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„The effect of natural products on the endothelial nitric oxide synthase in EA.hy926 cells“

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Gabriel Zirkovits

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Abstract

Endothelial cells line the vasculature and for this reason, they are permanently exposed to the circulation. Therefore, the endothelium is the most important organ to sense alterations in blood flow. It maintains the vascular tone, avoids excessive blood coagulation and immoderate vascular smooth muscle proliferation. Moreover, it mounts an anti-inflammatory environment. Irregularities in the endothelial cell layer are known to cause endothelial dysfunction and severe cerebrovascular and cardiovascular diseases. Increased endothelial nitric oxide synthase activity and elevation of its enzymatic product NO were shown to prevent such complaints. Several natural compounds, used as traditional medicines or in the daily diet, were shown to exert positive influence on the endothelium.

Leoligin is a promising drug to avoid restenosis after arterial surgery. The substance provokes G1-phase arrest in VSMC by influencing the tumor suppressor p27. Several leoligin derivatives were available in our laboratory. We investigated whether these semisynthetic substances cause an increase in endothelial nitric oxide release, which may contribute to cell cycle arrest *in vivo*. However, no significant effect was determined.

Previous studies provided evidence that rutaecarpine is primarily responsible for the vasodilatory effect of the TCM drug Wu-Chu-Yu. We conducted experiments to prove increased eNOS activity and NO release. Due to solubility problems we were not able to obtain reliable data. Thus, these solubility problems have to be solved, before further research is feasible.

Gallic acid derivatives are polyphenolic substances and are supposed to have a number of positive effects on health, including increased eNOS activity. In our study, we examined whether elevated conversion of L-arginine to L-citrulline in EA.hy926 cells stimulated with gallic acid derivatives results in increased NO release. Additionally, we investigated mechanisms that lead to eNOS activation. No rise in NO release or protein abundance was detected. Indeed, approximately fivefold augmentation in eNOS mRNA levels was found.

Zusammenfassung

Endothelzellen kleiden die Blutgefäße aus und sind aus diesem Grund ständig dem Blutkreislauf ausgesetzt. Das Endothelgewebe ist daher das wichtigste Organ, um Veränderungen im Blutfluss zu registrieren. Es erhält den Gefäßtonus, verhindert exzessive Blutgerinnung und übermäßige Proliferation von glatten Gefäßmuskelzellen. Zusätzlich sorgt es für eine antiinflammatorische Umgebung. Störungen des Endothelgewebes führen zu endothelialer Dysfunktion und zu schweren cerebrovaskulären und cardiovaskulären Erkrankungen. Erhöhte eNOS Aktivität und Steigerung der enzymatischen Produktion von NO können derartige Leiden verhindern. Viele pflanzliche Substanzen, die in der traditionellen Medizin oder in der täglichen Ernährung verwendet werden, üben positiven Einfluss auf das Gefäßendothel aus.

Leoligin ist ein vielversprechendes, experimentelles Therapeutikum, um Stenoserezidiven nach Operationen an Arterien zu verhindern. Duch Beeinflussung des Tumorsuppressorproteins p27 verursacht die Substanz G1-Phasenarrest in glatten Gefäßmuskelzellen. Uns lagen einige Derivate von Leoligin vor, die wir daraufhin untersuchten, ob sie die endotheliale NO Freisetzung erhöhen, was *in vivo* zu dem Zellzyklusarrest beitragen könnte. Eine derartige Wirkung konnte allerdings nicht festgestellt werden.

In früheren Studien konnte gezeigt werden, dass Rutaecarpine hauptsächlich verantwortlich für die gefäßerweiternde Wirkung der TCM Droge Wu-Chu-Yu ist. Wir versuchten daher erhöhte eNOS Aktivität und NO Freisetzung nachzuweisen. Wegen Löslichkeitsproblemem konnten wir allerdings keine zuverlässigen Daten gewinnen. Weitere Untersuchungen sind nur sinnvoll, wenn die Löslichkeit verbessert werden kann.

Derivate der Gallussäure sind polyphenolische Substanzen. Es wird vermutet, dass sie etliche positive Effekte auf die Gesundheit ausüben. In unserer Studie untersuchten wir, ob die durch Derivate der Gallussäure gesteigerte eNOS Aktivität in EA.hy926 Zellen auch eine Erhöhung der endothelialen NO Freisetzung bewirkt. Zusätzlich untersuchten wir Mechanismen, die zu dieser Aktivierung führten. Wir konnten keine erhöhte NO Freisetzung bzw. Steigerung der eNOS Proteinmenge feststellen. Allerdings wurde die eNOS mRNA um etwa das fünffache erhöht.

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Part I.

Introduction

1. The endothelium

Nearly 400 years ago, William Harvey established the model of circulating blood and Marcello Malpighi found the existence of a capillary system. Their findings adjusted Galen's paradigm of tidal flow and ebb, which was accepted for more than a millennium before.[1] Other crucial discoveries were made in the following centuries, when von Reckinghausen described the lining of blood vessels by cells and Heidenhahn characterized these as an active secretory system. Following electron microscope and physiological studies defined the endothelium both as a dynamic and heterogeneous organ, which essentially contributes to secretory, synthetic, metabolic and immunological functions.[2]

Since they line vessels in every organ, endothelial cells (EC) show broad phenotypic variation according to the needs of the surrounding tissue. However, in general they are linked to the basement membrane and to each other via tight junctions. These junctional properties and adaption of transcytosis regulate the permeability of fluids, solutes and macromolecules between circulation and tissue.[3] Their location not only provides barrier function, but also enables ECs to notice alteration in blood flow. Secretion of vasodilator (e.g. nitric oxide (NO), prostacyclin (PGI2), endothelium-derived hyperpolarizing factor (EDHF)) and vasoconstrictor (e.g. endothelin-I, angiotensin-II) substances essentially contribute to vasoregulation.[4] Some of these endothelium-derived signalling molecules prevent platelets from adhesion to the EC surface and mount an anti-thrombotic environment. Others affect leucocyte trafficking by modulating integrin expression.[2] EC also play a pivotal role in vasculogenesis and angiogenesis in the embryonic development and in adults, respectively.[5]

Intensive research over the past thirty years depicted nitric oxide as a key signalling molecule in the cardiovascular system. Depletion of NO may lead to endothelial dysfunction, which is hallmarked by perturbation in vasorelaxation and switching the endothelial environment to a prothrombotic, proinflammatory and proliferative state. Such pathological transformations favor cardiovascular and cerebrovascular diseases like hypertension, atherosclerosis, cardiac infarction and stroke.[6–8]

2. Nitric oxide synthases

2.1. Endothelium-derived relaxing factor

Furchgott and Zawadzky accidentally discovered the vasorelaxing activity of carbachol in ring preparations from rabbit thoracic aortas in 1980. The same effect was observed in response to acetylcholine later. First evidence arose that the endothelium is of particular importance for relaxation of vascular smooth muscle cells, but the mediating "endothelium-derived relaxing factor" remained unknown.[9] It took further seven years until Ignarro et al. and Palmer et al. simultaneously unraveled the mystery and determined nitric oxide as the mediator, although pharmacodynamics of organic NO-donors were already investigated by Murad et al. many years before.[10–12]

Robert F. Furchgott, Louis J. Ignarro and Ferid Murad received the Nobel Prize for Physiology and Medicine for their major discoveries, in the year 1998.[13]

2.2. Nitric oxide synthase isoforms

Despite distinct variations in the amino acid sequences among the human isoforms, the isozymes seem to be well conserved across species. They are named NOS 1 - 3 in order to their first purification and first isolation of their cDNAs.[14]

2.2.1. nNOS

Isoform I is termed neuronal NOS (nNOS), although it is also constitutively expressed in cells, other than nerve tissue. The catalytic activity of the predominantly soluble protein is partly calcium-dependent. NOS1 takes part in numerous physiological processes. It governs synaptic plasticity, contributes to regulation of blood pressure in periphery and is of paramount importance for reproduction of mammalians.[14]

2.2.2. iNOS

NOS2 is also called inducible NO synthase (iNOS). As this nomenclature indicates, it is not constitutively expressed in cells but the enzyme is induced upon

inflammation and infection in response to agents like cytokines and bacterial lipopolysaccharides, in many tissues. Unlike NOS1 and NOS3, its activity is not dependent on the intracellular calcium concentration which is due to the lack of an auto-inhibitory element.[14,15] Thus once expressed, large amounts of nitric oxide are released and contribute to immune reactions and tumor lysis by inhibiting iron-sulfur proteins, provoking DNA strand breaks and fragmentation.[16,17] The collapse in blood pressure in septic shock is also due to the excessive NO synthesis by NOS2.[18] Similar to nNOS, iNOS is predominantly soluble.[14]

2.2.3. eNOS

The endothelial NO synthase was discovered last. It is mostly expressed in the endothelium, but also present in cardiac myocytes, platelets and kidney tubular epithelial cells, respectively. Acylation by fatty acids associates eNOS to membranes, which is a major difference to the other two isozymes. Such as NOS1, its activity is partly calcium-dependent.[14] Physiological functions and regulation will be mentioned in detail below.

3. The endothelial NO synthase

3.1. Structural motifs

The NOS3 gene is encoded on chromosome 7 (7q35–7q36). Expression exhibits a 1,203 amino acid, homodimeric protein with a molecular weight of 133 kDa. Each of the monomers consists of a NH_2-terminal oxygenase and a COOH-terminal reductase domain, linked by a calmodulin-binding peptide sequence (amino acids 493-412). Several other cofactors are crucial for nitric oxide synthesis (see Fig. 1). Nicotinamide adenine dinucleotide (NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) are bound to the reductase domain, provide electrons and regulate their flow to the other monomer's oxygenase domain. Prosthetic heme, oxygen, tetrahydrobiopterin (BH_4, 5,6,7,8-tetrahydrobiopterin) and L-arginine are attached to the oxidising substructure. Additionally to their contribution to the catalytic activity of eNOS, heme, BH_4 and L-arginine are essential to ensure the active dimeric constitution. All NOS isoforms also contain two CXXXXC motifs, each contributed by a monomer. These cysteine residues tetracoordinate a zinc atom to structurally maintain binding sites for other cofactors.[19] The amino acids Gly^2, $Cys^{15}$and Cys^{26} provide the ability of acylation, allowing targeting of eNOS at membranes.[20,21] An approximately 50 amino acid autoinhibitory loop within the reductase domain also contributes to eNOS regulation. It is located next to the calmodulin binding region and restricts its accessibility at low intracellular Ca^{2+} concentration.[15,22,23]

3.2. Nitric oxide synthesis

Synthesis of the heterodiatomic free radical nitric oxide is based on a complex stepwise electron transport. NADPH donates the first electron. By forming stable semiquinone intermediates, FAD and FMN transfer it to the heme group located within the oxygenase domain of the opposite monomer. Ca^{2+}- calmodulin binding to eNOS (and nNOS) increases the electron flow by a multiple. This elevation in catalytic activity is controlled by an auto-inhibitory element, which restricts Ca^{2+}-calmodulin adhesion. Lacking this structural motif, iNOS can be activated at very low intracellular Ca^{2+}-concentrations.[15] Now, reduced heme

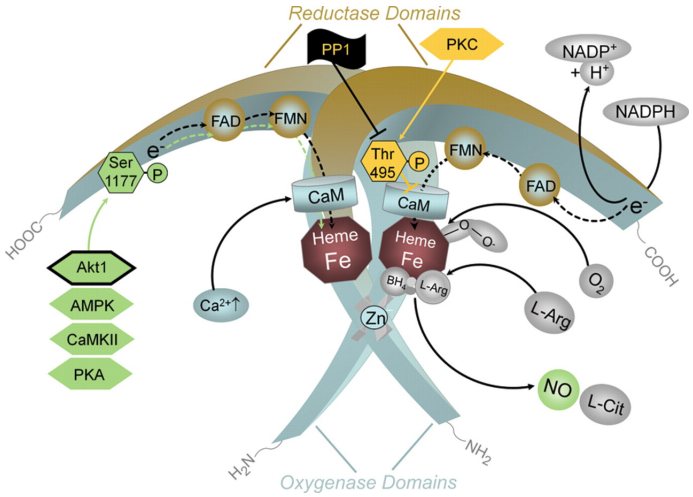


Figure 1.: Dimeric structure of eNOS and its cofactors
Förstermann, U and Sessa, WC, "Nitric oxide synthases: regulation and function", European Heart Journal (2012), 829–837[24]

iron can form ferrous-dioxy species, likewise in haemoglobin.[25] In a second step, either BH_4 or the reductase domain again, provides another electron to further reduce the intermediate.[24] Immediate creation of a reactive oxygen radical leads to hydroxylation of L-arginine. N^{ω}-hydroxy-L-arginine (NHA) is subsequently converted to L-citrulline and nitric oxide by a tetrahedral intermediate.[25]

3.3. Physiological functions of NO

Nitric oxide is produced under basal conditions as well as in response to agonist stimulation and frictional laminar shear stress. Initially discovered as a vasodilator, nowadays, NO is also known for its contributions to many other pivotal physiological functions.

