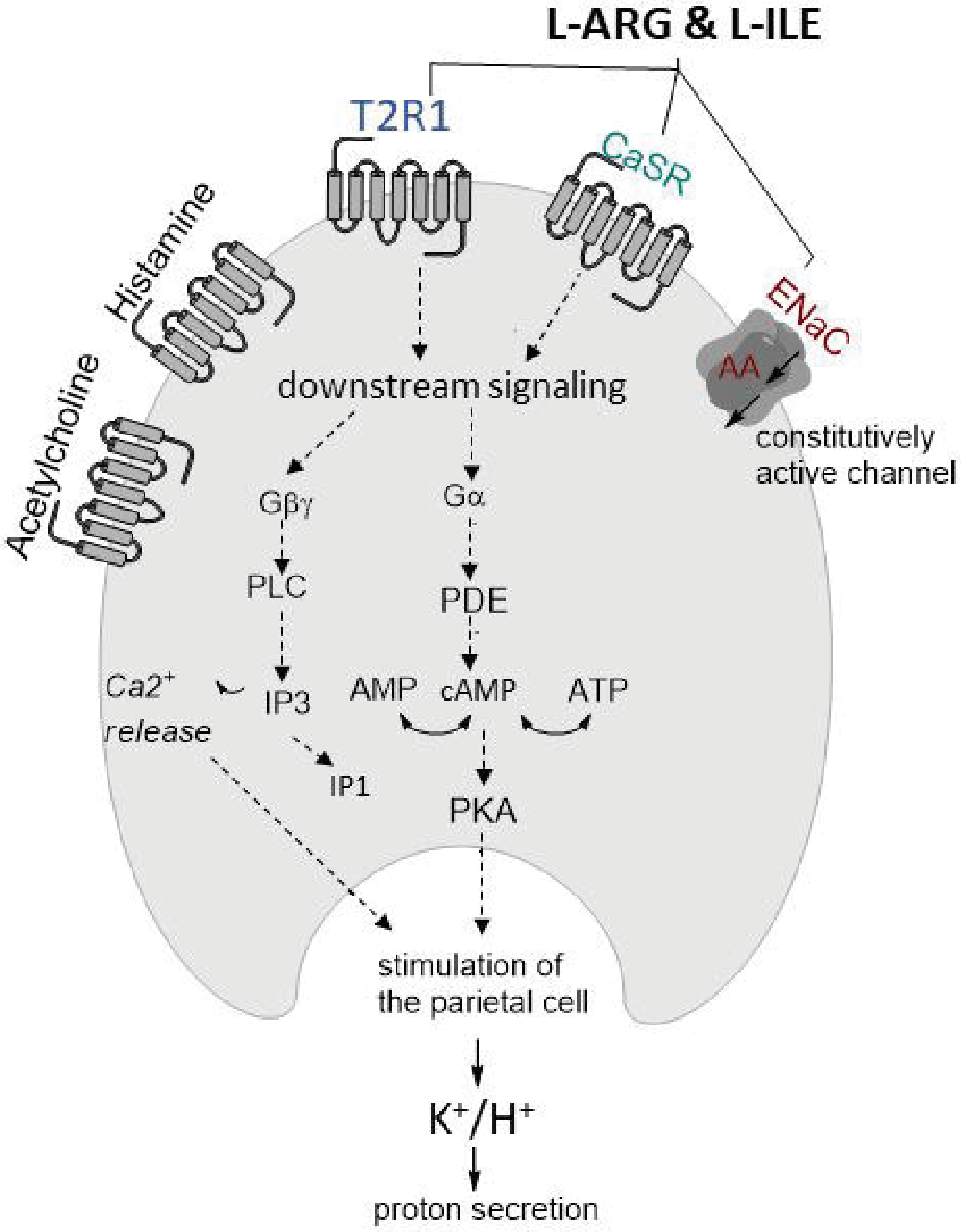
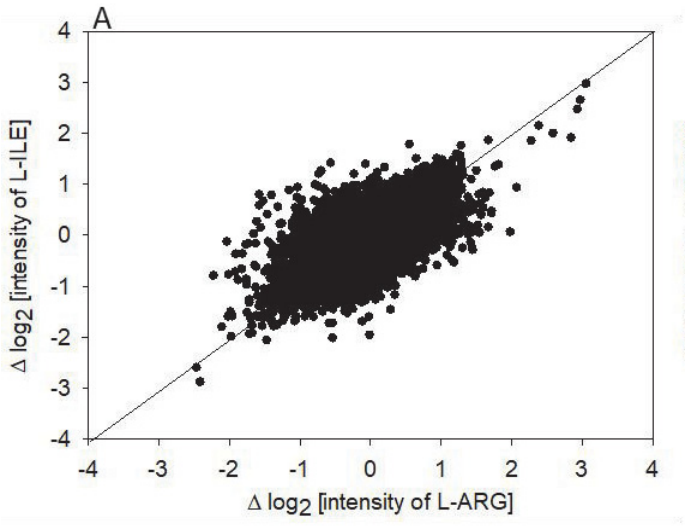
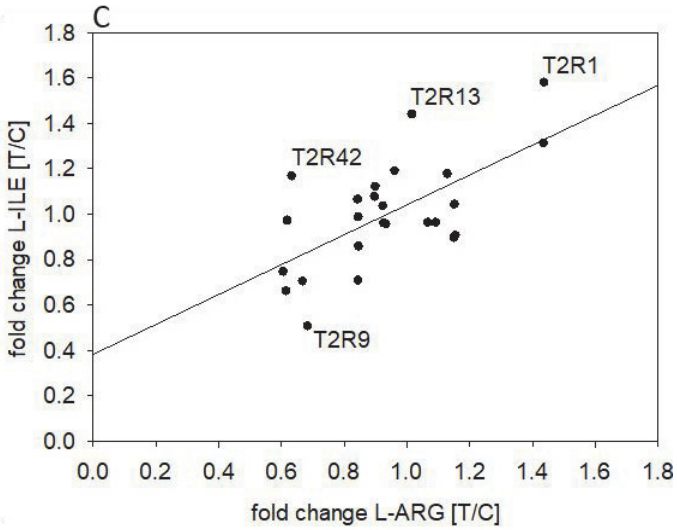

L-ARG & L-ILE
T2R1
CaSR
ENaC
Histamine
Acetylcholine
AA
downstream signaling
constitutively
active channel
Gβγ
Gα
PLC
PDE
Ca2+
release
IP3
AMP
cAMP
ATP
IP1
PKA
stimulation of
the parietal cell
K+/H+
proton secretion

Figure 2





B

Functional Annotation Analysis: G-protein coupled receptor signalling pathway Annotation Cluster 1		
	L-ARG	L-ILE
counts	32	11
enrichment score	6.68	7.90
p-value	1.8E-02	1.0E-09
Benjamini	5.5E-01	3.9E-07

Figure 3

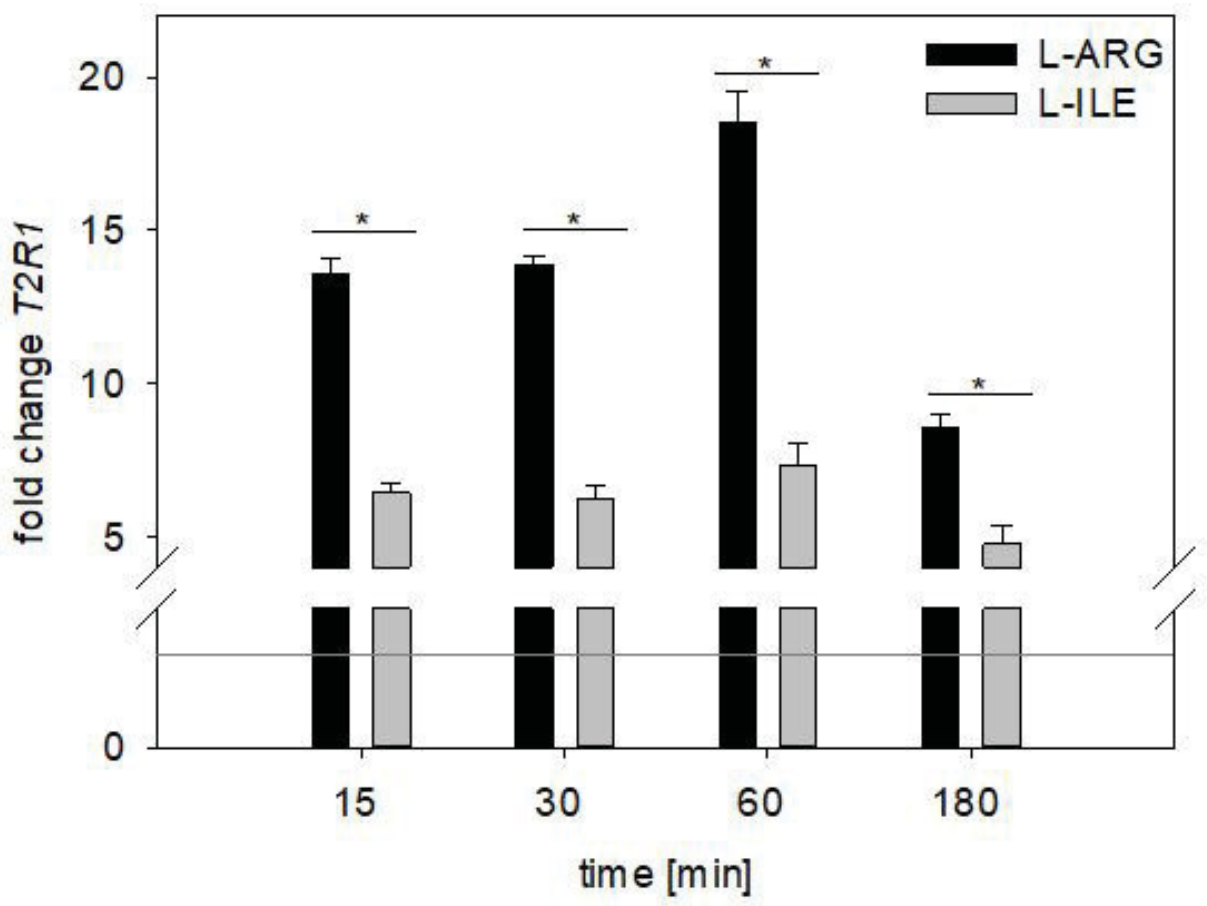


Figure 4

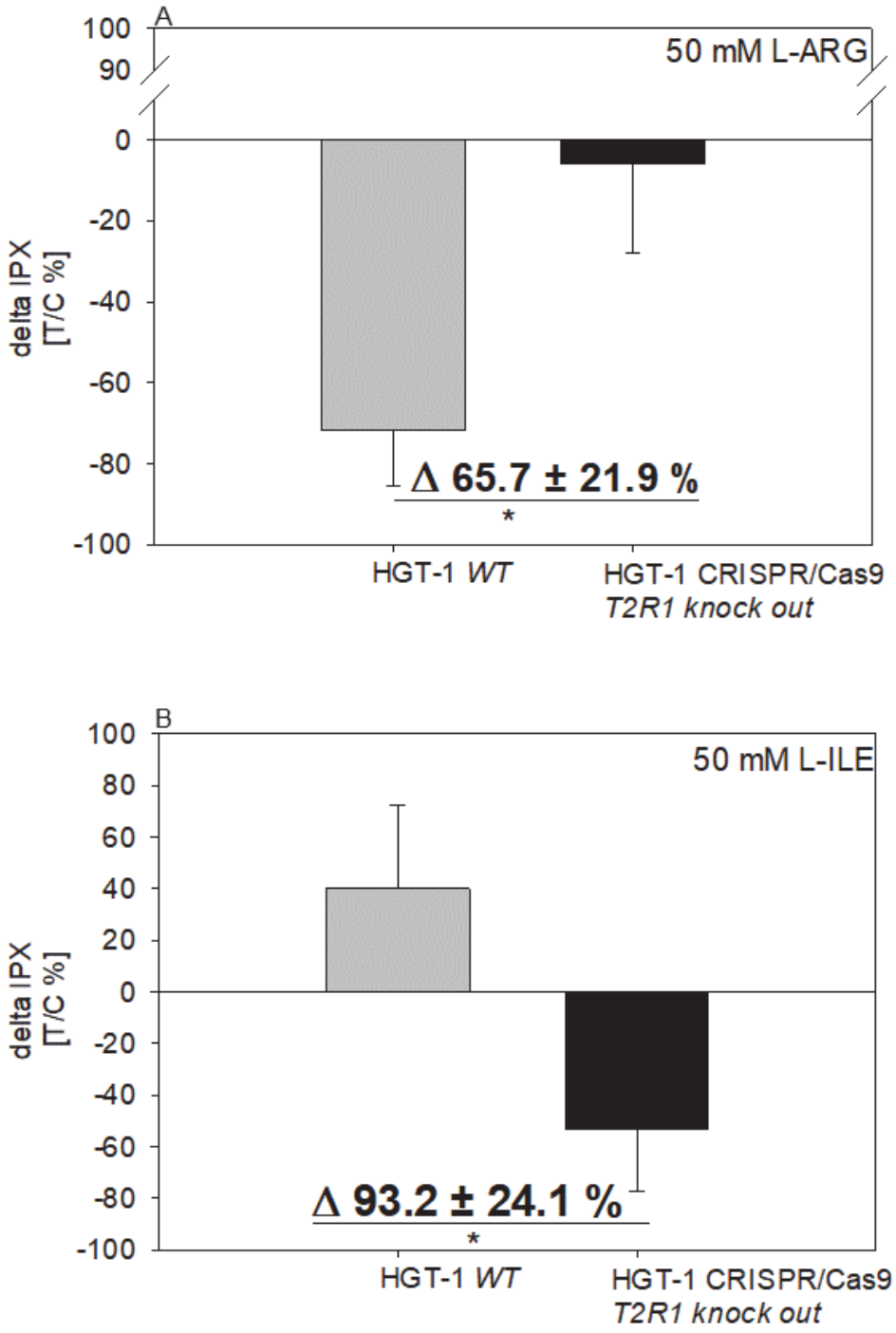

A
50 mM L-ARG
100
90
0
-20
-40
-60
-80
-100
delta IPX
[T/C %]
Δ 65.7 ± 21.9 %
*
HGT-1 WT
HGT-1 CRISPR/Cas9
T2R1 knock out
B
50 mM L-ILE
100
80
60
40
20
0
-20
-40
-60
-80
-100
delta IPX
[T/C %]
Δ 93.2 ± 24.1 %
*
HGT-1 WT
HGT-1 CRISPR/Cas9
T2R1 knock out

