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„The effect of gallic acid derivatives on intracellular reactive oxygen species in endothelial EA.hy926 cells“

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Abstract

The endothelium, a single cell lining covering the inner surface of blood vessels, is known as a main regulator of vascular homeostasis. It plays a major role in the control of vascular tone, blood fluidity and coagulation. Especially nitric oxide (NO), produced by the endothelial nitric oxide synthase (eNOS), is important for vasodilatation. An imbalance in the regulatory functions of endothelial cells is associated with endothelial dysfunction, which is considered to be the underlying event in the development of cardiovascular diseases. Oxidative stress, caused by different sources, can lead to an uncoupling of eNOS monomers thereby forcing the production of reactive oxygen species (ROS) instead of NO. A high level of ROS is also perceived as a risk factor for cardiovascular diseases.

Polyphenolic substances, originally found in natural sources as secondary plant metabolites, and their derivatives are in discussion to provide a beneficial effect in the prevention of cardiovascular diseases. Gallic acid and its derivatives are known for their positive effects, like inhibition of platelet aggregation, vasorelaxation and modulation of lipid metabolism. They also act as radical scavengers and showed an eNOS activating effect in a former study. As intracellular ROS levels and eNOS activation are known to be linked, we investigated in our work gallic acid and six derivatives (methyl gallate, ethyl gallate, propyl gallate, butyl gallate, isopentyl gallate and octyl gallate) for their ability to reduce intracellular ROS levels. Although there was no difference in radical scavenging ability between the gallic acid derivatives detectable, we were able to show a correlation between aliphatic chain length and intracellular ROS reduction. Increasing aliphatic chain correlated with a more pronounced effect in intracellular ROS reduction. In addition we investigated a possible correlation between ROS levels and eNOS activity after treatment with gallic acid derivatives. Unfortunately in our experimental setting we couldn't show a correlation, at least in unstressed, healthy cells.

Various posttranscriptional or posttranslational modifications, for example phosphorylation, are able to regulate eNOS activity. In a second part of this thesis the influence of gallic acid derivatives on the eNOS phosphorylation site Ser^{114} was investigated. Our data showed no significant change in eNOS protein expression and eNOS-Ser^{114} phosphorylation after treatment with butyl or ethyl gallate.

These results point to an additional antioxidative quality besides the radical scavenging effect of gallic acid derivatives.

Zusammenfassung

Das Endothelium bekleidet als Einzelzellschicht die innere Oberfläche der Blutgefäße und ist bekannt als Hauptregulator der Gefäßhomeostase. Es spielt eine große Rolle in der Kontrolle des Gefäßtonus, Blutflusses und der Koagulation. Besonders Stickstoffmonoxid (NO), das von der endothelialen Stickstoffmonoxid Synthase (eNOS) produziert wird, ist wichtig für die Vasodilatation. Ein Ungleichgewicht der regulierenden Funktionen von Endothelzellen wird mit dysfunktionalem Endothel in Verbindung gebracht, welches als zugrunde liegendes Ereignis für kardiovaskuläre Erkrankungen erachtet wird. Oxidativer Stress, der durch verschiedene Quellen hervorgerufen werden kann, kann zu einer Entkopplung der eNOS Monomere führen und die Produktion von reaktiven Sauerstoff Spezies (ROS) anstelle von NO fördern. Ein hoher Spiegel von ROS wird auch als Risikofaktor für kardiovaskuläre Erkrankungen erachtet.

Polyphenolische Substanzen, ursprünglich in natürlichen Quellen als sekundäre Pflanzenstoffe entdeckt, und deren Derivate sind in Diskussion einen positiven Effekt für die Prävention von kardiovaskulären Erkrankungen zu haben. Gallensäure und ihre Derivate sind bekannt für ihre gesundheitsfördernden Effekte, wie Plättchenaggregationshemmung, Vasorelaxation und Modulation des Lipidstoffwechsels. Außerdem haben sie eine Radikalfängerwirkung und zeigten einen eNOS aktivierenden Effekt in vorhergehenden Studien. Da ein Einfluss auf intrazelluläre ROS Spiegel durch eNOS Aktivität vermutet wird, haben wir in unserer Arbeit Gallensäure und sechs ihrer Derivate (Methylgallat, Ethylgallat, Propylgallat, Butylgallat, Isopentylgallat und Octylgallat) auf ihre intrazelluläre ROS reduzierenden Fähigkeiten getestet. Obwohl keine Unterschiede zwischen den Gallensäurederivaten in ihrer Radikalfängerwirkung festgestellt wurden, konnten wir einen Zusammenhang zwischen der aliphatischen Kettenlänge und der intrazellulären ROS Reduktion feststellen. Je länger die Kette ist, desto stärker ist der reduzierende Effekt. Wir versuchten auch einen Zusammenhang zwischen dem ROS Spiegel und der Aktivität von eNOS nach der Behandlung mit Gallensäurederivaten zu ermitteln. Leider konnten wir in unserem Versuchsaufbau keinen Zusammenhang feststellen, zumindest nicht im gesunden Zustand der Zelle.

Verschiedene posttranskriptionale und posttranslationale Modifikationen, zum Beispiel Phosphorylierung, sind in der Lage die eNOS Aktivität zu regulieren. Ein weiterer Teil dieser Arbeit war die Untersuchung des Effektes von Gallensäurederivaten auf die Phosphorylierungsstelle Ser^{114}. Unsere Daten zeigten keine signifikante Änderung in eNOS Proteinexpression und eNOS-Ser^{114} Phosphorylierung nach der Behandlung mit Butylgallat oder Ethylgallat. Die Ergebnisse unserer Untersuchungen lieferten Anzeichen für zusätzliche antioxidative Eigenschaften der Gallensäurederivate, neben dem reinen Radikalfänger-Effekt.

Contents

Part I

Introduction

1. The endothelium

The endothelium is a single cell lining covering the inner surface of blood vessels and the lymphatic system. The fact that the endothelium is semipermeable enables molecules to pass between blood and tissue. [20] But this is not the only function of endothelial cells. They are structurally heterogeneous and play a major role in regulating vascular tone, blood fluidity and coagulation, and are able to influence angiogenesis and immunity. [21]

To control the relaxation of blood vessels endothelial cells generate a so called endothelium derived relaxing factor (EDRF). In 1987/1988 Ignarro et al. and Palmer et al. first identified nitric oxide (NO) as EDRF. [22] [23]

In addition to its action as vasodilator, NO exhibits important endothelial functions like inhibition of platelet activation, adhesion and aggregation as well as leukocyte adhesion. [24]

Endothelial dysfunction occurs, if there is an imbalance or defect in the regulatory functions of endothelial cells. A dysfunctional endothelium leads to an increase in reactive oxygen species (ROS) and a decrease in NO levels and promotes cardiovascular diseases. [15] It is an early marker for atherosclerosis, which is regarded as the underlying pathology of cardiovascular diseases like peripheral vascular disease, stroke or coronary heart disease. [46] [47] Risk factors like diabetes, hypertension, hypercholesterolemia, genetic disposition or cigarette smoking are able to promote oxidative stress and endothelial dysfunction. [15]

2. Nitric oxide synthases

2.1. Nitric oxide synthase isoforms

There are three different isoform types of nitric oxide synthase. They are located on individual gene segments and the synthases themselves are expressed in different cell types. [7]

2.1.1. Neuronal nitric oxide synthase - nNOS

The neuronal nitric oxide synthase is mainly expressed in neurons in the brain and its activity is regulated by Ca^{2+} and calmodulin. nNOS derived NO acts as neurotransmitter and thereby influences memory function and neurogenesis. Neurodegenerative pathologies like stroke, multiple sclerosis, Alzheimer's disease or Parkinson's disease are linked to an abnormal NO signaling.
Nitric oxide produced by nNOS in nitrergic nerves acts like a neurotransmitter that stimulates NO-sensitive guanylyl cyclase and decreases the tone of smooth muscle cells, contained in various tissues, including blood vessels. nNOS also occurs in small amounts in vascular smooth muscle cells and is able to take over vasodilatation if eNOS is dysfunctional. [7]

2.1.2. Inducible nitric oxide synthase - iNOS

This isoform is not constitutively expressed but only expressed if induced by bacterial lipopolysaccharides or other pro-inflammatory stimuli like cytokines. Once expressed, iNOS is constantly active and not regulated by intracellular Ca^{2+} levels. [7] INOS is primarily located in macrophages, although expression can also be stimulated in other cells or tissues. Whereas the constitutive NOS isoforms nNOS and eNOS produce nitric oxide in nanomolar concentrations for seconds and minutes, iNOS generates amounts in the micromolar range for hours or days. [7] [8] [9]

2.1.3. Endothelial nitric oxide synthase - eNOS

The endothelial nitric oxide synthase is mainly expressed in endothelial cells. However, small amounts are also present in other tissues. The enzyme is constitutively expressed and is regulated by intracellular Ca^{2+}. eNOS is inevitable for a functional cardiovascular system, as

eNOS-derived NO acts as vasodilatator. Additionally NO inhibits platelet aggregation and adhesion, leukocyte adhesion, vascular inflammation and smooth muscle cell proliferation. [7]

3. Endothelial nitric oxide synthase

The eNOS enzyme consists of an oxygenase domain at the N-terminus and a reductase domain at the C-terminus. [35] Head to tail dimerization of two identical eNOS monomers results in an active enzyme. The dimer interface is stabilized via a zinc bound tetrathiolate cluster between cysteine 94, 99 on the respective monomers. [10] The functionality of eNOS is controlled by many cofactors. The reductase domain of the enzyme contains binding sites for FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide) and NADPH (nicotine adenine dinucleotide phosphate), whereas the oxygenase domain provides binding sites for heme, BH_4 (tetrahydrobiopterin) and L-arginine. Additionally both domains share a recognition site for CaM (calmodulin). [35]

3.1. Production of nitric oxide

The substrate for NO production by eNOS is the amino acid L-arginine. NADPH binds eNOS and delivers electrons to the enzyme, which pass via FAD and FMN to the reductase domain. [11] [35] The electron flow through the reductase domain requires the presence of bound Ca^{2+}/calmodulin. [35] These electrons are then transferred to the oxygenase domain, where they interact with the heme iron. (Figure 1) At the heme center, the electrons are used to reduce O_2 and catalyze the stepwise formation of NO and L-citrulline out of L-arginine. [11] [18] [35]

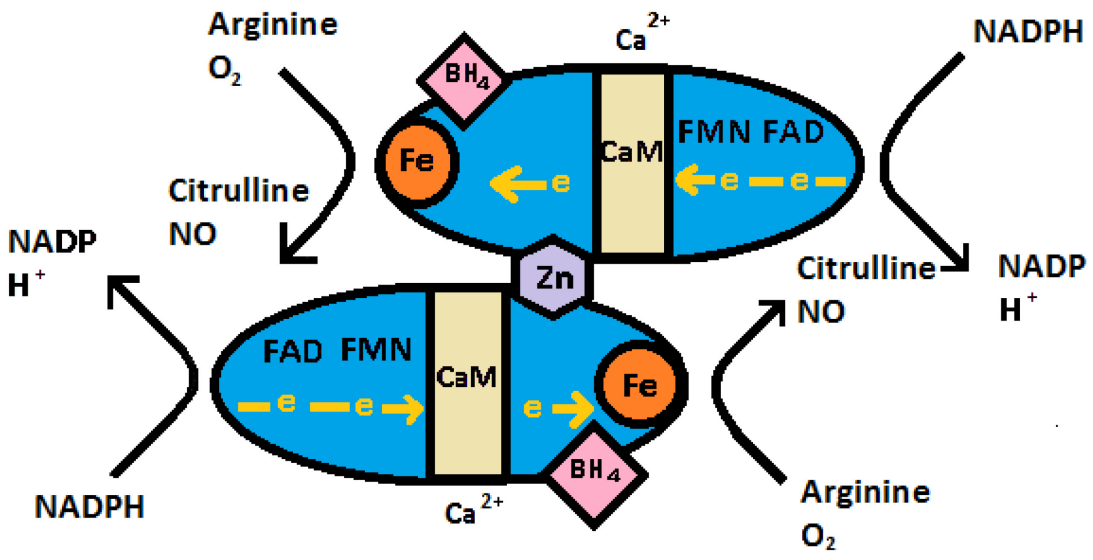


Figure 1: Formation of NO by the eNOS enzyme
modified from: Wendy K. Alderton, Chris E Cooper and Richard G Knowles. Nitric Oxide Synthases: structure, function and inhibition. Biochem. J. (2001) 357, 593-615
and: Bradley A. Maron, Thomas Michel. Subcellular Localization of Oxidants and Redox Modulation of Endothelial Nitric Oxide Synthase. Circ J., 2012, 76:2497–2512

3.2. Posttranslational regulation of eNOS

Besides posttranscriptional modifications, [57] the activity of the eNOS enzyme can be regulated by various posttranslational modifications.

3.2.1. Posttranslational regulation by phosphorylation

Phosphorylation on serine, threonine and tyrosine residues are known to regulate eNOS activity. (Figure 2)

Ser^{1177}

One of the best studied phosphorylation sites is Ser^{1177}. It is located in the reductase domain, near the C-terminus, and its phosphorylation plays an important role in the increase of eNOS activity. Several agonists like bradykinin, epidermal growth factor (EGF), histamine or thrombin stimulate phosphorylation on the Ser^{1177} residue. [12] [11] The kinases involved in this process vary with the stimuli applied. For example Akt (protein kinase B) and PKA (protein kinase A) are activated by shear stress, insulin or VEGF. CaMKII (Ca^{2+}/CaM-dependent protein kinase II) is modulated by bradykinin and increased intracellular Ca^{2+}

levels. [11] Also GlycNAc O-glycosylation of Ser^{1177} leads to a decreased Ser^{1177} phosphorylation (see chapter 3.2.5.)