3.3.1. Vasoregulation

Murad et al. were the first that reported increased guanylate cyclase activity in response to NO donors and knock-out experiments using eNOS deficient mice offered the link between the enzyme and its importance for vasoregulation.[8,12] Further studies revealed that cGMP-dependent protein kinase I (cGKI, protein kinase G) acts as a downstream mediator.[26] *In vivo*, NO synthesized by eNOS subsequently diffuses through the plasma membranes into vascular smooth muscle cells. There seem to be three major regulatory mechanisms that induce relaxation in VSMC ascribed to protein kinase G (PKG): reduction of free intracellular calcium, modulation of calcium sensitization and thin filament regulation.[27]

Reduction of free intracellular calcium: myosin light chain phosphorylation occurs in a Ca^{2+}-calmodulin dependent manner and is required for smooth muscle contraction. The inositol-1,4,5-triphosphate receptor (IP_3 receptor) is responsible for calcium release from the sarcoplasmatic reticulum. Phosphorylation of IRAG (IP_3 receptor-associated PKG-I substrate) and the IP_3 receptor itself, by protein kinase G, inhibits IP_3 receptor-dependent Ca^{2+} influx into the VSMC cytoplasm.[28,29] Increased activity of sarcoplasmatic calcium pumps and inhibition of voltage-gated calcium channels also reduce $[Ca^{2+}]$.[27]

Modulation of calcium sensitization: calcium sensitization describes the increase of contractile activity by G-protein-coupled agonists compared with potassium depolarisation. The underlying mechanism is regulated by RhoA-kinases, which inhibit myosin light chain phospatases (MLCP) by phosphorylation. PKG antagonizes this effect by direct interaction with MLCP (protein-protein interaction and phosphorylation of the myosin binding subunit) and additionally inhibition of RhoA-kinase.[27,29]

Thin filament regulation: thin filaments are polarized copolymers of actin subunits. Several regulatory proteins are attached to these structures and facilitate coordinated smooth muscle contraction.[30] Two of them, vasodilatory-stimulated phosphoprotein (VASP) and 20-kDa heat shock-related protein (HSP20) are apparently regulated by PKG. VASP is bound to actin in every cell and abundant at focal adhesion points. PKG provokes its phosphorylation, resulting in dissociation from the actin binding protein profilin and reduction in focal adhesions, followed by diminished VSMC contraction. Other studies also attributed HSP20 phosphorylation to smooth muscle relaxation.[27]

3.3.2. Platelet inhibition

Beside the endothelium, eNOS was also found in human platelets.[31] Radomski et al. reported first that L-arginine stimulates soluble guanylate cyclase (sGC) in collagen activated platelets, resulting in increased cGMP concentrations in the platelet cytosol. This leads to down-regulation of platelet aggregation. The same applies to the aggregating agents ADP and arachidonic acid. The fact that this mechanism is dependent on Ca^{2+}-calmodulin and furthermore, occurrence of decreased cGMP levels in response to N^G -monomethyl-L-arginine treatment, a non-specific NOS inhibitor, identified NOS3 activity in platelets. These findings induced them to propose that NOS3 activation acts as an intraplatelet negative feedback mechanism to restrict excessive platelet aggregation.[32,33] Since then, many other platelet NOS3 activating substances were found. Among them there

are β_2-agonists, Von-Willebrand-Faktor (vWF), insulin, α-tocopherol, high glucose concentration and shear stress.

In case of vessel injury, platelets become activated and change their shape rapidly. They become spherical with spiky pseudopods and an irregular membrane. At endothelium deficient sites, the subendothelial vWF is exposed to the blood flow. Activated platelets interact with this blood clotting protein via their constitutively expressed GPIb-IX-V receptor and GPIIb-IIIa receptor. Additionally, collagen, a structural protein of many various connective tissues, binds to its receptor and stabilizes thrombocyte adhesion. Platelets secret thromboxane A_2 (TxA_2), ADP, Ca^{2+} and fibrinogen during the subsequent release reaction. Thereby other platelets are recruited to form a thrombus. Conformational changes of GPIIb-IIIa receptor are essential for fibrinogen binding, which causes platelet cross-linking and irreversible aggregation.[34] Both platelet activation and structural changes of glycoprotein IIb-IIIa are partly Ca^{2+}-dependent events.[35,36]

Like in endothelial cells, nitric oxide activates platelet soluble guanylate cyclase. Accordingly, cGMP concentration augments in the cytosol. cGKI, a downstream mediator of cGMP, on the one hand accelerates sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase-dependent (SERCA) refilling of Ca^{2+} stores, and on the other hand decreases IP_3 - dependent sarcoplasmatic Ca^{2+} influx.[28,37] It inhibits both the TxA_2 receptor by intracellular phosphorylation and activation of GPIIb-IIIa receptor in a IP_3-kinase dependent manner.[38,39]

Futhermore, nitric oxide was shown to decrease Ca^{2+}-uptake into membrane vesicles and exocytosis of granules (dense, lysosomal and α -granules). These events inhibit the release phase and reduce platelet recruitment and leukocyte adhesion. The underlying pathway seems to be apart from the described cGMP-dependent pathways above. Thereby, thrombus formation decreases.[34] Since platelet inhibition prevents thrombus-mediated acute coronary events, nitric oxide allays late stages of atherosclerosis.[40]

3.3.3. Reduction of leukocyte adhesion

Leukocyte adhesion and migration across the endothelial cell layer into the intima is one of the main events in the early stages of atherosclerosis development. On the spot of laminar shear stress perturbations, endothelial cells change their morphology. Such variations appear preferentially at vessel bifurcations and result in increased permeability for macromolecules like low density lipoprotein (LDL). In healthy mammals, LDL supplies tissues with necessary cholesterol. High fat/high cholesterol nutrition elevates LDL blood levels and hence, LDL particles migrate

passively through EC junctions at previously mentioned sites. By trapping and oxidation inside the vessel wall, modified LDL causes upregulation of adhesion molecules in the overlying endothelial cell layer and secretion of chemotactic substances to recruit white blood cells. Leukocyte rolling across the endothelium is mediated by selectins, while intercellular adhesion molecule (ICAM)-I and vascular cell adhesion molecule (VCAM)-I facilitate firm binding. Upregulation of these adhesive proteins anchor monocytes and their migration into the vessel wall occurs. Once in the intima, monocytes proliferate, differentiate into macrophages and absorb oxidized LDL via a specific scavenger receptor. In that way formed so called "foam cells" contribute their lipid-filling to the necrotic core of atherosclerotic lesions if they die. These "fatty streaks" induce smooth muscle cells to migrate from the media into the intima and to secrete fibrous elements, thus developing plaques. They narrow or even occlude blood vessels and rupture of their thin fibrous caps promote thrombosis in later stages. This characterizes atherosclerosis as an inflammatory disease.[40]

The susceptibility to atherosclerotic lesions at sites of shear stress perturbations is partly attributed to decreased eNOS activity. Nitric oxide nitrosylates NF-κB at subunit p50, which suppresses NF-κB translocation to the nucleus and disables transcription factor activity.[41] The endothelial-leukocyte adhesion molecules E-selectin, P-selectin and cell adhesion molecules VCAM and ICAM exhibit NF-κB binding motifs within their promoter regions.[42–44] Thus enhanced NF-κB activity, due to decreased NO levels, results in greater adhesion molecule density. It was also shown that NO interferes with the leukocyte adhesion to blood vessel walls, either by down-regulation of CD11/CD18 glycoprotein or by interfering with the ability of CD11/D18 to bind VCAM and ICAM.[45,46] In addition, nitric oxide inhibits NF-κB-dependent expression of the chemotactic factors monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8).[47] This highlights the remarkable importance of nitric oxide in suppression of atherogenesis.

3.3.4. Vascular smooth muscle cell regulation

In most cases, blood vessel injury concurs with endothelium denudation. Exposure of the underlying cells to the blood flow initiates a cascade of healing processes. Seconds after injury, platelets aggregate and adhere to the marred site. Activated thrombocytes secrete paracrine and autocrine substances during the release phase. One of the released substances is the platelet-derived growth factor (PDGF), which represents a potent mitogenetic factor and stimulates VSMC migration and proliferation. Following platelet aggregation, leukocytes adhere

and likewise secrete VSMC stimulating substances (e.g. PDGF, IL-1). These processes and degradation of the extracellular matrix, which prevents VSMC migration in undamaged blood vessels, are the main events in neointimal hyperplasia. Due to restenosis, this exaggerated healing process limits the success of many vascular interventions including bypass grafting, endarterectomy and balloon angioplasty.[48]

As previously mentioned, nitric oxide exerts influence on the release phase of activated platelets and leukocyte trafficking. However, there are other pathways by which NO affects vascular smooth muscle cell proliferation. Garg et al. demonstrated that VSMC mitogenesis and proliferation is inhibited by NO-donors in a cGMP-dependent manner, since usage of 8-bromo-cGMP, an hydrolysis-resistant cGMP-analog, mimics NO effects.[49] Potential downstream events may be Ca^{2+}-channel blocking, increasing Ca^{2+}-pump activity of an endoplasmic reticulum calcium-sequestering ATPase and protein kinase A activation. Despite studies providing evidence that there is a relationship between cytosolic Ca^{2+}-levels and vascular smooth muscle proliferation, the precise mechanisms have to be elucidated.

In VSMC, activated NO/cGMP-pathway inhibits growth factor/cytokine signal transduction and late G_1-phase events in VSMC. The former is operated by interaction with tyrosine kinase receptors, G-protein coupled receptors, inhibition of the mitogen-activated protein (MAP-) kinase cascade, which results in decreased expression of the early response genes c-jun and c-fos, and inhibition of c-myc gene induction. The latter might be influenced at any stages, but there is evidence that NO/cGMP interferes with transcription factor c-myc and the cyclin-dependent kinase inhibitors p21, p27 and INK-1-4. These cell cycle regulating proteins control retinoblastoma protein (pRb) binding to transcription factor E2F, and thereby gene transcription. Among others, unphosphorylated tumor suppressor pRb sticks to E2F and represses transcription of DNA polymerase II, which is required for DNA replication. This is sufficient to cause cell cycle arrest at the G_1-S-boundary. However, cGMP-independent effects are not well understood.[48,50]

4. Endothelial NO synthase regulation

The endothelial NO synthase is regulated at different levels. These numerous regulatory mechanisms are needed to adapt to varying conditions and to maintain the physiological state. Disturbances may cause life-threatening diseases.[27]

4.1. eNOS promotor activity

Sequencing of the human genome revealed that only a small percentage of the genome contains protein information. Non coding regions account for a much larger fraction and are responsible for the complex nature of regulation of gene expression.[51]

The eNOS promotor sequence is highly conserved across species and similar to other constitutively expresses proteins it lacks a TATA box. However, it possesses binding motifs for a wide range of transcription factors (e.g. Sp1, GATA, NF-κB, AP1, KLF2). Additionally, basal promotor activity is dependent on many other regulatory cis-elements (e.g. shear stress response-, acute phase reactant regulatory-, sterol regulatory elements) as well as transacting factors and an enhancer region upstream 4,700 nt of the transcription start.[52,53] Other gene silencing mechanisms, especially intronic microRNA and DNA-methylation, also seem to play a role in eNOS transcription.[54,55]

Exposure of endothelial cells to shear stress leads to increased transcription of eNOS. On the one hand laminar blood flow suppresses inhibitor of kappa B (IκB) activity, thus representing a NF-κB-mediated pathway.[56,57] Following nitric oxide augmentation deactivates this transcription factor by nitrosylation of its subunit p50. This provides a negative feedback mechanism.[41] KLF2 upregulation and increased mRNA stability promotes eNOS transcription on the other side.[58,59] Newer data suggest cooperative interaction between NF-κB and KLF2.[60]

4.2. Post-transcriptional mRNA modifications

Even if genes are transcribed, translation does not necessarily happen. This step seems to be critically controlled by primary and secondary mRNA structure motifs that determine nucleo-cytoplasmic transport, translation efficiency,

sub-cellular localization and stability.[51] In eNOS, these patterns are basically attributed to 3′-mRNA untranslated regions (3′-UTR). In contrast to the large number of cis-elements in the eNOS DNA promotor region, only two (AU-rich elements) were found within the 3′-UTR of its mRNA transcript.[51] Such sequences usually regulate mRNA degradation in labile transcripts, but do not seem to affect eNOS mRNA stability.[51,61] Other studies identified at least two cytosolic proteins that appear upon reaching cell confluence or in response to lipopolysaccharides and cytokines. Their binding to a cytidine-rich region within the 3′-UTR changes mRNA configuration, so that RNAse-dependent degradation increases.[22] An antisense mRNA called sONE is known to be of particular importance in eNOS mRNA silencing. It derives from the opposite DNA strand of the one that codes for the eNOS protein located on chromosome 7. sONE was detected in a variety of cells but not in vascular endothelial cells.[62] Findings of Weber et al. highlighted another regulatory mechanisms. They reported elongation of a polyadenylation tail in the 3′-UTR in response to shear stress, HMG-CoA-reductase-inhibitors and hydrogen peroxide, resulting in increased eNOS mRNA half-life and translational activity.[63] Rho-GTPases and their downstream sensor actin are supposed to be involved in this phenomenon.[64,65]

4.3. Post-translational modifications

In addition to protein abundance, eNOS activity is controlled by post-translational modifications. The most important are mentioned below.

Membrane targeting and protein interactions

The endothelial NO synthase is specified as a membrane bound enzyme. Site-directed mutagenesis studies by Sessa et al. attributed this characteristic to N-myristoylation at Gly^2.[20] Ancillary palmitoylation at Cys^{15} and Cys^{26} stabilizes membrane anchoring and is necessary for correct targeting at caveolae (compartmentation effect).[21] Those special plasmalemmal invaginations are important for cell signaling, endocytosis, lipid and cholesterol homeostasis and tumor suppression and accommodate many receptors and channels.[66] eNOS is associated to caveolin-1 within these structures, which renders the enzyme inactive at low Ca^{2+}(clamp effect).[67] Thus, changes in caveolin-1 expression can alter eNOS activity. Agonist-induced depalmitoylation and increasing intracellular Ca^{2+}-concentrations translocate eNOS from caveolin-1 and the plasma membrane to subcellular regions and eNOS activity augments.[68] The golgi apparatus may be

the location of repalmitoylation and the protein is recycled to caveolae.[69] Studies using chelation of extracellular Ca^{2+} or a CaM - antagonists verified the Ca^{2+}-sensitivity.[70,71]

In addition to caveolin-1 and calmodulin, several other eNOS-associated proteins were identified so far. There are G-protein-coupled-receptors, like bradykinin B_2-, Angiotensin II AT_1-, and the endothelin ET_B-receptor, that directly inhibit eNOS in unstimulated cells.[72,73] The underlying mechanism by which eNOS becomes deactivated by the intracellular domain 4 of G-protein-coupled-receptors is still controversial. Interaction with L-arginine, BH_4 binding or influence on the electron flow are discussed.[22]

Heat shock protein 90, a 90 kDa chaperone, was shown to modulate protein folding and activity by weakening the clamp effect.[74] It is also reported that HSP90 is crucial for heme insertion into the immature protein.[75] Stimulation with vascular endothelial growth factor (VEGF), estrogen, laminar shear stress or histamine increases the interaction of HSP90 with eNOS, probably by tyrosine phosphorylation of HSP90.[76] Furthermore, HSP90 acts as a scaffold for eNOS and Akt kinase, thus facilitating eNOS phosphorylation. It also mediates the displacement of caveolin-1 from eNOS.[77] Other eNOS-associated proteins are eNOS interacting protein (NOSIP), endothelial nitric oxide synthase traffic inducer (NOSTRIN), platelet endothelial cell adhesion molecule (PECAM-1, CD31) and the motor protein dynamin. Their function and impact on eNOS regulation still remains partially unknown.[22]

Phosphorylation

eNOS provides multiple phosphorylation sites at serine, threonine and tyrosine residues, but Ser^{1177} and Thr^{495} are the most prominent ones. The phosphorylating kinase varies with the applied stimulus and the phosphorylation site (for examples see Tab. 1).

