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Regulation of the redox response dependent gene expression through endogeneous and exogeneous stimuli in human endothelial cells

Dissertation

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Ich danke meiner Mutter für Ihre Unterstützung in allen Lebenslagen während meiner Schulzeit, meines Studiums und meiner Promotion.

Summary

One of the main causes of atherosclerosis is endothelial dysfunction. It is associated with oxidative stress, an imbalance of the redox state of the endothelial cell (EC). Hence, modulating processes of the redox state of the EC in favor of the antioxidative response are a main part to prevent atherosclerosis.

Nrf2 belongs to the master regulators of redox homeostasis by regulating the expression of antioxidative enzymes like hemeoxygenase I (HO-1), NADPH dehydrokinase quinine 1 (NQO1) and glutamate cysteine ligase catalytic subunit (GCLC). Oxidative stress can lead to dysfunction of the NO/soluble guanylate cyclase (sGC)/ guanosine cyclic monophosphate (cGMP) pathway in the vasculature.

Therefore, the aim of this dissertation was to modulate the redox state of the human endothelial cell through endogenous and exogenous stimuli to gain a protection against oxidative cell damage. This work shows that the used endogenous gasotransmitters NO and H_2S enhanced the expression of the antioxidative enzymes HO-1 and NQO1. The simultaneous incubation of both molecules showed a potentiation of the effects observed after single incubation. The inhibition of endogenous NO production by the NOS inhibitor L-NAME decreased the effects of sulfide, indicating an interaction between sulfide and endogenous NO production in endothelial cells. In flow cytometry the used exogeneous electrophil (-)-epicatechin, which is contained in food, showed an enhancement of total free thiols in EC in a concentration dependent manner. The cellular GSH concentration was only increased by a high concentration of the H_2S donor as well as by the co – incubation of NO and H_2S. cGMP levels were augmented through incubation of simultaneous treatments of H_2S donor and NO donors.

This up-regulation of antioxidant enzyme levels as well as the up-regulation of GSH modulates the redox state of the EC in favor of an antioxidative response. Furthermore, the indirect interaction of the ubiquitously present gasotransmitters becomes apparent. Therefore we could show a modulation of the redox state of endothelial cells through exogenous stimuli such as (-)-epicatechin and endogenous stimuli such as NO and H_2S.

Zusammenfassung

Endotheliale Dysfunktion ist eine der wesentlichen Ursachen der Atherosklerose. Diese steht im Zusammenhang mit oxidativem Stress und einem Ungleichgewicht des Redoxstatuses der Endothelzelle. Somit sind modulierende Vorgänge der Redoxhomöostase zugunsten von Antioxidantien ein wichtiger Bestandteil zur Prävention und zur Behandlung der Atherosklerose.

Nrf2 gehört zu den wichtigsten Regulatoren dieser Homöostase durch Expression mehrerer antioxidativer Enzyme wie zum Beispiel der Hämoxygenase I (HO-1), der NADPH dehydrokinase quinone 1 (NQO1) und der katalytischen Einheit der Glutamatcysteinligase (GCLC).

Oxidativer Stress ist außerdem einer der Hauptfaktoren, die zur Fehlfunktion des Stickstoffmonooxid (NO)/lösliche Guanylatcyclase (sGC)/ cyclisches Guanosinmonophosphat (cGMP) – Kreislaufs in Gefäßen führt.

Das Hauptziel dieser Arbeit war es, den Redoxstatus der Endothelzelle durch exo- und endogene Stimuli zu modulieren, um einen Schutz gegen die oxidative Belastung der Zelle zu erreichen. Die Arbeit zeigt, dass die endogenen Gasotransmitter NO und Hydrogensulfid (H_2S) die Expression der antioxidativen Enzyme NQO1 und HO-1 in HUVECs erhöhen. Eine gemeinsame Inkubation der beiden Gasotransmitter zeigte eine noch deutlichere Steigerung der Enzymexpression. Die Ausschaltung der endogenen NO-Quelle durch den NOS-Inhibitor L-NAME ergab eine Reduktion der gesteigerten Proteinproduktion, was auf eine indirekte Interaktion der beiden Gasotransmitter NO und H_2S schließen lässt. Außerdem zeigten die HUVECs nach Inkubation mit der exogenen elektrophilen Substanz (-)-epicatechin, die in verschiedenen Nahrungsmitteln enthalten ist, in bestimmten Konzentrationen gesteigerte GSH – Level. Nur eine hohe Konzentration von H_2S konnte ebenfalls die zelluläre GSH – Konzentration steigern. Auch hierbei ergab eine simultane Inkubation der HUVECs mit NO und H_2S in bestimmten Konzentrationen höhere Werte. Auch die cGMP-Level in HUVECs wurden vor allem durch die gleichzeitige Inkubation der beiden Gasotransmitter erhöht.

Diese Ergebnisse bestätigen die Möglichkeit einer Modulation des Redoxstatus der Endothelzelle durch die hier verwendeten exo- und endogenen Stimuli. Darüber hinaus wird die indirekte Interaktion der beiden ubiquitär vorhandenen Gasotransmitter NO und H_2S deutlich. Folglich lässt sich die Endothelzelle zugunsten der antioxidativen Abwehr beeinflussen, wodurch diese vor weiteren oxidativen Zellschäden geschützt werden kann.