Figure 5

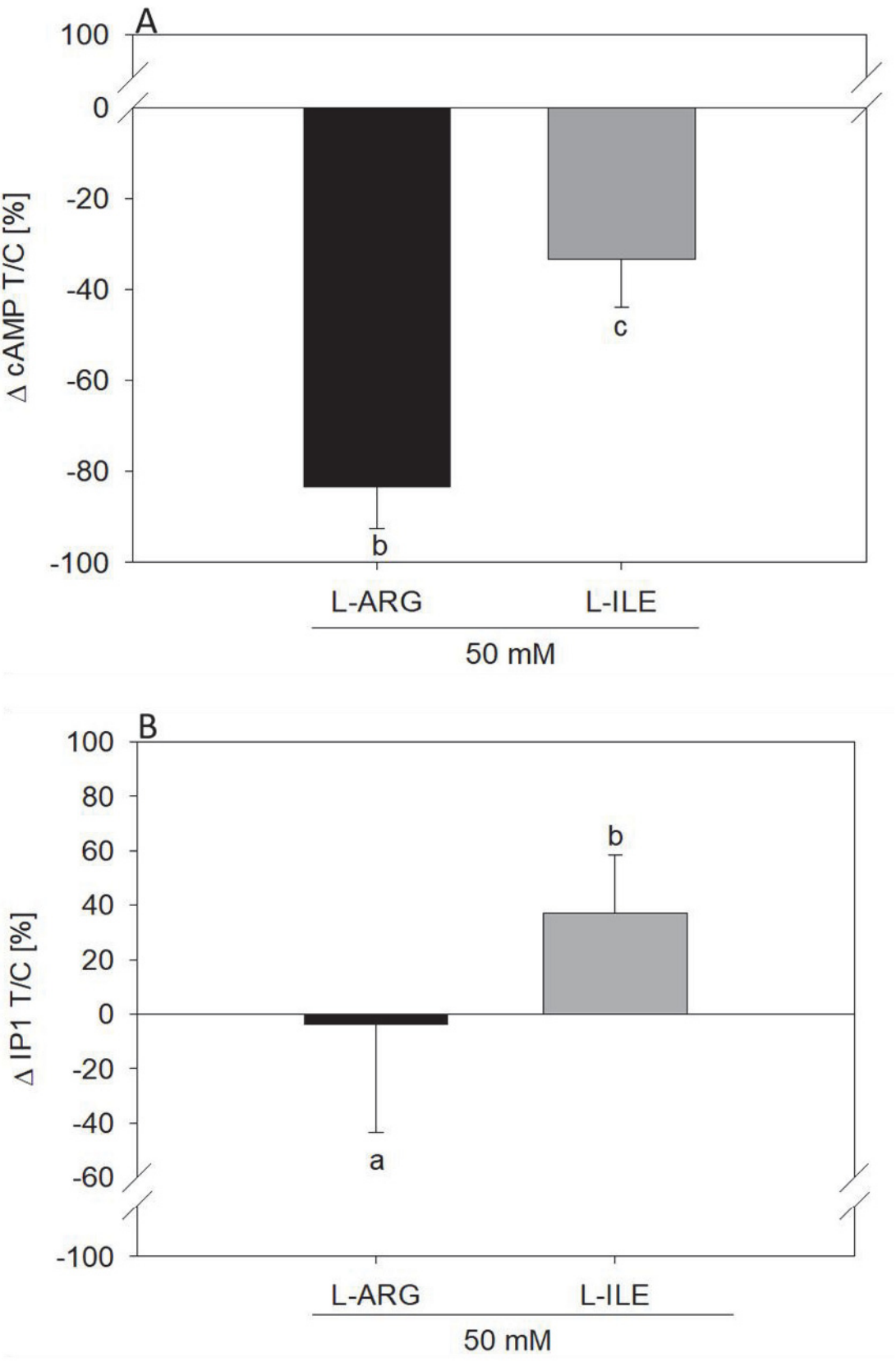
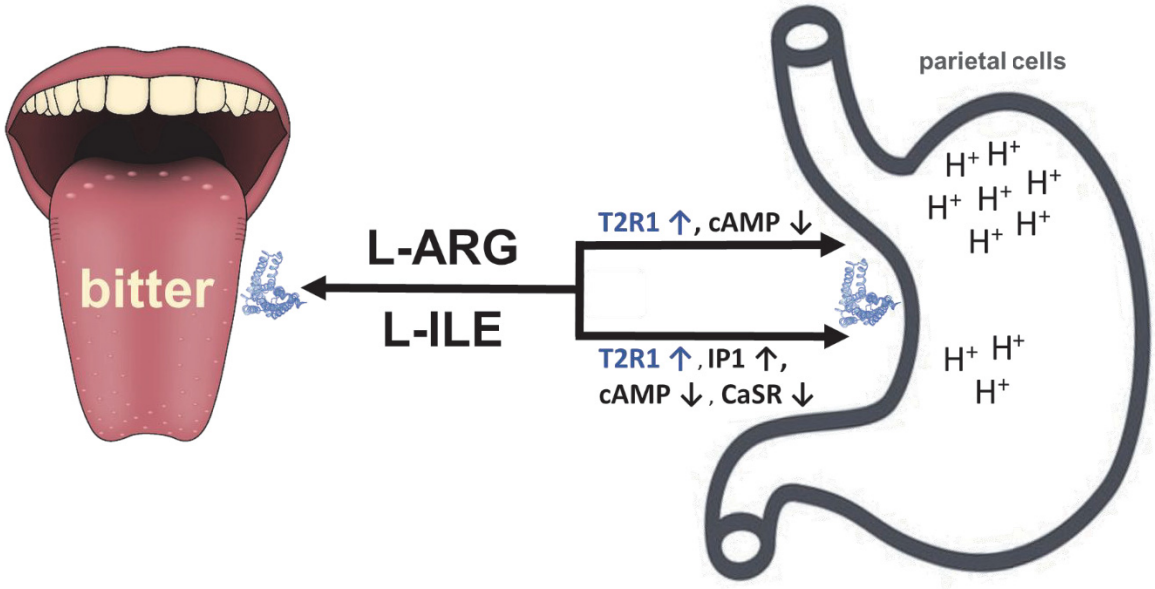

A
100
0
-20
-40
-60
-80
-100
Δ cAMP T/C [%]
b
c
L-ARG
L-ILE
50 mM
B
100
80
60
40
20
0
-20
-40
-60
-100
Δ IP1 T/C [%]
a
b
L-ARG
L-ILE
50 mM

Table of Content Graphic



Supplemental Material

Figure S1

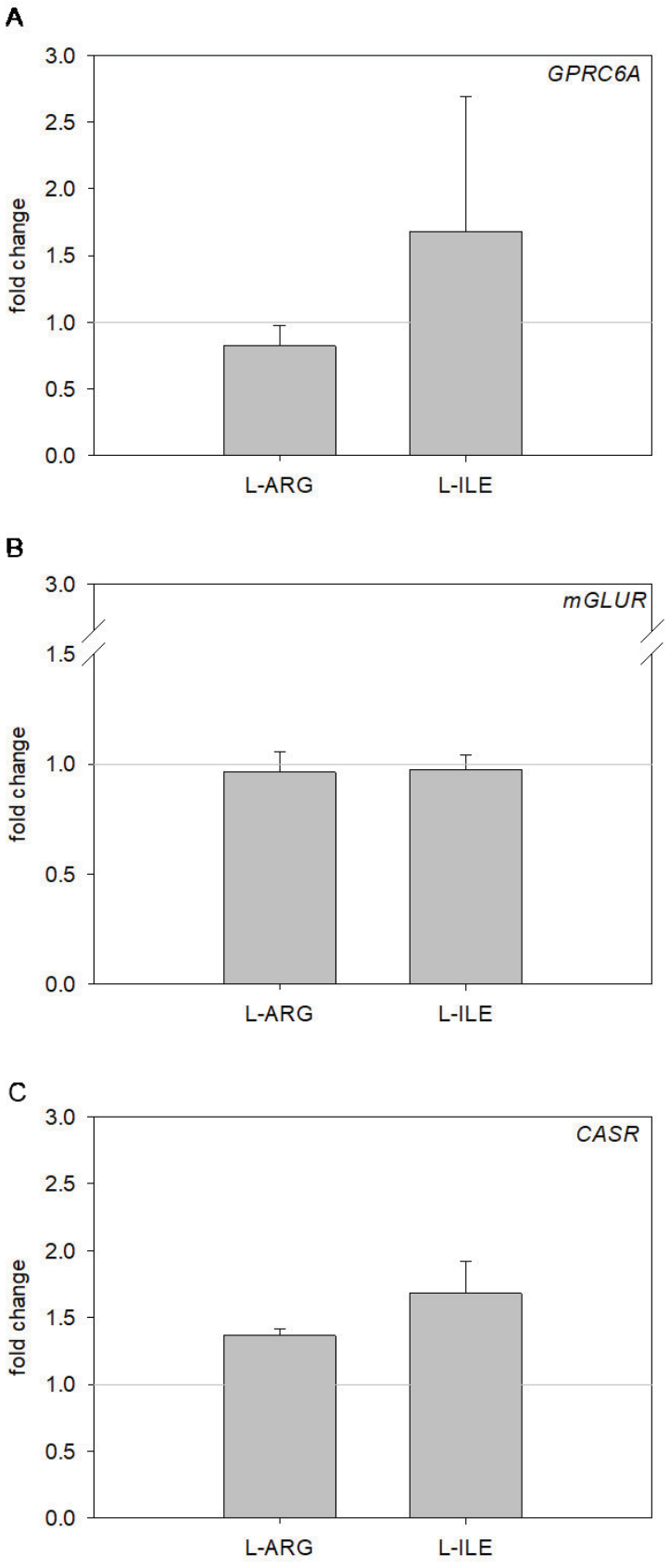


Figure S1: No influence on cDNA expression was detected by means of microarray method for *GPRC6A* (**A**), *mGLUR* (**B**) *and CASR* (**C**) in HGT-1 cells after incubation with L-ARG and L-ILE for 3 h. n = 2-3, $p > 0.05$.

Figure S2

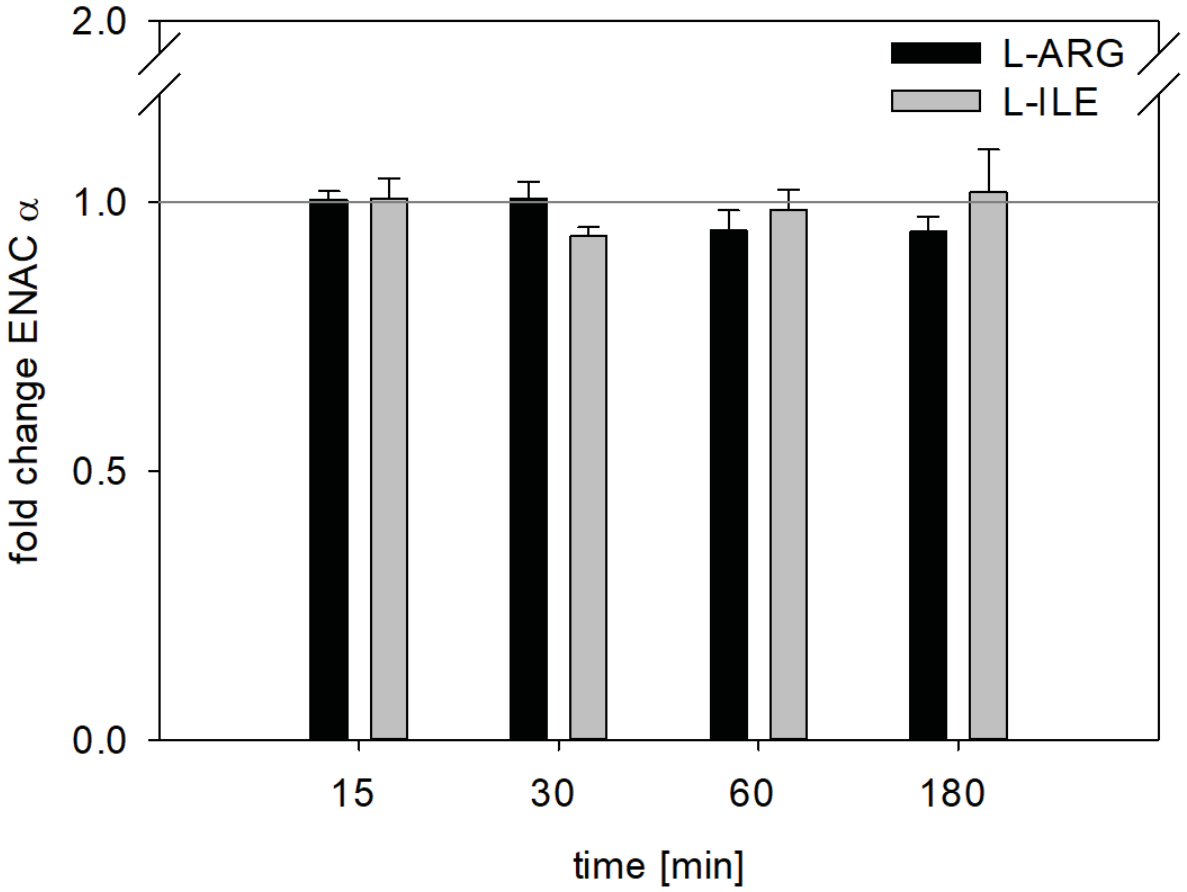


Figure S2: mRNA expression of *ENAC* α, after incubation with 50 mM L-ARG or L-ILE over time, n = 4,t.r. = 3. There were no statistical differences after testing by One Way ANOVA.