Ser^{615}

Another positive regulation site for eNOS is Ser^{615}. The function of this residue is not exactly known yet, but it's supposed to be important in regulating phosphorylation of other residues, protein-protein interactions and increases the Ca^{2+}/calmodulin sensitivity of eNOS. This phosphorylation site is regulated by PKA and Akt. [11]

Ser^{114}

This site is innately phosphorylated and the only one located in the oxygenase domain of the eNOS enzyme. [11] [12] Phosphorylation on Ser^{114} has an inhibitory effect on eNOS activation and its dephosphorylation is agonist dependent. [12] The ERK 1/2 MAP-kinase (ERK1/ERK2-MAP-kinase) has been reported for being responsible for Ser^{114} phosphorylation under basal conditions, leading to an increased Pin-1 (peptidyl-propyl isomerase NIMA interacting 1) interaction with eNOS. This interaction induces a conformational change in the eNOS enzyme and suppresses eNOS activity. [43]
Dephosphorylation of this site, for instance VEGF dependent, leads to an activation of eNOS. [13]

Ser^{633}

Phosphorylation of Ser^{633} has been reported to increase eNOS enzyme activity. This residue is located in the calmodulin (CaM) autoinhibitory domain [12] and one of the main kinases to phosphorylate this site is AMPK (AMP activated protein kinase). [14] Ser^{633} phosphorylation is stimulated by fluid shear stress, VEGF, bradykinin or 8-bromo cAMP and has a lower time course of phosphorylation than Ser^{1177} or Thr^{495}. [11]

Thr^{495}

Another phosphorylation site located in the CaM binding domain is Thr^{495} but in contrast to Ser^{633} its phosphorylation leads to a decrease in eNOS enzyme activity. [11] Thr^{495} is basally phosphorylated and reduces NO production by encumbering the association of CaM with its binding site. PKC (protein kinase C) maintains the phosphorylation on Thr^{495} in unstimulated endothelial cells. [44] [11] The appearance of agonists like bradykinin, histamine or an increased intracellular Ca^{2+} concentration results in an activation of eNOS via dephosphorylation of Thr^{495}. Protein phosphatase PP1 is suggested to be the responsible phosphatase. [44]

Tyr[81]

The phosphorylation of eNOS on tyrosine residues has not been extensively studied so far, but it was shown that phosphorylation on Tyr[81] is regulated by the tyrosine kinase Src and leads to an increase in NO production. Interestingly phosphorylated Tyr[81] does not seem to promote eNOS activity directly, but serves as a docking site for adapter molecules. [45]

Tyr[657]

On the other hand phosphorylation of Tyr [657], which is performed by c-Src and PYK2 (protein tyrosine kinase 2-beta), has a negative effect on eNOS activity. [11]

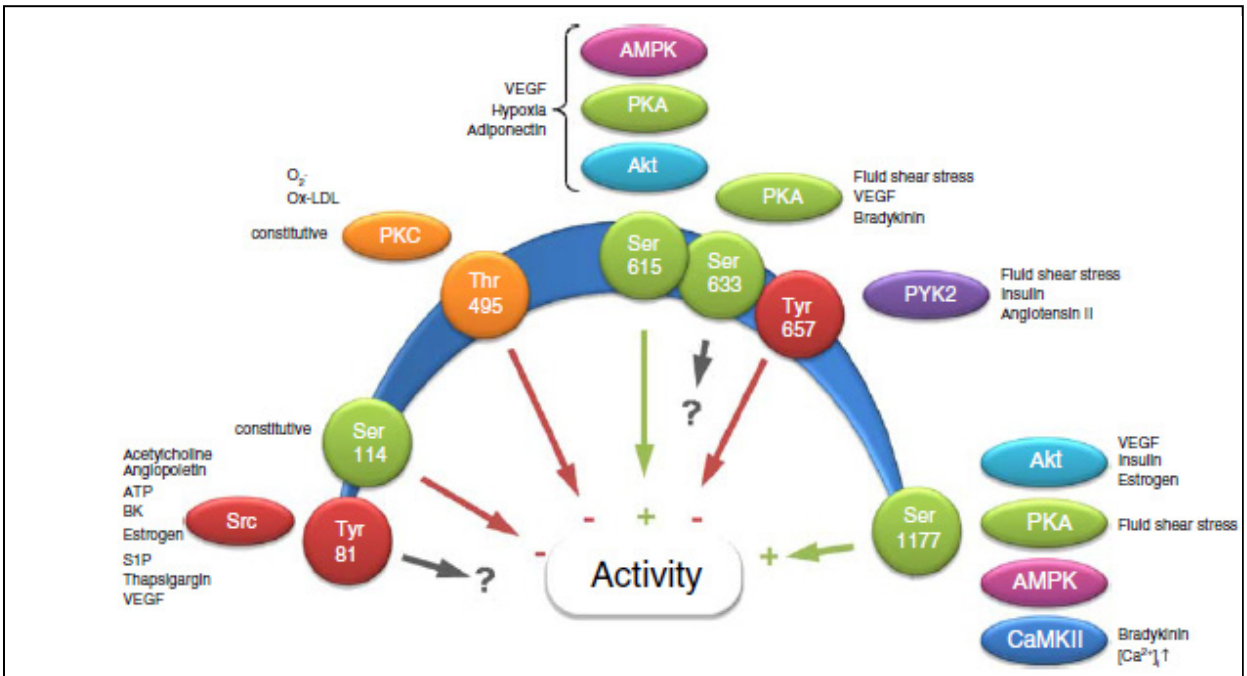


Figure 2: The regulation of eNOS by phosphorylation
(green arrows: activation, red arrows: inhibition, black arrows: no direct effect on enzyme activity)
modified from: Ingrid Flemming, Molecular mechanisms underlying the activation of eNOS, Pflugers Arch – Eur J Physiol (2010) 459:793-806

3.2.2. S-nitrosylation

Reversible S-nitrosylation at Cys[94] and Cys[99] is known as another posttranslational mechanism for regulation of eNOS activity. The cysteine residues Cys[94] and Cys[99] are part of the zinc tetrathiolate cluster in the eNOS homodimer interface. S-nitrosylation of these residues leads to a decrease of enzyme activity. Zinc can be released from the tetrathiolate cluster after S-nitrosylation, which suggests that the mechanism of inactivation depends on

dissociation of the eNOS homodimer into inactive monomers. As eNOS itself generates NO it thereby provides the substrate for S-nitrosylation. [33]

3.2.3. Protein interactions

Protein-protein interactions are important for the regulation of eNOS activity. Various different proteins are known to interact with eNOS. A selection of the most important ones is mentioned below.

Calmodulin

One of the first proteins known to regulate eNOS activity was calmodulin (CaM). [34] The activity of all three nitric oxide synthase isoforms is increased by binding at CaM. This binding depends on a high intracellular calcium concentration. Especially eNOS and nNOS activity is influenced by calmodulin. Both of them contain a Ca^{2+} depended 40-50 amino acid insert in the FMN binding domain, acting as autoinhibitory loop. [35] CaM binding increases the rate of electron transfer from NADPH to the reductase domain and the electron transfer from reductase domain to the heme center. [35]

Heat-shock protein 90 (Hsp-90)

The heat-shock protein 90 is a chaperone that is expressed in all eukaryotic cells. The main function of Hsp-90 is the proper folding of proteins. [34] The binding of Hsp-90 to eNOS leads to an increased eNOS activity via enhanced affinity of calmodulin binding. [33] Other stimuli that cause an increased eNOS-derived NO release, like vascular endothelial growth factor (VEGF), histamine, fluid shear stress or estrogen enhance the interaction between Hsp-90 and eNOS. [34]

Caveolin

Caveolins are major proteins of the caveolae. Caveolae are cholesterol- and sphingolipid-rich flask shaped invaginations of the plasma membrane, found in various cell types, especially in endothelial cells. [62] One function of caveolin-1 is to traffic, as cholesterol binding protein, cholesterol from the endoplasmatic reticulum through the golgi to the plasma membrane. It also influences the biogenesis of caveolae, although the mechanism is not known until now. [34] Additionally caveolin-1 negatively regulates eNOS activity. The binding motive in eNOS lies between the heme and calmodulin binding site. Caveolin-1 might interfere with the reduction of heme iron thereby disturbing NO production. [36] Furthermore caveolin has a binding site for NOSTRIN-protein (eNOS trafficking inducer), which regulates the localization

of eNOS. Overexpression of NOSTRIN leads to trafficking eNOS from the plasma membrane to cytosol, resulting in an additional decrease in NO production. [63]

Interestingly a connection between Hsp-90, calmodulin and caveolin has been suggested. Former studies showed that, *in vitro,* caveolin-1 and Hsp90 form a heterotrimeric complex with eNOS. Stimulating factors, like VEGF, increased Ca^{2+} levels or fluid shear stress, activate CaM and caveolin gets slightly displaced from the active CaM. [34] [37] [38] The presence of Hsp-90 facilitates CaM in displacing eNOS from caveolin-1. [37] The removal of the caveolin inhibitory clamp also leads to an enhanced Hsp-90 recruitment, continuing stabilization of the complex and supports binding of kinase Akt. The docking of Akt on eNOS forces the phosphorylation on Ser^{1177} (bovine Ser^{1179}) and maybe change in phosphorylation-state on Thr^{495} (bovine Thr^{497}). [38] (Figure 3)

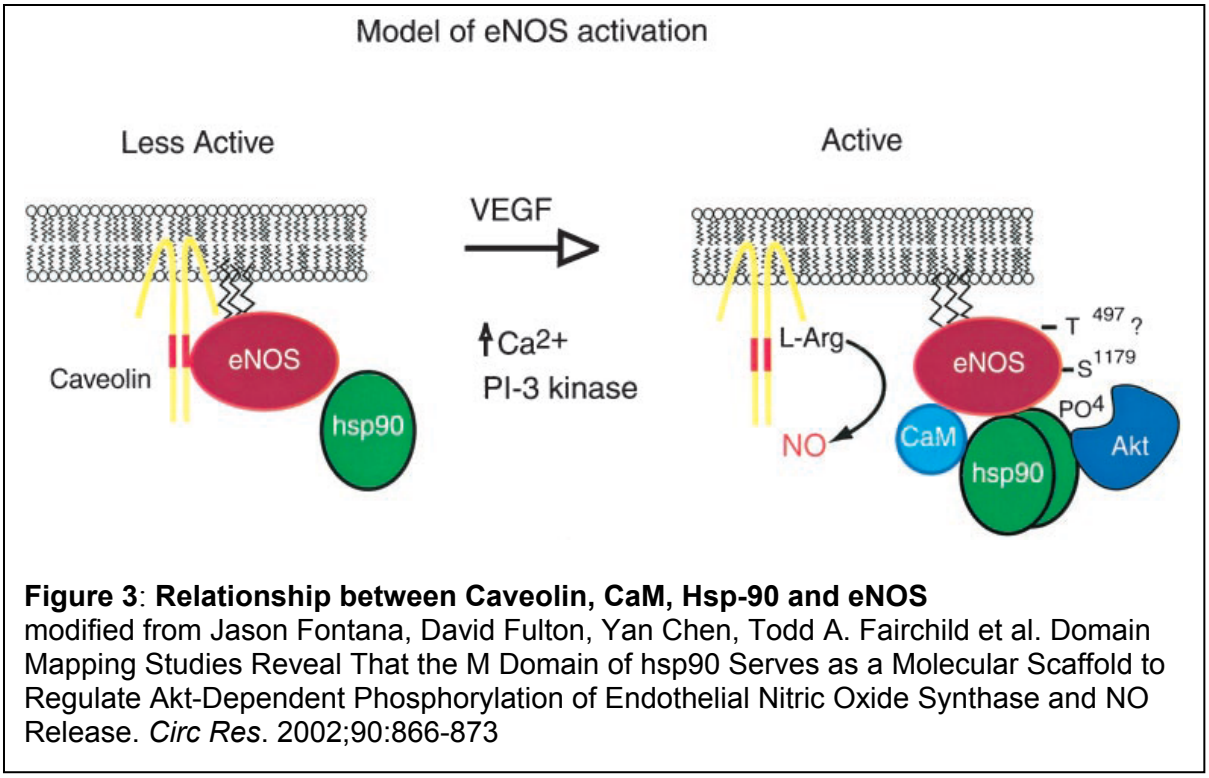


Figure 3: **Relationship between Caveolin, CaM, Hsp-90 and eNOS**
modified from Jason Fontana, David Fulton, Yan Chen, Todd A. Fairchild et al. Domain Mapping Studies Reveal That the M Domain of hsp90 Serves as a Molecular Scaffold to Regulate Akt-Dependent Phosphorylation of Endothelial Nitric Oxide Synthase and NO Release. *Circ Res*. 2002;90:866-873

3.2.4. Acetylation

Acetylation and deacetylation of eNOS at lysine residues are important mechanism of eNOS regulation. Former studies showed that deacetylation of eNOS, which is constitutively acetylated at Lys^{496} and Lys^{506} in the calmodulin binding domain, leads to an increase of eNOS enzyme activity. This deacetylation is performed by classIII NAD^+ dependent histone deacetylase SIRT1 (Sirtuin-1). [39] Saet-Byel Jung et al showed in their study an increase in eNOS activity after aspirin (acetylsalicylic acid) treatment via acetylation of the Lys^{609} residue.

This acetylation leads to an increased binding of CaM to eNOS thereby resulting in an increased eNOS activity. [40]

3.2.5. Glycosylation

A study from Du et al reported a lower basal level of NO production in endothelial cells, cultured under high glucose conditions. They showed that glucose leads to an increase in N-acetylglucosamine (GlcNAc) O-glycosylation and a decrease in eNOS phosphorylation on serine 1177 residues. These two mechanisms are correlated to each other and lead to a decrease in eNOS activity. [41][42]

4. Formation of ROS

Reactive oxygen species (ROS) are reactive oxygen-containing molecules, including free oxygen radicals, superoxide radicals, oxygen ions and peroxides, as well as non-radical species like hydrogenperoxide. ROS can be produced during many physiological processes like cell proliferation, inflammation, apoptosis, phagocytosis or intracellular signaling. Oxidative stress is characterized by an imbalance between reactive oxygen radicals and the ability of the body to neutralize them with antioxidative mechanism. Therefore it is perceived as a risk factor for cardiovascular diseases. [25] There are many enzyme systems involved in the production of ROS. NADPH (nicotinamide adenine dinucleotide phosphate) oxidases, xanthine oxidases, mitochondrial respiratory chain enzymes and in certain cases eNOS are the four most important systems. [15]

4.1. NADPH oxidases

For a long time the NADPH oxidase was considered to be only present in phagocytes, until homologs of the cytochrome subunit of the phagocyte NADPH oxidase were found. [54] NADPH oxidases are located in many tissues, including the vasculature. They are membrane associated enzymes that catalyze the reduction of oxygen using NADPH as electron donor. [53] In vascular cells Nox1 (NADPH oxidase 1) is overexpressed especially in the presence of increased angiotensin II levels. [15] Also thrombin, platelet derived growth factor (PDGF), tumor necrosis factor-α (TNF-α) and lactosylceramide activates NADPH oxidases. [53]

4.2. Xanthine oxidase

Xanthine oxidase is generated from xanthine dehydrogenase by proteolysis. Increased cholesterol levels have been reported to promote the release of xanthine oxidase from the liver. Xanthine oxidase donates electrons to molecular oxygen, thereby producing $O_2^{-\bullet}$ and hydrogen peroxide. [15] Vascular endothelial cells have glucosaminoglycan (GAG)-rich receptors which reversibly bind xanthine oxidase and concentrate the enzyme at the cell surface. This leads to an increased generation of ROS in the presence of its substrate xanthine or hypoxanthine. [17]

4.3. Mitochondrial respiratory chain enzymes

The mitochondrial electron transport chain consists of a series of electron carriers, for example flavoproteins, iron-sulfur proteins or cytochroms, which are organized into four complexes. Electrons are delivered by NADH or $FADH_2$ to the mitochondrial electron transport chain and during this transfer, some of the electrons leak out to molecular oxygen to form superoxide. [55] About 1% of the mitochondrially used oxygen is transformed into superoxide by single electron reductions. Reactive oxygen species can be produced by two distinct enzymes of the mitochondrial respiratory chain: NADH dehydrogenase (complex I) and ubiquinone-cytochrome b-c1 region (complex III). [15] Also the flavin mononucleotide (FMN) group, the first electron carrier of complex I, is primarily responsible for ROS generation. [55]

4.4. ROS generation by eNOS

In the healthy endothelium the two eNOS subunits are coupled and the substrate L-arginine is oxidized to NO and L-citrulline. Oxidative stress can lead to an uncoupling of the protein subunits resulting in the production of superoxide instead of NO.