Abbreviations

AB	Antibody
AChR	acetylcholine receptors
ARE	Antioxidant response element
AS	Angeli's salt
BAEC	Bovine aortic endothelial cells
Ca^{2+}	Calcium
CaM	Calmodulin
CBS	cystathionine-β-synthase, and 3-mercaptopyruvate sulfutransferase (3-MST)
CSE	cystathionine-γ-lyase
CTRL	Control
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
EC	endothelial cell
ED	endothelial dysfunction
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
FAD	flavin adenine dinucleotide
Fig.	Figure
FMD	flow mediated dilation
FMN	flavin mononucleotide
GCLC	Glutamate-cysteine ligase catalytic subunit
GSH	Glutathione
GSSG	Glutathione disulfide
GYY 4137	(*p*-methoxyphenyl)morpholino-phosphinodithioic acid
H_2O	water
H_2O_2	hydrogen peroxide
HepG2	hepato cellular carcinoma cell line
HO-1	Hemeoxigenase 1
HSNO	thionitrite
HUVECs	human umbilical vein endothelial cells
IgG	Immunoglobulin G
IU	International unit
IVR	Intervening region
kDa	Kilo dalton
LDL	Low density lipoprotein
L-NAME	N^G-Nitro-L-arginine-methyl ester
MAPK	Mitogen-activated protein kinase
max.	maximum
min.	minimum
mM	millimolar
µM	micromolar
n	Number
N_2O	nitrous oxide

Na_2S	Sodium disulfide
NaCl	Potassium chloride
NADP	Nicotinamide adenine dinucleotide phosphate
NF_KB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
$NO^{\cdot}$	nitric oxide
NQO1	NAD(P)H dehydrogenase (quinone 1)
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
p	Statistical probability value
Pa	Pascal
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pen	Penicillin
ROS	reactive oxygen species
RBC	Red blood cell
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
SD	standard deviation
SDS PAGE	sodium dodecyl polyacrylamite gel electrophoresis
SEM	standard error of the mean
sGC	Soluable guanylat cyclase
SIN 1	3-morpholinosydnonimine
SMC	smooth muscle cells
SNAP	S-nitroso-N-acetylpenicillamine
SNP	sodium nitroprusside
$SSNO^-$	nitrosopersulfide
SULFI/NO	dinitrososulfite (*N*-nitrosohydroxylamine-*N*-sulfonate)
Tab.	table
tBHQ	tert-Butyl Hydrochinon
TBS	Tris buffer saline
UV	ultra violet
VEGF	vascular endothelial growth factor
WT	wild type

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1 Introduction

1.1 Atherosclerosis and oxidative stress

Epidemiological and medical anthropological investigations suggest that flavanol-rich foods exert cardiovascular health benefits [1] as well as hydrogen sulfide (H_2S) [2]. The endothelium, which is the inner side of the blood vessel, plays a central role in regulation of vasodilatation and –constriction through the accumulation of nitric oxide (NO) as well as in homeostasis and thrombosis [3]. So, healthy endothelium has an important influence on cardiovascular control.

Thus, endothelial dysfunction may play a significant role in the pathogenesis of atherosclerosis [4]. It is defined by a "decreased bioactivity of nitric oxide and impaired flow-mediated vasodilation (FMD)" [1].

Oxidative stress can lead to endothelial dysfunction [5] and provoke reduced vasodilatation and increased vascular permeability [4, 6]. Consequently, vascular remodeling and thrombotic events can occur [4]. Oxidative stress is also known to lead to dysfunction of the NO/soluble guanylyl cyclase (sGC)/ guanosine cyclic monophosphate (cGMP) pathway in the vasculature [7].

Summarized cardiovascular diseases such as hypertension and atherosclerosis are associated to endothelial dysfunction [8].

Therefore, mechanisms which protect the endothelium from oxidative injury may play an important role in prevention of atherosclerosis. The transcription factor Nrf2 was identified as a general regulator of one of the defense mechanisms against oxidative stress [9].

1.1.1 The Nrf2 pathway

Many stress-induced proteins are regulated at the transcriptional level by consensus sequences for the antioxidant response elements (ARE) found in their promoter regions [10, 11]. Nrf2 was identified as one of the major regulator of ARE-mediated gene expression [12]. It is ubiquitous in most tissues and mammalian cells [13-15] and a central molecular target of indirect antioxidants by maintaining redox homeostasis [16, 17].

Nrf2 is a member of the ‘cap-n-collar’ class of basic leucine zipper transcription factors [15, 18]. It is bounded on Keap1 (Kelch-like ECH associating protein 1) and Cullin 3 and kept in the cytoplasm under basal conditions [19, 20]. Cullin 3 ubiquitinates its substrate Nrf2. Keap1 however, serves as a substrate adaptor and supports the ubiquitination of Nrf2 by Cullin 3. So Nrf2 has a short half-life that lasts only 20 min under normal conditions [21].

Keap1 consists mainly of three domains: the N-terminal BTB domain, an intervening region (IVR), and the C-terminal Kelch domain [22, 23]. The C-terminal Kelch domain acts mainly for the association of Keap1 to Nrf2 [24], whereas the BTB domain promotes the degradation of Nrf2 from Keap1 [21]. IVR possesses four cysteine residues, that binds to the electrophilic agent dexamethasone 21-mesylate. Cys273 and Cys288 were identified playing essential roles in sensing oxidative stress [25]. So, the IVR domain liberates Nrf2 from Keap1.

The main function attributed to the Nrf2-Keap1 system is the regulation of cellular defense against environmental insults like oxidative stress [26].