Figure S3

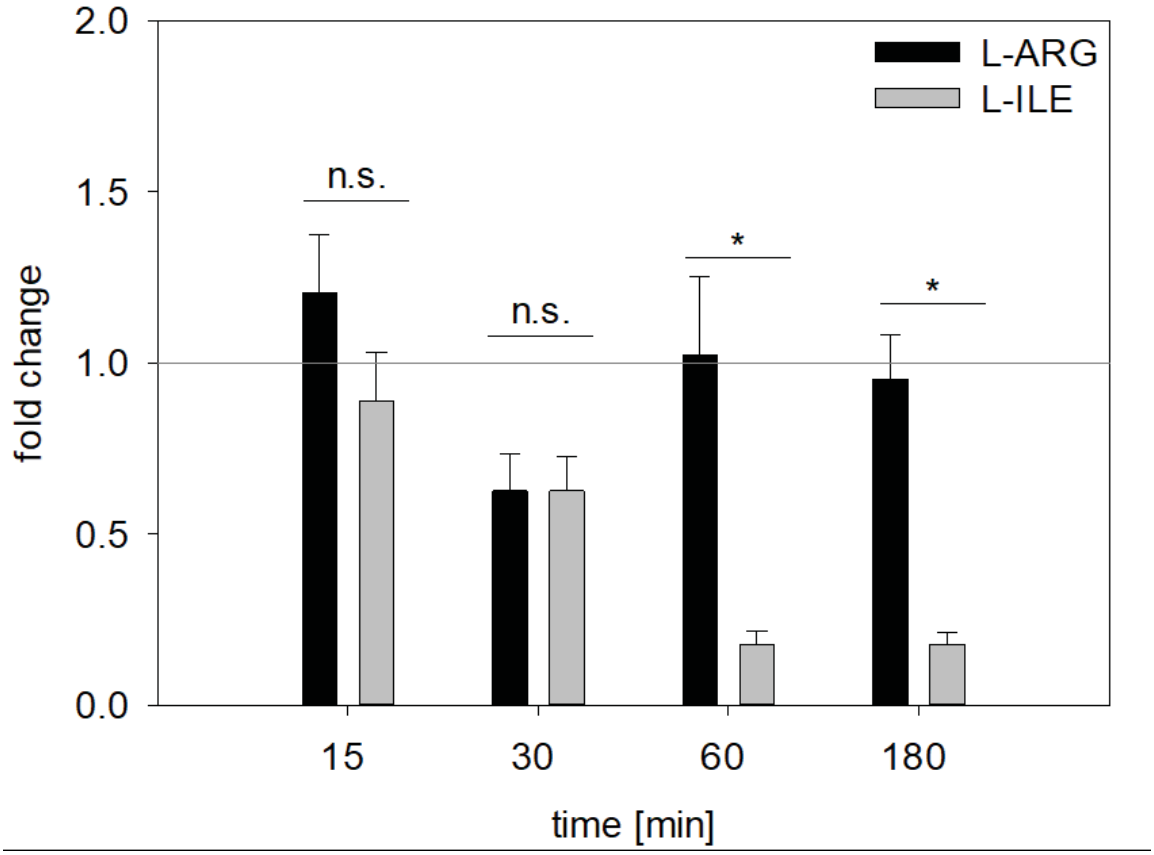


Figure S3: expression of the *CASR* after incubation with L-ARG and L-ILE from 15 min to 180 min, statistical differences have been tested by t-Test, $p < 0.05$, n = 3-4, t.r. = 3-6.

Figure S4

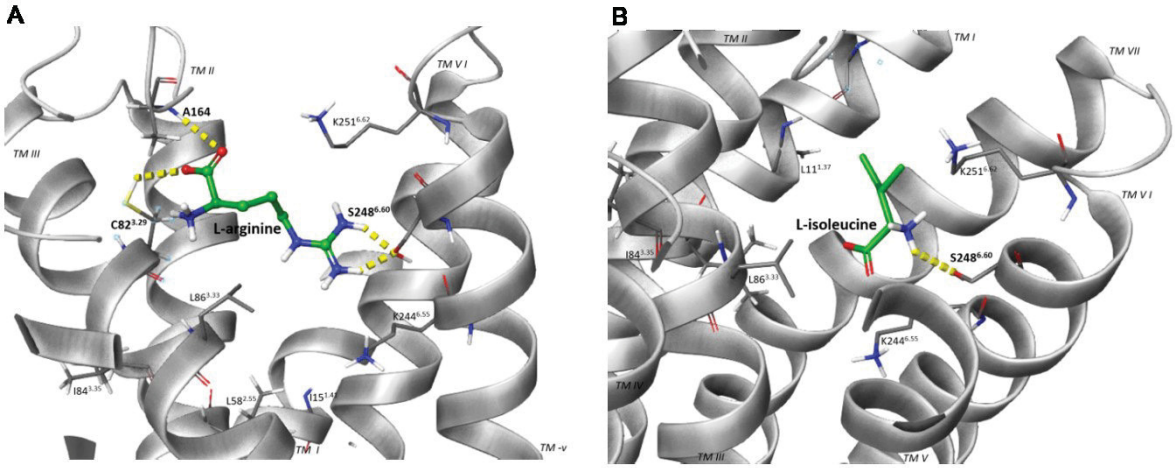


Figure S4: Predicted binding modes of (**A**) L-arginine (L-ARG) and (**B**) L-isoleucine (L-ILE) in the orthosteric binding site of T2R1. Hydrogen bonds between the ligands and the interacting residues (bold) are plotted as dashed yellow lines. T2R1 protein model shown as grey cartoon representation. Protein residues shown as grey sticks with per-element colouring. L-ARG and L-ILE shown as green sticks with per-element colouring. Nonpolar hydrogen atoms omitted for clarity.

Figure S5

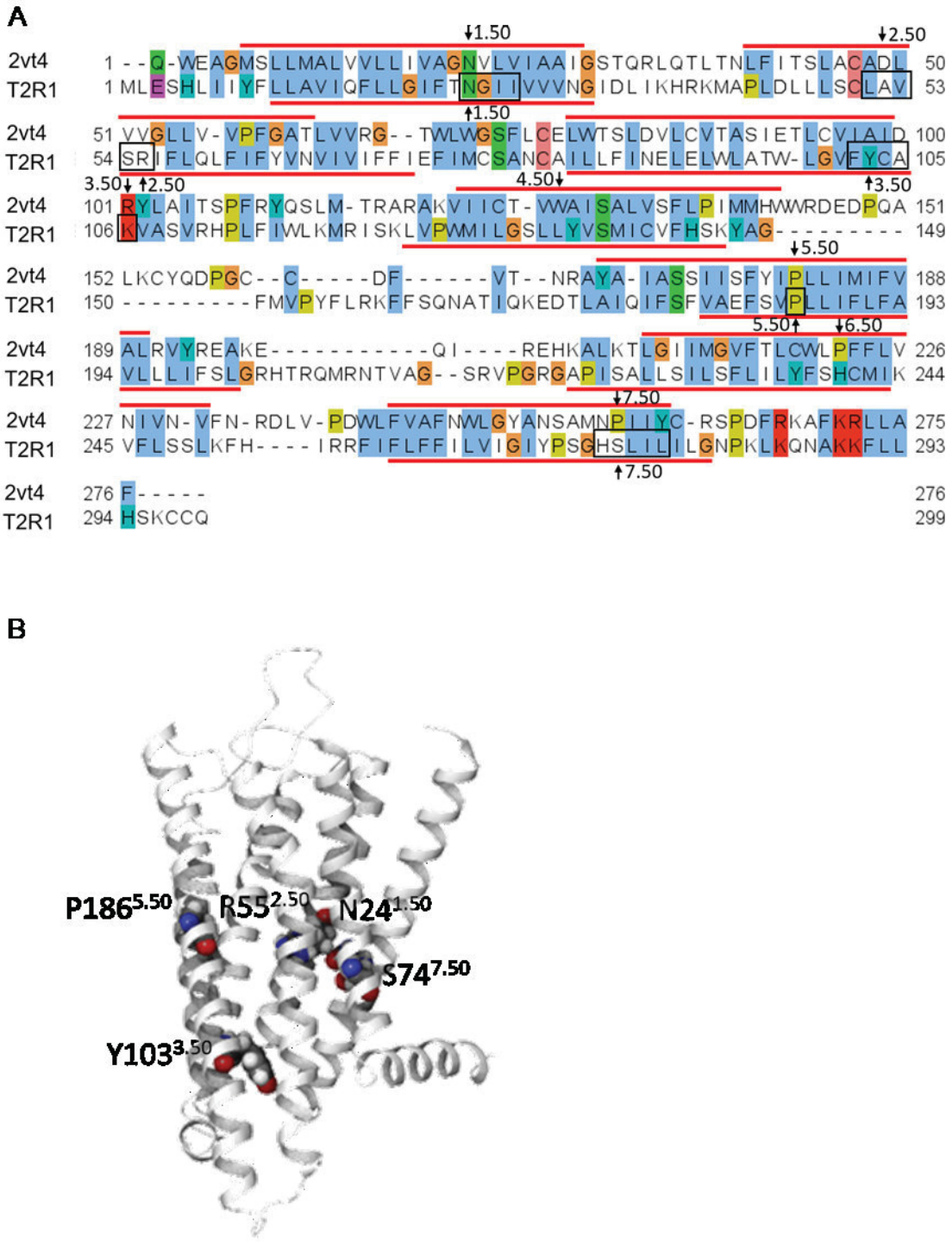


Figure S5 A depicts a pairwise sequence alignment between the model sequence and the template sequence (PDB: 2vt4). The alignment showed that transmembrane regions (red lines) of the 2vt4 protein and the T2R1 model are overlapping in the structure. An identity of 12 % as well as a similarity of 27 % was calculated for this

alignment. (**B**) Conserved motifs known for T2Rs (N1.50xxI$^{1.53}$/ L$^{2.46}$xxxR$^{2.50}$/ F$^{3.49}$Y$^{3.50}$xxK$^{3.53}$/ P$^{5.50}$/F$^{6.44}$xxxY$^{6.48}$/ H$^{7.49}$S$^{7.50}$xxL$^{7.53}$), were present in the T2R1 structure and were identified to be located at the same positions like the conserved motifs of the 2vt4 protein structure.

Figure S6

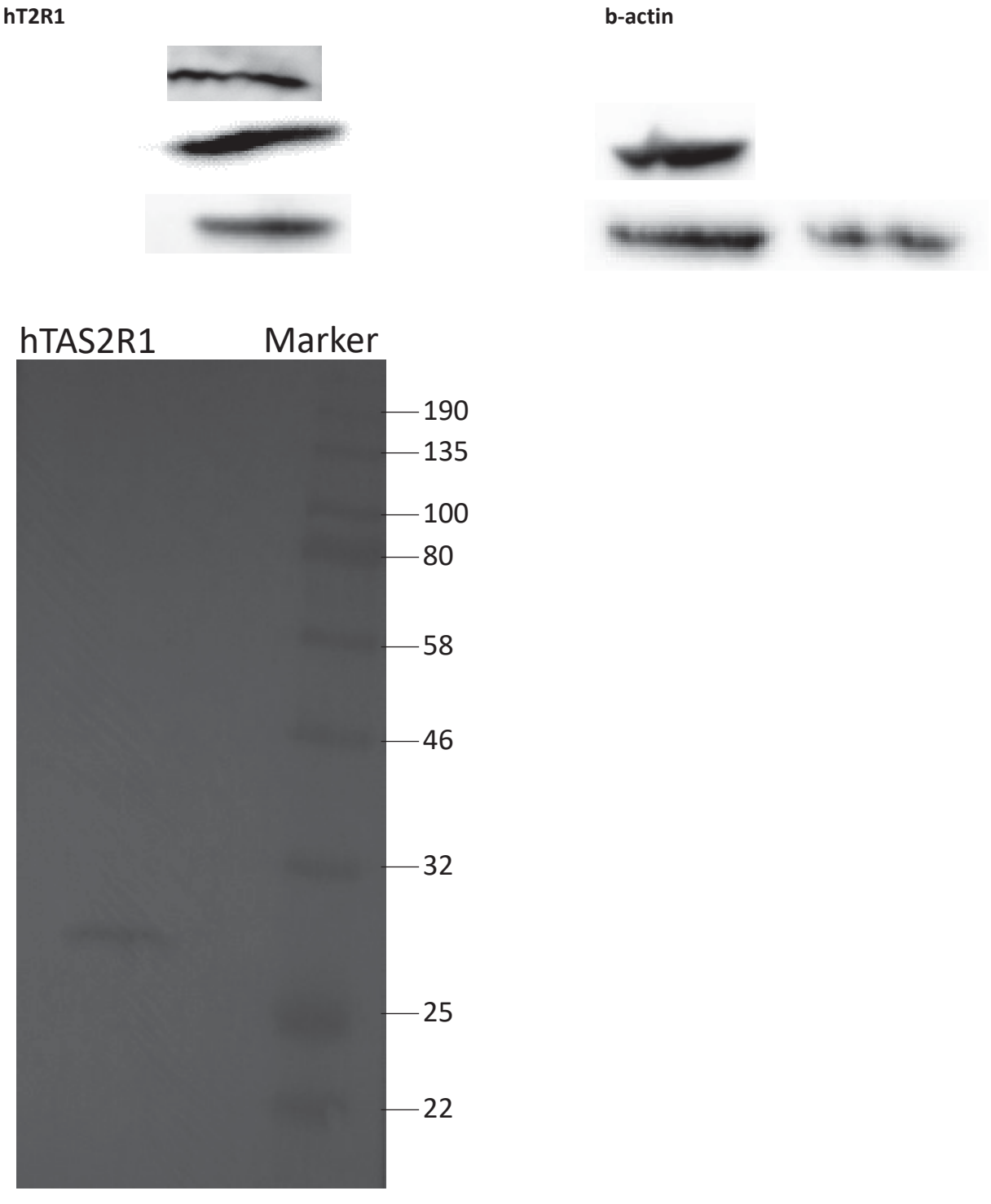


Figure S6 Detection of T2R1 on a protein level in three different passages by means of Western Blot in HGT-1 cells, n = 2-3, b-actin was used as control.