Low intracellular BH_4 levels as a consequence of BH_4 oxidation lead to the uncoupling of eNOS. Intracellular BH_4 levels depend on the balance of its *de novo* synthesis, loss of BH_4 by oxidation to BH_3 or BH_2 and the BH_4 regeneration capacity of the cell. [15] [19]

BH_3 and BH_2 can be reduced back to BH_4 by L-ascorbic acid if sufficient reducing equivalents are available. [15] Also the ubiquitary enzyme DHFR (dihydrofolate reductase) is able to regenerate BH_4 from BH_2 as part of a recycling pathway. [19]

The superoxide generated by different enzyme systems is able to interact with nitric oxide produced by eNOS and forms peroxynitrite. $ONOO^-$ is in turn able to oxidize BH_4 (tetrahydrobiopterin) to BH_3 and BH_2. The ratio between BH_4 and BH_2 determines the uncoupling of eNOS subunits. The fewer BH_4 is available the more eNOS uncoupling occurs thereby resulting in increased ROS production (Figure 4) [18] [19]

Additionally the zinc tetrathiolate cluster is highly sensitive to peroxynitrite. The oxidation of this cluster also results in monomerization of eNOS. [10]

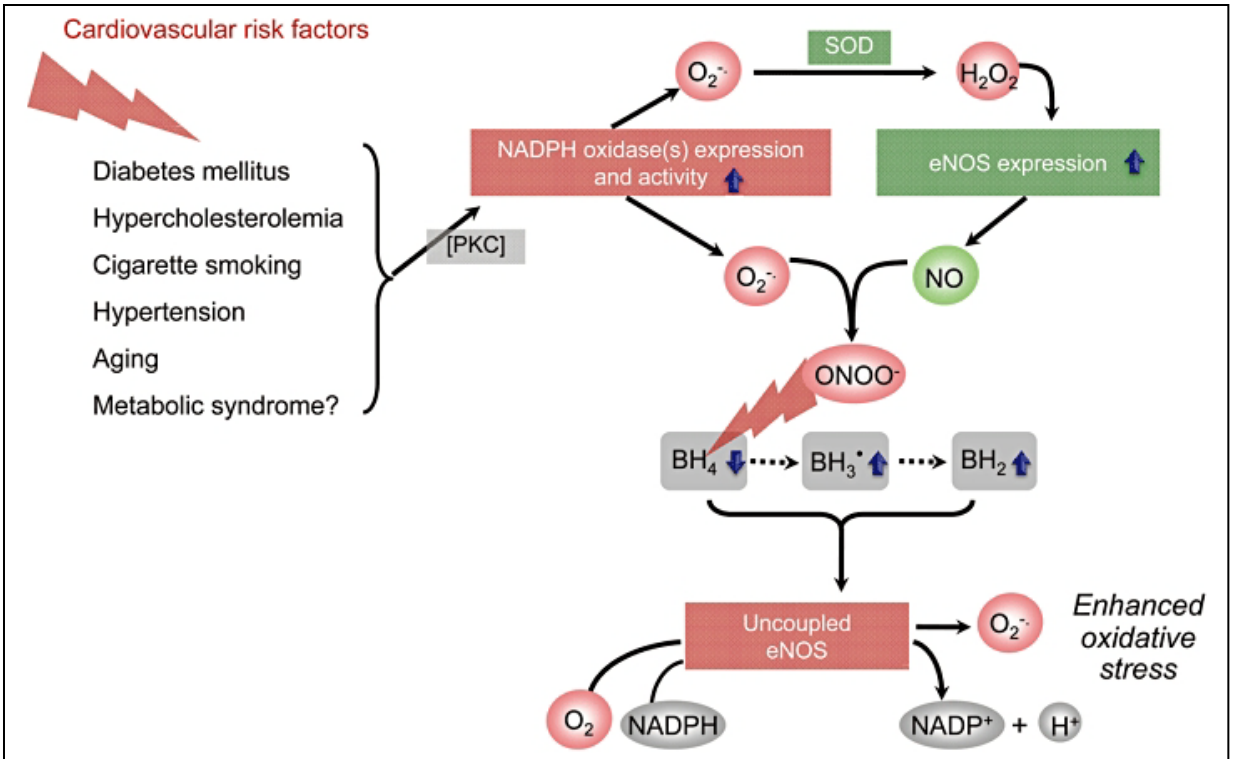


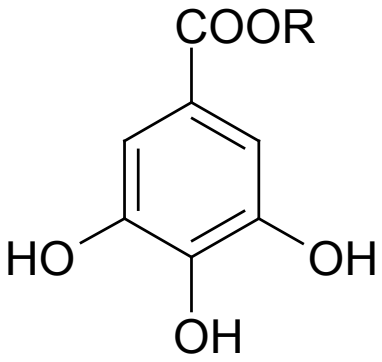
Figure 4: Oxidative stress depended uncoupling of eNOS
Modified from Ulrich Förstermann, Huige Li. Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling. Br J Pharmacol. 2011 Sep; 164(2): 213–223

5. Gallic acid and its derivatives

Gallic acid and its derivatives belong to a large family of plant secondary polyphenolic metabolites. [26] Gallic acid and ethyl gallate for example were isolated from grapes and wine. [59] [60] Additionally gallic acid was found in the green alga *Spirogyra sp.* and ethyl gallate in Galla Rhois, a gallnut present on *Rhus javanica L.* [58] [61]
The esters of gallic acid only differ in the number of carbon atoms in the aliphatic chain. (Figure 5) The length of the chain determines lipophilicity and chemical properties. [27] It is well established that gallic acid derivatives are able to scavenge free radicals. These derivatives, specifically methyl, propyl, octyl and dodecyl gallates are used as antioxidants in food, cosmetic and pharmaceutical industry. [27] [26]

A balanced diet is supposed to be a suitable prevention for cardiovascular diseases. [31] In this context the role of red wine consumption was investigated in previous studies. Red wine consumption might give an explanation for the lower incidence of coronary diseases despite high saturated fat intake in France (so called French paradox). [31][32] Several studies observed a beneficial effect of polyphenols from red wine, including gallic acid derivatives, in the prevention of cardiovascular diseases. [28] In addition to the antioxidative effect it could be shown that red wine polyphenols have functions like inhibition of platelet aggregation, vasorelaxing activity, modulation of lipid metabolism and inhibition of low-density lipoprotein oxidation. [28] Gallic acid derivatives showed in *in vitro* and *in vivo* assays antitumoral activity against several types of tumor cell lines by inducing apoptosis via different mechanisms depending on the cell type. [27]
A recent study linked beneficial neuroprotective effects of gallic acid derivatives to a decrease in intracellular ROS levels in neuronal cells. [26]
In the work of Oliver Donath [29] an increase of eNOS activity by gallic acid and seven of its derivatives was shown. Especially ethyl-, propyl-, butyl- and isopentyl-gallate treatment of endothelial cells led to an increase in eNOS activity. [29] (Figure 6) Therefore we were interested if gallic acid derivatives also lead to a decrease in intracellular ROS levels in endothelial cells.

Figure 5: Gallic acid and its derivatives
Modified from Claudriana Locatelli, Fabiola Branco Filippin-Monteiro, Tania Beatriz Creczynski-Pasa et al. Alkyl esters of gallic acid as anticancer agents: A review. European journal of medicinal chemistry 60, 233–9.



Gallates	-R
Gallic acid	-H
Methly gallate	$-CH_3$
Ethyl gallate	$-(CH_2)CH_3$
Propyl gallate	$-(CH_2)_2CH_3$
Butyl gallate	$-(CH_2)_3CH_3$
Pentyl gallate	$-(CH_2)_4CH_3$
Hexyl gallate	$-(CH_2)_5CH_3$
Heptyl gallate	$-(CH_2)_6CH_3$
Octyl gallate	$-(CH_2)_7CH_3$

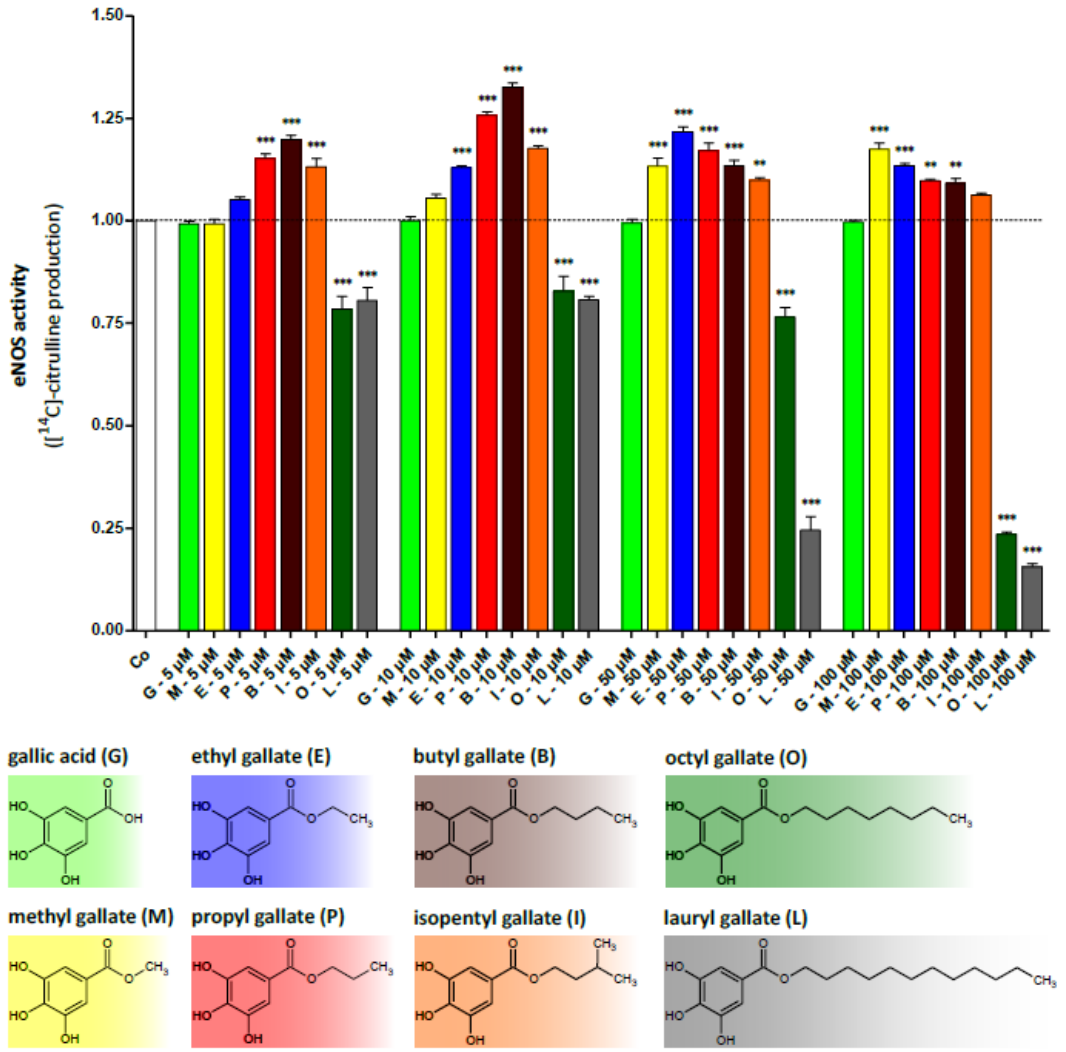


Figure 6: Effect of gallic acid alkyl esters on eNOS enzyme activity
Modified from: Mag. Pharm. Oliver Donath, Activity profiling of Austrian red wine targeting cardiovascular diseases: A contribution to understand the “French Paradox”, Doctoral Thesis, University of Vienna, 2015

6. Aim of the work

Oxidative stress and high ROS levels are known to be a risk factor for the generation of cardiovascular diseases. [64] On the other hand reactive oxygen species also plays an important role in cardiovascular physiology. ROS acts as a signaling molecule, which influences the correct function of many intracellular proteins and enzymes, like EGF-R (epidermal growth factor receptor), PDGF-R (platelet derived growth factor receptor), Ras (Rat sarcoma G-protein), Akt (Protein kinase B) or MAP-kinases (mitogen activated protein-kinases) [65] Physiological ROS levels are important for a healthy organism and imbalanced high ROS concentrations and oxidative stress are critical factors for generation of cardiovascular diseases. Therefore it is highly relevant to find strategies and prospects to reduce oxidative stress in disease related circumstances.

The aim of this work was to determine the effect of gallic acid and its derivatives on intracellular ROS levels in the endothelial cell line EA.hy926. During the dissertation of Oliver Donath gallic acid derivatives were identified to increase eNOS enzyme activity. Therefore we investigated if there is a connection between eNOS enzyme activity and intracellular ROS levels after treatment with gallic acid derivatives in our cell system. This seems to be especially interesting as gallic acid and its derivatives are known radical scavengers and an increased ROS levels can lead to uncoupling of eNOS. [15]

In previous investigations (by Dieu Linh Nguyen) the influence of gallic acid derivatives on many eNOS phosphorylation sites, with exception of Ser^{114}, was analyzed. That's the reason why the influence of gallic acid derivatives on this phosphorylation site was evaluated as part of this thesis.

Part II

Materials and Methods

1. Cell culture

In this study EA.hy926 cells were used. This cell line was created in 1983 from primary human umbilical vein endothelial cells [1], which were fused with A549/8 cells to immortalize them. A549/8 is a human lung cancer line which was established in 1972 out of a sample from a 58 year old Caucasian man. [2]
In order to select for hybridized cells HAT medium (hypoxanthine-aminopterin-thymidine) was used in the cell culture routine. The combination of hypoxanthine and thymidine, which are intermediates in DNA synthesis, and aminopterin, a folate metabolism inhibitor, provides selection for hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and thymidine kinase (TK) containing cells. [51]
This hybridized EA.hy926 cells have similar properties than human endothelial cells making them a useful tool as endothelial cell culture model. [3]

1.1. Cultivation

EA.hy926 cells were cultivated in DMEM medium supplemented with 4.5 g/l glucose, 2 mM glutamine, 10% HAT (100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine) and 10% fetal bovine serum (FBS). FBS was heat inactivated for 30 minutes at 56°C in a water bath. Additionally, 100 U/ml penicillin and 100 µg/ml streptomycin were added to avoid bacterial contamination.
For the cultivation 175 cm² flasks were used and placed in an incubator with 37°C and 5% CO_2.