Ser^{1177} is not phosphorylated in unstimulated, cultured endothelial cells, but in response to insulin, VEGF, estrogen, bradykinin and shear stress rapid phosphorylation occurs. This potentiates eNOS activity at resting Ca^{2+}-levels by improving electron flux between the reductase and the oxidase domain.[22,78] The constitutive phosphorylation of Thr^{495} influences the binding of CaM and inactivates eNOS. Dephosphorylation by protein phosphatase 1 increases NO synthesis by 10-20-fold over basal levels.[79] Additional known phosphorylation sites are Ser^{114}, Ser^{615}, Ser^{633}, Tyr^{81}, and Tyr^{567}.

Table 1.: Phosphorylation sites and stimuli-dependent kinases

Kinase	Site	Agonists
Akt	$S^{1177/1179}$	VEGF,IGF,shear stress,estrogen,statins
AMPK	S^{1177},[a] T^{495}	$[AMP]_i$
PKC	T^{495}	PMA
PKA	S^{1177}, S^{633}	Iloprost
PKG	S^{1177}, S^{633}	N.D.
?	S^{116}	Shear stress
MAP kinase	? (not 1177)	Bradykinin

N.D. not determined,[a] In the presence of CaM/Ca^{2+}
Adapted from Fulton, D, Gratton, J. P, & Sessa, W. C. (2001) Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? The Journal of pharmacology and experimental therapeutics 299, 818–24[80]

S-nitrosylation

Beside phosphorylation, S-nitrosylation at Cys^{94} and Cys^{99} exhibits another kind of reversible post-translational modification. These amino acid residues are nitrosylated in intact cells where eNOS is targeted at the plasma membrane. Agonist stimulation supports denitrosylation, which is associated with translocation and augmented enzyme activity. Since eNOS produces nitric oxide, it provides its own source for nitrosylation.[81]

Glycosylation

Non-enzymatic glycation of proteins is known to occur in diabetes and aging.[82] Such advanced glycosylation end products accumulate in the vascular subendothelium and quench nitric oxide activity, leading to defective endothelium-dependent vasodilation.[83] Later studies also illustrated O-glycosylation of endothelial NO synthase at sites where phosphorylation regulates enzyme activity.[84] This mechanism may contribute to endothelial dysfunction in diabetes mellitus.

4.4. Substrate and BH_4 availability

The basic amino acid L-arginine is the substrate of all NO synthases. Physiological L-arginine concentration in endothelial cells depends on cationic amino acid transporter-1 (CAT-1) influx and on substrate recycling from L-citrulline.[85,86] Despite L-arginine concentration in endothelial cells was estimated to be higher

than the K_M of isolated, purified eNOS in vitro, numerous studies show beneficial effects of substrate supplementation in endothelial dysfunction.[87,88] Antagonism of the endogenous eNOS inhibitor asymmetric dimethylarginine (ADMA) could explain this "L-arginine paradox".[87]

The intracellular BH_4 concentration depends on its balance between de novo synthesis, with GTP cyclohydrolase I acting as the rate-limiting enzyme, and degradation. Recycling of the cofactor is accomplished by reduction by eNOS, ascorbic acid or dihydrofolate reductase. Since it is a powerful reducing agent, it makes sense that excessive oxidative stress may lead to BH_4 depletion. As mentioned before, tetrahydrobiopterin ensures the active dimeric constitution of endothelial NO synthase. If oxidative stress overwhelms the antioxidative protective mechanisms, BH_4will vanish and eNOS uncoupling will appear. This highlights BH_4 as a crucial cofactor for physiologic endothelial function.[89]

5. Endothelial dysfunction

In healthy humans, the endothelium exhibits antithrombotic, antiinflammatory properties and tightly regulates vasorelaxation, vascular growth and vascular remodeling. Thus development of cardiovascular and cerebrovascular diseases is prevented. Several cardiovascular risk factors perturb these physiological endothelial functions, including diabetes mellitus, cigarette smoking, as well as hypercholesterolemia and hypertension. Thereby life-threatening conditions arise.

Cardiovascular risk factors (CVRF) are known to induce production of reactive oxygen species (ROS) in the vessel wall. Among other enzymes that are able to produce ROS, NADH/NADPH oxidases (Nox) are considered as the predominant initiator of superoxide (O_2^-).[89] Under physiological conditions, Nox-family proteins have low level of constitutive activity.[90] However, if CVRF act on the vasculature, activity and expression of NADH/NADPH oxidases will increase. For instance, elevated Nox protein abundance was detected in hypercholesterolemia. On the one hand, this may be due to increased oxidised LDL (oxLDL) or on the other hand due to exaggerated activation of the angiotensin renin system, since both oxLDL and angiotensin-II (AT-II) were shown to up-regulate/activate NADH/NADPH oxidases and are present at hypercholesterolemia. There is evidence that protein kinase C is an upstream mediator of this mechanism, as PKC inhibitors reversed this effect. Elevated AT-II levels were also detected in atherosclerotic lesions, collaborating with PDGF to rise Nox-family expression.[89,91]

Enhanced oxidative stress also affects the bioactivity of nitric oxide. Superoxide has the ability to directly interact with NO, thus depleting this essential antiatherogenic factor. To make matters worse, it undergoes a very quick radical-radical reaction, generating the toxic oxidant peroxynitrite ($ONOO^-$). This reaction is fast enough to outcompete the neutralisation of superoxide by its scavenging enzyme superoxide dismutase (SOD).[88] $ONOO^-$ oxidizes BH_4, an eNOS cofactor relevant to keep the active dimeric structure, first to its BH_3* radical and then to BH_2. This not only reduces BH_4 bioavailability, but also the emerging oxidation products compete with still available tetrahydrobiopterin for eNOS binding, although they do not feature any cofactor activity. Hereby, the electron transfer from the eNOS reductase domain becomes "uncoupled" from L-arginine oxida-

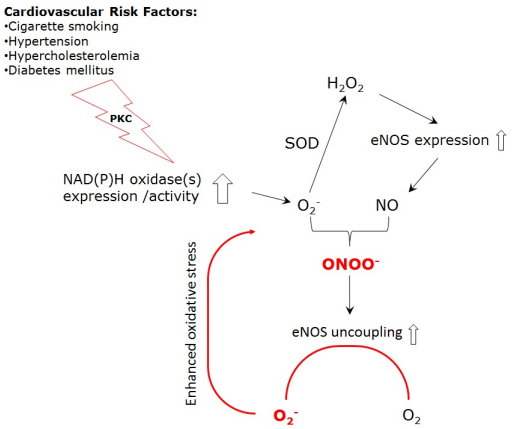


Figure 2.: Simplified illustration of endothelial dysfunction causing mechanisms
Adapted from Förstermann, Ulrich and Li, Huige, "Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling", British journal of pharmacology (2011), 213–23.

tion and subsequently eNOS turns into a superoxide producing protein. eNOS uncoupling is implicated in heart failure, ischaemia reperfusion injury, atrial fibrillation, diabetes, cigarette smoking, hypertension, and atherosclerosis.[92] Apart from O_2^-, other radicals and oxLDL were also shown to scavenge nitric oxide. The zinc-thiolat cluster inside of all eNOS isoforms is of particular importance for cofactor (BH_4) and substrate (L-arginine) binding and may also fall victim to peroxynitrite oxidation, potentiating endothelial NO synthase caused superoxide production.[93]

Despite the quick radical-radical reaction between NO and O_2^-, superoxide is partly defused by SOD, resulting in hydrogen peroxide occurrence.[89] In eukaryotes, this mechanism reduces oxidative stress, since H_2O_2 is further metabolized to water by the enzyme catalase, and additionally provides an important signaling molecule.[94] Hydrogen peroxide increases eNOS expression at least by elongation of a polyadenylation tail in the 3′-UTR, thus elevating eNOS mRNA half-life. This explains the fact, that eNOS protein is overabundant in most types of vascular diseases and closes the vicious circle.

6. Natural products used in this work

6.1. Leoligin derivatives

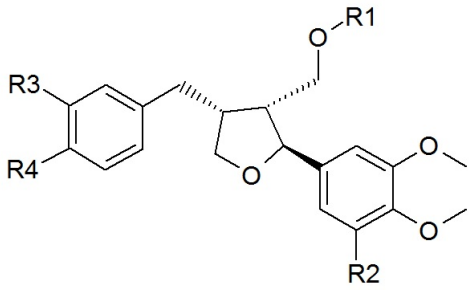


Figure 3.: Basic structure of leoligin derivatives
Used derivatives were altered in positions R1-R4

The alpine Edelweiss, *Leontopodium alpinum Cass.* (Asteraceae), is mostly found in the Alps, Carpathians, Pyrenees, the Tatra and the Balkan Peninsula. It is a well-known plant in folk medicine and has been used to ameliorate diarrhea, abdominal aches, bronchitis or fever. Recent studies also revealed anti-inflammatory and analgesic characteristics. Apart from the secondary plant products diterpenes, sesquiterpenes, benzofuranoids, *L. alpinum Cass.* synthesizes lignans.[95] Leoligin ([(2S,3R,4R)-4-(3,4-dimethoxybenzyl)-2-(3,4-dimethoxyphenyl)tetrahydrofuran-3-yl]methyl (2Z)-2-methylbut-2-enoat), a lariciresinol-type lignan, can be isolated from the roots of Edelweiss. The substance showed potent activity in an organ culture model, testing the inhibitory effect of various compounds on human saphenous vein intimal hyperplasia. Whilst leoligin was capable of inhibiting hyperplasia at 5 µM, the use of a higher concentration, 50 µM, even reversed the pre-existing pathologic transformation. The same study determined a G1-phase cell-cycle arrest of VSMC as the underlying mechanism and demonstrated decreased TNF-α-mediated VCAM-1 expression. Futhermore, no toxicity on endothelial cells was found.[96] Later *ex vivo* and *in vivo* experiments identified cholesteryl ester transfer protein activation (CETP) after treatment with leoligin as well.[97]

Leoligin was shown to cause cell-cycle arrest in the G1-phase, due to modulation of tumor suppressor p27. Since there were no toxic effects on EC, we examined whether leoligin derivatives increase endothelial NO release, which may contribute to inhibition of VSMC proliferation *in vivo*.

6.2. Natural compounds from traditional medicinal herbs

Investigation of plants used in traditional medicine is a major aim of our department and numerous pure substances have been isolated and characterized over the past years. Cell culture experiments provide the opportunity to determine the major active component in such herbal medicinal products. Thus, traditional indications can be confirmed or new ones highlighted. We obtained the following compounds from Prof. Liselotte Krenn (University of Vienna, Department of Pharmacognosy).

6.2.1. Eriosema laurentii

Eriosema laurentii De Wild, Leguminosae, is a herbaceous shrub and widespread in West and Central Africa. It is used as food and for the treatment of infertility, gynecological and menopausal problems.[98] We screened three substances derived from this plant for increased NO release in EA.hy926 cells (Tab. 2).

Table 2.: Tested substances isolated from *Eriosema laurentii*

SCI	Lupinalbin a
KMU B	5,7-Dihydroxy-2´-methoxy-6´´,6´´-dimethyl-pyrano(2´´,3´´:4´,5´)isoflavone
KMU C	6,8,3´-Triprenyl-dihydromorin

6.2.2. Ferula gummosa Boiss.

Ferula gummosa Boiss, Apiaceae, is common in Nothern and Western parts of Iran. Galbanum, the resin derived from *F. gummosa*, is commonly used in traditional Iranian medicine to treat Morbus Alzheimer.[99] Furthermore, antinociceptive, anti-inflammatory and antipyretic effects and smooth muscle relaxing activity was reported.[100] In the DAF-FM assay tested substances are listed in table 3.