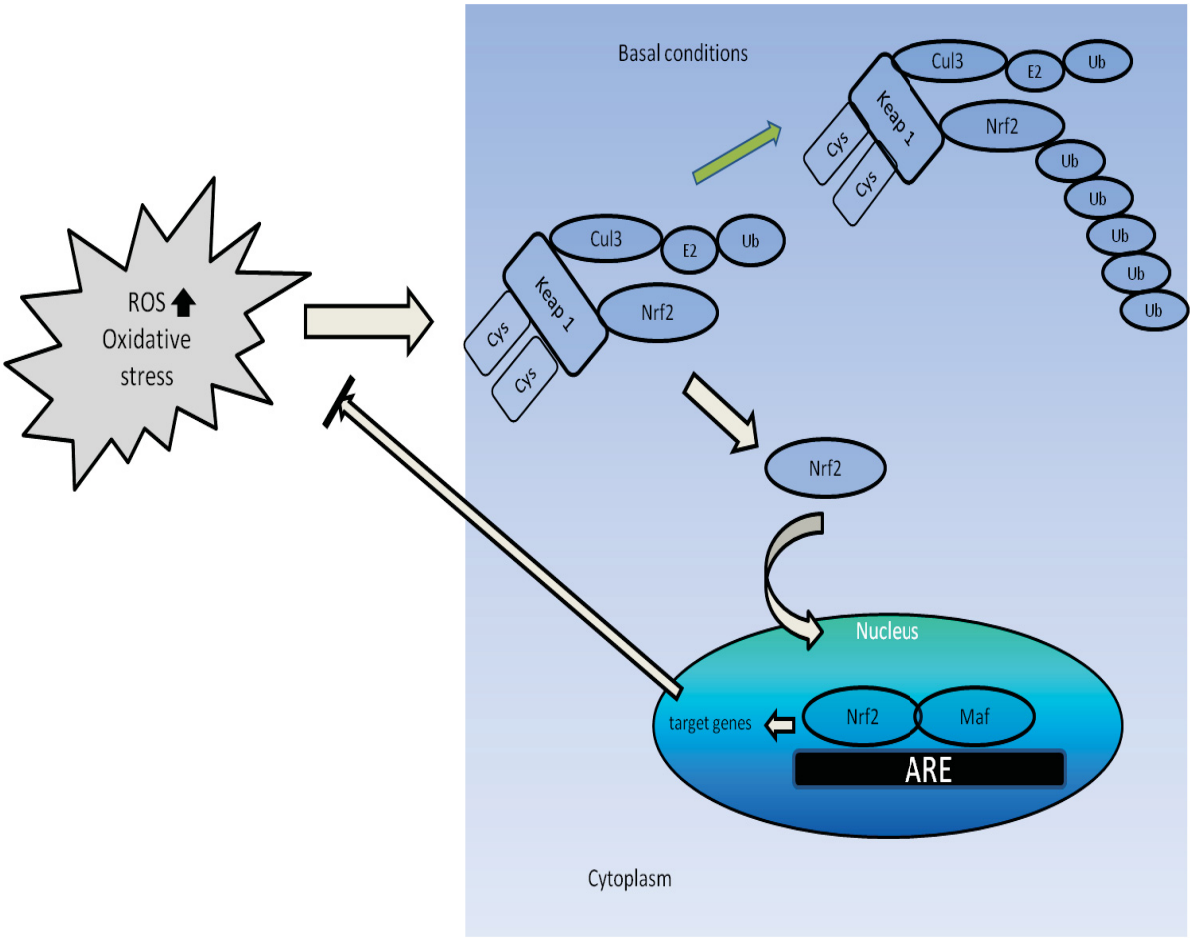


Fig. 1: Nrf2 translocation to the nucleus. As an answer to oxidative stress, Nrf2 translocates from cytoplasm to nucleus and combines with Maf and ARE to initiate the transcription of a number of antioxidative genes. Under basal conditions, Nrf2 is ubiquitinylated [24].

If Nrf2 is not ubiquitinylated, it is released from the Keap1 dimers. [27] Hereafter, Nrf2 translocates to the nucleus to form heterodimers with the small Maf proteins [28] binding to the ARE [29]. This initiates the transcription of many ARE dependent genes [30, 31] like the phase II detoxifying enzymes NAD(P)H:Quinone oxidoreductase1 (NQO-1), Heme oxygenase-1 (HO-1), Glutamate cysteine ligase catalytic and modulatory subunit (GCLC and GCLM), which play a crucial role in the defense against oxidative stress.

NQO1 is a flavoprotein which is able to detoxicate ROS in promoting two-electron reduction of quinones [32]. There are other genes that are induced together with NQO1 [33]. These include *glutathione S-transferases,* which conjugates hydrophobic electrophiles and reactive oxygen species with glutathione [34], and *γ-glutamylcysteine synthetase,* which is one of the crucial factors in the regulation of glutathione metabolism [33, 35].

HO-1 also plays an important role in protecting cells from oxidative stress [36] through catalyzing the first and rate-limiting step in the oxidative degradation of heme b to form the open-chain tetrapyrrole biliverdin-IXα [37]. It is induced by agents and chemicals that produce oxidative stress as well [38, 39].

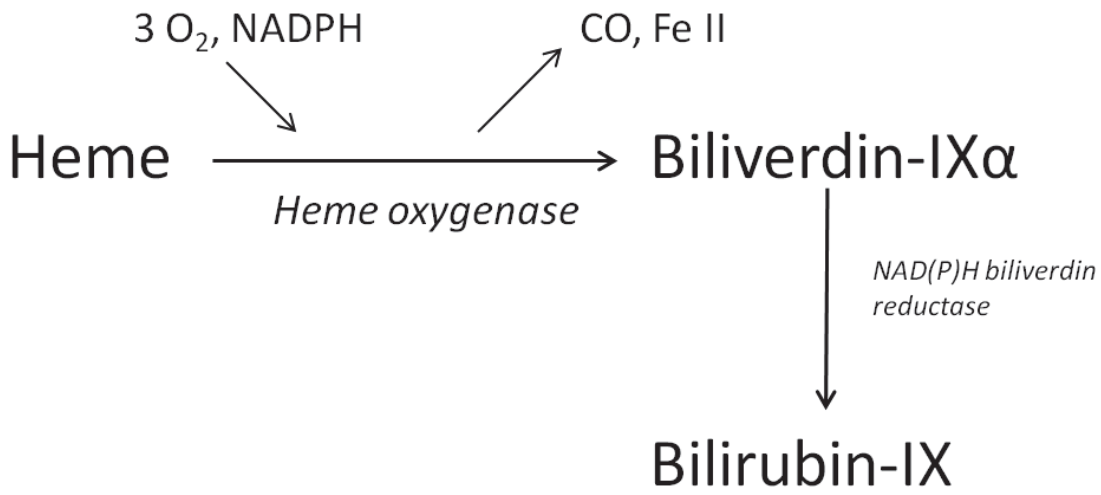


Fig. 2: HO-1 function [36]. HO-1 catalyzes heme b (Fe-protoporphyrin-IX) to form the open-chain tetrapyrrole biliverdin-IXα.