1.2. Passaging

To keep the cells growing they need to be passaged every seven days. First all solutions have to be pre-warmed in a 37°C water bath. Then the medium was removed from the flask and the cells were washed with PBS (123 mM NaCl, 10 mM Na_2HPO_4, 3.2 mM KH_2PO_4, pH 7.4).
Afterwards 6 ml trypsin/EDTA solution was added. The flask was placed for approximately 3 minutes in the incubator. To inactivate the trypsin solution 14 ml medium were added. The cell suspension was transferred in a falcon tube and the cells were counted via a cell viability analyzer (ViCell®). Then the cells were seeded in a concentration of 1.6 $x10^6$ cells in a new

flask. After approximately three days the medium was changed and after a week the cells were passaged again.

1.3. Solutions and buffers

PBS pH: 7.4	NaCl Na_2HPO_4 KH_2PO_4 ddH_2O	123 mM 10 mM 3.2 mM ad 1000 ml
Complete growth medium	DMEM – Glucose L-Glutamin HAT: • Hypoxanthine • Aminopterin • Thymidine FBS Streptomycin Penicillin	4.5 g/l 2 mM 100 µM 0.4 µM 16 µM 10% 100 µg/ml 100 U/ml
Trypsin/EDTA In PBS	Trypsin EDTA	0.05 % 0.02 %

2. Intracellular ROS detection - DCF-Assay

2.1. Principle

The principle of this assay relies on the use of a flow cytometer, which allows the measurement of a fluroescent marker for each cell individually. Therefore fluorescently labeled cells need to be vortexted briefly to produce a single cell suspension, which is then drawn through a microchannel. The small diameter of this channel allows only one cell at a time to pass through. Along the way every cell is touched by a laser and emits light in characteristic wavelengths. This allows the gathering of information about physical properties, like size, shape and internal complexity of measured cells.

In this thesis cells were labeled with the fluorescent dye 2',7'-dichlorodihydrofluorescein-diacetate (H_2DCF-DA). This dye is able to penetrate the cell membrane. Inside the cell esterases then cleave the diacetate moiety and thereby prevent the dye to migrate out of the cell. Oxidation of H_2DCF by intracellular reactive oxygen species (ROS) results in the formation of the highly fluorescent DCF.(Figure 7)

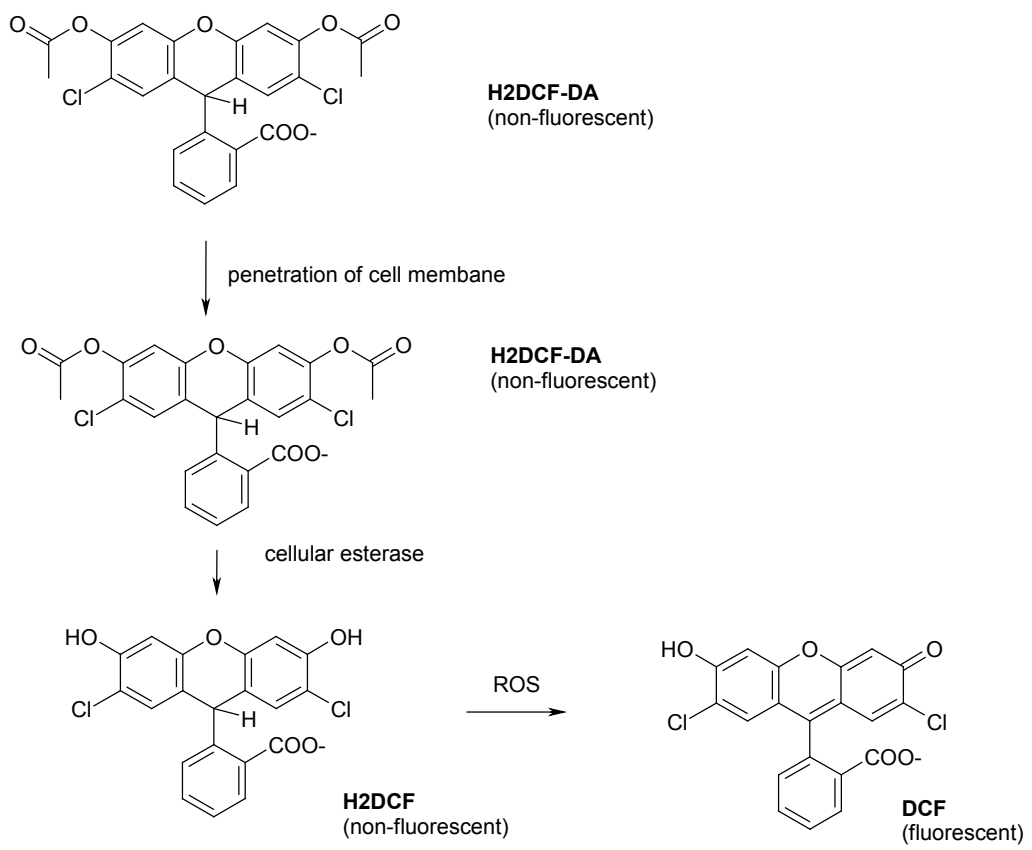


Figure 7: H_2DCF-DA-Assay principle

Modified from:
http://link.springer.com/protocol/10.1385%2F1-59259-424-7%3A169#page-2 (30.03.2016)
http://www.cellbiolabs.com/reactive-oxygen-species-ros-assay (13.11.2015)

2.2. Experimental Procedure

EA.hy926 cells were seeded in 12-well plates at a concentration of 1.6 x10^5 cells/well and incubated at 37°C and 5% CO_2 until the cells were confluent.

Cells were then treated with indicated compounds. First the medium in all wells, except of those for NDGA and its negative control, was changed to medium supplemented with 50 U catalase/ml. This step was performed to remove hydrogen peroxide produced in the cell medium that would falsify the outcome of the assay. Then the test compounds and positive controls were added and incubated for 24 hours.

For treatment with NDGA cells were washed with HBSS and incubated for 15 minutes with 20 µM NDGA or just the solvent DMSO as negative control.

After the incubation time was over cells were labeled with H_2DCF-DA. Regarding the light sensitive nature of the dye all steps were performed while avoiding daylight. Each well was

filled with 2 ml 20 µM H_2DCF-DA/HBSS solution and was incubated for 25 minutes, except the control wells, which were filled exclusively with HBSS. Then the cells were washed with HBSS and 300 µl trypsin/EDTA was added for 3 minutes. Afterwards trypsin was inactivated with 1000 µl PBS/2%BSA and the cells were transferred into FACS tubes. The obtained data was evaluated with geometric mean.

2.3. Solutions and buffers

Complete growth medium	DMEM – Glucose	4.5 g/l
	L-Glutamin	2 mM
	HAT:	
	• Hypoxanthine	100 µM
	• Aminopterin	0.4 µM
	• Thymidine	16 µM
	FBS	10%
	Streptomycin	100 µg/ml
	Penicillin	100 U/ml
Medium/catalase	Compete growth medium	
	Catalase	50 U/ml
HBSS	NaCl	140 mM
	KCl	5 mM
	Na_2HPO_4	0.14 mM
	KH_2PO_4	0.37 mM
	$CaCl_2 * 2H_2O$	1,2 mM
	$MgSO_4$	0,8 mM
	D-Glucose	5.5 mM
	HEPES	20 mM
	ddH_2O	ad 1000 ml
Trypsin/EDTA In PBS	Trypsin	0.05 %
	EDTA	0.02 %
PBS/BSA 2%	BSA	10 g
	PBS	500 ml

3. Protein detection by Western Blot

3.1. Principle

Western blotting is a multi-step method for the detection of proteins. First whole cell lysates have to be produced and proteins solubilized. For this purpose a lysis buffer containing strong detergents, like CHAPS is used. Sonification can be used additionally to destroy cell membranes. Afterwards the samples are boiled and treated with ß-mercaptoethanol to denature proteins and reduce disulfide bonds. The addition of the detergent SDS (sodium dodecylsulfate) to the lysate applies a negative charge to the protein in a uniform mass-charge ratio. Now it is possible to separate the proteins of the lysate according to their molecular weight when applied on a polyacrylamide gel. After the separation via discontinuous gel electrophoresis the proteins are transferred to a PVDF-membrane (Polyvinylidene difluoride). To avoid unspecific binding of the membrane with the antibody, the membrane was blocked with fat free 5% milk/TBS-T. Then the membrane is treated with the primary antibody, which binds directly and specifically at the protein of interest. Afterwards a secondary antibody is added to visualize the first one. On the one hand these secondary antibodies are able to bind the primary antibody and on the other hand, they are linked to the enzyme horseradish peroxidase, which oxidizes luminol, to produce a luminescence signal. The intensity of this signal gives a relation about the amount of protein, present in the cell lysate.

3.2. Experimental Procedure

3.2.1. Cell lysates

First EAhy926 cells were seeded in 6-well plates in a concentration of 0.1 x 10^6 cells/wells. After confluence was reached, the medium was changed to 2 ml fresh growth medium and the test compounds were added and incubated as indicated.
Now all steps were performed on ice. First cells were washed two times with cold PBS. Then 100 µl freshly prepared lysis buffer was added and the cells were lifted from the bottom by gently scratching with a cell scraper. These lysates were transferred to Eppendorf tubes and after brief vortexing they were incubated for 15 min on ice. Then each tube was sonicated for 10 seconds with the highest intensity. Subsequently the lysates were centrifuged with 13000

rpm at 3°C for 40 minutes. 5 µl of the thereby obtained supernatants were mixed with 95 µl H_2O and stored for protein quantification.
Additionally 70 µl of the supernatants were mixed with 35 µl Laemmli buffer. This mixture was boiled at 95°C for 10 minutes on a dry block heater.

3.2.2. SDS-PAGE

To separate the proteins in the lysates SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used. The used gels consisted of a 7.5 % separation gel and a 5% stacking gel. The gels were installed in the electrophoresis chamber and the slots were loaded with 30 µg protein. For the separation a setting of 20 mA per gel for approximately 90 minutes was used.

3.2.3. Western blotting

After electrophoresis, the proteins were transferred to a PVDF membrane. For this a tank blot system was used and the transfer was performed at 100 V for 90 min. Then the membranes were blocked for 90 minutes with 5% fat free milk/TBS-T solution, followed by washing three times for 8 minutes with TBS-T.

3.2.4. Protein detection

For protein detection the membrane was incubated with primary antibody solution over night at 4°C. The next day the membrane was washed three times for eight minutes with TBS-T and incubated with the secondary antibody solution for 90 minutes at room temperature. After washing with TBS-T for an additional three times, fresh made ECL-solution was added for one minute. Blots were detected with a LAS 3000 luminescent image analyzer.
Actin levels were used as loading control. For this purpose the membrane was stripped for 15 minutes with 0.5 N NaOH and treated the same way with primary actin antibodies and horseradish peroxidase conjugated secondary antibodies.

3.2.5. Protein quantification

The Bradford assay was used to quantify protein levels in the cell lysates. The previously removed 5 µl supernatant, which was mixed with water, was shortly vortexed and 10 µl of this solution were put in triplicates in a 96-well plate. Also BSA standard dilutions (0 – 25

µg/ml final concentration) were transferred into the plate and then 190 µl Bradford reagent was added. After an incubation of five minutes the samples were measured with the microplate reader Sunrise Tecan at 595 nm.

3.3. Solutions and buffers

PBS pH 7.4	NaCl Na_2HPO_4 KH_2PO_4 ddH_2O	123 mM 10 mM 3.2 mM ad 1000 ml
Lysis buffer	Lysis buffer stock Phosphatase inhibitor Complete stock	637 µl 35 µl 28 µl
Lysis buffer stock	Tris-HCl CHAPS EDTA EGTA DTT Gluthatione Glycerol ddH_2O	50 mM 20 mM 0.5 mM 0.5 mM 2 mM 7 mM 10% ad 25 ml
Phosphatase inhibitor	NaF Na_3VO_4 $Na_4P_2O_7$ * 10 H_2O ddH_2O	0.2 mM 40 mM 0.1 mM ad 5 ml
Laemmli solution	ß-Mercaptoethanol 3xSB-stock	45 µl 255 µl
3xSB-stock (Westernblot sample buffer)	Tris-HCl pH 6.8 SDS Glycerol Bromphenol blue ddH_2O	187.5 mM 0.2 mM 30 % 0.2 mM ad 1000 ml
Stacking gel	PAA 30% 1.5M Tris-HCl pH: 6.8 SDS 10% TEMED	5 % 125 mM 0.1 % 0.2 %

	APS 10%	0.1 %
	ddH_2O	ad 3.75 ml
Separation gel	PAA 30%	7.5 %
	1.25M Tris-HCl pH: 8.8	375 mM
	SDS 10%	0.1 %
	TEMED	0.1 %
	APS 10%	0.05 %
	ddH_2O	ad 7.5 ml
SDS-Running buffer (1x)	SDS-Running buffer (10x)	100 ml
	ddH_2O	ad 1000 ml
SDS-Running buffer (10x)	Tris-base	248 mM
	Glycine	1.9 M
	SDS	35 mM
	ddH_2O	ad 1000 ml
Blotting buffer (1x)	Blotting buffer (5x)	200 ml
	Methanol	200 ml
	ddH_2O	ad 1000 ml
Blotting buffer (5x)	Tris-base	125 mM
	Glycine	971 mM
	ddH_2O	ad 1000 ml
TBS-T pH: 8.0	Tris-base	248 mM
	NaCl	1.9 M
	Tween 20	1 %
	ddH_2O	ad 1000 ml
ECL-solution	Tris-base pH: 8.5	100 mM
	luminol	1.24 mM
	p-coumaric acid	0.2 mM
	H_2O_2	0.018 %
	ddH_2O	ad 10 ml

3.3.1. Antibody solutions

Type	Target	Dilution	Species	Provider
Primary Antibody	eNOS	1:2500 in 1xTBS-T	Mouse	BD
Primary Antibody	eNOS-P Ser^{114}	1:1000 in 5% BSA/TBS-T	Rabbit	Cell Signaling
Primary Antibody	Actin	1:1000 in 1x TBS-T	Mouse	Millipore
Secondary Antibody	Anti-mouse-HRP	1:3000 in 5 % milk/TBS-T		Cell Signaling
Secondary Antibody	Anti-rabbit-HRP	1:1000 in 5 % milk/TBS-T		Cell Signaling

4. DPPH-Assay

4.1. Principle

The DPPH-Assay is a method to determine the antioxidative capacity of chemical compounds. For this purpose DPPH (2,2-diphenyl-1-picrylhydrazyl) is used as a substrate, which is a stable, free radical, with a purple absorption maximum at 520 nm. If it gets in contact with an antioxidant, it receives hydrogen and turns to a non-radical stable form [4], with the colour yellow. (Figure 8) The stronger this decrease of colour intensity, the higher is the antioxidative activity of the sample solution.