2.3 "Wheat protein hydrolysate fortified with L-arginine enhances satiation induced by the capsaicinoid nonivamide in moderately overweight male subjects."

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RESEARCH ARTICLE

Molecular Nutrition & Food Research
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Wheat Protein Hydrolysate Fortified With L-Arginine Enhances Satiation Induced by the Capsaicinoid Nonivamide in Moderately Overweight Male Subjects

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Scope: Increasing the intake of satiety-enhancing food compounds represents a promising strategy for maintaining a healthy body weight. Recently, satiating effects for the capsaicinoid nonivamide have been demonstrated. As various proteins and amino acids have also been demonstrated to decrease energy intake, oral glucose tolerance test (oGTT)-based bolus interventions of 75 g glucose + 0.15 mg nonivamide (NV control) are tested with/without combination of a wheat protein hydrolysate (WPH: 2 g) and/or L-arginine (ARG: 3.2 g) for their satiating effects in 27 moderately overweight male subjects.

Methods and Results: Compared to NV control intervention, ARG and WPH + ARG treatment both reduce ($p < 0.01$) total calorie intake from a standardized breakfast by –5.9 ± 4.15% and –6.07 ± 4.38%, respectively. For the WPH + ARG intervention, increased mean plasma serotonin concentrations (AUC: 350 ± 218), quantitated by ELISA, and delayed gastric emptying, assessed by ^{13}C-Na-acetate breath test (−2.10 ± 0.51%, $p < 0.05$), are demonstrated compared to NV control. Correlation analysis between plasma serotonin and gastric emptying reveals a significant association after WPH ± ARG intervention ($r = -0.396$, $p = 0.045$).

Conclusion: Combination of WPH and ARG enhances the satiating effect of nonivamide, providing opportunities to optimize satiating food formulations by low amounts of the individual food constituents.

1. Introduction

Overweight, obesity, and associated diseases represent a rapidly growing problem in worlds' population, causing burdens not only to the affected persons but also for the health care system.[1] One feasible strategy that is currently in focus of research is to develop foods that combine nutrients to induce an early and more intensified feeling of satiety, leading to a reduced energy intake.[2] The usage of an optimized combination of ingredients has the advantage that the satiating effect does not depend on the amount of a single compound, thereby limiting the risk of side or adverse effects. Such satiating products are thought to limit dissatisfaction during dieting, which often lead to poor compliance and effectiveness.

In general, feelings of satiety and hunger are regulated via a brain–gut signaling, which involves secretion of hormones and neurotransmitters from the stomach, duodenum, colon, and pancreas mediated by the hypothalamus.[3,4] Following food intake, blood glucose levels are regulated by the hormone insulin, which is immediately released from the pancreas to stimulate glucose uptake by

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peripheral tissues.[4,5] Moreover, intestinal satiety hormones such as Peptide YY (PYY), Glucagon-like peptide-1 (GLP-1), and Cholecystokinin (CCK) are secreted in response to nutrient intake and delay gastric emptying, thereby leading to gastric distension and to satiation.[4] Gastric emptying is also regulated by peripheral serotonin, a neurotransmitter stored and released by enterochromaffin cells of the intestine.[6] Central serotonin, in addition, also promotes satiety.[7,9]

Food intake is a complex process that is regulated by psychological and physiological factors. Whereas short-term regulation of food intake involves mechanisms that are effective during a meal to promote satiation and thereby terminate food intake, long-term factors require continuous metabolic activities that mediate satiety and determine the time between meals. From a physiological perspective, the feeling of hunger represents the need for food and is controlled by central and peripheral signals. Central factors include activation of hypothalamic neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons and GLP-1 neurons in the nucleus of the solitary tract that project directly to the ventral tegmental area and nucleus accumbens to control food intake. Moreover, gastric afferent nerve fibers and stretch receptors translate the state of full- or emptiness of the stomach to the hypothalamus region in the brain. In contrast, peripheral factors include the secretion of gastrointestinal and peripheral hormones, as well as nutrient concentrations in the blood. High concentrations of amino acids, fatty acids, and glucose suppress the feeling of hunger, whereas a secretion of the hormone ghrelin is known to stimulate food intake. Furthermore, ghrelin stimulates gastric emptying,[10] which is suggested to be mediated via activation of the vagus nerve or ghrelin receptors. In addition, ghrelin has been shown to be involved in the inhibition of insulin release via central melanocortin signaling.[11]

Consumption of proteins has been associated with prolonged feelings of satiety in comparison to fats and carbohydrates when applied in equicaloric amounts.[12] Moreover, protein consumption is known to retard gastric emptying[13–16] and to stimulate gastric acid secretion (GAS).[15] Secretion of gastric acid facilitates the degradation of proteins into amino acids, which may contribute to the satiating effect of proteins. Dietary L-arginine, i.e., has been shown to reduce energy intake in rodents[16] and to delay and inhibit gastric emptying.[17] Furthermore, we recently demonstrated that L-arginine stimulated mechanisms of GAS[18] and increased the release of serotonin in a parietal cell model.

Besides proteins, also pungent compounds, e.g., capsaicin and its less pungent structural analogue nonivamide, have been well investigated for their satiating effects. Studies from our own group demonstrated a reduced energy intake and accompanied by increased plasma serotonin levels after a 0.15 mg bolus administration of nonivamide in a cross-over human intervention trial.[19] Furthermore, administration of a milk shake fortified with 0.15 mg nonivamide for 12 weeks to overweight, but otherwise healthy subjects, increased peripheral serotonin levels and prevented a dietary-induced body fat gain.[20] Also, on the cellular level, nonivamide has been shown to promote serotonin secretion by neural SH-SY5Y cells.[21]

Since it has been demonstrated that a combination of satiety enhancing ingredients can be a feasible strategy for combating overweight and obesity,[2] in the current study, a combination of a wheat protein hydrolysate and L-arginine was tested on its potential to enhance the satiating effect of nonivamide. The amino acid L-arginine was chosen due to its published benefits on obesity by decreasing plasma glucose levels and fatty acids,[22] but also by its ability to reduce food intake.[16] Moreover, since a satiating potential of proteins is well known, we investigated whether a fortification of a wheat protein hydrolysate with L-arginine additionally enhances satiety. A wheat protein hydrolysate was chosen since wheat protein presents the lowest amount of L-arginine among dietary legumes and grains,[23,24] allowing to study the satiating effect of L-arginine fortification. Moreover, wheat is one of the world's most commonly consumed cereal grains. The amount of wheat protein hydrolysate administered as a bolus was limited to 2 g due to its unpleasant taste, although other proteins for which a satiating effect has been shown in previous studies had been administered in higher amounts.[25] For L-arginine, an amount of 3.2 g L-arginine was chosen, which is also in accordance with common doses used for dietary L-arginine supplementation,[26] and is far below the No Observed Adverse Effect Level (NOAEL) of 30 g d^{-1} for healthy young adults.[26]

The aim of the present study was to investigate whether a combination of a wheat protein hydrolysate and L-arginine enhances the satiating effect of nonivamide in a short-term human intervention study with healthy male subjects. Hence, in this study, we hypothesized a wheat protein hydrolysate fortified with L-arginine enhances the satiating effect of nonivamide when administered in an oral glucose tolerance test (oGTT).

2. Experimental Section

2.1. Characteristics of the Subjects

From December 2017 to April 2018, a total of 50 men were recruited for a medical screening by advertisements in web forums and billboards at Universities in Vienna. An age between 20 and 45 years, a BMI between 25 and 30 kg m^{-2}, but neither tobacco consumption nor alcohol abuse or medical treatment were allowed for metabolically healthy, sensorially untrained study subjects. Subjects with a BMI > 25 kg m^{-2} were categorized as moderately overweight, although body composition (fat mass, free fatty mass) was not considered. Due to estrogen fluctuations during the menstrual cycle and their influence on serotonin levels, women were excluded from this study.[27,28] Within the medical screening after recruitment, hematological parameters, plasma thyroid hormone status, plasma lipids, and glucose concentrations after an oGTT were analyzed by Ihr Labor 1220 (Medical diagnostics laboratory including microbiology, Dr. Gabriele Greiner, Vienna, Austria) to ensure recruited subjects were metabolically healthy. Moreover, a fasting blood glucose < 120 mg dL^{-1} was mandatory for enrolment. Body height was measured by a stadiometer (Seca, Germany) with precision of 0.1% and body weight was assessed by a digital scale within 100 g exactly (Weinberger, Germany). By means of the open source software GPower 3.1, a number of 50 volunteers was identified when a statistically significant difference of total energy intake of 0.49 MJ between two dependent means and a power of 80% was taken into account. The effect size of 0.49 MJ was based on one of

Table 1. Study subjects' characteristics.

N	27
Gender	male
Age [years]	25.7 ± 0.7
Height [m]	1.82 ± 0.0
Body weight [kg]	89.0 ± 2.1
BMI [kg m^{-2}]	26.8 ± 0.4
fasting blood glucose [mg dL^{-1}]	74.4 ± 1.4

Data are depicted as mean ± SEM.

our previous human intervention studies, in which the satiating effect of a 0.15 mg nonivamide bolus administration was investigated and demonstrated to reduce total energy intake from an ad libitum breakfast from 4.75 ± 0.45 MJ after control intervention to 4.26 ± 0.33 MJ.[23] Hence, 50 male volunteers were recruited and a total of 27 volunteers completed the study per protocol (**Table 1**).

Overall, the study protocol adhered to the guidelines of the Declaration of Helsinki. Although dietary intervention studies at the University of Vienna do not require approval by the local ethics board, the study protocol using the pungent compound nonivamide was approved by the ethics committee of the city of Vienna (registration no. EK 12–084–0812).

2.2. Dosage Information/Dosage Regimen' in the Experimental Section

In the current human intervention study, a bolus of 0.15 mg nonivamide[19] alone or in combination with either 2 g of a wheat protein hydrolysate and/or 3.2 g L-arginine was administered to healthy young males in an oGTT (75 g glucose/300 mL water). Participants were asked to drink the test solution within 5 min.

The bolus administration of 0.15 mg nonivamide had been chosen as reference intervention since it had previously been demonstrated to effectively decrease energy intake and increase plasma serotonin concentrations in healthy volunteers, whereas post-load plasma concentrations were below the limit of detection (5 fMol).[19] Although data on habitual dietary intake of nonivamide are lacking, a daily intake of total capsaicinoids between 0.5 and 4 mg kg^{-1} body weight has been estimated by the European Council.[29]

Nonivamide (Flavor & Extract Manufacturers Association (FEMA) 2787) is classified as a Generally Recognized As Safe (GRAS) aroma compound by the Food and Drug Administration (FDA), and can be used for food fortification up to 10 ppm.[30] According to risk assessment published by the European Food Safety Agency (EFSA), the maximum daily intake is 6 µg per capita.[34] Furthermore, a NOAEL (No Observed Adverse Effect Level) of 8.4 mg kg^{-1} body weight d^{-1} has been established.[31] In the current study, the amount of 0.15 mg nonivamide applied together with a 300 mL glucose solution correspond to 0.5 mg L^{-1} or 0.5 ppm or 500 ppb. Since this concentration is below the NOAEL and also meets the FDA recommendations for food fortification, the nonivamide in the amount administered did not bear any risk to the study subjects. Because of its limited solubility in water, the administered amount of 150 µg nonivamide was pre-dissolved in 15 µL ethanol absolute. The final concentration of the ethanol in the study solution was <0.001% and can also not be considered as harmful to the study participants.