Glutathione (GSH) plays an essential role in defense against oxidative stress. It has the ability to scavenge ROS and free radicals directly or indirectly by being oxidized to GSSG [40]. After this reaction, glutathione reductase can induce the reduction from GSSG to GSH using NADPH as a co-factor [41].

Another way to fill up the GSH pools is the de novo synthesis. Glutamate cysteine ligase (GCL) as another phase II detoxifying enzymes, which is composed of a catalytic (GCLC) and modulatory (GCLM) subunit, catalyzes the first and also rate limiting step of GSH biosynthesis [42]. The second step is then catalyzed by the GSH synthase [43].

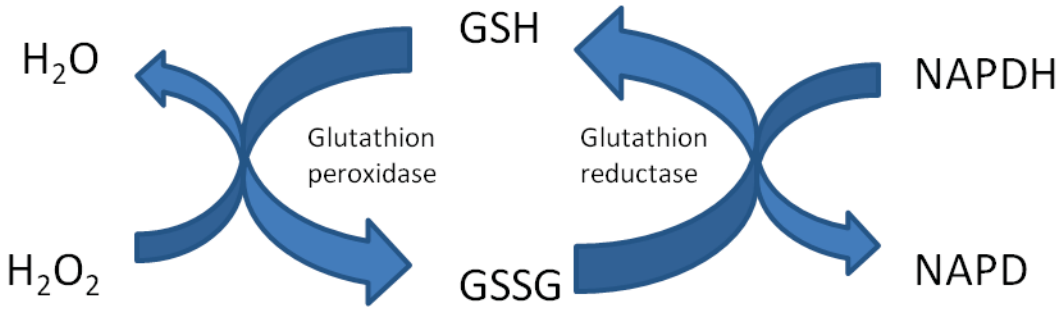


Fig. 3: GSH pathway. Through oxidation of GSH it is possible to detoxify ROS. By using NADPH as a cofactor, the dimer GSSG can react back into the monomer GSH [40].

1.2 The effects of (-)-epicatechin, nitric oxide and hydrogen sulfide to oxidative stress

1.2.1 (-)–epicatechin

Schroeter, Heiss et al showed in 2006 that flavonoids like (-)-epicatechin and its metabolites mediate beneficial effects on vascular function in humans [1]. Since then there were several studies investigating the molecular short and long-time mechanisms [44]. The modulation of the redox state of mammalian cells was considered as a promising cause of these long-time effects.

(-)-epicatechin is known to have ROS [45, 46] and reactive nitrogen species (RNS) [47] scavenging activities, which prevent oxidative stress through direct antioxidative effects.

In vivo tissues need to be exposed to high concentrations of polyphenols to effect direct antioxidant actions, but in blood, (−)-epicatechin and its metabolites can reach concentrations in the low-micromolar range [48]. (−)-epicatechin is likely to neutralize radicals at a rate about 25 times lower than ascorbate for example [49]. In comparison to other compounds e.g., glutathione, albumin and tocopherols, in most other tissues the concentrations of flavanols and procyanidins are too low to provide any relevant direct antioxidant action [48].

Antioxidant activity is correlated to the membrane surface potential and the resistance to detergent disruption [50]. Flavanol metabolites and procyanidines protect the membrane lipids from oxidation and so have an effect on membrane surface potential [50, 51]. Furthermore, (-)-epicatechin inhibits the activity NADPH-oxidase in human umbilical vein endothelial cells (HUVECs) [52, 53]. NADPH-oxidase enzymes catalyze the formation of superoxide which could then react with NO to form peroxynitrite and so form ROS. By inhibition of this process (-)-epicatechin reduces ROS production and indirectly increases NO bioavailability within minutes [54]. Accordingly, the mechanism presents a reasonable explanation for short term effects of (-)-epicatechin in vitro [54].

1.2.2 Nitric oxide

NO signaling has been one of the most rapidly developing areas in biology. It is able to regulate a multitude of biological processes [55].

EC in normal blood vessels secrete NO tonically. They can increase the NO production in response to increased shear stress [56] or oxidative stress. NO plays an important role in the contribution of vessel homeostasis by inhibiting vascular smooth muscle tone [57] and growth [58], platelet aggregation [59], and leukocyte binding to the endothelium [60].

1.2.2.1 Endothelial nitric oxide synthase – The pathway

The endothelial nitric oxide synthase (eNOS) is part of the first mammalian pathway known to synthesize a gas as a signalling molecule [61]. eNOS catalyzes the oxidation of L-arginin to NO and L-citrullin in EC [62, 63] with oxygen using calcium, calmodulin (CaM), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), NADPH, and tetrahydrobiopterin as cofactors [64].