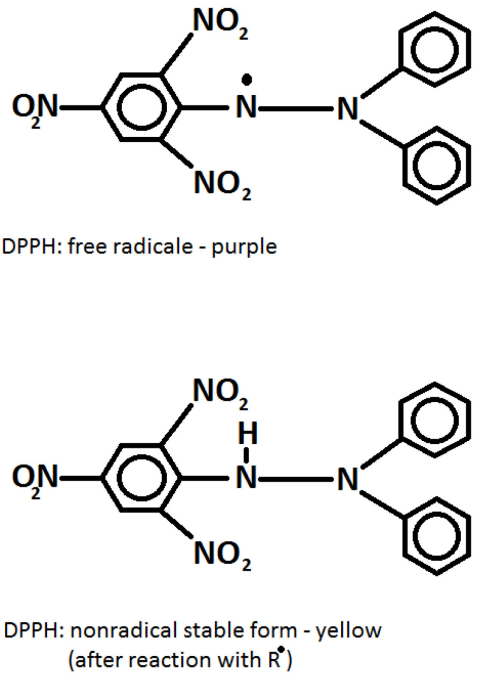


Figure 8: DPPH-Assay principle
Sagar B. Kedare and R. P. Singh. Genesis and development of DPPH method of antioxidant assay. J Food Sci Technol. 2011 Aug; 48(4): 412–422.

4.2. Experimental Procedure

First a dilution series of the test compound was prepared from a 100 mM stock solution (solved in DMSO). For comparison a reference solution of 100 µM ascorbic acid and a negative control (DMSO/EtOH) were used.

Then a 500 µM DPPH (2,2-diphenyl-1-picrylhydrazyl) solution in 80% EtOH was freshly prepared and mixed 1:1 with the samples in quadruplicates. Afterwards the plate was immediately measured with the microplate reader Sunrise Tecan at 550 nm. Thirteen cycles were measured in five minute intervals for one hour. Data after 30 min was used for further analysis.

5. Statistical analysis

For statistical analysis GraphPad Prism software was used. One-way ANOVA or two-way ANOVA were used to compare different treatment groups. For statistical evaluation also t-test was used. P values < 0.05 were considered significant. All graphs are showing means ± SD.

Part III
Results

1. Antioxidative capacities of gallic acid derivatives as determined by the DPPH- Assay

To investigate the direct radical scavenging properties of gallic acid derivatives seven substances were analysed: gallic acid, methyl gallate, ethyl gallate, propyl gallate, butyl gallate, isopentyl gallate and octyl gallate. (Figure 5) First a dilution series of the respective test compounds was prepared from a 100 mM stock solution (dissolved in DMSO). The following concentrations were prepared in 80% ethanol: - 100 µM, 50 µM, 30 µM, 10 µM, 5 µM, 3 µM, 1 µM and 0.5 µM.

Each gallic acid derivative was able to scavenge radicals in a more or less pronounced fashion. The biggest difference in data curves is between methyl gallate and octyl gallate (Figure 9).

When looking at Figure 10 the IC_{50} values for intracellular ROS scavenging did not correlate with the length of the aliphatic chain. Especially the IC_{50} values obtained from ethyl-, propyl-, butyl- and isopentyl gallate are very close together. (Figure 10)

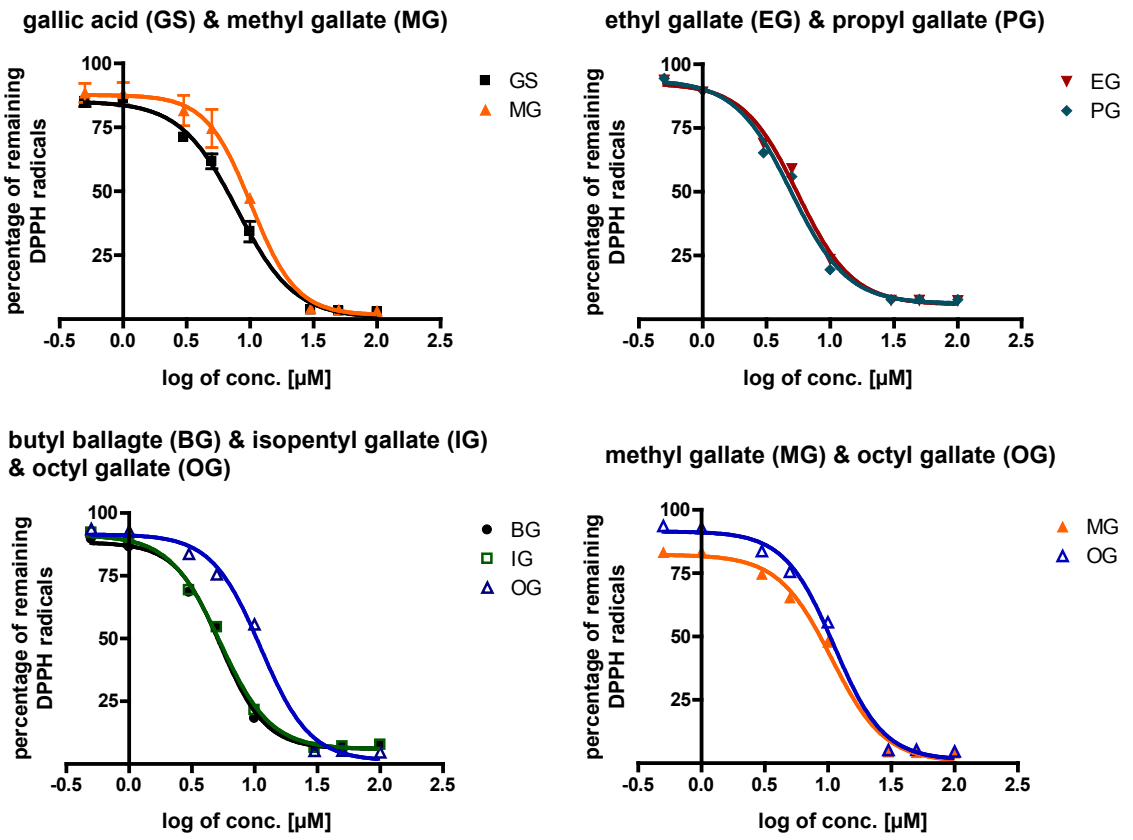


Figure 9: Antioxidant capacity of gallic acid derivatives
Solutions of 100 µM, 50 µM, 30 µM, 10 µM, 5 µM, 3 µM, 1 µM and 0.5 µM of GS (gallic acid), MG (methyl gallate), EG (ethyl gallate), PG (propyl gallate), BG (butyl gallate), IG (isopentyl gallate) and OG (octyl gallate) were subjected to a DPPH Assay as described in the material and methods section. 100 µM ascorbic acid was used as reference compound. The determined data were shown as nonlinear regression (curve fit) of logarithmic transformed data.
(n=3, Mean ± SD, not significant)

Figure 10: Radical scavenging IC_{50} values of gallic acid derivatives
Data out of Figure 9

Gallic acid derivate	IC_{50} [µM]
Gallic acid	7.913
Methyl gallate	10.23
Ethyl gallate	5.581
Propyl gallate	4.945
Butyl gallate	5.358
Isopentyl gallate	5.309
Octyl gallate	11.17

2. Effect of gallic acid derivatives on intracellular ROS levels

2.1. Determination of suitable controls

In previous studies the ability of PMA (phorbol 12-myristate 13-acetate) for increasing and of NDGA (nordihydroguaiaretic acid) for decreasing intracellular ROS levels were shown. [5] [6] Treatment with PMA leads to the repression of catalase expression and therefore to an increase in intracellular ROS levels. [5]

NDGA is a selective inhibitor of 12-lipoxygenase which produces ROS during arachidonic acid metabolism. [6] To determine the suitability of these compounds as positive controls EA.hy926 cells were treated with 2 nM PMA for 24 hours and 20 µM NDGA for 15 minutes. DMSO was used as solvent control. The obtained data confirmed both of them as suitable controls in EA.hy926 cells (Figure 11). In every following experiment PMA and NDGA were used as controls also if not depicted in the graphs.

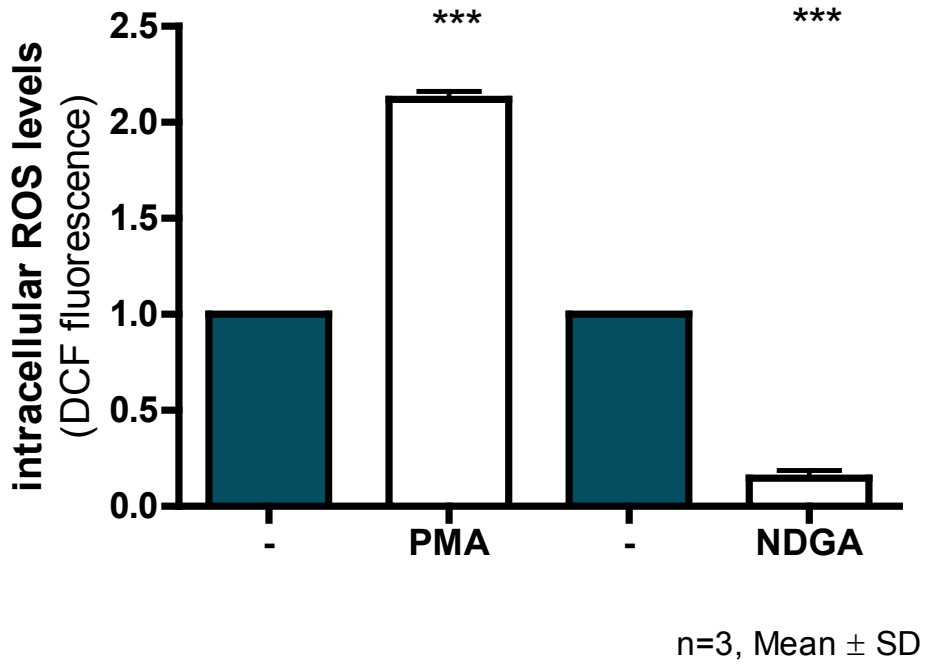


Figure 11: H_2DCF-DA-Assay controls
EA.hy926 cells were treated with 2 nM PMA for 24 hours, 20 µM NDGA for 15 minutes and 2 µl DMSO for 24 hours as solvent control
(n=3, Mean ± SD, *** p<0.001)

2.2. Effect of butyl gallate and ethyl gallate on intracellular ROS levels in EA.hy926 cells

Out of all other gallic acid derivatives used in this study, which were gallic acid, methyl gallate, ethyl gallate, propyl gallate, butyl gallate, isopentyl gallate and octyl gallate, especially butyl gallate and ethyl gallate were chosen for investigation. The effect of butyl gallate was interesting, because previously Oliver Donath could show in his dissertation that butyl gallate in concentrations of 5 µM and 10 µM leads to the highest increase in eNOS activity when compared to all other investigated gallic acid derivatives. (Figure 6) Interestingly the natural compound ethyl gallate, isolated from red wine, was also able to increase eNOS activity. [29]

Because eNOS activity and intracellular ROS levels influence each other (mentioned in part I 4.4) it is interesting to investigate the influence of butyl gallate and ethyl gallate on intracellular ROS levels. To determine a possible effect of these two gallic acid derivatives on intracellular ROS levels EA.hy926 cells were treated with two different concentrations of these compounds. The obtained results showed that butyl gallate at 5 µM and ethyl gallate at 10 µM were able to significantly decrease intracellular ROS levels, however the reduction did not exceed 15 %. (Figure 12)

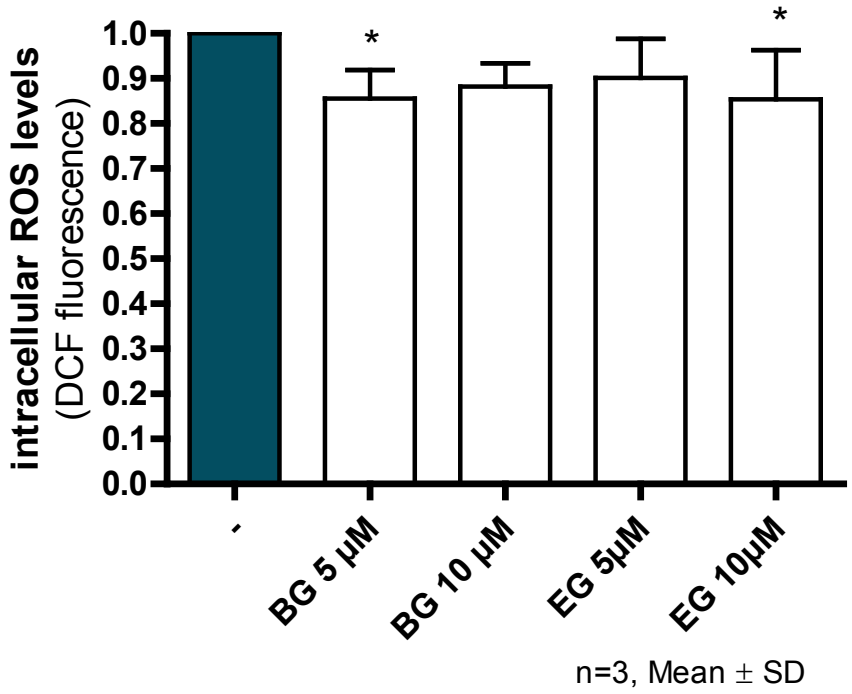


Figure 12: Effect of butyl gallate and ethyl gallate on intracellular ROS levels determined by H_2DCF-DA-Assay
EA.hy926 cells were treated with 5 µM, 10 µM BG (butyl gallate) and 5 µM, 10 µM EG (ethyl gallate) for 24 hours and data were normalized to the control cells treated with DMSO
(n=3, Mean ± SD, *p<0,05)

2.3. Dose response of butyl gallate

As butyl gallate was able to significantly decrease intracellular ROS levels (Figure 12) we now investigated the concentration dependence of this effect. For this purpose a dilution series with six different concentrations of butyl gallate was prepared. (Figure 13)
There was a significant decrease of intracellular ROS levels at 5 µM, 10 µM, 30 µM and 50 µM, although the effect was a little smaller at 10 µM. The biggest response was at 50 µM with a reduction of 29.5% in contrast to the other significant concentrations with an average reduction of about 13%.