Table 3.: Tested substances isolated from *Ferula gummosa Boiss.*

VF1	Deacetylkellerin
VF3	Aurapien
VF4	Kellerin
VF5	Farnesiferol A
VF6	5´-Acetoxyaurapien
VF7	5´-Hydroxyaurapien

6.2.3. Adhatoda vasica

Adhatoda vasica, Acanthaceae, is common in Asia. Because of its bronchodilator effect it has been used to treat pulmonary diseases. We tested two compounds (vasicine, vasicinon) on their NO-releasing effect.[101]

6.3. Rutaecarpine

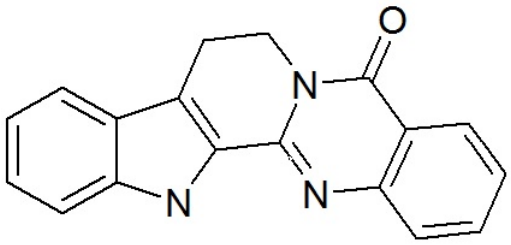


Figure 4.: Chemical structure of rutaecarpine

The dried unripened fruits of *Evodia rutaecarpa*, Rutaeceae, (Chinese name: Wu-Chu-Yu) have been used for a long time in traditional Chinese medicine (TCM) to treat headache, amenorrhea, gastrointestinal disorders and postpartum hemorrhage. Additionally, their positive inotropic/chronotropic effect and central stimulant effect are very well appreciated. Scientific phytochemical studies revealed a wide variety of compounds, present in this herbal medicinal product. They can be classified into an alkaloid and a non-alkaloid fraction (see Table 2). Rutaecarpine (7,8-dihydro-13H-indolo [2'3':3,4] pyrido [2,1-b] quinazolin-5-one) is attributed to the former, occurs as colorless needles (melting point 259-260 °C) and is soluble in alcohol, benzene, chloroform and ether but hardly in water. Its molecular weight is 287.3 g/mol.[102]

Several *in vitro* and *in vivo* studies proposed rutaecarpine as one of the main active compounds in *Evodia rutaecarpa*. It showed dose-dependent (0.1 µM - 0.1

mM) relaxation of phenylephedrine precontracted isolated rat mesenteric arterial segments. The effect was inhibited by endothelium denudation, treatment with the eNOS inhibitor L-N^G-nitro-arginine and the guanylate cyclase inhibitor methylene blue, suggesting an nitric oxide mediated mechanism.[103] The vasodilating activity was affirmed by further *in vitro* and *in vivo* studies by Wang et al. Bolus injection of rutaecarpine lowered mean arterial pressure in anesthetized rats and prevented tension development induced by norepinephrine in rings from rat aortae in an endothelium-dependent manner. Furthermore, they attributed this effect to increased intracellular Ca^{2+}concentration in endothelial cells by increased influx and to diminished Ca^{2+} concentration in the cytosol of VSMC due to decreased Ca^{2+} release from sarcoplasmatic stores.[104] However, other studies by Hu et al. attributed the vasorelaxing activity of rutaearpine to the transient receptor potential vanilloid subfamily member 1 binding properties, which is associated with calcitonin gene related peptide release, a powerful vasodilator.[105] Hence, multiple pathways are probable. In addition, Sheu et al. demonstrated an anti-platelet effect *in vivo*.[106]

These findings revealed rutaecarpine as a potential antiatherogenic drug and we therefore investigated its influence on eNOS activity.

6.4. Gallic acid derivatives

At the beginning of the 1990s, the WHO MONICA Project (Monitoring of Trends and Determinants in Cardiovascular Disease) gathered data about the incidence of cardiovascular diseases (CVD) and their causal relationship to dietary intakes in 21 countries. Despite a diet relatively rich in saturated fats, lower atherosclerotic-related death rates were highlighted in the French population. Media called this observation "The French paradox". Whether wine consumption or the associated moderate intake of ethanol accounts for this effect remained unclear. Later phytochemical investigations determined red wine as a rich source of bioactive compounds.[107] Among the non-

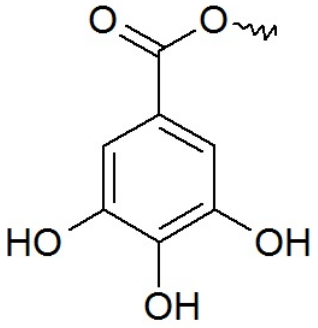


Figure 5.: Basic structure of gallic acid esters

flavonoid fraction are polyphenolic acids like gallic acid (3,4,5-trihydroxybenzoic acid, GA) and its derivative ethyl gallate.[108] Gallic acid esters only differ in atom number of the aliphatic side chain, which determines lipophilicity and physicochemical properties. Several GA esters are widely used as antioxidants in food manufacturing, as well as in pharmaceuticals and cosmetics. Antifungal, antibacterial, antimalarial, antiherpetic and ROS scavenging characteristics turn them into potent drugs.[109] Additionally, selective cytotoxicity against a variety of tumor cells enables their potential usage as antitumor agents.[109] Gallates also reduce cytokine induced NF-κB translocation to the nucleus, expression of leukocyte adhesion molecules and prevent LDL from oxidation.[110–112] Each of these mechanisms restrict atherosclerosis progression.

Since previous work from Oliver Donath (University of Vienna, Department of Pharmacognosy, not published yet) showed increased eNOS activity in ethyl gallate, propyl gallate, butyl gallate and isopentyl gallate stimulated EA.hy926 cells, we investigated the underlying regulatory pathways.

7. Aim of the work

Wealth is thought to bear the blame for the increasing incidence of "diseases of affluence" in the western society. The high caloric, high fat, modern diet and little exercise are important risk factors that contribute to the development of cardiovascular diseases, type 2 diabetes, high blood pressure, obesity, acne and depression. Atherosclerosis, the primary source of heart disease and stroke, accounts for about 50 % of all deaths in the first world.[40]

Nowadays, it is established that supernutrition, unhealthy diet and physical inactivity influence the endothelium negatively and account for the progression of atherosclerotic stages. Despite not all underlying molecular mechanisms are fully understood, decreased eNOS expression/activity plays a major role in chronic vascular disease development. eNOS upregulation and increasing the NO synthesis rate seems to be an auspicious therapeutic option for the treatment of atherosclerosis.

In our study, we therefore screened natural compounds for potential eNOS activating effects. Futhermore, we aimed at identifying the pathways upstream of eNOS activation.

Part II.

Material & Methods

1. Cell culture

1.1. EA.hy926 cells

The human endothelial cell line, EA.hy926, was created by hybridization of primary human umbilical vein endothelial cells (HUVEC) and the human lung carcinoma cell line A549/8. A549/8 is a hypoxanthine phosphoribosyltransferase (HPRT) deficient clone, which allows selection with HAT medium (hypoxanthine-aminopterin-thymidine medium). These hybridoma cells express vWF in the same morphologic distribution pattern like HUVECs, which is maintained for more than 100 cumulative population doublings. This characterises EA.hy926 cell as a permanent cell line.[113]

EA.hy926 provide several advantages over primary HUVECs. They exhibit a longer lifespan under culture conditions and grow in less complex medium. Since primary HUVEC vary genetically from donor to donor, the EA.hy926 cell line is better characterised. However, when interpreting data from cell culture experiments using cell lines, one should always consider changes in cell signaling pathways due to the hybridization process and prolonged cultivation.

EA.hy926 cells were a gift from Dr. C. -J. S. Edgell University of North Carolina, Chapel Hill (USA).[113]

1.2. Cultivation

For cultivation of the EA.hy926 cell line, we used Dulbecco´s Modified Eagle Medium (DMEM) without phenol red. We additionally supplemented 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, HAT (100 µM hypoxanthine, 16 µM thymidine, 0.4 µM aminopterin) and 10 % fetal bovine serum (FBS). FBS was not heat-inactivated. EA.hy926 cells were cultivated in 175 cm^2 in an humidified incubator at 37 °C and 5 % CO_2.

1.2.1. Thawing

EA.hy926 cells were stored in cryovials containing one million cells. After taking it out of the liquid nitrogen container, the vial was quickly defrosted in a 37 °C waterbath until a weak film of liquid medium was visible at the wall. The

still frozen cells were diluted in a previous prepared 50 ml falcon containing 15 ml pre-warmed complete medium. After resuspension with a sterile pipette, the whole amount of liquid was transferred into a 75 cm^2 flask, also containing 15 ml pre-warmed complete growth medium. The medium was replaced by fresh culture medium on the next day. Cells were passaged after about one week when confluence was reached.

1.2.2. Passaging

The EA.hy926 cells were passaged after approximately one week, when confluence was reached. Cells were washed with 30 ml pre-warmed PBS before 3 ml Trypsin/EDTA were added for trypsinisation. Subsequently, the flask was stored in the incubator for 3 minutes. The reaction was stopped by 12 ml complete pre-warmed growth medium and the whole liquid was poured into a falcon. About 0.5 ml cell suspension were transferred into the cell viability analyzer (ViCell®, Beckman Coulter) to count the cells. For cultivation 1.6 x 10^6 cells were seeded to reach confluence after one week and medium was changed after 3 days (if passaging took place on friday) or 4 days (if passaging took place on monday).

1.2.3. Solutions and buffers for cell culture

Table 4.: Solutions and buffers for cell culture

Complete growth medium (EA.hy926)	DMEM	500 ml
	L-Glutamine	2 mM
	penicillin	100 U/ml
	streptomycin	100 µg/ml
	FBS	10 %
	hypoxanthine	100 µM
	aminopterin	0.4 µM
	thymidine	16 µM
PBS pH 7,4 (autoclaved)	NaCl	123 mM
	Na_2HPO_4	10 mM
	KH_2PO_4	3.2 mM
	ddH_2O	ad 1000 ml
Trypsin/EDTA solution (sterile-filtered)	trypsin	0.05 %
	EDTA	0.02 %
	in PBS	

2. Quantification of nitric oxide by 4-Amino-5-Methylamino-2',7'-Difluorofluorescein (DAF-FM)

2.1. Principle

Low levels of biosynthesized nitric oxide, degradation by superoxide as well as the short half life complicate reliable measurements of this multi functional signaling molecule *in vivo* and *in vitro*. Kojima et al. developed a specific and sensitive method to detect and quantify nitric oxide in cell culture experiments. They designed and synthesised diaminofluoresceins as fluorescence indicators. The reaction is based on the reactivity of NO with two aromatic vicinal diamines in the presence of dioxygen, yielding a highly green-fluorescent triazole form (DAF-2T). Kojima et al. reported a detection limit of 5 nM for this method, which is low enough to enable quantification of nitric oxide in cell culture studies. DAF-2-NO-measurement is also hallmarked by significant selectivity, as the dye does not react with other reactive oxygen of nitrogen species, like peroxynitrite or superoxide.[114] Otherwise, bivalent cations (like Ca^{2+}) increase fluorescence signals. Since the cells were incubated with calcium ionophore A23187 during the DAF-FM assay, a substance that facilitates Ca^{2+} influx into the cytosol, we used the NOS inhibitor L-NAME to detect Ca^{2+} caused non-specific fluorescence.[115] The assay was optimized for 96-well plates. A resazurin assay following the DAF-FM assay normalized seeding variations.

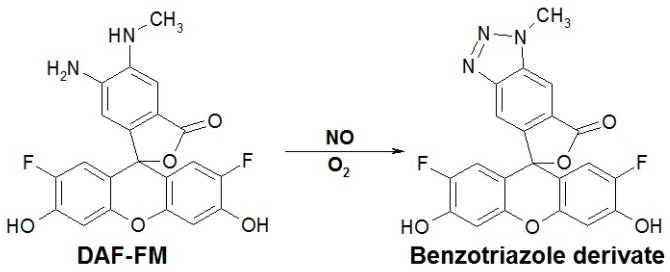


Figure 6.: Structure of DAF-FM and its benzotriazole derivative

2.2. Experimental Procedure

EA.hy926 cells were seeded in 96-well plates at a density of approximately 2.5 x 10^4 cells/well and incubated for approximately 72 h. At confluence, cells were treated with test compounds (positive control = 3 µM Mevastatin, vehicle = 0.1% DMSO), following a 24 h incubation period. The compound dilutions were prepared right before use. On the fourth day, the treated cells were washed two times with PBS^+, containing 100 µM arginine and equilibrated for 10 minutes. Then calcium ionophor A23187 and DAF-FM were added at a final concentrations of 1 µM and 0.1 µM, respectively. Additionally, some rows were stimulated with L-NAME to detect non-specific fluorescence. After 1 h incubation at 37 °C the supernatant was transferred into black 96-well plates and measurement of the fluorescence with an excitation wavelength of 485 nm and an emission wavelength of 520 nm was executed. To normalize the obtained values to the cell count, a resazurin assay followed. We therefore washed the cells wit PBS, added 0.1 mg/ml resazurin in PBS and incubated for another hour. Fluorescence was measured at an excitation wavelength of 535 nm an an emission wavelength of 590 nm.

2.3. Solutions and buffers

Table 5.: Solutions and buffers for DAF-2 assay

PBS+ (autoclaved)	NaCl	8.0 g
	KCl	0.2 g
	Na_2HPO_4	1.15 g
	KH_2PO_4	0.2 g
	$MgCl_2$ x 6 H_2O	0.1 g
	$CaCl_2$ x 2 H_2O	0.1 g
	ddH_2O	ad 1000 ml
PBS^+ + Arg	100 mM L-arginine stock solution diluted 1:1000 in PBS^+	
DAF-FM solution	DAF-FM stock solution diluted in 1:250 in PBS^+	
DAF-FM stock	5 mM DAF-FM in DMSO, stored at -80 °C	

3. Cell viability assay

3.1. Principle

Several different cell viability assays have been established to determine cytotoxic effects in treated cells. They range from simple dye exclusion assays that measure membrane integrity and the effect of drugs on cell growth, to other techniques that determine cell viability indirectly, by measuring the ability of cells to reduce compounds.[116] In our work we used the resazurin cell viability assay (also known as Alamar Blue assay). The method is ascribed to the metabolic activity assays, since usually mitochondrial NADPH/NADH dehydrogenases facilitate the reaction of the non-fluorescent dye resazurin to the pink and highly fluorescent resorufin. Cytotoxic substances lower the metabolic activity, as well as the redoxpotential in cells. This causes diminished or depleted reduction of resazurin.[117]

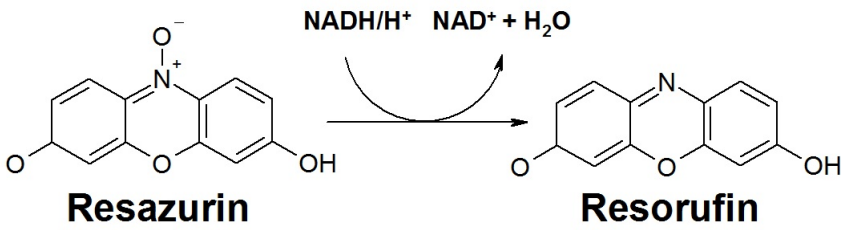


Figure 7.: Reduction of resazurin to resorufin

3.2. Experimental Procedure

EA.hy926 cells were seeded at a density of 1.0 x 10^4 cells/well in 96-well plates and incubated for approximately 72 h. When confluence was reached, the cells were treated with selected compounds. The compound dilutions were prepared right before use. After 24 h, the medium was aspirated and 0.01 mg/ml resazurin solution was added and another incubation period of 2 h (37 °C) followed, before fluorescence measurement was performed (excitation wavelength at 535 nm, emission wavelength at 590 nm)

4. Measurement of eNOS mRNA levels by quantitative real-time PCR

4.1. Principle

Quantitative real-time polymerase chain reaction (qPCR) is a powerful method to obtain reliable data on gene expression levels in cell culture studies. The experimental procedure consists of several steps. First of all, the cells have to be lysed before the RNA can be purified. In our study we used a kit based on spin columns to simplify isolation and to avoid toxic chemicals (peqGOLD total RNA Kit (peqlab)). Then extracted mRNA is converted into cDNA by reverse transcription. The cDNA population accurately reflects the mRNA population in terms of transcript abundance. During the following rt-qPCR, performed with the LightCycler™ LC480 (Roche Diagnostics) system, the cDNA undergoes many temperature cycle repeats. At the beginning, the double-stranded DNA is denatured at 95 °C. In a second step, primers are annealed at lower temperature. The chosen temperature depends on the primer sequence. Finally, the synthesized DNA is elongated by taq-polymerase, a heat-stable DNA polymerase originally from the thermophilic bacterium *Thermus aquaticus*. During each cycle, the amount of DNA is doubled, which leads to exponential increase in PCR products. This amplification can be monitored by DNA double-strand-specific fluorescence dye and the resulting fluorescence curves allow drawing conclusions from the originally present mRNA levels in the sample. Additionally, melting point analysis can be performed to check the quality of the qPCR reaction. The product is slowly heated up until a sharp drop in SYBR® green fluorescence is observed, at the temperature when DNA double strands denaturation occurs. Such melting temperatures provide information on the accuracy of the measurement, as they are specific for every DNA sequence.