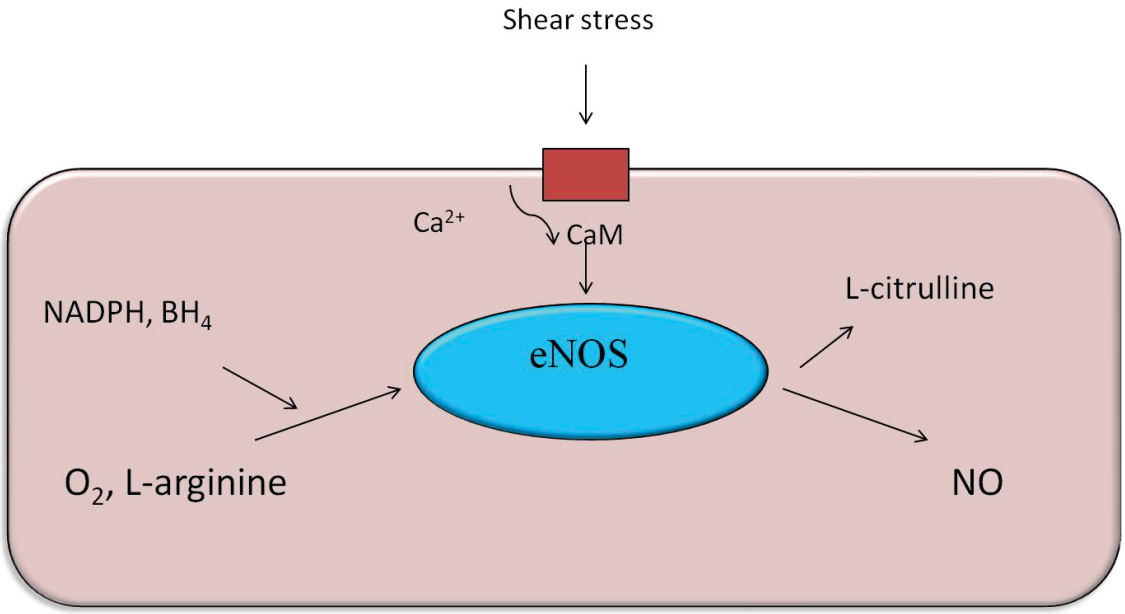


Fig. 4: Synthesis of NO in EC. eNOS catalyzes the oxidation of L-arginine to L-citrulline synthesizing NO. Amongst others Ca^{2+}, CaM, NADPH and BH_4 serve as cosubstrates [64].

eNOS is only active as a homodimer [65]. Phosphorylation as one post translational modification of the eNOS isoform has an activating effect on eNOS activity. eNOS can be phosphorylated at different sites: tyrosine, serine and threonine [66]. Fluid shear stress for example causes tyrosine phosphorylation of eNOS and an increase in calcium-independent NOS activity, so it generates a higher NO outcome to protect the vessel homeostasis [56]. Oxidative stress as well induces eNOS activation through phosphorylation of S1177 [66]. However, S-nitrosylation of the cysteine residues as another post translational modification inhibits eNOS activity, for example through the presence of exogenous NO [67].

1.2.3 Hydrogen sulfide

Hydrogen sulfide (H_2S) belongs, together with NO and carbon monoxide (CO), to a family of gasotransmitters [68], which are produced endogenously. It is involved in multiple processes of the human body like cardiovascular function, gastrointestinal system, inflammatory response, the regulation of nervous system, and renal function [69]. Gasotransmitters all have in common that they are lipid soluble and are able to freely permeate the plasma membrane of a cell to pass the signal directly to an intracellular target [70]. Additionally, they do not need the normal sequences of regulatory mechanisms to transmit a signal [69].

However, H_2S has recently turned up to be produced enzymatically in all mammalian species at low micromolar levels (10 µM to 100 µM) by cysteine metabolic enzymes: cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercaptopyruvate sulfutransferase (3-MST) [71].

Nevertheless, there is an significant progress in our understanding of H_2S biochemistry, signalling mechanisms and physiological role [68]. For example, H_2S mediate vasculoprotective and antihypertensive effects of dietary garlic (*Allium sativum*) [72].