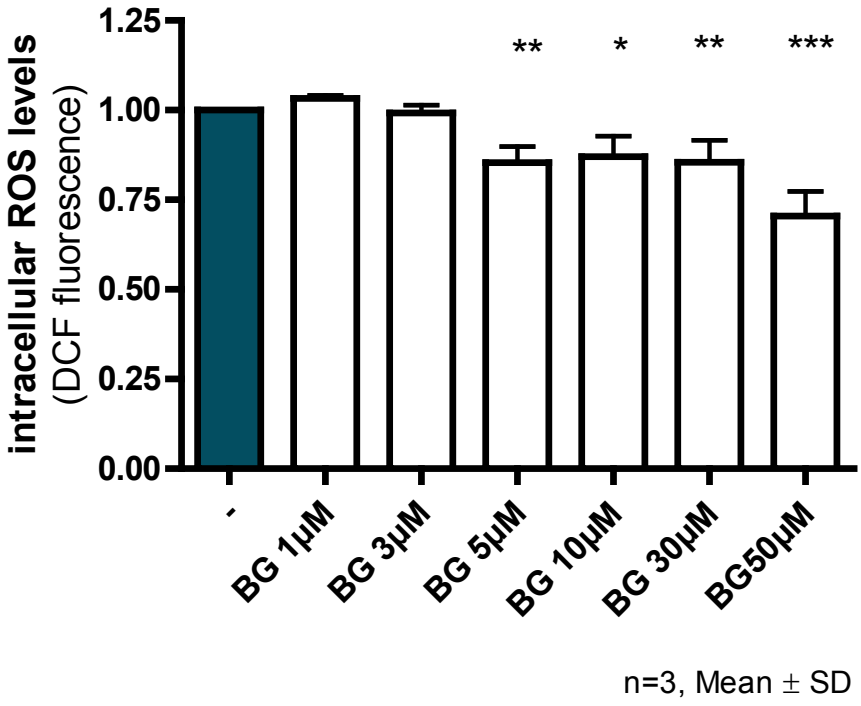


Figure 13: Dose response of butyl gallate
EA.hy926 cells were treated with 1 µM, 3 µM, 5 µM, 10 µM, 30 µM and 50 µM BG (butyl gallate) for 24 hours. The data were determined by H_2DCF-DA-Assay and normalized to the control cells treated with DMSO
(n=3, Mean ± SD, *<0.05, **<0.01, ***<0.001)

2.4. Influence of gallic acid derivatives on intracellular ROS levels

Now we were interested to what extent other gallic acid derivatives, besides butyl and ethyl gallate, have an influence on intracellular ROS reduction. Seven different gallic acid derivatives (gallic acid, methyl gallate, ethyl gallate, propyl gallate, butyl gallate, isopentyl gallate, octyl gallate) were investigated for their effect on intracellular ROS levels in EA.hy926 cells. EA.hy926 cells were treated with 10 µM of each test compound for 24 hours. There was a significant decrease of intracellular ROS levels with propyl gallate, butyl gallate, isopropyl gallate and octyl gallate. Interestingly although no clear difference of radical scavenging potential between the derivatives could be seen, (Figure 9, Figure 10) the intracellular ROS levels seem to be more effectively downregulated the longer the hydrocarbon chain is. (Figure 14)

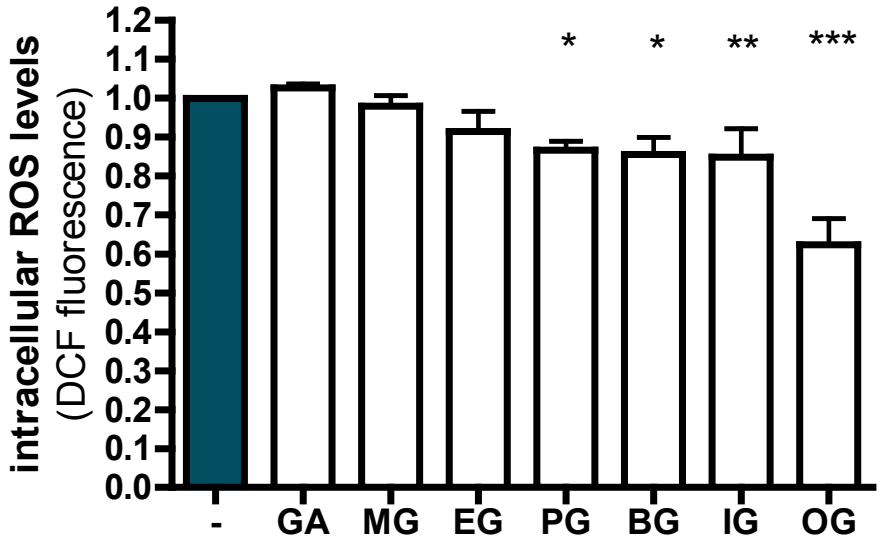


Figure 14: Effects of gallic acid derivatives on intracellular ROS levels determined by H_2DCF-DA-Assay
EA.hy926 cells were treated with 10 µM GA (gallic acid), MG (methyl gallate), EG (ethyl gallate), PG (propyl gallate), BG (butyl gallate), IG (isopropyl gallate) and OG (octyl gallate) for 24 hours.
(n=3, Mean ± SD, *<0.05, **<0.01, ***<0.001)

2.5. Influence of the NOS inhibitor L-NAME on intracellular ROS levels

L-NAME (N-nitro-L-arginine methyl ester) is a well-established inhibitor of nitric oxide synthases. It is not specific and inhibits all three isoforms of NOS. [48] However, in endothelial cells mainly eNOS is expressed, so L-NAME could be used to block eNOS in EA-hy926 cells.

EA.hy926 cells were treated with 5 µM butyl gallate for 24 hours and with 200 µM L-NAME for one hour. Additionally cells were treated only with 5 µM butyl gallate for 24 hours as well as 200 µM L-NAME for one hour.

There was a significant decrease in intracellular ROS levels with butyl gallate and the co-treatment of butyl gallate and L-NAME. The biggest effect was in cells, just treated with butyl

gallate. The sole treatment with L-NAME showed no significant change in intracellular ROS levels versus control cells treated with DMSO. (Figure 15)

Based on this data it seems that the decrease in intracellular ROS levels by butyl gallate is not dependent on eNOS activity in unstressed endothelial cells.

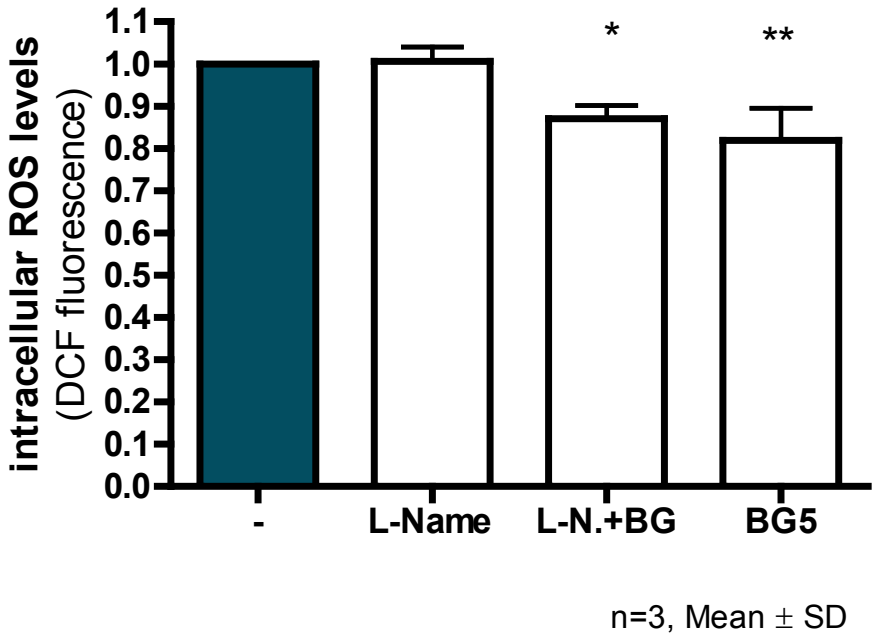


Figure 15: Effect of L-NAME on intracellular ROS levels
EA.hy926 cells were treated with 5 µM BG (butyl gallate) for 24 hours and with 200 µM L-NAME (L-N.) for one hour. The data were determined by H_2DCF-DA-Assay and normalized to the control cells treated with DMSO
(n=3, Mean ± SD, *<0.05, **<0.01)

3. Effect of butyl gallate and ethyl gallate on the eNOS phosphorylation site Ser^{114} in EA.hy926 cells

3.1. Evaluation of troglitazone as control for eNOS-Ser^{114} phosphorylation

Troglitazone was reported to increase NO production amongst others via dephosphorylation of Ser^{114}. Proteinkinase C (PKC) is supposed to play an important role in troglitazone-induced eNOS-Ser^{114} dephosphorylation. [49] To determine the suitability of this compound as positive control, EA.hy926 cells were treated with 5 µM, 10 µM and 20 µM troglitazone for 24 hours and protein levels were determined by Western blot.

There was no significant effect on of eNOS-Ser^{114} phosphorylation detectable. (Figure 16)

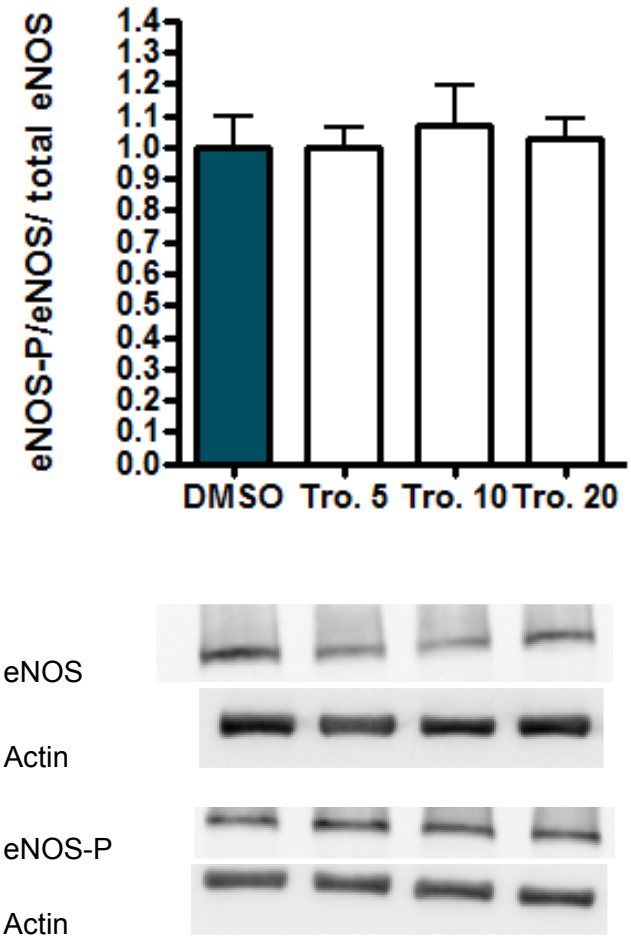


Figure 16: Evaluation of troglitazone as control for eNOS-Ser^{114} phosphorylation
EA.hy926 cells were treated with 5 µM, 10 µM and 20 µM Tro. (troglitazone) for 24 hours and eNOS and eNOS-PSer^{114} protein levels were determined by Western blot. The obtained band intensities were normalized to DMSO and compared with actin
(n=3, Mean ± SD)

3.2. Evaluation of VEGF as control for eNOS-Ser^{114} phosphorylation

Because troglitazone turned out to be an inappropriate control treatment another compound was tested. Also known to promote dephosphorylation of eNOS- Ser^{114} is vascular endothelial growth factor (VEGF). [50] EA.hy926 cells were treated with 20 ng/ml and 40 ng/ml VEGF for different intervals (5, 15 and 30 min). Against our expectation 20ng/ml VEGF did increase the phosphorylation of eNOS at Ser114 after 30 min. (Figure 17)

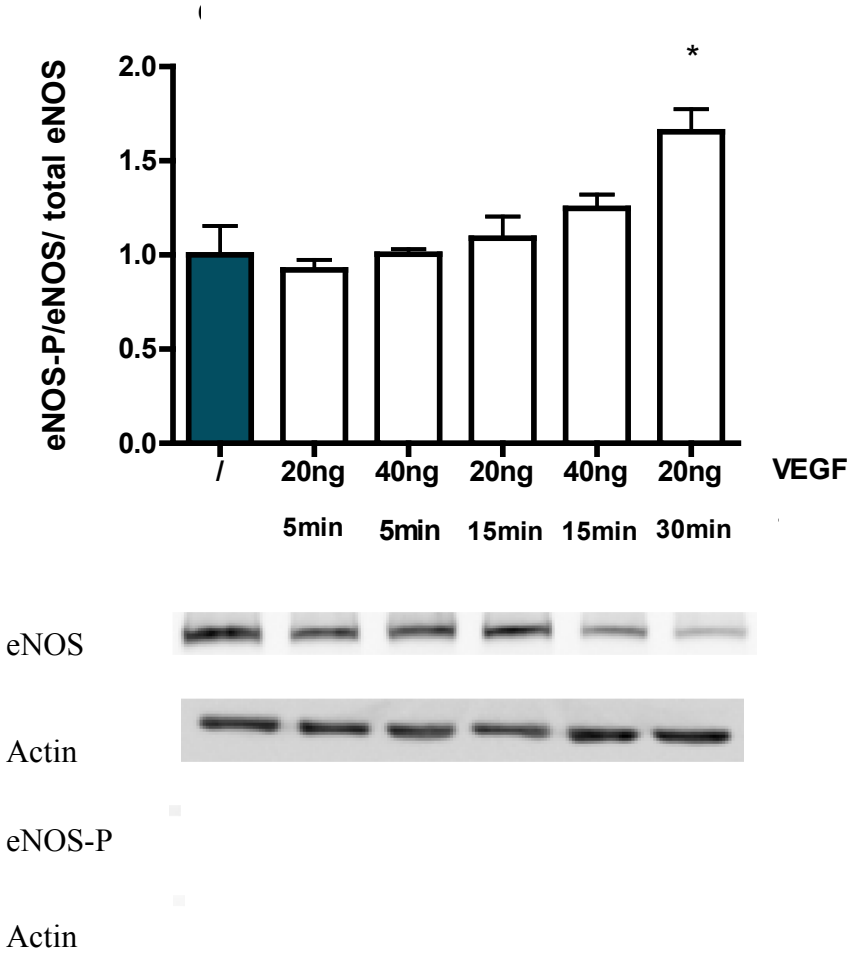


Figure 17: Evaluation of VEGF as control for eNOS-Ser^{114} phosphorylation
EA.hy926 cells were treated with 20 ng/ml and 40 ng/ml VEGF for different times (5-30 min). eNOS and eNOS-PSer^{114} protein levels were determined by Western blot and band intensities were normalized to actin
(n=3, Mean ± SD, *<0.05)

3.3. Influence of butyl gallate and ethyl gallate on eNOS expression and eNOS-Ser^{114} phosphorylation

3.3.1. Effect of butyl gallate and ethyl gallate on eNOS expression

Before investigating the effect of butyl and ethyl gallate on eNOS-Ser114 phosphorylation a possible influence on total eNOS expression was determined. For that EA.hy926 cells were incubated with 5 µM and 10 µM butyl gallate and 5 µM and 10 µM ethyl gallate for 24 hours. Unfortunately we could not observe a significant effect. (Figure 18)