The amount of 2 g of the wheat protein hydrolysate (food-grade quality) applied in this study was assessed in sensorial pretests and found to be palatable (data not shown). According to regulation no. 1169/2011 by the EFSA, wheat protein hydrolysate is not subjected to food declarations, although its content as food allergen has to be labeled.[32]

In addition to nonivamide and a wheat protein hydrolysate 3.2 g of free L-arginine HCl (FEMA 3819) were administered. The amount of 3.2 g L-arginine was chosen, since this is a common amount for L-arginine supplements and is in the range of habitual daily intake of up to 5 g.[33]

2.3. Study Design

A single blinded cross over study with four randomized groups according to four interventions (**Figure 1**) was conducted. Test subjects received the following interventions in a randomized order on four study days after an overnight fast, applied within an oGTT: Nonivamide control (**NV**, 0.15 mg nonivamide, 75 g glucose, 15 µL ethanol, and 150 mg ^{13}C-Na-acetate), wheat protein hydrolysate (**WPH**) (2 g wheat protein hydrolysate 0.15 mg nonivamide, 75 g glucose, 15 µL ethanol, and 150 mg ^{13}C-Na-acetate), L-arginine hydrochloride (**ARG**) (3.2 g L-arginine, 0.15 mg nonivamide, 75 g glucose, 15 µL ethanol, and 150 mg ^{13}C-Na-acetate) or **WPH +ARG** (2 g wheat protein hydrolysate, 3.2 g L-arginine, 0.15 mg nonivamide, 75 g glucose, 15 µL ethanol, and 150 mg ^{13}C-Na-acetate). The four visits were carried out 1 week apart. In addition, the test subjects were asked not to consume foods containing naturally higher amounts of ^{13}C, e.g., corn, tequila, or cane sugar, 48 h prior to intervention. On each study day, subjects were asked to rate their subjective feeling of hunger on a 100 mm visual analogue scale (VAS), followed by collection of two baseline breath samples and baseline blood collection. Further blood samples were collected 15/30/60/90 and 120 min after administration of the oGTT. In parallel, breath samples were collected every 10 min during the first hour, followed by time points 80/120 and 140 min. After the last breath sample was taken, the subjective feeling of hunger was assessed again by means of a VAS, and a standardized continental breakfast with ≈3000 kcal was served ad libitum. The breakfast consisted of: four rolls, three slices whole grain rye bread, 3.5 slices of cheese (≈125 g), eight slices of ham (≈99 g), 80 g butter, 100 g jam, 60 g honey, three pieces of coffee cream, 4 g sugar, 180 g yoghurt, 200 mL water, and 200 mL coffee or tea. Short-term energy intake from the ad libitum breakfast was analyzed using nut.s v1.32.50 by weighing the remaining food on each subject's tray. Dietary intake data were collected by performing repeated 24-h-recalls using the software GloboDiet. GloboDiet was developed by the International Agency for Research on Cancer (IARC) within the European Investigation into Cancer and Nutrition Study (EPIC-Study) and adopted to Austrian nutrition habits by the Department of Nutritional Sciences to carry out standardized 24-h-recalls. The reported foods assessed during the interviews were linked to the German food composition database Bundeslebensmittelschlüssel 3.02.[34,35]

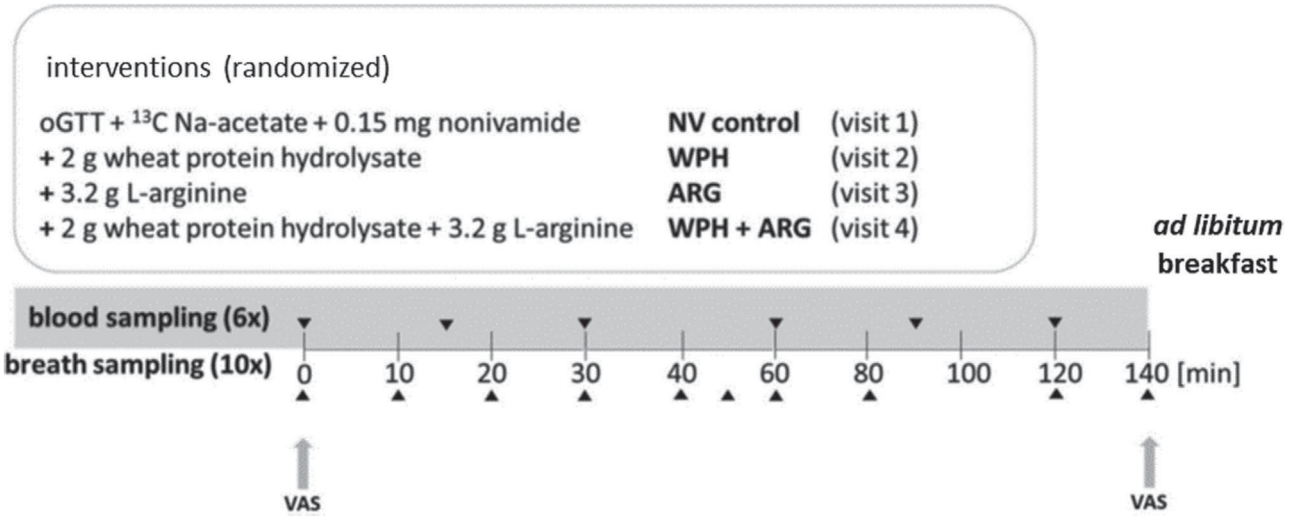

Figure 1. Study design of this cross-over human intervention study. A total of 27 volunteers underwent each of the four interventions with a 7 day interval between interventions and set their subjective feeling of hunger by means of a visuale analgoue scale (VAS).

2.4. Blood Sample Collection

Venous blood samples for plasma serotonin concentrations were collected in EDTA-coated tubes and immediately centrifuged at 1800 × *g* at 4 °C for 15 min. For avoiding platelets in plasma, samples for serotonin were additionally centrifuged for 1 min at 7000 x *g* at 4 °C. For increasing ghrelin stability during storage, whole blood samples were mixed with AEBSF (4-[2-aminoethyl benzene] sulfonyl fluoride, Sigma, Germany), centrifuged at 1800 × *g* at 4 °C for 15 min and stabilized with hydrochloric acid (Sigma, Germany).

2.5. Plasma Concentrations of Total Ghrelin, Total GLP-1, Serotonin, Insulin, and Glucose

Ghrelin (LOD: 50 pg mL^{-1}) and GLP-1 (LOD: 2 pM) were determined by means of a sandwich ELISA (Merck Millipore, Germany), whereas serotonin was quantified by means of a competitive ELISA (LOD: 2.6 ng mL^{-1}). Serotonin samples under this LOD were additionally analyzed using the serotonin high sensitive ELISA with an LOD of 0.39 pg per sample (both DLD Diagnostika, Hamburg, Germany). Insulin concentrations were assessed using a sandwich ELISA (LOD: 50 pg mL^{-1}) obtained from IASON (Graz, Austria). Glucose concentrations were quantitated by a colorimetric assay, presenting an LOD of 0.23 mg dL^{-1} (Cayman Europe, Tallinn, Estonia).

2.6. Gastric Emptying

The study drink was labeled with ^{13}C-Na-acetate,[36] produced according to the guidelines for clinical trial material by Euroisotop (France). Breath bags for sample collection were obtained from Cambridge Isotope Laboratories, Inc. (Massachusetts, USA).

Table 2. Influence of WPH and ARG alone or in combination on Δ DOB AUC considering the body surface of each study subject, n = 22–26. Data are presented as ΔAUC values of the Delta Over Baseline (DOB) presenting $^{13}CO_2/^{12}CO_2$ ratio and are depicted as mean ± SEM.

Intervention	ΔDOB AUC [treatment – NV control]	*p*-Value [*t*-test vs NV control]
WPH	−1000 ± 343	0.01
ARG	−634 ± 398	0.12
WPH+ARG	−1214 ± 295	0.001

Breath sample collection was standardized as follows: after the breath was hold for 20 s, the breath bag had to be fully inflated by one subsequent exhale. Twenty-six subjects per intervention provided adequate breath samples (CO_2 > 3 vol%) in which $^{12}CO_2$ and $^{13}CO_2$ concentrations were quantitated by infrared spectroscopy (POCone, Otsuka Pharmaceutical S.A., Barcelona, Spain).

In detail, each study subject had to fill two baseline breath bags (breath air before intervention was applied) and sample breath bags (breath air containing $^{13}CO_2$) for every time point (t_{10}–t_{140}). These bags were analyzed and the device detected DOB (Delta Over Baseline)-values ($[^{13}CO_2/^{12}CO_2]_{sample} - [^{13}CO_2/^{12}CO_2]_{baseline\ sample}$; n = 22–26 in Figure S6 and n = 1 in Figure S5, Supporting Information) and the $^{12}CO_2$-concentrations (%) of the sample as well as the baseline. For considering the body surface area of each study subject, the DuBois formula[37] was used and the $^{13}CO_2$ in mol m^{-2}/precursor in mol (%) of the DOB-value was calculated, based on these data the area under the curve (AUC) as well as the ΔAUC (subtracting the NV control from each intervention) were generated and are plotted as **Table 2** and Figure S6, Supporting Information.

2.7. Statistical Analyses

Statistical analyses were performed by SigmaPlot 12 and figures were generated either by SigmaPlot 12 or GraphPad Prism 8. A normal distribution of the data was determined by Shapiro–Wilk test. Statistical differences were defined with a p-value < 0.05. To test if WPH + ARG increased the satiating effect in comparison to NV, a one-tailed t-test or Mann–Whitney rank sum test, for non-normally distributed data sets, was conducted. Intergroup differences of WPH, ARG and WPH + ARG and NV were tested by means of a One Way ANOVA or a One Way ANOVA on Ranks followed by Student–Newman–Keuls method post hoc, respectively. Δ-Values were calculated by subtracting the NV control from the treatment. For serotonin and gastric emptying, total AUC was calculated over time. A correlation between serotonin and gastric emptying was assessed by Pearson Product Moment Correlation. Calculation of individual $^{13}CO_2$ was performed by using DuBois formula.[37]

3. Results

3.1. Assessing the Subjective Feeling of Hunger by Means of VAS

In comparison to the NV control (mm after intervention – mm before intervention = Δmm set to 0 for calculating the effects of WPH, ARG, and WPH+ARG interventions), ARG and the combination of WPH + ARG decreased the subjective feeling of hunger (ARG Δ: −0.97 ± 0.31, WPH + ARG Δ: −0.57 ± 0.31, p < 0.05) (**Figure 2**A).

3.2. Total Energy Intake After an Ad Libitum Breakfast

ARG as well as WPH + ARG intervention decreased total energy intake from the standardized breakfast in comparison to NV control treatment, with mean reductions of –83 ± 60 kcal (−5.9 ± 4.15%, $p < 0.05$) and 86 ± 63 kcal (−6.07 ± 4.38%, $p < 0.05$). Moreover, the WPH + ARG intervention decreased energy intake more effectively than the WPH intervention ($p < 0.05$; Figure 2B). Total kcal intake from the standardized ad libitum breakfast in the NV control group ranged between 753 to 2067 kcal.

3.3. Plasma Concentrations of Glucose, Insulin, Ghrelin, and GLP-1

Statistical analysis demonstrated no intergroup differences for plasma glucose, insulin, ghrelin, and GLP-1 levels after WPH, ARG, and WPH + ARG intervention compared to NV control ($p > 0.05$; **Figure 3**A–D).

3.4. Plasma Serotonin Levels

WPH + ARG intervention increased Δ-plasma AUC values up to 350 ± 218 in comparison to NV control intervention set to 0

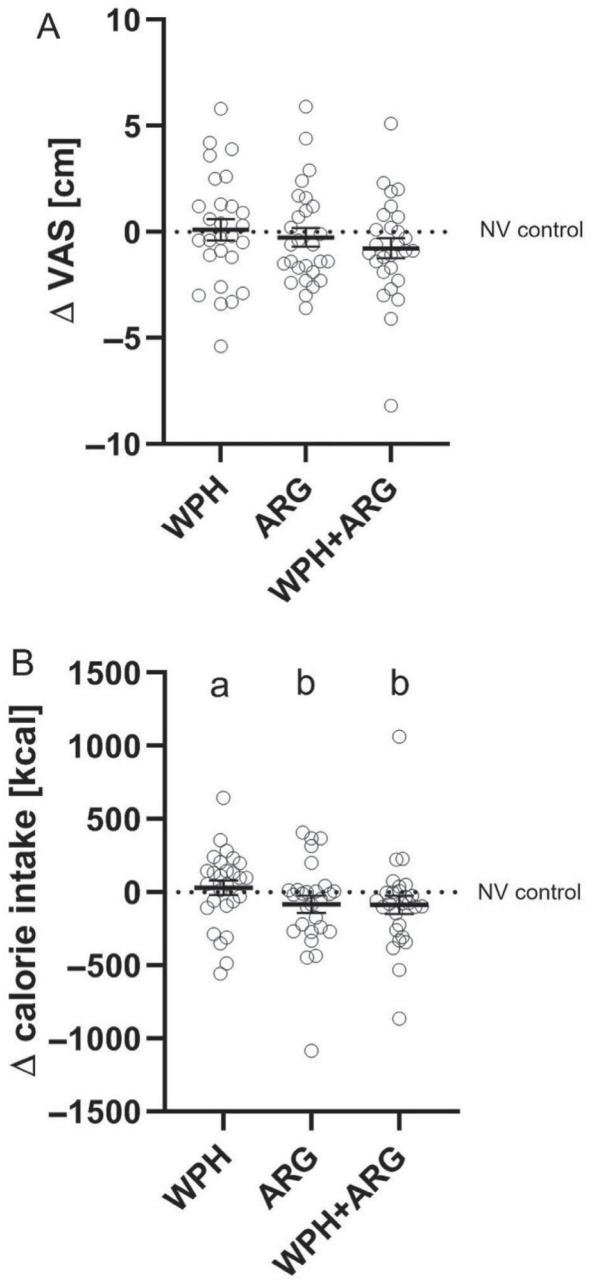

Figure 2. A) Differences in subjective ratings of hunger before and after administration of 75 g glucose solution supplemented with either 0.15 mg NV (control), and 2 g wheat protein hydrolysate (WPH) and/or 3.2 g L-arginine (ARG), were adjusted to values for NV control (= 0; $n = 27$). Group mean of WPH+ARG was significantly lower than those of NV control as identified by Mann–Whitney Rank Sum Test ($p < 0.05$). Every symbol represents a study participant and means are shown as a black line. B) Total calorie intake from a standarized ad libitum breakfast served 140 min after oral adminstration of 75 g glucose in combination with either NV (NV control), WPH, ARG, or WPH + ARG in 27 healthy males. Letters indicate significant differences between treatments by One Way ANOVA on Ranks followed by Student–Newman–Keuls post hoc test, $p < 0.05$. Group mean of WPH+ARG (–86 ± 63 kcal) was decreased by –6.07 ± 4.38 % compared to NV control as identified by Mann–Whitney Rank Sum test ($p < 0.01$). Total kcal intake of the NV control group ranged between 753 to 2067 kcal. Every symbol represents a study participant and means are shown as a black line.