We used constantly expressed 18S ribosomal RNA as an internal control to normalize RNA levels.

4.2. Experimental procedures

EA.hy926 cells were seeded in 6-well plates at a density of 0.5 x 10^6 cells/well. Once confluence was reached after approximately 72 h, the cells were treated with selected compounds for 1 h, 4 h, 8 h, 12 h and 24 h, respectively.

4.2.1. RNA extraction

We used the extraction kit peqGOLD Total RNA KIT (peqlab®) and followed the manufacturer's recommended instructions. To lyse the cells, 400 µl RNA lysis buffer were added to each well, containing treated EA.hy926 cells. Additionally, we scraped the cells with a cell scraper. Lysates were stored under nitrogen at -80 °C until RNA isolation. DNA was removed with DNA removing columns at the beginning of the RNA purification process. By adding 400 µl of 70 % ethanol to the column and vigorously vortexing, the RNA was attached to the RNA binding columns. DNA remnants were digested by peqGOLD DNase I Digest Kit (peqlab®) to improve RNA quality. After several washing steps, the RNA was eluted with 50 µl RNAse free water. The quality of the purified RNA was checked spectrophotometrically with a Nanodrop® 2000c. Values for ratio of absorbance at 260 nm and 280 nm usually lay above 2, which reflects high purity. RNA extracts were stored at -80 °C.

4.2.2. cDNA synthesis

For reverse transcription of the purified RNA to cDNA we used the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®). An aliquot of the isolated RNA, up to 500 ng RNA, was mixed with 2.0 µl 10X RT Buffer, 0.8 µl 25X dNTP Mix (100 mM), 2.0 µl 10X RT Random Primers, 1.0 µl MultiScribe™ Reverse Transcriptase, and 1.0 µl RNase Inhibitor in a PCR strip and filled up with Nuclease-free H_2O to a total amount of 20 µl. After sealing and gentle mixing, the liquid was spinned down. Afterwards, the strips were transferred to a thermal cycler. The cDNA was processed immediately or stored under nitrogen at -80 °C.

Table 6.: Reaction settings for cDNA synthesis

	step 1	step 2	step 3	step 4
temperature	25 °C	37 °C	85 °C	4 °C
duration	10 min	120 min	5 min	∞

4.2.3. Quantitative real-time PCR

LightCycler® LC480 (Roche Diagnostics) and the LightCycler® 480 SYBR Green I Master reagent, a fluorescence dye that binds to double stranded DNA by intercalating with the minor groove, was used for quantitative real-time polymerase chain reaction. For this method, eNOS cDNA was diluted 1:10 with RNAse-free water. First, 2 µL of the 1:10 diluted DNA were pipetted into 96-well PCR plates, followed by addition of 13 µL reaction mix, containing 7.5 µL SYBR®Green I Master Mix, 1.5 µL primer solution and 4 µL PCR-grade water. eNOS primer were obtained from Quiagen (QuantiTect® Primer assays) and 18S primer from Invitrogen. The reaction conditions accorded to manufacturer's instructions. Sampels were measured in triplicate. Data were analyzed using LightCycler® LC480 and further evaluation was executed in Microsoft Excel.

Table 7.: Amino acid sequences of the used primers

gene	direction	sequence (5' to 3')
18S	forward	GAA TTG ACG GAA GGG CAC CAC CAG
	reverse	GTG CAG CCC CGG ACA TCT AAG G
eNOS	forward	CAC TGT GAT GGC CGA GCG AAG GTT G
	reverse	GCT TAG GGA GAG CGA GCT GGT GTT

Table 8.: rt-qPCR reaction conditions

step	temperature	time
denaturation	95 °C	10 min
cycling (50 x)	95 °C	5 sec
	61 °C	5 sec
	72 °C	15 sec
melting	95 °C	5 sec
	60 °C	10 sec
	97 °C	0.5 sec
cooling	40	∞

5. Western blotting

5.1. Principle

Western blotting is an important method in molecular biology to identify specific proteins among others. At first, cells are lysed and proteins solubilized. Since eNOS sticks firmly to other regulatory proteins (e.g. caveolin-1), the use of the strong zwitterionic detergent CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) in the lysis buffer is necessary. The substance is commonly used as a non-denaturing solvent for purifying membrane proteins. Furthermore, cells were sonicated for better lysis results. By boiling and addition of β-Mercaptoethanol (and dithiothreitol (DTT) in the lysis buffer), the proteins become denatured and disulfide bonds are reduced to thiols. Sodium dodecyl sulfate (SDS), another detergent, binds to the proteins, forces them to take a rod-like form and charges them negatively in a uniform mass-charge-ratio. Therefore discontinuous gel electrophoretic separation by their molecular weight is possible. Subsequent, the separated proteins are transferred to polyvinylidene difluoride (PVDF) membrane. To avoid unspecific antibody binding, the membranes are blocked with bovine serum albumin or milk. Detection of the protein of interest requires two antibodies. The primary antibody targets the specific protein, whilst the secondary antibody, binds to the primary one. Enzymatic activity of the reporter enzyme horseradish peroxidase, conjugated to the secondary antibody, enables measurement of protein abundance, by producing a luminescence signal. The signal intensity depends on the protein amount.

5.2. Experimental procedures

5.2.1. Cell lysis

EA.hy926 cells were seeded at a density of 0.5 x 10^6 in 6-well plates. After an incubation period of approximately 72 h at 37 °C, confluence was reached and cells were treated with compounds for 24 h. Afterwards, cell layers were washed twice with ice-cold PBS and put on ice. Then 100 µl lysis buffer were added, followed by thoroughly scraping with cell scrapers. The lysates were incubated for 15 minutes on ice after transfer to reagent tubes. After sonicating the lysates

for 30 seconds, the tubes were put in the centrifuge for 40 minutes (4 °C, 16000 g). To determine the protein concentration using the Bradford method, 5 µl of the supernatant were taken off and transferred to 0.5 ml reagent tubes. The remaining supernatant (about 80 µl) was mixed with 3x sample buffer (80 µl lysate : 40 µl 3x sample buffer = 2 : 1) and boiled for 10 minutes at 95 °C on a dry block heater. The finished whole cell lysates were stored at -20 °C.

5.2.2. Protein quantification

5 µl of the EA.hy926 cell lysate were diluted 1:20 with ddH_2O to a total volume of 100 µl. 10 µl of this dilution were transferred to 96-well plates in triplicate as well as BSA standards (0 - 25 µg/ml final BSA concentration). Finally, 190 µl Bradford reagent (1:5 dilution of Roti®-Quant, Carl Roth GmbH) were added to measure protein concentration at 595 nm at a Tecan Sunrise™ (Tecan) microplate reader after 10 minutes incubation on a shaker.

5.2.3. SDS-PAGE

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were prepared using a 30 % polyacrylamide (PAA) solution (Rotiphorese®Gel 30, Carl-Roth GmbH, contains 0.8 % bisacrylamide) and a Mini-PROTEAN™ 3 Cell System (BIO-RAD). The final concentration of polyacrylamide (PAA) in the resolving gel was 7.5 %. Every lane was loaded with 30 µg protein. Proteins were separated at 20 mA per gel for 90 min.

5.2.4. Western Blotting

A Mini Trans-Blot® Electrophoretic Transfer Cell System was used to transfer the proteins to a PVDF membrane. Transfer conditions were 100 V for 85 min. The membrane was blocked in 5 % fat-free milk powder in TBS-T for two hours and washed three times for 10 min in TBS-T.

5.2.5. Detection

Antibodies were diluted as listed in Tab. 9. Membranes were incubated with primary antibody solutions at 4 °C over night and washed the next day 3 times with TBS-T for 10 min. Afterwards, incubation for 2 h with secondary antibody at room temperature and again washing 3 times with TBS-T for 10 min followed. For detection, the membranes were then incubated in homemade ECL-solution

Table 9.: Antibodies used for Western Blotting

type	target	kDA	dilution	species	provider
primary antibodies	eNOS	140 kDa	1:2500 in 1x TBS-T	mouse	BD Biosciences
	actin	42 kDa	1:1000 in 1x TBS-T	mouse	MP Biochemicals
secondary antibody	mouse IgG	-	1:1000 in 1x TBS-T	goat	MP Biochemicals

for 1 min. Luminescence signals were detected by a LAS-3000™ (Fujifilm) luminescent image analyzer and quantified by densitometric analysis using AIDA software (raytest). eNOS-protein band intensities were normalized to actin levels on the same lane.

5.2.6. Solutions and buffers

Table 10.: Solutions and buffers used for Western Blotting

PBS pH 7.4 (autoclaved)	NaCl	123mM
	Na_2HPO_4	10 mM
	KH_2PO_4	3.2 mM
	ddH_2O	ad 1000 ml
lysis buffer stock pH 7.5	Tris-HCl	50 mM
	CHAPS	20 mM
	EDTA	0.5 mM
	EGTA	0.5 mM
	DTT	2 mM
	glutathione	7 mM
	glycerol	10 %
	ddH_2O	ad 25 ml

mixture	ingredients	concentration
phosphatase inhibitor stock 20x	NaF	0.2 M
	Na_3VO_4	40 mM
	$Na_4P_2O_7$ * 10 H_2O	0.1 M
	ddH_2O	ad 5 ml
lysis buffer (prepared right before use)	lysis buffer stock	637 µL
	complete™ protein inhibitor 25x	28 µL
	phosphatase inhibitor stock 20x	35 µL
Western blot sample buffer 3x	Tris-HCl pH 6.8	187.5 mM
	SDS	0.2 M
	glycerol	30 %
	bromphenol blue	0.2 mM
	ddH2O	ad 1000 ml
	β-Mercaptoetnahol (added right before use)	15 %
resolving gel	PAA (Rotiphorese®Gel 30, 30 % PAA)	7.5 %
	Tris-HCl pH 8.8	375 mM
	SDS	0.1 %
	TEMED	0.1 %
	APS	0.05 %
	ddH_2O	ad 7.5 ml
stacking gel	PAA (Rotiphorese®Gel 30, 30 % PAA)	5 %
	Tris-HCl pH 6.8	125 mM
	SDS	0.1 %
	TEMED	0.2 %
	APS	0.1 %
	ddH_2O	ad 3.75 ml
SDS-Running buffer 10x	Tris-base	248 mM
	glycine	1.9 M
	SDS	35 mM
	ddH_2O	ad 1000 ml

mixture	ingredients	concentration
Blotting buffer 5x	Tris-base	125 mM
	glycine	971 mM
	ddH_2O	ad 1000 ml
Blotting buffer 1x	Blotting buffer 5x	200 ml
	methanol	200 ml
	ddH_2O	ad 1000 ml
TBS-T pH 8.0 10x	Tris-base	248 mM
	NaCl	1.9 M
	Tween-20	1 %
	ddH_2O	ad 1000 ml
homemade	Tris-base pH 8.5	100 mM
ECL-solution	luminol	1.24 mM
	p-coumaric acid	0.2 mM
	H_2O_2	0.018 %
	ddH_2O	ad 10 ml

6. Statistical analysis

We used GraphPad PRISM™ software (GraphPad Software Inc) for statistical analysis. One-way analysis of variance (one-way ANOVA) was used to compare multiple treatment groups with the control. $P < 0.05$ was considered significant. All graphs are showing means ± SD.

Part III.

Results

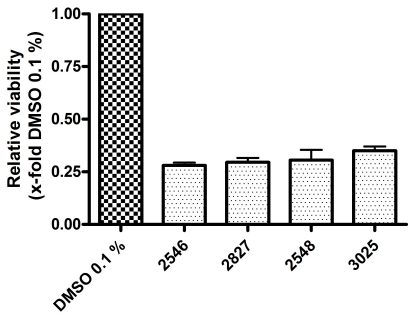
1. Screening of eNOS modulating natural compounds

1.1. No influence of Leoligin derivatives on endothelial NO release

It was demonstrated that leoligin causes G1-phase cell-cycle arrest in vascular smooth muscle cells.[96] Physicians may take advantage of this effect to prevent intimal hyperplasia after coronary artery bypass grafting and percutaneous coronary intervention. Such treatment avoids restenosis and increases the chance of success after surgery. Both, leoligin and nitric oxide cause cell-cycle arrest due to modulation of tumor suppressor p27. Therefore, we investigated whether NO is a potential downstream signaling molecule of leoligin, leading to G1-arrest.