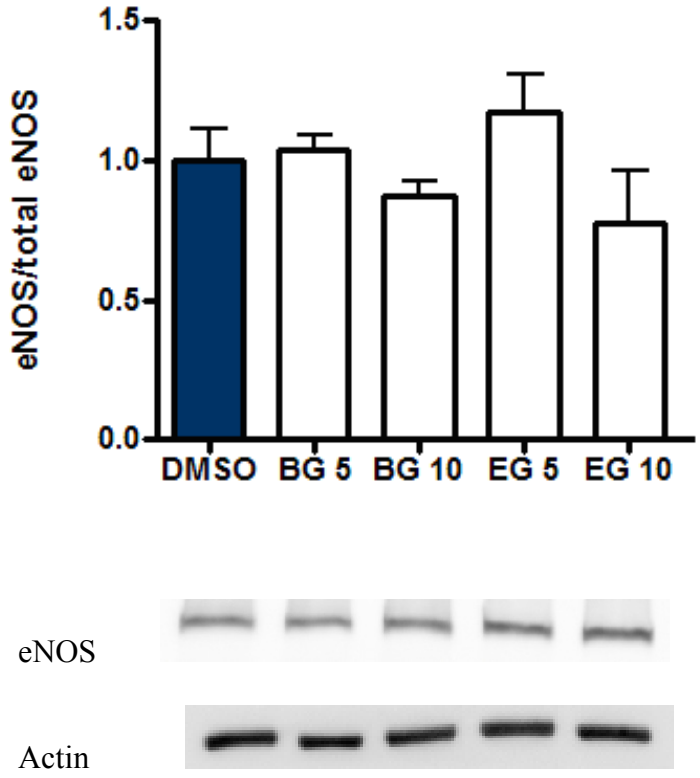


Figure 18: Effect of butyl gallate and ethyl gallate on eNOS
EA.hy926 cells were treated with 5 µM BG (butyl gallate), 10 µM BG, 5 µM EG (ethyl gallate) and 10 µM EG for 24 hours. eNOS protein levels were determined by Western blot and band intensities were normalized to actin. (n=4, Mean ± SD)

3.3.2. Influence of butyl gallate and ethyl gallate on eNOS-Ser^{114} phosphorylation

To investigate the effect of butyl gallate and ethyl gallate on eNOS phosphorylation at Ser^{114} EA.hy926 cells were treated with 5 µM and 10 µM butyl gallate and 5 µM and 10 µM ethyl gallate for 24 hours and eNOS and eNOS-P-Ser^{114} protein levels were determined by Western blot. (Figure 19)

There was no significant effect on eNOS-Ser^{114} phosphorylation detectable.

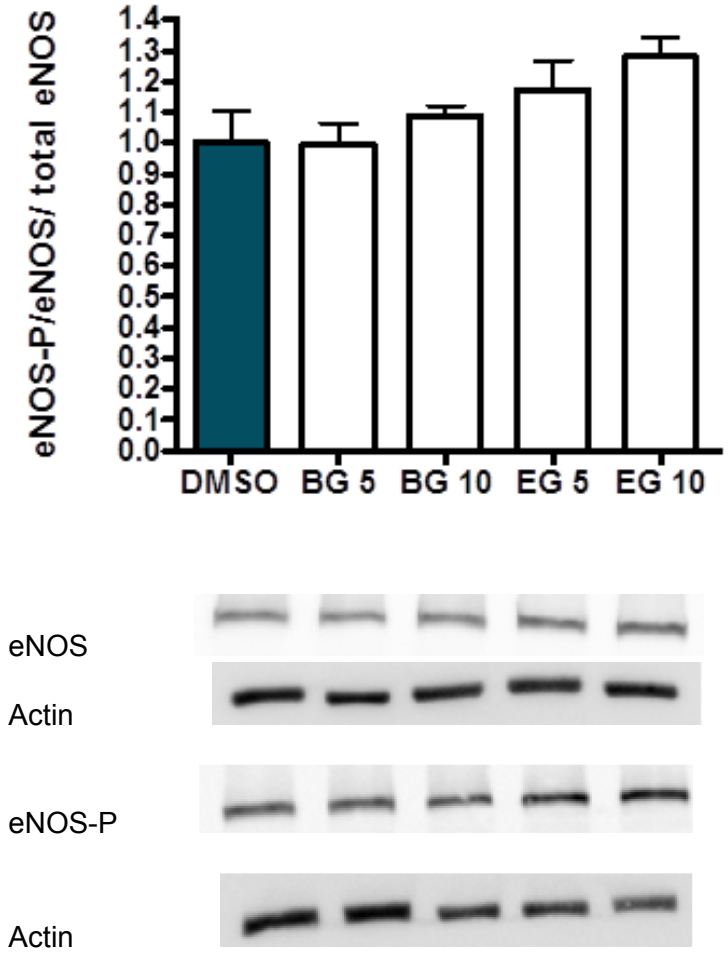


Figure 19: Effect of butyl gallate and ethyl gallate on eNOS-P-Ser ^{114}
EA.hy926 cells were treated with 5 µM BG (butyl gallate), 10 µM BG, 5 µM EG (ethyl gallate) and 10 µM EG for 24 hours. eNOS protein levels were determined by Western blot and band intensities were normalized to actin.
(n=4, Mean ± SD)

Part IV
Discussion

1. Radical scavenging effect of gallic acid derivatives

The antioxidative ability of gallic acid and its derivatives has been evaluated in former studies. [30] But not every derivative that was used in our study was tested before. To complete this data and for a better comparison the antioxidative capacities of the here tested gallic acid derivatives were determined via a DPPH-Assay. All tested substances showed a radical scavenging ability, but there was no significant difference in antioxidant activity between the tested derivatives, suggesting that the different length of the aliphatic chain is not important for scavenging DPPH-radicals. This raises the question how much this radical scavenging ability of gallic acid derivatives influences the intracellular ROS reduction in endothelial cells. To investigate a correlation, intracellular ROS levels were determined with the H_2DCF-DA-Assay.

2. Gallic acid derivatives and intracellular ROS levels

Gallic acid derivatives showed an increase in eNOS activity in Oliver Donaths work. [29] To investigate a possible effect of these eNOS activators on intracellular ROS levels seven derivatives out of this group of substances were used. In particular butyl gallate and ethyl gallate were investigated for their influence on intracellular ROS levels. Ethyl gallate was chosen because it is a natural compound, isolated from red wine, and butyl gallate was interesting because it elicited the highest eNOS activation among these seven derivatives, as detected by Oliver Donath. (Figure 6)

In contrast to the results obtained from the DPPH-Assay the data from intracellular ROS levels seemed to correlate with the length of the aliphatic chain. Octyl gallate, the derivative with the longest aliphatic chain tested in this thesis, was able to elicit the strongest effect in this cell based assay. Propyl and butyl gallate showed a significant, but lower reduction in intracellular ROS levels. Also the incubation with 10 µM ethyl gallate elicited a low, but significant, decrease in intracellular ROS levels. For butyl gallate no dose-dependent effect could be observed. Although the study of Oliver Donath reported the biggest increase in eNOS activity with 5 µM and 10 µM, data in this investigation showed an almost similar decrease in intracellular ROS levels at 5 µM, 10 µM and 30 µM and the biggest effect at 50 µM.

Interestingly, although there was no significant difference in DPPH-radical scavenging detectable among the different derivatives, this was not the case for the reduction of

intracellular ROS levels. This led us to the conclusion that there might be an additional effect influencing intracellular ROS levels besides radical scavenging. It is possible that compounds with a longer aliphatic chain and therefore higher lipophilicity are more likely to migrate into cells. [66]

It is possible that the observed effects are elicited by a synergistic mechanism resulting in the reduction of intracellular ROS levels via stimulating eNOS activation and radical scavenging. On one side there is an antioxidative radical scavenging ability of gallic acid derivatives, which reduces oxidative stress and promotes coupled eNOS activity and on the other side eNOS activity might be directly increased. However it is not clear if the reduction in intracellular ROS levels by gallic acid derivatives leads to an increase in eNOS activity, or if these both effects are related at all.

Interestingly in a recent study by Dieu Linh Nguyen [56] gallic acid derivatives were shown to enhance eNOS activation via a different mechanism. This study reported a butyl gallate dependent dephosphorylation of eNOS at Thr^{495} which leads to an increased eNOS activity. Additionally butyl gallate lowers caveolin-1 expression, thereby enhancing eNOS activity. [56]

It would be interesting for further investigations to determine the underlying mechanism of this effect. Caveolin-1 levels as well as dephosphorylation of Thr^{495} are correlated to a high intracellular Ca^{2+} concentration, [38] [44] suggesting an effect of butyl gallate on intracellular Ca^{2+} levels.

Considering the co-treatment experiment with L-NAME intracellular ROS reduction seems not to be dependent on eNOS enzyme activity, at least in healthy unstressed endothelial cells. Further investigations would be necessary to elucidate if eNOS enzyme activity is necessary for the effect of gallic acid derivatives on intracellular ROS levels during the state of increased oxidative stress. For this EA.hy926 cells could be treated with ROS stimulating substances like TNF-α or PDGF [53] to simulate a “diseased state”.

3. Effects of gallic acid derivatives on eNOS phosphorylation site Ser^{114}

ENOS enzyme activity can be regulated by phosphorylation on different residues. Phosphorylation at Ser^{114} has a negative influence on enzyme activation and a dephosphorylation on this residue leads to an increase in eNOS activity. [12][13] This phosphorylation site was of particular interest in this thesis as it was not investigated in previous experiments (by Dieu Linh Nguyen [56]), in contrast to many other phosphorylation sites. Troglitazone (dephosphorylates eNOS at this site) and VEGF (enhances dephosphorylation of eNOS-Ser^{114}) were described in the studies by Du-Hong Cho et al. [49]

and Ruqin Kou et al. [50] to modulate this phosphorylation site. In our hands troglitazone was not able to elicit the published effect, probably because these studies were performed with bovine aortic endothelial cells. Although VEGF was able to significantly increase eNOS phosphorylation at Ser^{114} the effect was not very strong. To optimize the response of our cell culture model to VEGF additional experiments are required like changes in incubation time and concentration.
Nevertheless butyl gallate and ethyl gallate were tested to get a first idea if there is an effect. Neither the eNOS protein expression nor the eNOS phosphorylation at Ser^{114} was significantly influenced by butyl or ethyl gallate.

The results in this work suggest that gallic acid derivatives have additional antioxidative qualities beside the sole radical scavenging effect. This thesis serves as a basis for further investigations to find the mechanism of intracellular ROS reduction by gallic acid derivatives. Moreover additional investigations would be necessary to determine if the increase in eNOS activity and the reduction of intracellular ROS levels are linked or if these effects are independent of each other.

Bibliography

[1]
Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc Natl Acad Sci USA, 1983 Jun, Vol 80: 3734-3737.

[2]
https://www.lgcstandards-atcc.org/products/all/CCL-185.aspx?geo_country=at&slp=1#characteristics (06.10.2015)

[3]
http://www.lgcstandards-atcc.org/products/all/CRL-2922.aspx?geo_country=at#characteristics (06.10.2015)

[4]
Sagar B. Kedare and R. P. Singh. Genesis and development of DPPH method of antioxidant assay. J Food Sci Technol, 2011 Aug, 48(4): 412–422.

[5]
Ojima Y, Duncan MT, Nurhayati RW, Taya M, Miller WM. Synergistic effect of hydrogen peroxide on polyploidization during the megakaryocytic differentiation of K562 leukemia cells by PMA. Exp Cell Res, 2013 Aug 15, 319(14): 2205-2215.

[6]
Guzmán-Beltrán S, Pedraza-Chaverri J, Gonzalez-Reyes S, et al. Nordihydroguaiaretic acid attenuates the oxidative stress-induced decrease of CD33 expression in human monocytes. Oxidative Medicine and Cellular Longevity, 2013, 375-893.

[7]
Förstermann U and Sessa WC. Nitric oxide synthases: regulation and function.
Eur Heart J, 2012 Apr, 33(7): 829-37, 837a-37d

[8]
Frederica Vanini, Khosrow Kashfi and Niharika Nath. The dual role of iNOS in cancer. Redox Biol. 2015 Dec, 6: 334–343

[9]
Bruckdorfer R. The basics about nitric oxide. Mol Aspects Med, 2005, 26(1-2): 3-31

[10]
Ruslan Rafikov, Fabio V Fonseca, Sanjiv Kumar, et all. eNOS activation and NO function: Structural motifs responsible for the posttranslational control of endothelial nitric oxide synthase activity. Journal of Endocrinology, 2011, 210: 271-284

[11]
Ingrid Flemming. Molecular mechanisms underlying the activation of eNOS. Pflugers Arch – Eur J Physiol, 2010, 459: 793-806

[12]
Jayanth Ramadoss, PhD, Mayra B. Pastore, B.S, and Ronald R. Magness, PhD
Endothelial Caveolar Subcellular Domain Regulation of Endothelial Nitric Oxide Synthase
Clin Exp Pharmacol Physiol. 2013 Nov, 40(11): 753–764.

[13]
Kou R, Greif D. and Michel T. Dephosphorylation of endothelial nitric-oxide synthase by vascular endothelial growth factor. Implications for the vascular responses to cyclosporin a. J Biol Chem, Aug 2002, 277(33): 29669-29673

[14]
Thomas Michel, Paul M. Vanhoutte. Cellular signaling and NO production. Pflugers Arch - Eur J Physiol, 2010, 459: 807–816

[15]
Ulrich Förstermann. Nitric oxide and oxidative stress in vascular disease. Pflugers Arch - Eur J Physiol, 2010, 459: 923–939

[16]
Matsuno K, Yamada H, Iwata K et al. Nox1 is involved in angiotensin II-mediated hypertension: a study in Nox1-deficient mice. Circulation. 2005 Oct, 112(17): 2677-85.

[17]

C. Roger White, Victor Darley-Usmar, William R. Berrington; Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. Proc. Natl. Acad. Sci. USA, 1996 August, Vol.93: 8745-8749

[18]

Ulrich Förstermann, Huige Li. Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling. Br J Pharmacol, 2011 Sep, 164(2): 213–223

[19]

Mark J. Crabtree, Keith M. Channon. Synthesis and recycling of tetrahydrobiopterin in endothelial function and vascular disease. Elsevier, 2011 Aug, 81–88

[20]

Aird WC. Phenotypic heterogeneity of the endothelium: I. structure, function, and mechanisms. Circ Res, 2007 Feb, 100(2): 158-173

[21]

Félétou M. The Endothelium: Part 1: Multiple Functions of the Endothelial Cells - Focus on Endothelium-Derived Vasoactive Mediators. Morgan & Claypool Life Sciences, 2011

[22]

Ignarro, L & Buga, G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci U S A, 1987 Dec, 84(24): 9265–9269

[23]

Ignarro LJ, Byrns RE, and Wood KS. Biochemical and pharmacological properties of edrf and its similarity to nitric oxide radical. Raven Press New York, 1988, in p.M. Vanhoutte ed. Mechanism of Vasodilation: 427-435

[24]

Ulf Landmesser, Burkhard Hornig and Helmut Drexler. Endothelial Function: A Critical Determinant in Atherosclerosis? Circulation, American Heart Association 2004, 109: II-27-II-33

[25]

NikolaosI Goutzourelas, Dimitrios Stagos, Ypatios Spanidis et al. Polyphenolic composition of grape stem extracts affects antioxidant activity in endothelial and muscle cells. Mol Med Rep., 2015 Oct, 12(4): 5846–5856

[26]

Zhongbing Lu, Guangjun Nie, Peter S. Belton, et al. Structure-activity relationship analysis of antioxidant ability and neuroprotective effect of gallic acid derivatives. Neurochemistry International, 2006, 48: 263-274

[27]

Claudriana Locatelli, Fabiola Branco Filippin-Monteiro, Tania Beatriz Creczynski-Pasa et al. Alkyl esters of gallic acid as anticancer agents: A review. European journal of medicinal chemistry, 2012, 60: 233–9.