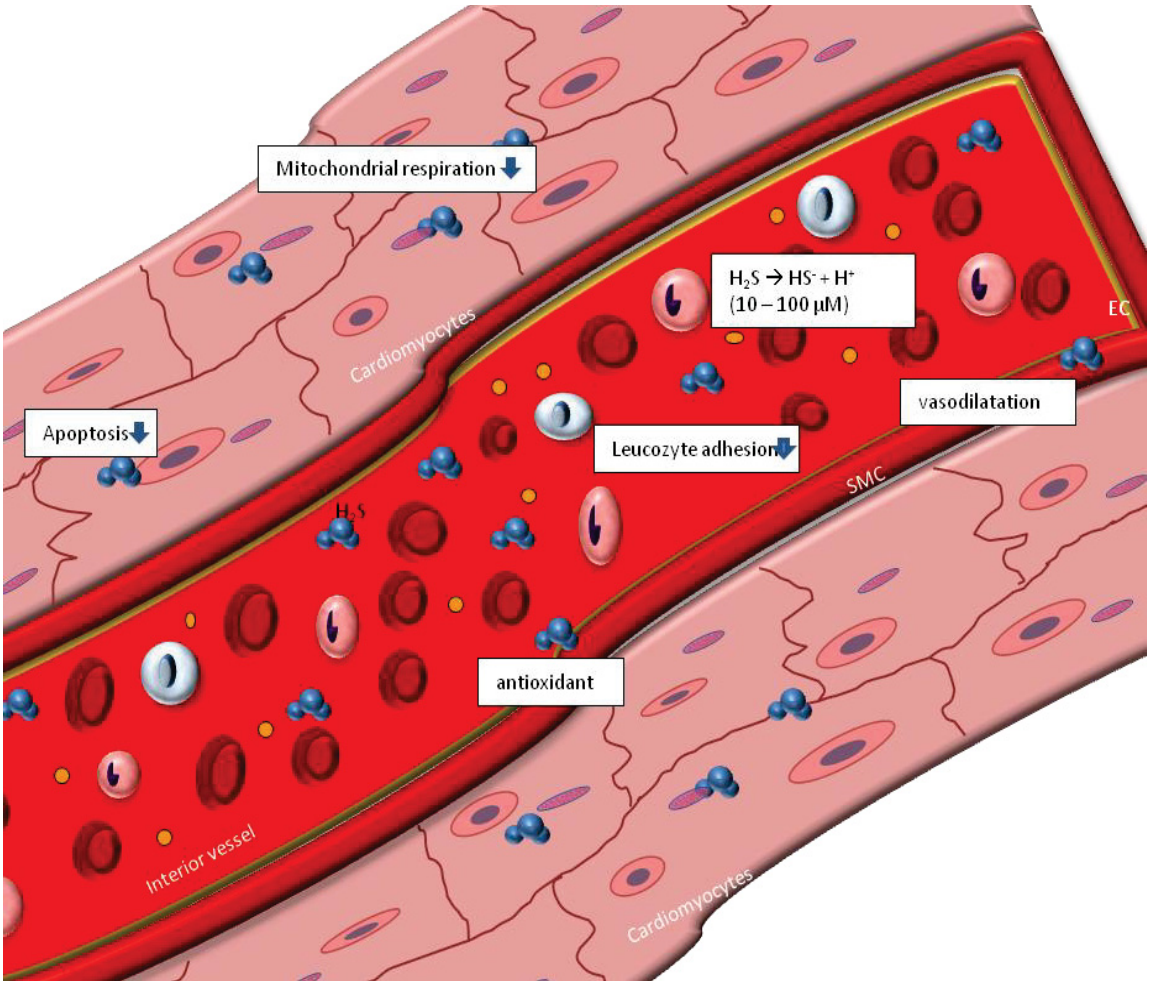


Fig. 5: Summary of the physiological actions of H_2S [73]. It can lead to vasodilatation and be used as antioxidant. Also, H_2S is able to down regulate leucocyte adhesion in the vascular system, apoptosis as well as mitochondrial respiration [73].

H_2S up-regulates the antioxidant defense [74]. It is also an inhibitor of leukocyte-endothelial cell interactions [75] and is able to reduce apoptotic signalling [74]. Like NO, it induces vascular smooth muscle relaxation [76], and reversibly modulates mitochondrial respiration [77]. These functions reveal that H_2S has multiple cytoprotective effects.

1.2.4 Gasotransmitter-dependent vasorelaxation

It is generally known that NO stimulates the sGC in EC [78] to increase the cellular concentration of cGMP [79], where its biological functions remain largely unexplored. In EC, it might be a target to modulate neovessel formation [78]. By the stimulation of muscarinic acetylcholine receptors (AChRs) on EC, Ca^{2+}-calmodulin (CaM) is activated. This however stimulates eNOS, CSE and HO_2 to produce NO, H_2S and CO. (see Fig. 6) [80]. H_2S then diffuses into smooth muscle cells where it activates K^+ channels leading to vasodilatation [81].

The sGC is an intracellular receptor for NO and CO. The binding of NO to the Fe^{2+}-heme of sGC in EC activates this enzyme leading to a subsequent increased production of cGMP from guanosine-5-triphosphate (GTP). The activation of sGC and the followed enhancement of the cGMP concentration conduct to downstream elements of the signaling cascade. This includes amongst others cGMP-dependent protein kinases, cGMP-gated cation channels, and cGMP-regulated phosphodiesterase. Finally, the signaling cascade influences the actin-myosin cross-bridge and leads to vasorelaxation [82].

Summarized, all three gases lead to vasodilatation.