Several semisynthetic leoligin derivatives were available at our department. Substances were ranked by their inhibitory effect on VSMC proliferation, which was assessed from our working group in previous studies (data not shown). The 20 most promising compounds were tested. EA.hy926 cells were treated with 30 µM leoligin derivatives for 24 hours and NO release was determined.

Obtained data from our experiments showed no increase in endothelial NO release. Since toxic compounds yield false-positive effects, these results are not shown in Fig. 9. Viability data from the toxic substances are shown in Fig. 8.



EA.hy926 cells were treated with 30 µM leoligin derivatives for 24 h and resazurin conversion was measured. The figure shows the normalized (DMSO 0.1 %) cell viability data of the 4 toxic compounds. (mean ± SD, n = 2)

Figure 8.: Toxic leoligin derivatives

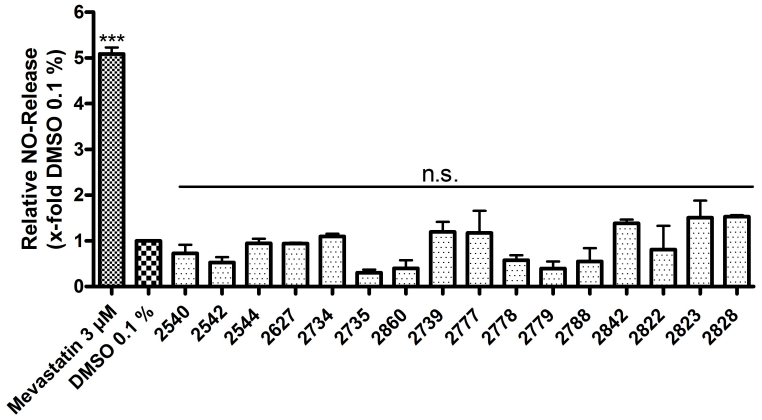


Figure 9.: Leoligin derivatives showed no effect on endothelial NO release
EA.hy926 cells were stimulated with 30 µM leoligin derivatives for 24 h and endothelial NO release was determined. NO production was normalized to the untreated control group (0.1 % DMSO). Toxic compounds are not shown. (***, $p < 0.001$; mean ± SD; n = 3)

1.2. Natural compounds from traditional medicinal herbs showed no influence on endothelial NO release

EA.hy926 cells were treated with the indicated compounds for 24 h. However, we could not observe a significant effect of these compounds on endothelial NO release (Fig. 10, 12,13). Viability data from the toxic compound KMU C are shown in Fig. 11.

EA.hy926 cells were stimulated with 30 µM SCI, KMU/B and KMU/C for 24 h and endothelial NO release was determined. NO production was normalized to the untreated control group (0.1 % DMSO). The toxic compound KMU/C is not shown. (mean ± SD; n = 3)

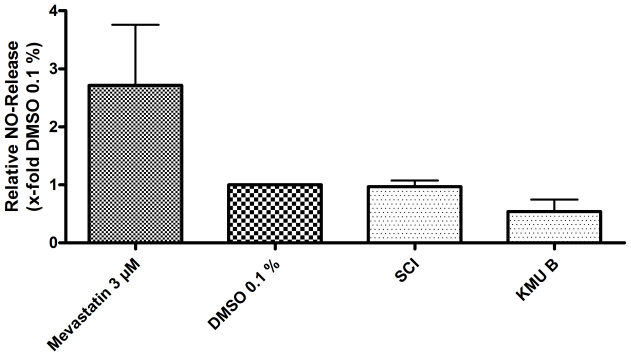
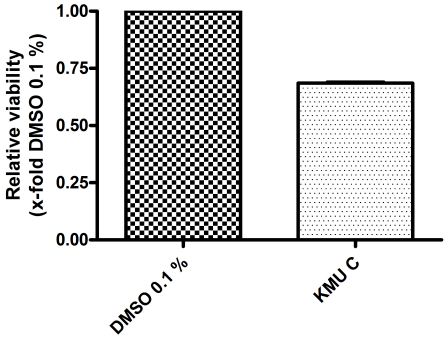
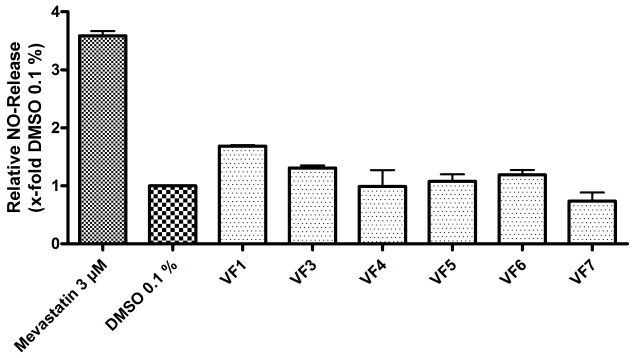


Figure 10.: Substances from *Eriosema laurentii* showed no increase in endothelial NO release



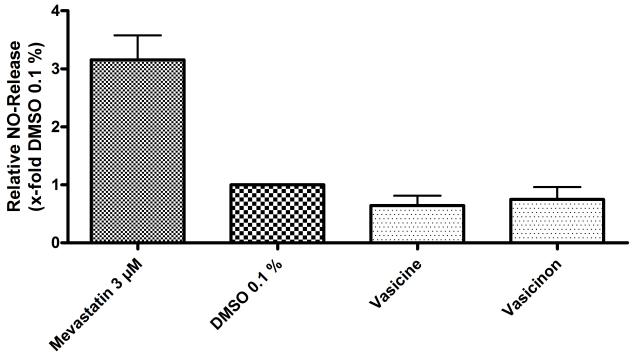
EA.hy926 cells were treated with 30 µM KMU C for 24 h and resazurin conversion was measured. The figure shows the normalized (DMSO 0.1 %) data from the cell viability assay. (n = 2)

Figure 11.: Toxic compound from *Eriosema laurentii*
EA.hy926 cells were treated with 30 µM KMU C for 24 h and DAF-FM experiments were conducted. The figure shows the normalized (DMSO 0.1 %) cell viability data of the 4 toxic compounds. (mean ± SD, n = 2)



EA.hy926 were stimulated with 30 µM VF1, VF3, VF4, VF5, VF6, VF7. After incubation for 24 h endothelial NO release was determined. NO production was normalized to the untreated control group (0.1 % DMSO). (mean ± SD, n = 2)

Figure 12.: Substances from *Ferula gummosa* showed no increase in endothelial NO release



EA.hy926 were stimulated with 30 µM Vasicine and Vasicinon. After incubation for 24 h endotheial NO release was determined. NO production was normalized to the untreated control group (0.1 % DMSO). (mean ± SD, n = 2)

Figure 13.: Substances from *Adhatoda vasica* showed no increase in endothelial NO release

1.3. Controversial data from rutaecarpine experiments

Previous *in vitro* and *in vivo* studies have shown that rutaecarpine dilates arteries. Since denudation of the endothelium and treatment with the NOS inhibitor L-N^G-nitro-arginine decreased vasorelaxation, the effect seems to be endothelium-dependent and suggests an at least partly eNOS involving mechanism.[103] In hitherto studies from our working group, endothelial NO release, performing DAF-FM assay, as well as eNOS activity, performing [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay, were measured. These experiments were carried out by Dieu Linh Nguyen in the course of her PhD thesis and showed a significant effect on eNOS activity and endothelial NO release (Fig. 14 & 15). We planned to investigate upstream mechanisms that cause eNOS activation after stimulation with rutaecarpine. Therefore, DAF-FM and [^{14}C]L-arginine/[^{14}C]L-citrulline conversion experiments were repeated to confirm former results. Cells were treated with 10 µM, 5 µM, 3 µM and 1 µM rutaecarpine for 24 h.

Obtained data from the first DAF-FM experiment matched with the former results (Fig. 16) and showed an increase in released endothelial nitric oxide. However, elevation of endothelial NO release (Fig. 17) and eNOS activity (data not shown) completely disappeared in further experiments.

EA.hy926 cells were stimulated with 5 µM, rutaecarpine for 24 h and endothelial NO release was determined. NO production was normalized to the untreated control group (0.1 % DMSO). Experiments were performed by Dieu Linh Nguyen (**, $p < 0.01$; mean ± SD; n = 3)

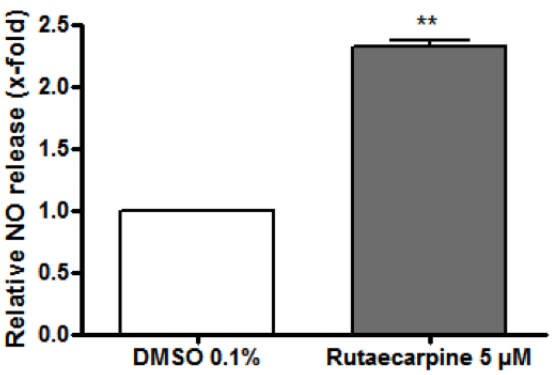
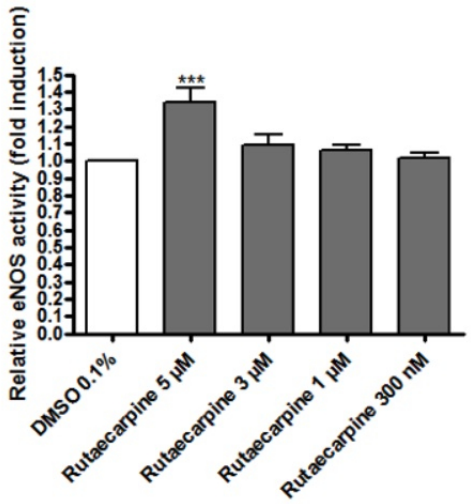
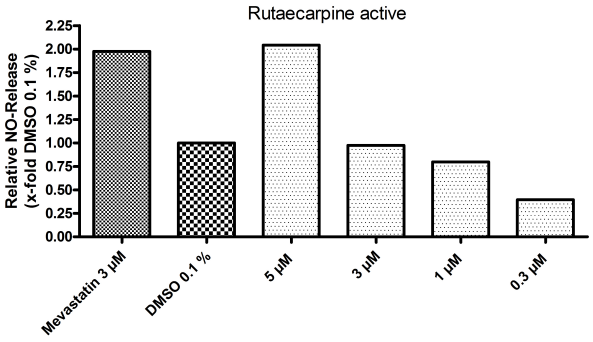


Figure 14.: Significant effect of rutaecarpine on NO release



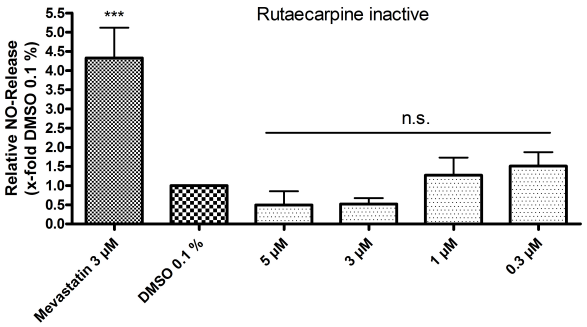
EA.hy926 cells were stimulated with 5 µM, 3 µM, 1 µM and 0.3 µM rutaecarpine for 24 h and conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline was determined. Data was normalized to the untreated control group (0.1 % DMSO). Experiments were performed by Dieu Linh Nguyen. (***, $p < 0.001$; mean ± SD; n = 3)

Figure 15.: Significant effect of rutaecarpine on eNOS activity



EA.hy926 cells were stimulated with 10 µM, 5µM, 3 µM and 1 µM rutaecarpine for 24 h and endothelial NO release was determined. NO production was normalized to the untreated control group (0.1 % DMSO). The figure shows the outcome of the first dose-response experiment. (n = 1)

Figure 16.: Effect of rutaecarpine on endothelial NO release could be reproduced



EA.hy926 cells were stimulated with 10 µM, 5µM, 3 µM and 1 µM rutaecarpine for 24 h and endothelial NO release was determined. NO production was normalized to the untreated control group (0.1 % DMSO). The figure shows representative results of further attempts using rutaecarpine from different stock solutions. (***, $p < 0.001$; n.s., not significant, n = 3)

Figure 17.: Significant effect of rutaecarpine on NO release was not reproducible

1.4. Rutaecarpine did not affect cell viability at applied concentrations

Simultaneously to the DAF-FM and [^{14}C]L-arginine/[^{14}C]L-citrulline experiments, the resazurin conversion method was performed to determine cell viability after treatment with different concentrations of rutaecarpine. EA.hy926 cells were treated with 5 µM, 3 µM, 1 µM and 0.3 µM rutaecarpine for 24 hours. At the applied concentrations, Rutaecarpine did not significantly influence the cell viability (Fig. 18).

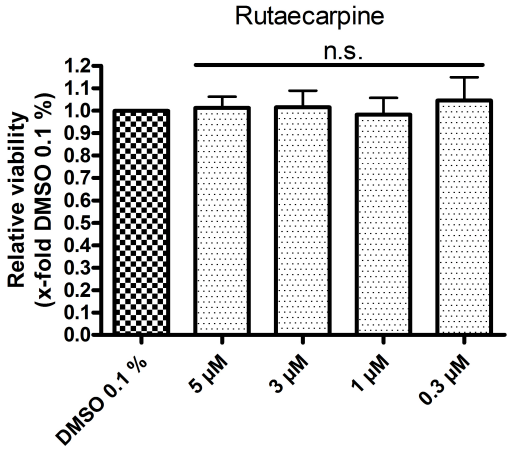


Figure 18.: Rutaecarpine did not affect cell viability
EA.hy926 cells were treated with 5 µM, 3 µM, 1 µM and 0.3 µM rutaecarpine for 24 h and cell viability, performing the resazurin conversion method, determined. Data were normalized to the untreated control group (DMSO 0.1 %). No toxic effect was shown. (n.s., not significant; n = 3)

Microscopic examination of the stimulated EA.hy926 cells showed crystallization in each well. We hypothesized that this is due to the weak solubility of rutaecarpine in aqueous medium. The effective concentrations in our study thereby differed from the stimulated concentration. Since these solubility problems made further experiments unreliable, we stopped the investigation of the effect of rutaecarpine on eNOS.