[28]

Mario Dell'Agli, Alessandra Buscialà, Enrica Bosisio. Vascular effects of wine polyphenols. European society of cardiology, 2004, 593-602

[29]

Mag. Pharm. Oliver Donath. Activity profiling of Austrian red wine targeting cardiovascular diseases: A contribution to understand the “French Paradox”, Doctoral Thesis, University of Vienna 2015

[30]

Hiroe Kikuzaki, Masashi Hisamoto, Kanae Hirose, et al. Antioxidant Properties of Ferulic Acid and Its Related Compounds. Journal of Agricultural and Food Chemistry, 2002, 50: 2161-2168

[31]

Emile Andriambeloson, Céline Magnier, Gisèle Haan-Archipoff, et al. Natural Dietary Polyphenolic Compounds Cause Endothelium-Dependent Vasorelaxation in Rat Thoracic Aorta. The journal of nutrition, 1998, Vol 128 nr2: 2324-2333

[32]

Christine N. Galinski, Jeffrey I. Zwicker, Daniel R. Kennedy. Revisiting the mechanistic basis of the French Paradox: Red wine inhibits the activity of protein disulfide isomerase *in vitro*. Thrombosis Research, 2016, 137: 169–173

[33]

David Dudzinski, M.D., J.D and Thomas Michel, M.D., PhD. Life History of eNOS: Partners and Pathways. Cardiovasc Res., 2007 Jul, 75(2): 247–260

[34]

David Fulton, Jean-Philippe Gratton and William C. Sessa. Post-Translational Control of Endothelial Nitric Oxide Synthase: Why Isn't Calcium/Calmodulin Enough? The journal of pharmkology and experimental therapeutics, 2001, 299: 818-824

[35]

Wendy K. Alderton, Chris E Cooper and Richard G Knowles. Nitric Oxide Synthases: structure, function and inhibition. Biochem. J., 2001, 357: 593-615

[36]

Guillermo García-Cardeña, Pavel Martasek, Bettie Sue Siler Masters, et al. Dissecting the Interaction between Nitric Oxide Synthase (NOS) and Caveolin; functional significance of the NOS caveolin binding domain *in vivo*. The Journal of Biological Chemistry, 1997, 272: 25437-25440.

[37]

Jean-Philippe Gratton, Jason Fontana, Daniel S. O'Connor et al. Reconstitution of an Endothelial Nitric-oxide Synthase (eNOS),hsp90, and Caveolin-1 Complex *in Vitro.*
Evidence that Hsp-90 facilitates calmodulin stimulated dislacement of eNOS from Caveolin-1. The Journal of Biological Chemistry, 2000, Vol. 275: 22268–22272

[38]

Jason Fontana, David Fulton, Yan Chen, Todd A. Fairchild et al. Domain Mapping Studies Reveal That the M Domain of hsp90 Serves as a Molecular Scaffold to Regulate Akt-Dependent Phosphorylation of Endothelial Nitric Oxide Synthase and NO Release. *Circ Res*., 2002, 90: 866-873

[39]

Brittany N. Albaugh, Kevin M. Arnold and Prof. Dr. John M Denu. KAT(ching) Metabolism by the Tail: Insight into the links between lysine acetyltransferases and metabolism. Chembiochem, 2011 Jan, 12(2): 290–298

[40]

Saet-Byel Jung, Cuk-Seong Kim, Asma Naqvi et al. Histone Deacetylase-3 antagonizes Aspirin-stimulated Endothelial Nitric Oxide production by reversing Aspirin-induced lysine acetylation of Endothelial Nitric Oxide Synthase. Circ Res., 2010 Oct, 107(7): 877–887

[41]

Junsuke Igarashi and Thomas Michel. More sweetness than light? A search for the causes of diabetic vasculopathy. J Clin Invest, 2001 Nov, 108(10): 1425–1427

[42]

Xue Liang Du, Diane Edelstein, Stefanie Dimmeler et al. Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. J Clin Invest, 2001 Nov, 108(9): 1341–1348

[43]

Ling Ruan, Christina M. Torres, Jin Qian et al. Pin1 Prolyl Isomerase Regulates Endothelial Nitric Oxide Synthase. Arterioscler Thromb Vasc Biol., 2011 Feb, 31(2): 392–398

[44]

Ingrid Fleming, Beate Fisslthaler, Stefanie Dimmeler et al. Phosphorylation of Thr^{495} Regulates Ca^{2+}/Calmodulin-Dependent Endothelial Nitric Oxide Synthase Activity. American heart association, 2001*,* 88: e68-e75

[45]

David Fulton, Jarrod E. Church, Ling Ruan et al. Src Kinase Activates Endothelial Nitric-oxide Synthase by Phosphorylating Tyr-83. The Journal of Biological Chemistry, 2005 Oct, 280: 35943-35952

[46]

Jean Davignon, Peter Ganz. Atherosclerosis: Evolving Vascular Biology and Clinical Implications - Role of Endothelial Dysfunction in Atherosclerosis. Circulation, 2004, 109(23 Suppl 1): III27-32

[47]

A. Chatterjee, J.D. Catravas. Endothelial nitric oxide (NO) and its pathophysiologic regulation. Vascul Pharmacol, 2008, 49(4-6): 134–140

[48]

Silvia Pfeiffer, Eva Leopold, Kurt Schmidt, et al. Inhibition of nitric oxide synthesis by NG-nitro-L-arginine Methyl ester (L-NAME): requirement for bioactivation to the free acid, NG-nitro-L-arginine. British Journal of Pharmacology, 1996, 118: 1433-1440

[49]

Du-Hyong Cho, Yoon Jung Choi, Sangmee Ahn Jo and Inho Jo. Nitric Oxide Production and Regulation of Endothelial Nitric oxide synthase phosphorylation by prolonged treatment with troglitazone. The journal of biological chemistry, 2004, Vol 279: 2499-2506

[50]

Ruqin Kou, Daniel Greif and Thomas Michel. Dephosphorylation of endothelial nitric oxide synthase by vascular endothelial growth factor. The journal of biological chemistry, 2002, Vol 277: 29669-29673

[51]

http://cellgro.com/products/cell-culture-reagents/hybridoma-reagents/hat-hypoxanthine-aminopterin-thymidine.html (14.02.2016)

[52]

Bradley A. Maron, Thomas Michel. Subcellular Localization of Oxidants and Redox Modulation of Endothelial Nitric Oxide Synthase. Circ J., 2012, 76: 2497–2512

[53]

Kathy K. Griendling, Dan Sorescu, Masuko Ushio-Fukai. NAD(P)H Oxidase - Role in Cardiovascular Biology and Disease. Circulation Research, 2000, 86: 494-501

[54]
Karen Bedard, Karl-Heinz Krause. The NOX Family of ROS-Generating NADPH Oxidases: Physiology and Pathophysiology. Physiological Reviews, 2007 Jan, Vol.87 no.1: 245-313

[55]
Yuanbin Liu, Gary Fiskum and Daviv Schubert. Generation of reactive oxygen species by the mitochondrial electron transport chain. Journal of Neurochemistry, 2002, 80: 780–787

[56]
Dieu Linh Nguyen, Natural Products and their Influence on the Endothelial Nitric Oxide Synthase System, Doctoral Thesis (in preparation) University of Vienna, 2016

[57]
Searles CD. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression. Am J Physiol Cell Physiol, 2006 Nov, 291(5): C803-C816

[58]
Nalae Kang, Ji-Hyeok Lee, Won Woo Lee et al. Gallic acid isolated from Spirogyra sp. Improves cardiovascular disease through a vasorelaxant and antihypertensive effect. Environmental Toxicology and Pharmacology, 2015

[59]
Pierre L Teissedre, Edwin N Frankel, Andrew L Waterhouse et al. Inhibition of *In Vitro* Human LDL Oxidation by Phenolic Antioxidants from Grapes and Wines. J Sci Food Agric, 1996, 70: 55-61

[60]
Jianping Sun, Feng Liang, Yan Bin et al. Screening Non-colored Phenolics in Red Wines using Liquid Chromatography/Ultraviolet and Mass Spectrometry/Mass Spectrometry Libraries. Molecules, 2007, 12: 679-693

[61]
Pil-Hoon Park, Jin Hur, Youn-Chul Kim et al. Involvement of Heme Oxygenase-1 Induction in Inhibitory Effect of Ethyl Gallate Isolated from Galla Rhois on Nitric Oxide Production in RAW 264.7 Macrophages. Arch Pharm Res, 2011, Vol.34 No.9: 1545-1552

[62]
http://www.bms.ed.ac.uk/research/others/smaciver/Cyto-Topics/caveolae.htm (03.04.2016)

[63]
Kirstin Schilling, Nils Opitz, Anja Wiesenthal et al. Translocation of Endothelial Nitric-Oxide Synthase Involves a Ternary Complex with Caveolin-1 and NOSTRIN. Mol Biol Cell, 2006 Sep, 17(9): 3870–3880

[64]
Koichi Sugamura and John F. Keaney, Jr. Reactive Oxygen Species in Cardiovascular Disease. Free Radic Biol Med, 2011 Sep, 51(5): 978–992

[65]
Kathy K. Griendling, Dan Sorescu, Bernard Lassègue, Masuko Ushio-Fukai. Modulation of Protein Kinase Activity and Gene Expression by Reactive Oxygen Species and Their Role in Vascular Physiology and Pathophysiology. American Heart Association, 2000, 20: 2175-2183

[66]
Tom Peck, Sue Hill and Mark Williams. Drug passage across the cell membrane, Pharmacology for Anaesthesia and Intensive Care. Third Edition, Cambridge University Press, 978-0-521-70463-2

Nomenclature

Akt .....proteine kinase B
AMPK .....AMP activated protein kinase
BH_2 .....dihydrobiopterin
BH_3 .....trihydrobiopterin
BH_4 .....tetrahydrobiopterin
BSA .....bovine serum albumin
CaM.....calmoduil
CaMKII .....calcium/calmodulin dependent protein kinase II
cAMP.....cyclic adenosine monophosphate
DCF .....dichlorofluorescein
DHFR .....dihydrofolate reductase
DMEM .....Dulbecco's Modified Eagle's medium
DPPH 2,2-diphenyl-1-picrylhydrazyl
ECL .....enhanced chemiluminescence
EDRF.....endothelium derived relaxing factor
EDTA.....ethylendiamintetraacetat
EGF .....epidermal growth factor
EGF-R .....epidermal growth factor receptor
eNOS.....endothelial nitric oxide synthase
eNOS-P .....endothelial nitric oxide synthase-phosphorylated
ERK.....extracellular-signal regulated kinases
FAD .....flavin adenine dinucleotide
FBS .....fetal bovine serum
FMN.....flavin mononucleotide
GAG .....glucosaminoglycan
GlycNac.....N-acetylglucosamine
HAT .....hypoxanthine-aminopterin-thymidine
HBSS.....Hank's Balanced Salt Solution
H_2DCF-DA .....dichlorodihydrofluorescein-diacetate
HGPRT.....hypoxanthine-guanine phosphoribosyltransferase
Hsp-90.....heat-shock protein 90
iNOS.....inducible nitric oxide synthase
L-NAME.....N-nitro-L-arginine methylester
MAP-kinase .....mitogen activated protein-kinases

NADPH.....nicotinamide adenine dinucleotide phosphate
NDGA.....nordihydroguaiaretic acid
nNOS.....neuronal nitric oxide synthase
NO.....nitric oxide
NOX.....NAD(P)H oxidase
PAA.....polyacrylamide
PBS.....phosphate buffered saline
PDGF.....platelet derived growth factor
PDGF-R.....platelet derived growth factor receptor
Pin-1.....peptidy-propyl isomerase NIMA interacting1
PKA.....protein kinase A
PKC.....proteinkinase C
PMA.....phorbol 12-myristate 13-acetate
PP1.....protein phosphatase 1
PVDF.....polyvinylidene difluoride
PYK2.....protein tyrosine kinase 2-beta
Ras.....Rat sarcoma G-protein
ROS.....reactive oxygen species
SDS.....sodium dodecylsulfate
SIRT-1.....sirtuin-1
TBS-T.....Tris-buffered saline with Tween20
TEMED.....tetramethylethylenediamine
TK.....thymidine kinase
TNF-α.....tumor necrosis factor-α
VEGF.....vascular endothelial growth factor

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Products, suppliers and stock solutions

product	stock	suppliers
DMEM (4.5g/L glucose)	-	Lonza, Verviers, Belgium
L-glutamine	200 mM in 0.85 % NaCl	Lonza, Verviers, Belgium
Penicillin/streptomycin	10,000 U/mL penicillin, 10,000 U/mL streptomycin	Lonza, Verviers, Belgium
Trypsin	2.5 % 10x in HBSS without Ca, Mg	Cambrex Bio Science, Verviers, Belgium
HAT	50 x in BSS (balanced salt solution)	Biochrom AG, Berlin, Germany
FBS	-	Gibco via Invitrogen, Paisley, UK
L-NAME	1 M in ddH_2O	Sigma-Aldrich, Vienna, Austria
Gallic acid	100 mM in DMSO	Fluka Chemika, Switzerland
Methyl gallate	100 mM in DMSO	Department Pharmacognosy, University of Vienna
Ethyl gallate	100 mM in DMSO	Sigma Aldrich, Steinheim, Germany
Propyl gallate	100 mM in DMSO	Sigma Aldrich, Steinheim, Germany
Butyl gallate	100 mM in DMSO	ABCR, Karlsruhe, Germany
Isopentyl gallate	100 mM in DMSO	ABCR, Karlsruhe, Germany
Octyl gallate	100 mM in DMSO	Sigma Aldrich, Steinheim, Germany
Roti®-Quant Bradford reagent	5x	Carl Roth, Karlsruhe, Germany
ViCell® cell viability Analyser	-	Beckman Coulter, Vienna, Austria
LAS-3000	-	Fujifilm, Düsseldorf,

Luminescent analyser		Germany
Tecan Sunrise	-	Tecan, Grödig, Austria
FACS Calibur	-	BD, Biosciences, Franklin Lakes, NJ, USA

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