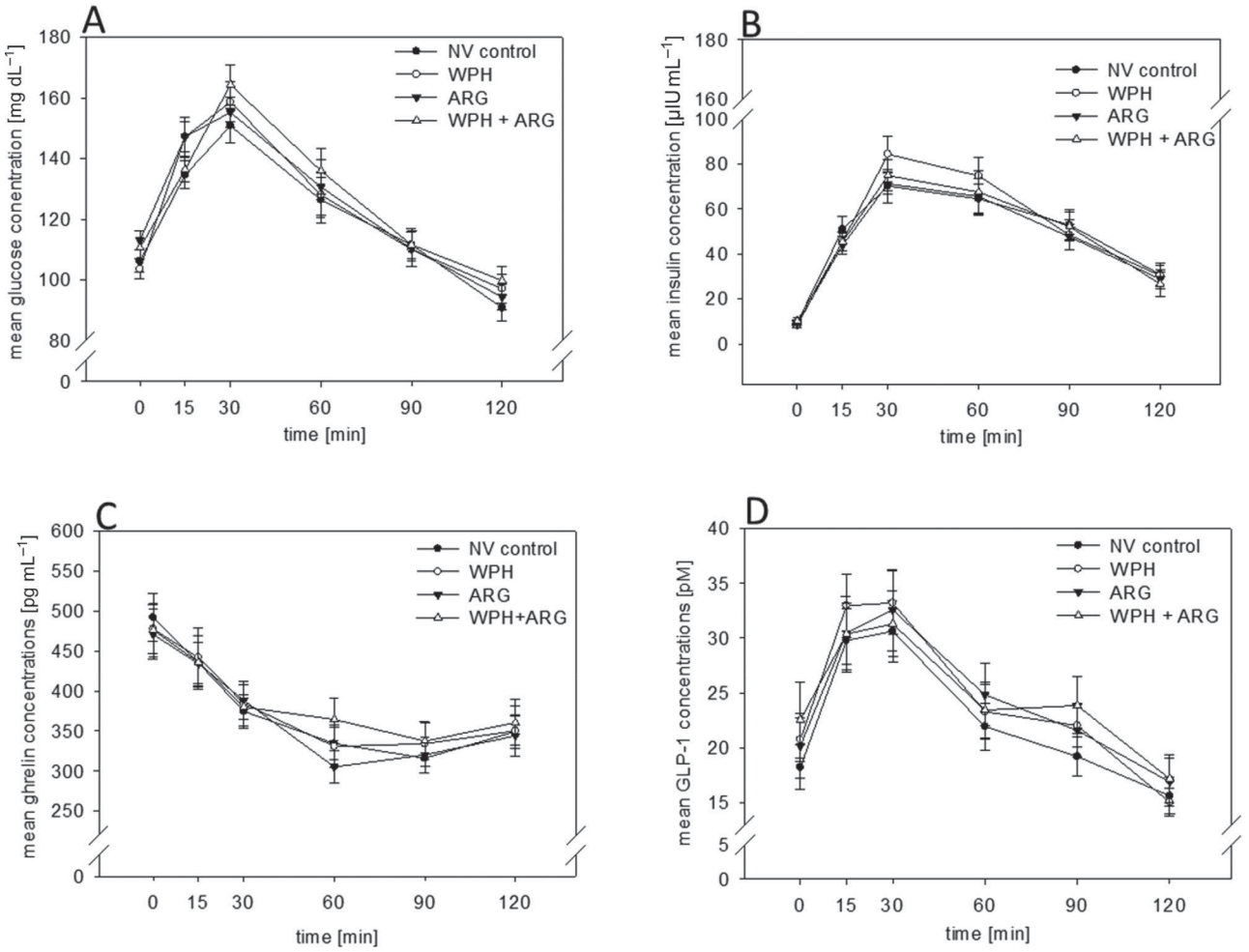

Figure 3. A–D) Administration of WPH, ARG and its combination WPH + ARG did not influence plasma glucose A), insulin B), ghrelin C), and GLP-1 D) concentration ($p > 0.05$). Statistical significances were tested by One Way ANOVA and *t*-test on plasma concentrations over time as well as on AUC values.

($p < 0.05$; **Figure 4**A). No intergroup differences were calculated by means of ANOVA (Figure 4B). Serotonin concentrations in subjects of the NV control group ranged from 0.40 to 42 pg mL^{-1}.

3.5. Gastric Emptying

By using DuBois[37] formula considering body surface and Nalimov outlier test, a delay in gastric emptying was detected in 22–26 subjects after WPH as well as WPH + ARG intervention, in comparison to NV control intervention (Δ AUC: WPH: 1000 ± 343 ≙ –1.72 ± 0.60%, $p < 0.01$; WPH + ARG: 1213 ± 295 ≙ –2.10 ± 0.51%, $p < 0.001$; Table 2). ANOVA analyses did not reveal any intergroup differences.

3.6. Pearson Correlation Between Gastric Emptying and Plasma Serotonin

Correlation analysis by means of Pearson Product Moment Correlation between ΔAUC of $^{13}CO_2/^{12}CO_2$ % × min and serotonin revealed a significant association for the WPH + ARG intervention ($r = –0.396$, $p = 0.045$; **Figure 5**A–C).

3.7. Twenty-Four Hours Calorie Intake Calculated by Globodiet

Only WPH + ARG intervention reduced the calorie intake 24 h post load in comparison to NV control intervention ($\Delta_{before-after}$ kcal intake: –360 ± 147; $p < 0.05$) (**Table 3**).[34,35] No intergroup differences were calculated by means of ANOVA. The total mean calorie intake of the subjects after NV control intervention was 2951 ± 141 kcal.

4. Discussion

Dietary intake of foods containing constituents to promote satiation present a promising strategy to reduce daily energy intake and to combat the increasing problem of obesity.[2,38] The aim of the current study was to identify whether the addition of a wheat protein hydrolysate and L-arginine, or a combination thereof, enhances the satiating effect of the pungent compound NV. This

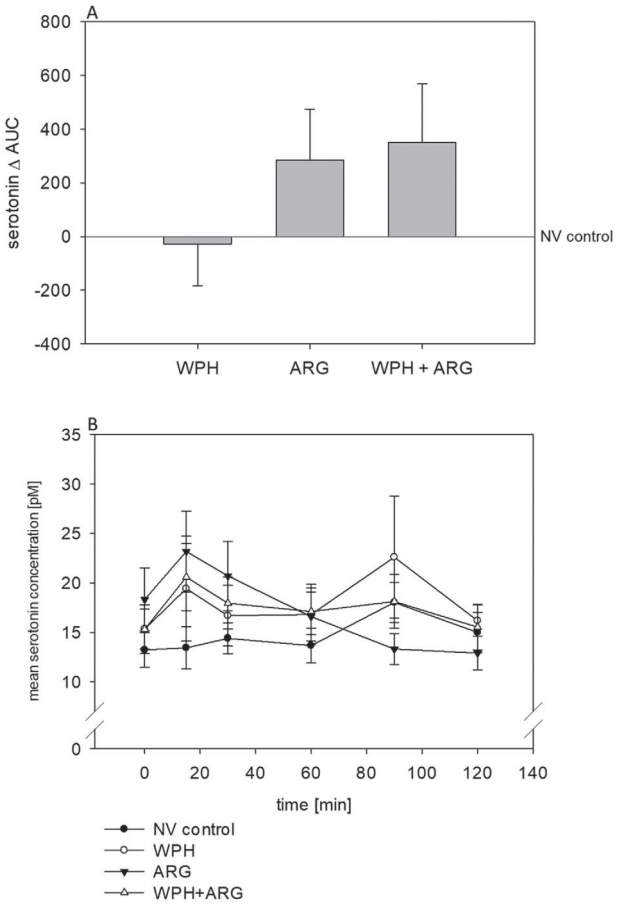

Figure 4. A) ARG + WPH intervention increased plasma serotonin levels (expressed as AUC [pM × min]) in 27 healthy volunteers over time in comparison to NV control (WPH + ARG: 350 ± 218, NV set to 0). Statistical significance between treatments was calculated by One Way ANOVA ($p > 0.05$). Group mean after WPH+ARG intervention was significantly higher than that after NV control treatment as identified by Mann–Whitney Rank Sum test ($p < 0.05$). Values are shown as mean ± SEM. Plasma serotonin concentrations of the NV control group ranged from 0.40 to 42 pg mL^{-1}. B) Influence of NV control, WPH, ARG, and WPH + ARG intervention on plasma serotonin levels. Statistical significances were tested by One Way ANOVA and *t*-test on plasma concentrations over time as well as on AUC values ($p > 0.05$). Plasma serotonin concentrations are depicted as mean ± SEM.

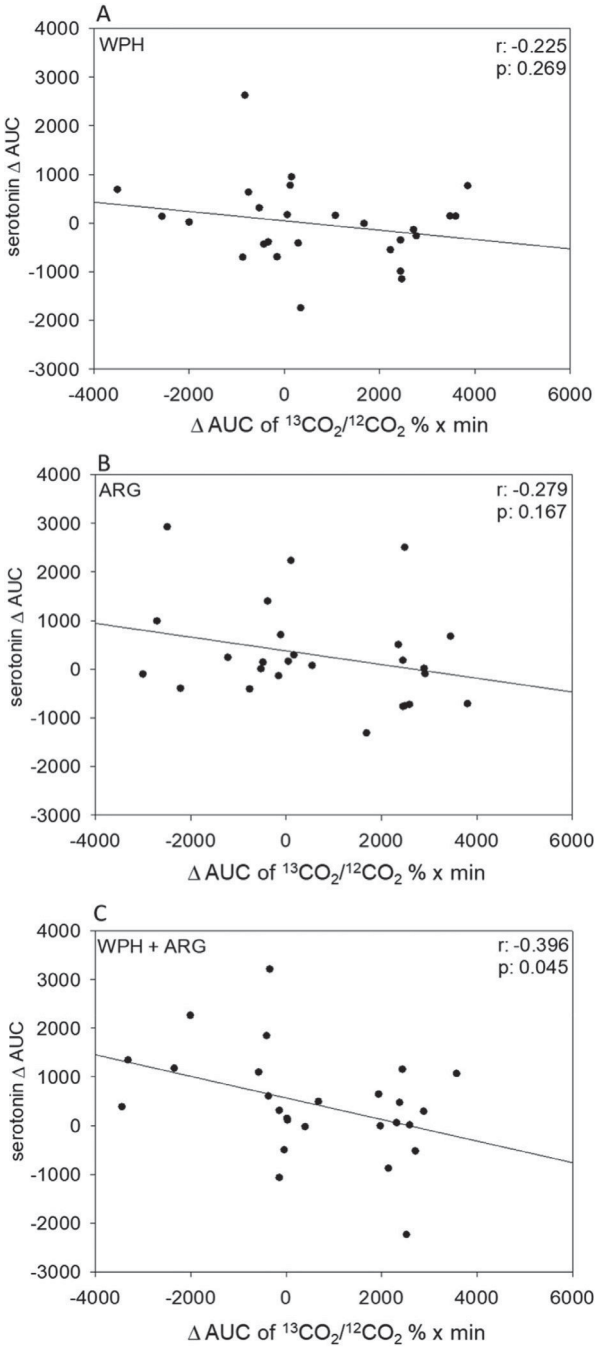

Figure 5. A–C) Correlation analysis between Δ AUC plasma serotonin and Δ AUC of $^{13}CO_2/^{12}CO_2$ % × min rates (NV control was subtracted from interventions) after WPH, ARG, or WPH + ARG intervention. Correlation coefficients were computed by means of Pearson Product-Moment Correlation method. For intervention, a significant correlation was demonstrated, with WPH+ARG ($r = -0.396$, $p = 0.045$).

structural analogue of capsaicin has recently been shown to decrease the subjective feeling of hunger, to reduce total energy intake from a standardized breakfast, and to increase plasma serotonin levels when applied in an amount of 0.15 mg in an oGTT.[19] In this study, total energy intake and satiety-associated gastro-intestinal and peripheral signals were investigated after administration of 0.15 mg NV (control intervention) in combination with 2 g wheat protein hydrolysate (WPH intervention) and/or 3.2 g L-arginine (WPH + ARG or ARG intervention) concomitant with 75 g glucose as a caloric load. The amount of WPH administered as bolus dose was limited to 2 g due to its unpleasant taste, although other proteins for which a satiating effect has been shown in previous studies, were administered in higher amounts.[25] A wheat protein hydrolysate was chosen due to its low L-arginine content in comparison to other proteins,[23,24] and was therefore suitable for investigating the satiating effect of an L-arginine fortification. Currently, a NOAEL of dietary added L-arginine up to 30 g d^{-1} is proposed for healthy young adults.[26] For L-arginine, an amount of 3.2 g was chosen, which is also in accordance with common amounts used for dietary L-arginine

Table 3. A decrease of the total calorie intake on the study day was detected by the 24-h-recall method GloboDiet, Mann–Whitney Rank Sum Test showed a significance of $p < 0.05$, $n = 27$. Data are presented as mean ± SEM. Δ Kcal has been calculated by subtracting NV control from interventions. The NV control intervention resulted in a mean kcal intake of 2951 ± 141 kcal.

Intervention	Δ Intake [Kcal]	*p*-Value [*t*-test vs NV control intervention]
WPH	−249 ± 149	0.459
ARG	−487 ± 170	0.082
WPH+ARG	−360 ± 147	0.025

supplementation.[26] Outcome measures included the subjective feelings of hunger, the total energy intake right after an ad libitum breakfast as well as over a period of 24 h post-intervention. In addition, changes in plasma glucose, insulin, serotonin, GLP-1, and ghrelin concentrations were analyzed over time for 140 min after intervention. Furthermore, all interventions included the administration of ^{13}C-Na-acetate for analyzing the ratio of $^{13}CO_2/^{12}CO_2$ in breath samples as an indicator of gastric emptying.