1.5. No effect of gallic acid derivatives on NO release

In previous *in vitro* studies, NO-dependent S-nitrosylation of NF-κB subunit p50 in ethyl gallate treated HUVECs was demonstrated.[110] Futhermore, it was shown that NOS isozymes facilitate inhibition of the NF-κB pathway, by S-nitrosylation

of subunit p65.[112] These post-translational modifications regulate cytokine induced inflammation processes in cells. Oliver Donath (University of Vienna, Department of Pharmacognosy) isolated ethyl gallate from red wine. Furthermore, he investigated the effect of several GA esters on eNOS activity in EA.hy926 cells by the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion method. The cells were stimulated with the compounds at concentrations of 10 µM and 50 µM for 24 h. Ethyl gallate, propyl gallate, butyl gallate and isopentyl gallate were able to significantly increase eNOS activity in these experiments (data from Oliver Donath not shown). We therefore examined whether eNOS activation also results in increased NO release. EA.hy926 cells were stimulated with 10 µM of GA derivatives to identify the most active substance.

Obtained data did not show any significant increase in endothelial NO release (Fig. 19).

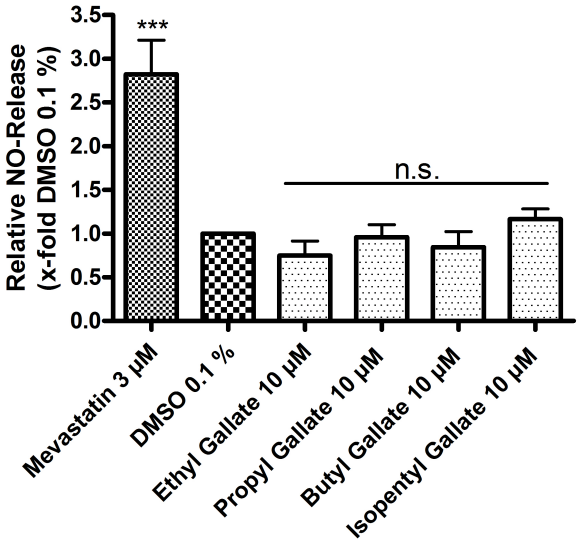


Figure 19.: No effect of GA derivatives on endothelial NO release
EA.hy926 cells were stimulated with 10 µM gallic acid derivatives for 24 h and endothelial NO release was determined. NO production was normalized to the untreated control group (0.1 % DMSO). (***, $p < 0.001$; n.s., not significant, $n = 4$)

Simultaneously, the derivatives were screened for eNOS activity in the [^{14}C]L-arginine/[^{14}C]L-citrulline assay to confirm former results. Experiments were conducted by Dieu Linh Nguyen. Propyl gallate and butyl gallate exhibited the strongest effect on eNOS activity (Fig. 20).

Although, the detection limit for NO in the DAF-FM assay is low, we supposed that higher concentrations of GA derivatives are needed to determine an effect

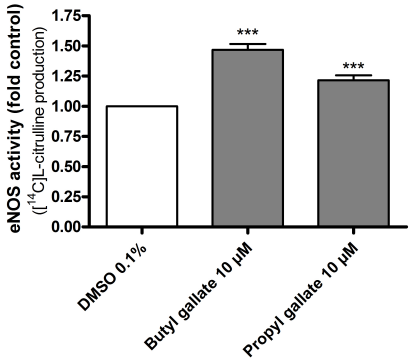


Figure 20.: Most active gallate derivatives in the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion method

EA.hy926 cells were stimulated with 10 µM gallate derivatives for 24 h and conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline was determined. Data were normalized to the untreated control group (0.1 % DMSO). Data from the most active substances are shown. Experiments were performed by Dieu Linh Nguyen. (***, $p < 0.001$; mean ± SD; $n = 3$)

under the chosen conditions. Such concentrations are not contradictory to feasible *in vivo* concentrations, since studies addressing the bioavailability of gallic acid in humans revealed that it is very well absorbed.[118] Therefore, we conducted dose-response experiments for the most active substances in the DAF-FM assay. The highest concentration used was 30 µM (Fig. 21 & 22).

Even EA.hy926 cells stimulated with higher concentrations of gallic acid derivatives did not show an increase in endothelial NO release (Fig. 21 & 22). Since there was no dose-response effect visible in our results, we supposed that the DAF-FM method is not ideal to detect NO induced by gallates, as also stated in the discussion. Hence, experiments were conducted just in duplicate.

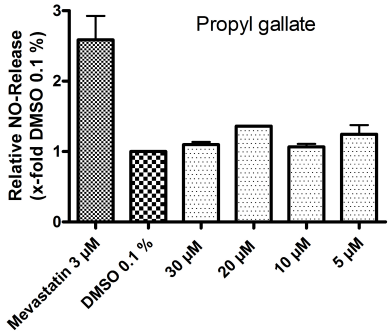


Figure 21.: Dose-response experiments for propyl gallate

EA.hy926 cells were stimulated with 30 µM 20 µM, 10 µM and 5 µM propyl gallate for 24 h and endothelial NO release was determined. NO production was normalized to the untreated control group (0.1 % DMSO).(mean ± SD, $n = 2$)

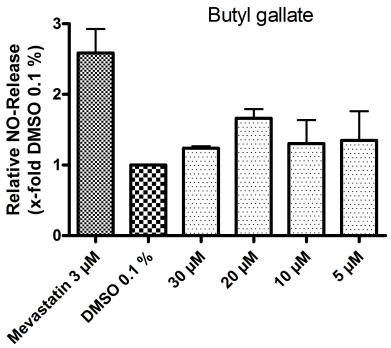


Figure 22.: Dose-response for butyl gallate in endothelial NO release experiments
EA.hy926 cells were stimulated with 30 µM 20 µM, 10 µM and 5 µM butyl gallate for 24 h and endothelial NO release was determined. NO production was normalized to the untreated control group (0.1 % DMSO). (mean ± SD, n = 2)

1.6. Gallic acid did not affect cell viability at applied concentrations

A good pharmaceutical substance not only exhibits strong activity, but also shows minimal toxic effects in therapeutical dosage. Therefore, we treated EA.hy926 cells with GA derivatives and cell viability was assessed by the resazurin conversion method. Data were provided by our technical assistant Daniel Schachner.

No cell toxicity on EA.hy926 cells was observed (Fig. 23). Our results confirm other reports where selective toxicity of gallic ester derivatives on tumor cells was highlighted.[109]

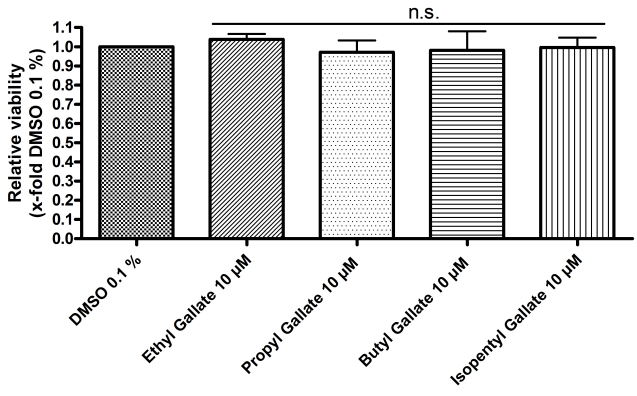


Figure 23.: Cell viability of EA.hy926 cells treated with gallic acid derivatives
EA.hy926 cells were treated with 10 µM ethyl gallate, propyl gallate, butyl gallate and isopentyl gallate for 24 h and cell viability was determined. Data were normalized to the untreated control group (DMSO 0.1 %). Experiments were conducted by Daniel Schachner. No toxicity was shown. (mean ± SD, n = 3)

2. Underlying mechanisms of eNOS activation by gallic acid derivatives

There are several limitations for the determination of an increase in endothelial NO release via the detection of chemiluminescence using DAF-FM reagent (see discussion). Quenched signals by nitric oxide scavenging reactive oxygen species are a main problem. Aliphatic esters of gallic acid have several pharmacological properties, especially antitumoral activity against lung cancer and other tumor cell lines. A major mechanism by which gallic acid esters cause apoptosis is the induction of ROS.[109] Therefore, we relied on the data from the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay and continued with the most effective substance butyl gallate.

2.1. Butyl gallate did not increase eNOS protein abundance

Significant conversion of L-arginine to L-citrulline by the endothelial NO synthase is not necessarily associated with elevated enzyme activity. Increased eNOS protein abundance causes the same output in this assay. Hence, a rise in eNOS activity is undistinguishable from an increase in eNOS quantity. Western blotting is not only a useful molecular biological technique to identify proteins, but also to investigate changes in protein levels. Therefore, EA.hy926 cells were treated with 10 µM, 5 µM, 3 µM and 1 µM butyl gallate for 24 hours and western blot analysis was performed. Mevastatin was used as a positive control.
As shown in Fig. 24, no elevation in eNOS protein abundance after 24h stimulation was observed.

2.2. Butyl gallate increased eNOS mRNA levels in EA.hy926 cells

Correlation between mRNA and protein abundance was found to be weak in biological systems, because there is no linear relationship between transcription and translation.[119] We therefore performed rt-qPCR experiments to investigate whether treatment of butyl gallate affects eNOS mRNA levels. EA.hy926 cells

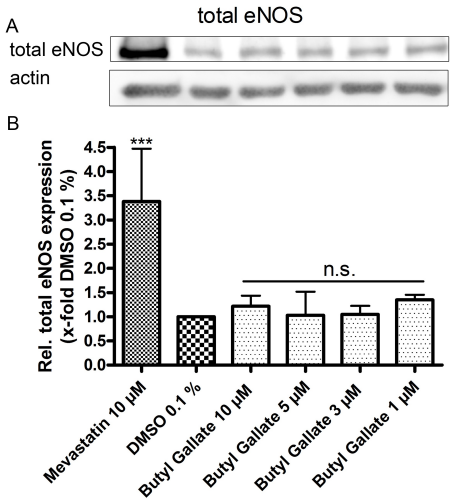


Figure 24.: Total eNOS protein levels are not changed by treatment of endothelial cells with butyl gallate

EA.hy926 cells were treated with 10 µM, 5 µM, 3 µM and 1 µM butyl gallate for 24 h and Western Blot analysis was performed. (A) Band intensities were normalized to actin and expressed as fold untreated control. (B) Results from three different experiments were normalized on the untreated control group (DMSO 0.1 %). No significant changes in protein abundance were detected. (***, $p < 0.001$; n.s., not significant; mean ± SD; n = 3)

were treated with 10 µM butyl gallate for 1 h, 4 h, 8 h, 12 h and 24 h.

eNOS mRNA levels were elevated by approximately fivefold after 8 h and 24 h in our experiments. After an incubation period of 12 h, eNOS mRNA content dropped to basal levels (Fig. 24).

These findings suggest that although eNOS mRNA levels are increased, protein expression is not influenced. An interpretation of this data is very difficult as these results cannot explain the increase in eNOS activity. We recommend to repeat the experiments to confirm these results.

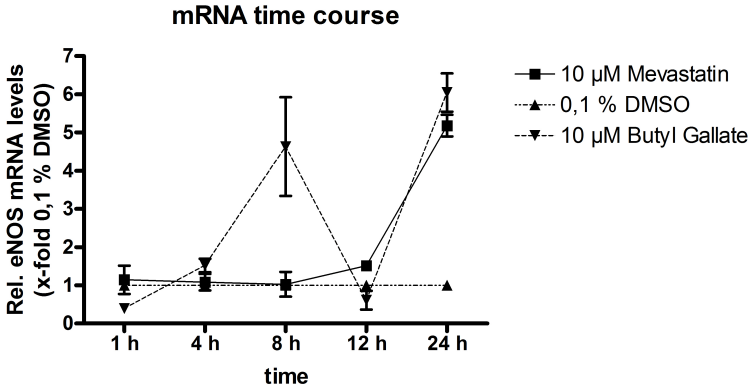


Figure 25.: eNOS mRNA level in butyl gallate stimulated EA.hy926 cells
EA.hy926 cells were treated with 10 µM butyl gallate for 1 h, 4 h, 8 , 12 h and 24 h and rt-qPCR experiments were performed. Results were normalized to the untreated control group (DMSO 0.1%). (mean ± SD, n = 2)

Part IV.

Discussion

1. DAF-FM limitations

The DAF-FM assay is technically simple to perform and a relatively quick method to detect real-time endothelial NO formation. Using low DAF-FM concentrations (0.1 µM) and subtracting the DAF-FM auto-fluorescence from the total fluorescence, reliable measurement of low level NO release from endothelial cells is possible.[120] However, there are some limitations using this fluorescence probe. First, DAF-FM is an hydrophilic reagent and not able to diffuse across the cell membrane. Hence, just extracellular nitric oxide is detectable. For measurement of intracellular nitric oxide, cell membrane permeable DAF derivatives (e.g. DAF-2 diacetate, DAF-FM diacetate) should be used. Nevertheless, we used the non-membrane permeable DAF-FM derivative, since high intracellular fluctuations of divalent cation (CA^{2+}, Mg^{2+}) concentrations affect fluorescence signals.[121] Second, DAF-FM does not directly react with nitric oxide to form the highly fluorescent benzotriazole. NO oxidation to N_2O_3 is essential, because DAF-FM does not interact with other oxidized forms of NO, e.g. $ONOO^-$, NO_3^-, NO_2^- or NO itself.[114,122] Hence, increase in NO release by reducing agents (e.g. ascorbic acid, polyphenolic substances like gallic acid derivatives) is likely to be not detectable by this assay.[121] Additionally, polyphenolic substances donor a hydrogen atom to radicals and the so formed phenoxy radical intermediate is resonance stabilized.[123] Kojima et al. highlighted a mechanismsn by which hydrogen donors may quench DAF-FM signals. They demonstrated that protonating the phenolic OH group in the DAF-FM benzotriazole derivative completely inhibits the fluorescence reaction.[114] Finally, intracellular nitric oxide scavenging by ROS may consequently falsify the results. Therefore, DAF-FM experiments should be conducted subsequent to the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion method, as this technique directly determines enzyme activity. Both together provide an informative basis about the eNOS coupling state and protein activity. However, they are not selective for eNOS.