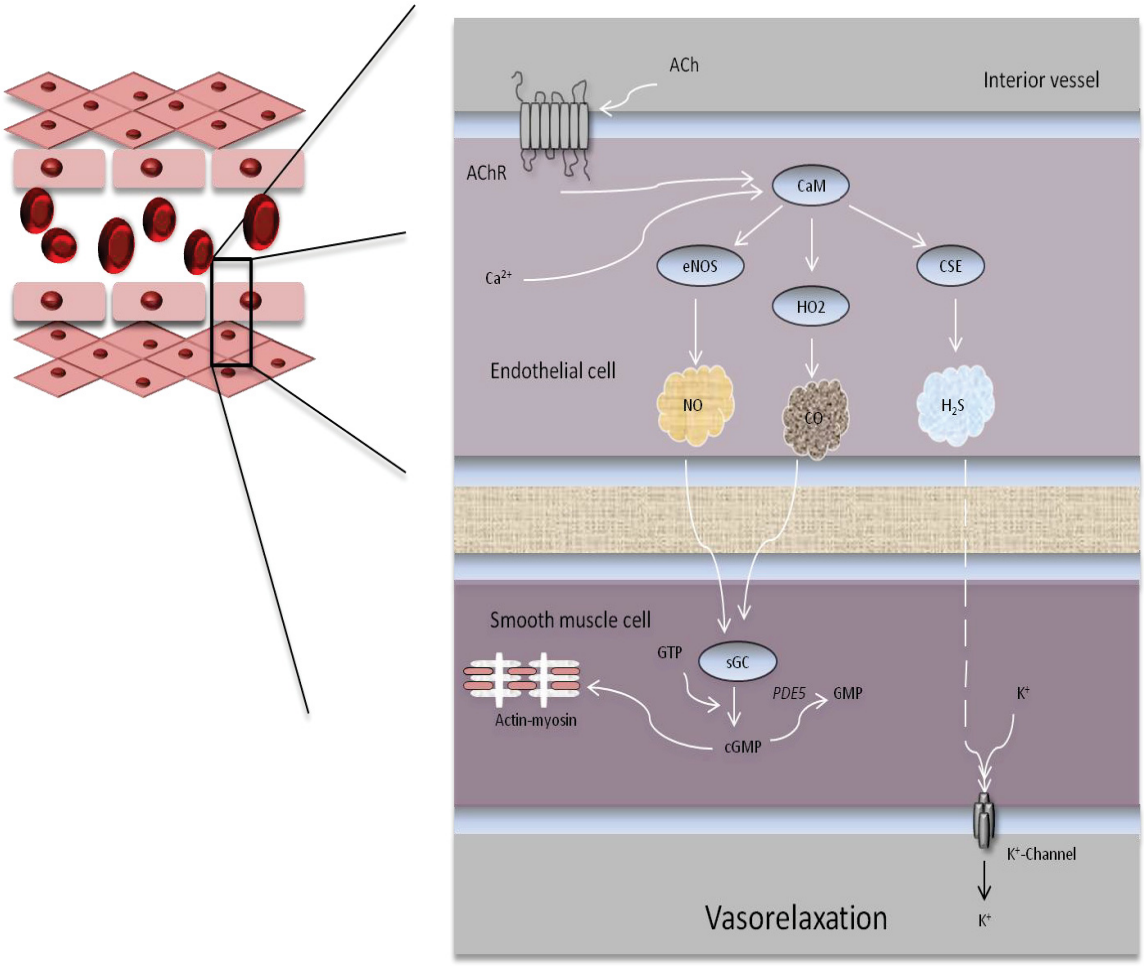


Fig. 6: Gasotransmitter-dependent vasorelaxation. By the stimulation of muscarinic acetylcholine receptors (AChRs) on EC, Ca^{2+}-calmodulin (CaM) is activated. CaM however binds to eNOS, cystathionine γ-lyase (CSE), and HO_2 to enhance the production of NO, H_2S, and CO. After diffusion in smooth muscle cells and by inducing either the sGC or K^+-Channels, the gases are leading to vasorelaxation. [80].

1.3 Cross talk of nitric oxide and hydrogen sulfide

The biological profile of hydrogen sulfide seems reminiscent of nitric oxide. Coletta et al showed 2012 that the angiogenic and vasorelaxant effects of NO and H_2S are dependent on each other's presence [83]. H_2S is able to form species that show distinct biological effects compared to H_2S and NO by reacting with NO or oxidized forms of NO and NO donors in vitro [84]. Those were identified from Cortese-Krott et al. as three main bioactive products: nitrosopersulfide ($SSNO^-$), polysulfides, and dinitrososulfite [*N*-nitrosohydroxylamine-*N*-sulfonate (SULFI/NO)]. In their study they identified $SSNO^-$ as a potent NO donor, resistant to the reducing milieu of the cell, and able to release both NO and HS_n^-, whereupon SULFI/NO was released as only a weak combined NO/HNO donor and generator of N_2O with potent effects on the heart [85].

However, Yoon, P.J. et al showed that a NO donor and a sulfide donor mixed together have a cumulative effect to NOS activation in interstitial cells of Cajal [86].

Altaany et al. demonstrated that H_2S enhances NO production in EC by activating a phosphorylation cascade, starting from p38 MAPK, Akt to eNOS [87]. Thus, there are NO-dependent as well as independent mechanisms where H_2S is able to stimulate EC tube formation, proliferation and angiogenesis. Thereby, H_2S can be seen as a key regulator for angiogenic signalling pathways, even with the absence of NO [87, 88]. Through further knowledge of the H_2S–NO relationship in the vascular biology, we could understand more of the pathogenic mechanisms for cardiovascular disease like artherosclerosis for example [87].

2 Aims of the study

The Nrf2 pathway is generally considered to play a major role in cellular defense and augments the production of the phase II detoxifying proteins such as NQO1, GCLC and HO-1.

This study aims to investigate whether exogenous and endogenous stimuli are able to modulate the redox state of the EC in order to prevent oxidative damage. Therefore, we used mainly activators of the Nrf2 pathway. The aim was to investigate the possible exogenous and endogenous redox signaling and especially the cross talk of H_2S and NO as two of the most important gasotransmitters, which has not been demonstrated so far in human umbilical vein endothelial cells.

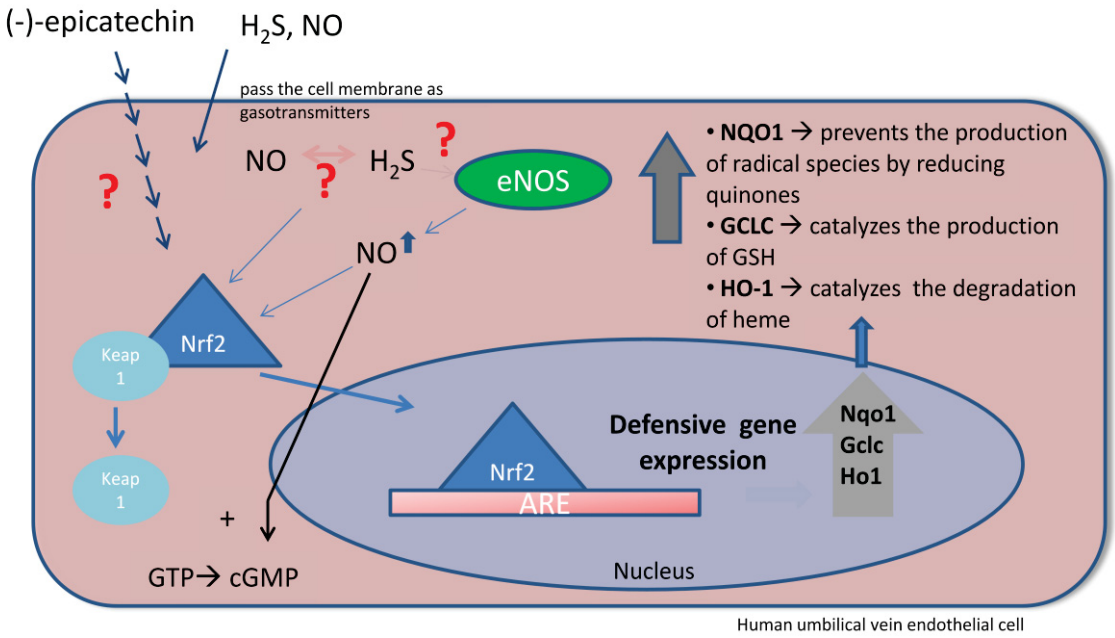


Fig. 7: Aims of this study. The study had the major aim to investigate the possible exogenous and endogenous redox signaling and the cross talk of H_2S and NO to reduce cell damage and oxidative stress.