For ARG and WPH + ARG interventions, a reduced subjective feeling of hunger in comparison to the NV control intervention was demonstrated, indicating a satiating effect of which the effect size was similar for both interventions. Whether bolus amounts of >2 g of a wheat protein hydrolysate with or without L-arginine fortification could further decrease the subjective feelings of hunger needs to be investigated in future studies.

The demonstrated effect of the ARG and WPH + ARG intervention is reflected by a lower energy intake calculated from the remaining food after a standardized ad libitum breakfast 2 h post load compared to NV control intervention. Plasma concentrations of glucose, insulin, ghrelin, and GLP-1 concentrations did not change after any of the intervention, which has been previously demonstrated for nonivamide in a comparable study design.[19] Since only 27 study subjects completed all four interventions, whereas G-Power analysis had revealed a number of 50 study participants, the low samples size of the study might have limited the effects on plasma hormones. Furthermore, the low number of study participants might be one reason for the reduced effect size of the ad libitum energy intake from the standardized breakfast of 0.17 MJ, in comparison to the expected effect size of 0.47 MJ. Overall, these results indicate a satiating effect of wheat protein hydrolysate and L-arginine, which is in accordance with current literature, stating that protein intake[39] in human and L-arginine[16] in rodents, induces a decrease in energy intake.

In addition to a reduction of the subjective feeling of hunger and energy intake, plasma serotonin levels in this study were also increased after WPH + ARG intervention. We previously demonstrated that administration of 0.15 mg nonivamide increased plasma serotonin levels after a bolus dose,[19] as well as after a daily dose in a 12 week human intervention study.[20] In the present study, we demonstrate plasma serotonin levels to further increase after administration of a combination of 0.15 mg nonivamide with either 3.2 g L-arginine and/or 2 g wheat protein hydrolysate. These results indicate a role of L-arginine not only on energy intake, but also on plasma serotonin levels. A sustained increase of central serotonin release after L-arginine superfusion has been demonstrated by Sinner and colleagues.[40] In addition, there is also growing evidence for peripheral serotonin to promote satiety via nerval activation, although its satiating pathways are not yet fully understood.[7,41] Moreover, there is growing evidence supporting a regulating role of peripheral serotonin in the systemic energy homeostasis.[7,41] In one of the previous studies, an oral application of 50 mg encapsulated L-arginine resulted increased plasma serotonin levels in healthy subjects.[42] Also the data of the present study strengthen the hypothesis of a modulating role of peripheral serotonin on energy homeostasis. Up to now, it can just be speculated how L-arginine and a wheat protein hydrolysate stimulate the release of serotonin from enterochromaffin (EC) cells. In the gastrointestinal tract, EC cells act also as nutrient sensors. Hence, it could be hypothesized that EC cells act as peptide and amino acid sensors, which might respond by 5-hydroxytryptamine (5-HT) release during meal intake. A significant release of 5-HT after a protein-rich meal in comparison to carbohydrate- or fat-rich meals was demonstrated by Blum and colleagues.[43] Recently, Lund and colleagues suggested a paracrine mechanism for stimulating EC cells by nutrient metabolites in the gut.[44] Furthermore, an increased 5-HT release could be mediated by an increased Ca^{2+} influx via voltage activated calcium channels,[45] which might have been induced by WPH+ARG in this study. Extracellular serotonin concentrations are regulated by serotonin-selective reuptake transporters (SERT) to cross the plasma membrane, where it is enzymatically degraded.[46] From the current human intervention study, it can be hypothesized that ARG and WPH may partly inhibit der SERT system, possibly resulting in increased plasma serotonin concentrations, which are currently discussed to promote satiety in addition to central serotonin.[41]

Peripheral serotonin is also involved in the control of gastric motility,[47] which regulates gastric empyting.[48] A delay in gastric emptying is known to promote early satiety.[49] However, evidence for L-arginine's effect on gastric emptying in humans is still conflicting, by describing a delay[17,50] on the one hand, and an accelerated gastric emptying[51] on the other, which may be due to application of different detection methods and study models. In addition, proteins differentially regulate gastric emptying depending on their origin, with a mostly increased gastric emptying for whey protein in comparison to cod, gluten, or casein, when administrated within a standardized test meal in an amount of 45 g.[14] In the present human intervention study, both the WPH as well as the WPH + ARG intervention delayed gastric emptying, which was analyzed using ^{13}C-Na-acetate as a marker.

The satiating effect of a wheat protein with and without L-arginine fortification demonstrated in our study is suggested to be supported by plasma serotonin levels correlating with gastric emptying. Furthermore, our results indicate that high plasma serotonin levels are favorable for a delay in gastric emptying. Thus, we hypothesize that the increased plasma serotonin levels lead to a retard in gastric emptying, which promotes the feeling of satiety and helps to reduce total energy intake. However, further research investigating the interaction between gastric emptying and plasma serotonin levels is needed. Overall, the combined administration of nonivamide, L-arginine, and wheat protein hydrolysate in an oGTT was most effective in triggering this

proposed satiety cascade, demonstrating the importance of an optimized nutritional composition of foods to achieve the most effective satiating effect.

To summarize, in the present human intervention study, we demonstrate that the combined bolus administration of 0.15 mg nonivamide, 3.2 g L-arginine and 2 g wheat protein hydrolysate in an oGTT reduced subjective feelings of hunger and total energy intake from a standardized breakfast, which might be due to increased plasma serotonin levels that lead to a delay in gastric emptying. Combinations of nutrients are favorable for potentiating the satiating effects of foods without having to apply high concentrations of the individual ingredients in the food's formulation. The use of nonivamide, for example, in large amounts, is limited due to its pungency,[19] and excessively high-protein diets may pose long-term health risks to the kidneys,[52] whereas a common side effect of high arginine doses above 9 g d^{-1} is diarrhea.[53] In the present study, each active agent was applied in an amount that is unlikely to induce any adverse effects, and the combined application of nonivamide, arginine, and wheat protein hydrolysate can be hypothesized to be more effective than the individual compounds.

Future studies including women are needed for a more representative study population to substantiate the demonstrated effect as basis for the development of satiating food formulations. In addition, long-term effects on satiety signals and body composition have not been investigated yet, but are needed to conclusively assess the efficacy of this approach to maintain a healthy body weight.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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V. St., J. H., J.P.L., G.E.K., and V.So. designed and conducted the human intervention study. V.St. and J.R. did VAS and kcal calculations. V.St. J.R., and P.R. collected and calculated data for GloboDiet. V.St., K.S., J.Hoi, and J.R. did blood sample preparations and performed experiments in plasma samples. V.St. took breath samples. V.R. and M.K. did $^{13}CO_2$ measurements. V.St., V.R., and V.So. did calculations for gastric emptying. V.St, B.L., and V.So. wrote the manuscript. All authors edited the manuscript, provided comments, and approved the final version of the manuscript. The authors thank Kristina Englert and Verena Hasenegger from the Department of Nutritional Sciences for support with data collection and applying GloboDiet, and Sebastian Bayer for technical support. The financial support by the Christian Doppler Research Association, the Austrian Federal Ministry for Digital and Economic Affairs, and the National Foundation for Research, Technology and Development is gratefully acknowledged. The authors also thank A. Costantino & C. S.P.A (Favria, Italy) for kindly providing the wheat protein hydrolysate.

Conflict of Interest

The authors J. Hans, J. P. Ley, G. E. Krammer are employees at Symrise AG, Holzminden, Germany, which holds IP on nonivamide in the context of satiety management.

Keywords

arginine, food satiety, gastric emptying, nonivamide, wheat protein hydrolysate

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3 Conclusions and Perspectives

Proteins are known to have a stronger impact on satiety in comparison to carbohydrates or fats. For the digestion of proteins, secretion of gastric acid is required, which degrades the proteins into absorbable peptides and free amino acids. Whereas L-enantiomers of amino acids are mainly described as bitter-tasting, their respective D-forms are predominantly described as sweet.

From the current literature, there is evidence that proteins and various bitter-tasting compounds regulate mechanisms of satiety, and that bitter-tasting L-amino acids activate T2Rs. Therefore, the question arises, which key structural characteristics of bitter-tasting amino acids regulate mechanisms of satiety via T2R-mediated secretion of gastric acid, and whether such stimulation of mechanisms of gastric acid secretion *in vitro* translates into a regulation of food intake and satiety *in vivo*.

In publication *(1) Identification of bitter-taste intensity and molecular weight as amino acid determinants for the stimulating mechanisms of gastric acid secretion in human parietal cells in culture,* structural determinants of amino acids stimulating gastric acid secretion in HGT-1 cells were identified. The current literature did not provide conclusive data regarding the taste qualities of amino acids, and also no taste intensities of amino acids had been published that allow a direct comparison. Thus, a sensory study with method-trained panelists was carried out. L-tryptophan and D-tryptophan were revealed to be the most intense bitter and sweet-tasting amino acids, respectively, whereas arginine and isoleucine were identified to be bitter-tasting in both isomeric forms. In HGT-1 cells, arginine most potently stimulated the secretion of protons, a marker of gastric acid secretion, in both enantiomeric forms. Correlation analysis revealed that a high bitter taste intensity and a high molecular weight of L-amino acids promote secretion of protons. Based on this outcome, it can be assumed that a high bitter taste intensity and a high molecular weight are favorable for a satiating effect of L-amino acids. Experiments with bitter- and sweet taste antagonists support the hypothesis that the bitter taste, but not the sweet taste, of amino acids is involved in mechanisms regulating proton secretion.

Since arginine and isoleucine induced a different effect on proton secretion, but were bitter-tasting in both enantiomers, the L-form of these two candidates were chosen for a mechanistical approach. In publication *(2), The bitter-tasting amino acids L-arginine and L-isoleucine differentially regulate proton secretion via T2R1 signaling in human parietal cells in culture,* L-arginine (L-ARG) and L-isoleucine (L-ILE) were tested in co-incubation experiments using specific antagonists for ENaC, CaSR, and T2R for their effects on proton secretion in HGT-1 cells. Results of these experiments indicate that neither CaSR nor ENaC, play a pivotal role in the mechanism of L-ARG and L-ILE-induced proton secretion in HGT-1 cells. In contrast, a different regulation of

proton secretion via T2R signaling was detected by co-incubation experiments with T2R antagonists.

Since an influence of T2R signaling on proton secretion was detected in HGT-1 cells, a mechanistic approach was conducted. Hence, a screening on cDNA expression by means of a microarray was conducted. Subsequent analysis of this data using the online software tool "DAVID"[93, 94], further demonstrated that L-ARG and L-ILE target T2Rs in the mechanism of proton secretion in HGT-1 cells. Therefore, *TAS2Rs* cDNA expression data were subjected to correlation analysis, which revealed T2R1 as a prominent candidate for L-ARG and L-ILE being involved in mechanism of cellular proton secretion. Validation by RT-qPCR demonstrated a strong regulation of *TAS2R1* expression induced by L-ARG and L-ILE. The effect induced by L-ARG on *TAS2R1* expression was more pronounced in comparison to L-ILE. Also, on a protein level, the presence of T2R1 in HGT-1 cells was detected by Western Blot. For providing further evidence that T2R1 is functional in the mechanisms of proton secretion in HGT-1 cells, a CRISPR/Cas9 knock-out approach was performed. This experiment demonstrated that T2R1 is functional in the mechanism of proton secretion in HGT-1 cells, however, whether there is an interaction with further T2Rs in the mechanism of proton secretion cannot be excluded. Different binding residues for L-ARG and L-ILE were predicted by using a homology model of the T2R1 receptor. These different binding residues possibly are also involved in the different effect of these amino acids on the mechanism of proton secretion. In addition, a different regulation of GPCR signaling was detected by the two amino acids. L-ARG decreased cAMP and IP1 concentrations, whereas L-ILE regulated cAMP concentrations to a smaller extent and induced an augmentation of IP1 concentrations. Overall, these results indicate a different regulation on mechanisms of proton secretion mediated via T2R signaling in HGT-1 cells induced by the amino acids L-ARG and L-ILE. This outcome is in accordance with the study published by Brockhoff and colleagues[95], who demonstrated that sesquiterpene lactones, which are structural similar have agonistic as well as antagonistic effects on T2R activation.[95] Since the mechanism how structurally similar compounds activate or inhibit T2R has not been elucidated yet, further mechanistical studies should be conducted in the future. Results presented in publication *(2), The bitter-tasting amino acids L-arginine and L-isoleucine differentially regulate proton secretion via T2R1 signaling in human parietal cells in culture,* demonstrated that L-ARG and L-ILE differentially target T2R signaling involved in mechanisms of proton secretion. Moreover, a pivotal role was identified for T2R1 signaling. This is the first time that L-ARG and L-ILE have been identified to target T2R1. Since other groups could not detect an effect of T2R activation by measuring Ca^{2+} mobilization in transfected HEK cells[75], analysis of other second messengers should be considered. Furthermore, investigations of further signaling pathways that indirectly involve T2R1 are suggested. These results further support HGT-1 cells as a native cell model for investigating T2R signaling for characterizing bitter-tasting compounds by modulation of proton secretion. In addition, this outcome supports our hypothesis that bitter-

tasting amino acids regulate gastric acid secretion *in vitro*. Since the bitter-tasting L-arginine had the strongest impact on proton secretion in HGT-1 cells and targeted T2R1 in mechanisms of proton secretion, this amino acid was chosen for a proof-of concept study with healthy subjects. We previously demonstrated that the capsaicinoid nonivamide increases satiety by a decrease of food intake from an *ad libitum* standardized breakfast, and increases plasma serotonin levels in moderately overweight healthy human.[13] Therefore, in the present thesis, a follow-up human intervention study *(3)* investigated whether the satiating effect of nonivamide can be enhanced by adding 3.2 g of L-arginine or 2 g of a wheat protein hydrolysate fortified with L-arginine. A wheat protein hydrolysate was chosen due to its low L-arginine content. A bolus administration of 0.15 mg nonivamide and 2 g of wheat protein hydrolysate fortified with 3.2 g L-arginine decreased the food intake from a standardized *ad libitum* breakfast. In addition, the combination of the wheat protein hydrolysate in combination with L-arginine augmented plasma serotonin concentrations. Neuronally, a delay of gastric emptying was detected after bolus administration of the fortified wheat protein hydrolysate in comparison to the nonivamide control. Results on metabolic, hormonal and neuronal parameters of satiety after oral application of the wheat protein hydrolysate and L-arginine, demonstrated that satiety was enhanced in comparison to nonivamide alone. This outcome supports our hypothesis that the bitter-tasting amino acid L-arginine is able to promote satiety.