The small amounts of bioactive nitric oxide in biological systems are otherwise detectable by more complex methods.[124]

2. Leoligin derivatives and natural compounds from *Eriosema laurentii*, *Ferula gummosa* and *Adhatoda vasica*

Leoligin coated stents may show great promise for the treatment of intimal hyperplasia after arterial surgery.[96] Not only because of the antiproliferative effect on VSMC, but also due to the absence of toxicity on endothelial cells.[96] Additionally, recent *in vitro* cell culture studies, as well as animal experiments in rats have demonstrated that 5-Methoxyleoligin (5-ML) is a potent inducer of arteriogenesis in infarcted myocardial ischemic area. These findings depict 5-ML as a potential post-MI therapeutic.[125] However, no increase in NO release in EA.hy926 cells treated with leoligin derivatives was detectable in our study. As one can see in Fig. 9, hardly any compound exceeded the untreated control group. Furthermore, four substances were toxic. They provide false-positive results and are therefore not shown in the figure. Although there were many more leoligin derivatives available, we stopped experiments with these compounds since our results were not promising. However, in order to verify our outcomes, we recommend to screen the tested substances in the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay too. Additionally, time course experiments should be conducted since short-term activation of eNOS is not measurable after an incubation period of 24 h.

Screening of isolated *Eriosema laurentii* substances for increased endothelial NO release did not result in reliable data, since we did not obtain significant results from the positive control. Contamination can be ruled out as reason for these outcomes, because wells were checked right before every working step and no abnormalities were visible. Therefore, we suppose that wrong pipetting was the reason for these outcomes. However, as one can see in Fig. 10 the endothelial NO release of EA.hy926 cells stimulated with SCI and KMU B did not exceed the DMSO 0.1 % group. The tested substances from *Ferula gummosa* and *Adhatoda vasica* also showed no increase in endothelial NO release. However, we recommend to verify the data in the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay and to perform time course experiments too.

3. Rutaecarpine

Several results of prior studies revealed rutaecarpine as a potent vasodilating substance. Since the effect seems to be endothelium-dependent, our working group conducted experiments to investigate the involvement of endothelial nitric oxide synthase in the underlying mechanism.[103,104] Previous experiments showed a concentration-dependent increase in eNOS promotor activity, NO release as well as eNOS activity in EA.hy926 cells stimulated with rutaecarpine. However, these experiments could not be reproduced although the positive control worked as expected in all experiments.

Rutaecarpine stock solutions were diluted in DMSO and stored at minus 20 °C. Thus aliquots were thawed numerous times (melting point DMSO = 18.45 °C). Kozikowski et al. investigated the effect of freeze/thaw cycles on stability of compounds diluted in DMSO. Their data depicted a loss of compound due to solid precipitation forming after several freeze/thaw cycles.[126] These observations agree with ours. Microscopic examination of the EA.hy926 cells, stimulated with rutaecarpine, revealed crystallization at the bottom of each well (more distinct at higher concentrations). This may explain the "reverse dose-response" effect shown in Fig. 17. To avoid this problem, rutaecarpine could be dissolved right before stimulation in subsequent experiments. However, this approach was also not successful.

Although there are no ester bonds or other labile functional groups in the chemical structure of rutaecarpine, we approached a local specialist (Dr. Martin Zehl, University of Vienna, Department for Pharmacognosy) to check the purity and stability of our compounds. No impurity or artifacts from decomposition reactions were detected (data not shown).

Therefore, we hypothesize that solubility problems are at the basis of our controversial results, because rutaecarpine is practically insoluble in water or aqueous medium.[102] The fact, that other working groups did not report any crystallization in their cell culture experiments may be due to differences in the composition of the solvent. Rutaecarpine was dissolved in a mixture of DMSO and ethanol (2:8) in the attempts performed by Wang et al. and Hu et al.[104,105] We suppose that the solubilizing effect of ethanol is significantly involved in prevention of crystallization. Differences in storing conditions may be another reason.

An explanation for the conflicting data between our study and previous experiments performed by our working group may be caused by changes in pH-value. We used DMEM without phenol red for our cell culture experiments. Such a setting was necessary, because the color of the pH indicator interferes with the photometric determination of DAF-FM benzotriazole derivative. Hence, changes in pH-value could not be visually noticed. Since pH-shifts may influence solubility, such variations cannot be excluded. However, involvement of FBS batch-to-batch variations can be ruled out, since the same LOT was used in all experiments.

Crystallization not only occurred in 96-well plates stimulated for NO release detection, but also in those prepared for cell viability measurement. Thus, it is possible that the effective dissolved rutaecarpine concentration was far below the assumed one.

Since rutaecarpine is a promising drug for the treatment of endothelial dysfunction, research into this compound should be continued. Several approaches are feasible to avoid solubility problems. To name but a few, ethanol co-solvency, microemulsions, inclusion complexes with cyclodextrins, or nanonization are established methods to increase solubility of hydrophobic substances.[104,127,128]

4. Necessary considerations regarding gallic acid results

As mentioned in the introduction, EA.hy926 cells are hybridoma cells, generated by fusion of human umbilical vein cells and the adenocarcinomic human alveolar basal epithelial cell line A549.[113] It is the presently best characterized endothelial cell line, grows in large quantities and propagation does not cause a loss of viability.[129] Furthermore, EA.hy926 cells produce many endothelium-specific surface proteins like vWF, ICAM-1, CD31 (PECAM-1) and CD34.[130] However, additionally to the other limitations (e.g. alteration in cell phenotype due to prolonged cultivation) one has to deal with if cancer cells are used for cell culture studies, gallic acid derivatives were shown to feature potent antitumoral activity. A major mechanism by which apoptosis is induced in tumor cell lines, is the production of reactive oxygen species.[109] Recent findings by You et al. confirmed ROS induction in A549 cells, especially O_2^- after 24 h treatment with GA. However, concentrations used were much higher than in our experiments.[131] A recent study has shown that propyl gallate decreases ROS levels in human umbilical endothelial cells.[132] Therefore, it would be valuable to determine ROS levels after gallate treatment in EA.hy926 cells too. Interestingly, in contrast to our study, their experiments were performed with exponentially proliferating cells.[131] Since we used EA.hy926 cells up to passage 25, alteration in cell phenotype are possible. Thus, results should be validated in primary endothelial cells.

5. Relationship between mRNA and protein levels

Every cell in the body contains the genetic information for all proteins. Nevertheless, cells differentiate depending on the tissue type and develop a specific proteome. Several complex transcriptional, post-transcriptional and translational regulating steps face this challenge. Thus, there is no linear relationship between mRNA levels and protein abundance.[119] In our study, we showed that incubation of EA.hy926 for 8 h and 24 h with butyl gallate results in increased eNOS mRNA levels. After an incubation period of 12 h EA.hy926 eNOS mRNA content was similar to the control group. This drop may be due to post-transcriptional mRNA degradation.[133] As deadenylation of mRNA is the major trigger of mRNA decay in mammalian cells, determination of the poly-(A) tail may provide information about mRNA half-life.[134] However, we challenge our results since this strange drop did not appear in EA.hy926 cells treated with mevastatin and no such event is reported in literature. Western blots were performed after stimulation of EA.hy926 cells for 24 h and no increase in protein abundance was detectable. Suppressed translation initiation or elevated activity of the ubiquitin-proteasome system may account for our findings. In future studies, increased proteasome activity could be identified by the use of proteasome inhibitors

Due to lack of time, we were not able to investigate further mechanisms, which could be responsible for an increased eNOS activity. eNOS is tightly regulated at numerous levels. Thus, several pathways have to be investigated. First of all, one could check enzyme phosphorylation at amino acid residue S^{1177} and dephosphorylation at amino acid residue T^{495}, as these post-translational modifications are well known to increase eNOS activity. Expression levels of caveolin-1, BH_4 stabilization and recruitment of other cofactors, like HSP90, are also worthy of investigation.

Since no relationship between eNOS mRNA and protein was detectable, further rt-qPCR dose-response experiments are not advisable. Furthermore, future findings should be confirmed in primary cells, because they provide more physiologically relevant data.

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Abbreviations

AP1 ..... activator protein 1
AT-II ..... angiotensin-II
BH4 ..... tetrahydrobiopterin
cDNA ..... complementary deoxyribonucleic acid
CETP ..... cholesteryl ester transfer protein
CVD ..... cardiovascular disease
CVRF ..... cardiovascular risk factor
EC ..... endothelial cells
eNOS ..... endothelial nitric oxide synthase
FAD ..... flavin adenine dinucleotide
FMN ..... flavin mononucleotide
HSP90 ..... heat shock protein 90
HUVEC ..... human umbilical vein endothelial cells
IkB ..... Inhibitor of kappa B
iNOS ..... inducible nitric oxide synthase
IRAG ..... IP3 receptor-associated PKG-I substrate
KLF2 ..... Krüppel-like Factor 2
LDL ..... low density lipoprotein
MCP-1 ..... monocyte chemotactic protein-1
MLCP ..... myosin light chain phosphatase
NADPH ..... nicotinamide adenine dinucleotide phosphate
NF-kB ..... nuclear factor kappa-light-chain-enhancer of activated B cells
NHA ..... N-hydroxyl-L-arginine
nNOS ..... neuronal nitric oxide synthase
NO ..... nitric oxide
NOS ..... nitric oxide synthases
NOSIP ..... eNOS interacting protein
NOSTRIN ..... endothelial nitric oxide synthase traffic inducer
Nox ..... NADPH oxidase
PDGF ..... platelet-derived growth factor
PECAM ..... platelelt endothelial cell adhesion molecule
PKG ..... protein kinase G

PVDF ..... polyvinylidene difluoride
ROS ..... reactive oxygen species
SDS ..... sodium dodecyl sulfate
sGC ..... soluble guanylate cyclase
SOD ..... superoxide dismutase
Sp1 ..... specificity protein 1
TCM ..... traditional Chinese medicine
UTR ..... mRNA untranslated regions
VASP ..... vasodilatory-stimulated phosphoprotein
VEGF ..... vascular endothelial growth factor
vWF ..... Von-Willebrand-Faktor

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Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

A. Products, suppliers, stock solutions

product	**stock**	**supplier**
DMEM (4.5 g/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-supplement	50x in BSS (balances salt solution)	Biochrom AG, Berlin, Germany
FBS	-	Gibco via Invitrogen, Paisley, UK
A23187	10 mM in DMSO	Alexis Biochemicals, Lausen, Switzerland
DAF-FM	5 mM in DMSO	Sigma-Aldrich Vienna, Austria
L-arginine	100 mM in ddH_2O	Fluka, Sigma-Aldrich, Vienna, Austria
L-NAME (N^{ω}-Nitro-L-arginine methyl ester)	1M in ddH_2O	Sigma-Aldrich, Vienna, Austria
Resazurin	10 mg/ml in PBS	Sigma-Aldrich, Vienna Austria

product	stock	supplier
Mevastatin	6 mM in DMSO, 10 mM in DMSO	Sigma-Aldrich, Vienna, Austria
Rutaecarpine	30 mM in DMSO	Sigma-Aldrich, Vienna, Austria
Leoligin-derivatives	30 mM in DMSO	DNTI (Drugs from Nature Targeting Inflammation)
Ethyl gallate	30 mM in DMSO	Sigma Aldrich, Steinheim, Germany
Propyl gallate	30 mM in DMSO	Sigma Aldrich Steinheim, Germany
Butyl gallate	30 mM in DMSO	ABCR, Karlsruhe, Germany
Isopentyl gallate	30 mM in DMSO	ABCR, Karlsruhe, Germany
peqGOLD Total RNA kit	-	peglab Biotechnologie, Erlangen, Germany
eNOS primers	-	Invitrogen, Paisley, UK
18S rRNA primers	-	Invitrogen, Paisley, UK
Roti®-Quant Bradford reagent	5x	Carl Roth, Karlsruhe, Germany
Immun-Blot® PVDF membrane	-	Bio-Rad Laboratories, Hercules, CA, USA
ViCell® cell viability analyzer	-	Beckman Coulter, Vienna, Austria
LAS-3000 Luminescent analyzer	-	Fujifilm, Düsseldorf, Germany
Mini-PROTEAN® 3 Cell-system	-	Bio-Rad Laboratories, Hercules, CA, USA
Power Supply PowerPac HC	-	Bio-Rad Laboratories, Hercules, CA, USA

product	**stock**	**supplier**
Mini Trans-Blot® Electrophoretic Transfer Cell system	-	Bio-Rad Laboratories, Hercules, CA, USA
LightCycler® 480 real-time PCR system	-	Roche Diagnostics, Vienna, Austria
Tecan Genios Pro	-	Tecan, Grödig, Austria
Tecan Sunrise	-	Tecan, Grödig, Austria

B. Curriculum vitae

Personal Data

Name:	Gabriel ZIRKOVITS
Date of birth:	29.04.1986
Place of birth:	Vienna
Nationality:	Austria

Education

09/1992 - 06/1996	Elementary school, VS Rodaun, Vienna
09/1996 - 06/2000	Grammar school, Bundesrealgymnasium Eisenstadt, Burgenland
09/2000 - 06/2006	Commercial academy, BHAK Eisenstadt, Burgenland
01/2007 - 09/2007	Alternative civilian service, CARITAS Altenwohn- u. Pflegezentrum Haus St. Martin, Eisenstadt, Burgenland
10/2007	Inscription at the Universitiy of Vienna, Diploma study pharmacy
03/2014 - 08/2014	Diploma thesis at the "Molecular Targets" group, Department of Pharmacognosy, University of Vienna

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