To summarize, the results of this PhD thesis, provide evidence for the bitter-tasting amino acid L-arginine to stimulate mechanism of gastric acid secretion via T2R1 activation, and to increase satiety, leading to a reduced food intake in healthy subjects after bolus administration in an amount of 3.2 g.

Hence, within this PhD thesis it could be demonstrated that bitter taste of L-amino acids is associated with their impact on mechanisms regulating proton secretion, which is linked to reduced food intake and increased satiety in humans. Whether and to which extend this effect is mediated via gastric acid secretion and T2R signaling has to be verified in healthy subjects.

IV. Abstract

Current numbers for the prevalence of overweight and obesity demonstrate an epidemic extent, indicating an urgent need for efficient measures. Since overweight arises from a positive energy balance, an optimization of foods to enhance satiety is a promising non-invasive strategy.

Proteins and certain free amino acids are known to stimulate mechanisms of satiety and gastric acid secretion. Most of the L-amino acids are described as bitter-tasting, and the bitter-tasting compound caffeine has been shown to stimulate gastric acid secretion via T2R signaling. Since application of bitter-tasting compounds to healthy subjects promoted satiety, the question arises if also bitter-tasting amino acids induce satiety. Therefore, the present PhD thesis aimed to identify a possible link between mechanisms regulating satiety and gastric acid secretion, induced by bitter-tasting amino acids.

For this purpose, in Part I of the present thesis, methods for characterizing and investigating a link between gastric acid secretion and satiety were established. Thus, *in vitro*, a T2R43-mediated proton secretion in HGT-1 cells was detected by a CRISPR/Cas9 knock-out approach. Furthermore, an influence on satiety induced by the bitter-masking compound HED was demonstrated in a human intervention study. In addition, treatment with HED regulated gastric motility in human stomach specimens. In a mechanistical approach studying gastric mechanisms of satiety, HGT-1 cells were established as a suitable cell model for analyzing peripheral serotonin release after treatment with L-arginine.

In Part II of this thesis, 20 amino acids were tested for their effects on proton secretion in HGT-1 cells and bitter as well as sweet taste intensities of these candidates were assessed by a method-trained panel. Correlation analysis identified bitter taste intensities and molecular weight of L-amino acids as key determinants for proton secretion. Furthermore, co-incubation experiments of L-arginine and L-isoleucine with bitter and sweet taste antagonists enlightened a T2R involvement in HGT-1 cells. Moreover, a *TAS2R1* knock out by means of CRISPR/Cas9 in HGT-1 cells indicates a role also on a functional level. Since L-arginine induced the most pronounced effect on proton secretion and is also known to increase satiety, this amino acid was chosen for administration in a human intervention study. In this study a fortified wheat protein hydrolysate with L-arginine further enhanced the satiating effect induced by the capsaicinoid nonivamide in healthy male subjects.

To summarize, within this PhD thesis, the bitter-tasting amino acid L-arginine was identified to regulate a T2R1-mediated secretion of protons *in vitro*, and to induce mechanisms regulating satiety and food intake *in vivo*. Whether and to which extend this effect is mediated via gastric acid secretion and T2R signaling has to be verified in healthy subjects.

V. Zusammenfassung

Aktuelle Zahlen zur Prävalenz von Übergewicht und Adipositas zeigen epidemische Ausmaße und begründen eine dringende Umsetzung neuer Strategien. Eine erfolgversprechende Strategie stellt die Optimierung von Lebensmittelinhaltsstoffen zur Steigerung der Sättigung dar, welche die Erhaltung eines gesunden Körpergewichts fördern können.

Sowohl für Proteine als auch für bestimmte freie Aminosäuren ist ein stimulierender Effekt auf die Magensäuresekretion als auch auf die Sättigung bereits bekannt. Darüber hinaus gibt es Evidenz, dass Aminosäuren vorwiegend in der L-Form bitter und die D-Form hauptsächlich süß schmecken. Nachdem kürzlich ein Anstieg der Sättigung nach Verabreichung von bitter-schmeckenden Substanzen bei gesunden Probanden gezeigt werden konnte, war das Ziel der vorliegenden Arbeit, einen Zusammenhang zwischen bitter-schmeckenden Aminosäuren, der Magensäuresekretion und der Sättigung zu untersuchen.

Für diesen Zweck wurde in Teil I dieser Arbeit die entsprechenden Methoden etabliert. Mittels CRISPR/Cas9 Experiment in HGT-1 Zellen konnte eine Beteiligung des T2R43 Rezeptors in der Regulation der Protonensekretion, nach einer Behandlung mit Koffein identifiziert werden. Weiters konnte ein Einfluss der bittermaskierenden Substanz HED auf gastrische Parameter des Sättigungsmechanismus in einer Humaninterventionsstudie sowie in humanen Gewebeproben gezeigt werden. Darüber hinaus konnte die HGT-1 Zelllinie als Model für die Untersuchung der peripheren Serotoninausschüttung nach Behandlung mit L-Arginin etabliert werden.

In Teil II der vorliegenden Doktorarbeit wurde der Einfluss von L- und D-Aminosäuren auf die Protonensekretion in HGT-1 Zellen bestimmt, sowie deren Bitter- als auch Süßintensitäten von einem methoden-trainierten Panel ermittelt. Korrelationsanalysen identifizierten die Bitterintensität und das Molekulargewicht von L-Aminosäuren als Determinanten für eine Stimulierung von Mechanismen der Magensäuresekretion. Darüber hinaus zeigte die Verwendung von Bitter- als auch Süßgeschmack-Antagonisten einen Einfluss von T2Rs im Mechanismus der Protonensekretion. Ein knock-out des *TAS2R1* Gens in HGT-1 Zellen mittels CRISPR/Cas9 führte zu einer Modulierung der Protonensekretion. In der anschließenden Sättigungsstudie wurde gezeigt, dass ein mit L-Arginin angereichertes Weizenprotein-Hydrolysat, die sättigende Wirkung des Capsaiciniods Nonivamid in gesunden Probanden weiter steigern.

Im Rahmen der vorliegenden Arbeit konnten somit die bitter-schmeckende Aminosäure L-Arginin als T2R1-abhängiges Stimulans der Protonensekretion sowie der Sättigung identifiziert werden. In welchem Ausmaß und ob eine T2R-abhängige Stimulierung der Magensäuresekretion stattfindet, muss in gesunden Studienteilnehmern validiert werden.

VI. Curriculum Vitae

Education

2013 – 2019

PhD studies in Chemistry at the Department of Physiological Chemistry, Faculty of Chemistry, University of Vienna, Supervisor: Univ.-Prof. Dr. Veronika Somoza
Research interest: Influence of bitter-tasting compounds on mechanisms of gastric acid secretion and satiety *in vitro* and *in vivo*

2010 – 2013

Master studies in Molecular Nutrition at the Department of Physiological Chemistry, Faculty of Chemistry, University of Vienna, Supervisor: Univ.-Prof. Dr. Veronika Somoza
Master Thesis: *„Einfluss von trans-Resveratrol und des antineoplastischen Wirkstoffes KP-1339 auf den Energiestoffwechsel in HepG2 Zellen“*

2007 – 2010

Bachelor studies in Nutritional Sciences at the Department of Nutritional Sciences, Faculty of Lifesciences, University of Vienna, Supervisor: Univ.-Prof. Dr. Karl-Heinz Wagner
Bachelor Thesis*: „Diabetes & DNA Damage“*

Grants

2019

ECRO travel grant
funding for participation and poster presentation at the Annual Meeting of the European Chemoreception Research Organization 2019 (Trieste, Italy)

short-term scientific mission
COST action Multi-target paradigm for innovative ligand identification in the drug discovery process (MuTaLig)
funding for 1.5 months for the research stay at the Hebrew University of Jerusalem

2018

short-term fellowship
European Molecular Biology Organization (EMBO)
funding for 3 months for the research stay at the Hebrew University of Jerusalem

2017

travel grant

Danone GmbH Germany
funding for participation and oral presentation at the Annual Meeting of the American Chemical Society 2017 (Washington, DC)

2016
KWA (Kurzfristige wissenschaftliche Auslandsaufenthalte) grant,
University of Vienna
funding for 3 months for a research stay at the Queen Mary University (London)

Publications:

First author

"Identification of bitter-taste intensity and molecular weight as amino acid determinants for the stimulating mechanisms of gastric acid secretion in human parietal cells in culture."
V. Stoeger, KI. Liszt, B. Lieder, M. Wendelin, M. Zopun, J. Hans, JP. Ley, GE. Krammer, and V. Somoza, 2018, 66 (26), Journal of Agricultural and Food Chemistry, DOI: 10.1021/acs.jafc.8b01802.

"The bitter tasting amino acids L-arginine and L-isoleucine differentially regulate proton secretion via T2R1 signaling in human parietal cells in culture."
V. Stoeger, AK. Holik, K. Hölz, T.Dingjan, J. Hans, JP. Ley, GE. Krammer, M. Niv, MM. Somoza, V. Somoza, submitted to Journal of Agricultural and Food Chemistry, *in review*

"Wheat protein hydrolysate fortified with L-arginine enhances satiation induced by the capsaicinoid nonivamide in moderately overweight male subjects."
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Co-author:

„Human sweet receptor T1R3 is functional in human gastric parietal tumor cells (HGT-1) and modulates cyclamate and acesulfame K-induced mechanisms of gastric acid secretion."
M. Zopun, KI. Liszt, **V. Stoeger**, M. Behrens, U. Redel, JP. Ley, J. Hans, V. Somoza, Journal of Agricultural and Food Chemistry 2018 ,66(19), DOI: 10.1021/acs.jafc.8b00658

"Appetite-Inducing Effects of Homoeriodictyol: Two Randomized, Cross-Over Interventions."

CM. Hochkogler, KI. Liszt, B. Lieder, **V. Stoeger**, A. Stübler, M: Pignitter, J. Hans, S. Widder, JP. Ley, GE. Krammer, V. Somoza, Molecular Nutrition and Food Research, 2017, 61(12), DOI: 10.1002/mnfr.201700459.

"Caffeine induces gastric acid secretion via bitter taste signaling in gastric parietal cells."
KI. Liszt, JP. Ley, B. Lieder, M. Behrens, **V. Stoeger**, A. Reiner, CM. Hochkogler, E. Köck, A. Marchiori, J. Hans, S. Widder, GE. Krammer, GJ. Sanger, MM. Somoza, W. Meyerhof, V. Somoza, Proceedings of the National Academy of Sciences of the United States of America, 2017, 114(30), DOI: 10.1073/pnas.1703728114.

"Impact of free Nε-carboxymethyllysine, its precursor glyoxal and AGE-modified BSA on serotonin release from human parietal cells in culture."
AK. Holik, **V. Stoeger**, K. Hölz, MM. Somoza, V. Somoza, Food and Function, 2018, 9, DOI: 10.1039/c8fo01045e

"**Serotonin biosynthesis and release from human gastric tumor cells (HGT-1).**"
AK. Holik, **V. Stoeger**, B. Lieder, J. Hoi, M. Zopun, GJ. Sanger, V. Somoza, *Submitted to Journal of FASEB in revision*

"Influence of gallic acid on mechanism of proton secretion in HGT-1 cells."
S. Sterneder, **V. Stoeger**, KI. Liszt, C. Dugulin, M. Eder, V. Somoza, *Journal of Agricultural and Food Chemistry in preparation*

Conference Paper

„Caffeine-induced activation of oral and gastric bitter taste receptors regulates gastric acid secretion."
KI. Liszt, JP. Ley, B. Lieder, **V. Stoeger**, E. Köck, A. Stuebler, CM. Hochkogler, MM: Somoza, S. Widder, J. Hans, GE. Krammer, V. Somoza, 37t^{h} Annual Meeting of the Association-for-Chemoreception-Sciences, Volume: 40

"Identification of amino acid structural determinants for activating mechanisms of gastric acid secretion."
V Stoeger, K Liszt, B Lieder, M Zopun, M Wendelin, J Hans, J P. Ley, G E. Krammer, V Somoza, Annual Meeting of the American Chemical Society 2017, Washington DC, Cornucopia, AGFD 245

"L-Arginine and L-Isoleucine interactions with bitter taste receptor T2R1 and relevance for gastric acid secretion."

V Stoeger, T Dingjan, Y Ben-Shoshan-Galeczki, J Hans, J P. Ley, G E. Krammer, M Niv, V Somoza, 29th Annual Meeting of the European Chemoreception Research Organization, Abstract booklet, p54, Th-P1-036

Patent Application

"Zubereitungen enthaltend argininreiche Proteine sowie Nonivamid", (PCT/EP/2019/051301) G. Krammer, J. Ley, J. Hans**, V. Stoeger**, V. Somoza

Reviewing Activity

Journal of Agriculture and Food Chemistry