This work aims modulating the endothelial cell to protect it from oxidative stress by posing following questions: 1) Are the phase II detoxifying enzymes upregulated in the EC by incubating with different stimuli influencing the Nrf2 pathway? 2) Does this also affect the GSH/GSSH pathway performing antioxidative protection? 3) Do oxidative conditions affect the cGMP level in ECs? 4) Does a co-incubation with H_2S and NO provoke an even higher enhancement of the phase II detoxifying enzymes, the GSH levels and the cGMP levels in EC?

3 Material

3.1 Cells

HUVECs Lot 1110701	Promocell	Heidelberg, DE
HUVECs Lot 1122701	Promocell	Heidelberg, DE

3.2 Media und Antibiotics

DMEM	Promocell	Heidelberg, DE
Dulbeccos PBS	PAA/GE Healthcare	Buckinghamshire, UK
Endothelial Cell Basal Medium	Promocell	Heidelberg, DE
Endothelial Cell Growth Medium SupplementMix	Promocell	Heidelberg, DE
FCS	Promocell	Heidelberg, DE
human Fibronectin	Biochrom/Merck KGaA	Darmstadt, Germany
Penicillin/Steptomycin	PAA/GE Healthcare	Buckinghamshire, UK
Trypsin EDTA	PAA/GE Healthcare	Buckinghamshire, UK

3.3 Western blot - Solutions and Buffer

NuPage 3-8% Tris-Acetate Gel	Novex	California, USA
Tris Acetat running buffer		
Transfer buffer		
Image Quant	GE healthcare	Buckinghamshire, UK
Membrane	GE healthcare	Buckinghamshire, UK
Whatman paper		
MagicMark^TM 220 / 120 / 100 / 80 / 60 / 50 / 40 / 30 / 20 kDa, unstained	life technologies	Carlsbad,USA
TBS (20x) pH 7,4	 1 l	Trizma base NaCl H_2O_{dd}
T-TBS	50 ml 1 ml 950 ml	TBS (20x) Tween 20 H_2O_{dd}
Running buffer	50 ml 950 ml	Tris Acetat running buffer(20x) H_2O_{dd}
Inner chamber buffer	200 ml 1 ml	running buffer antioxidant

Transfer buffer	50 ml 25 ml 425 ml	Methanol running buffer (20x) H_2O_{dd}

3.4 Chemicals

(-)-Epicatechin	Sigma-Aldrich	St. Louis, USA
10x PVP-Solution	Mecatronic	Netherlands
StemPro® Accutase® Cell Dissociation Reagent	Thermo Fisher Scientific	Massachusetts, USA
acetic acid	Carl Roth GmbH	Karlsruhe, Germany
Aqua bidest	Merck Millipore	Darmstadt, DE
beta-Mercaptoethanol	Sigma-Aldrich	St. Louis, USA
Bovine serum albumin	Sigma-Aldrich	St. Louis, USA
Calciumchlorid ($CaCl_2$)	Sigma-Aldrich	St. Louis, USA
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	St. Louis, USA
Ethanol	Merck KGaA	Darmstadt, Germany
GYY 4137	Cayman	Michigan, USA
Hybond P membrane	GE healthcare	Buckinghamshire, UK
Hydrochloride acid (HCl) 25%	Merck KGaA	Darmstadt, Germany
Hydrogen peroxide 30% (H_2O_2);	Carl Roth GmbH	Karlsruhe, Germany
L-Arginin	Carl Roth GmbH	Karlsruhe, Germany
L-NAME	Enzo Life Sciences GmbH	Lörrach, Germany
Magic Mark XP Western Protein Standard	Invitrogen	Carlsbad, USA
Magnesium sulfate (MgSO4	Sigma-Aldrich	St. Louis, USA
Methanol	Merck KGaA	Darmstadt, Germany
Methanol, β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate	Sigma-Aldrich	St. Louis, USA
Milkpowder blotting grade	Sigma-Aldrich	St. Louis, USA
Neutralred	Sigma-Aldrich	St. Louis, USA
NuPAGE LDS Sample Buffer	Invitrogen	Carlsbad, USA
NuPAGE Reducing Agent	Invitrogen	Carlsbad, USA
NuPAGE Transferbuffer	Invitrogen	Carlsbad, USA
NuPAGE Tris acetate running buffer	Invitrogen	Carlsbad, USA
Protease-Phosphatase-Inhibitor Cocktail	Bio-Rad	Munich, Germany
RIPA Buffer	Thermo Fisher Scientific	Massachusetts, USA
SNAP	Cayman	Michigan, USA
Sodium chloride (NaCl)	Sigma-Aldrich	St. Louis, USA
Sodium dihydrogen phosphate ($Na_2H_2PO_4$)	Sigma-Aldrich	St. Louis, USA
Sodium hydrogencarbonat ($NaHCO_3$)	Sigma-Aldrich	St. Louis, USA
Sodium hydroxide (NaOH)	Merck KGaA	Darmstadt, Germany
tBHQ	Sigma-Aldrich	St. Louis, USA
Thiol Tracker Violet	Thermo Fisher Scientific	Massachusetts, USA

Trizma(R) Base (Tris)		
Trypsin EDTA	PAA/GE Healthcare	Buckinghamshire, UK

3.5 Consumables

12 well plates	Greiner Bio One	Frickenhausen, DE
12 well plates	Corning	NY, USA
6 well plates	Greiner Bio One	Frickenhausen, DE
6 well plates	Corning	NY, USA
cell scraper 18mm blade	Becton, Dickinson and Company (BD)	Franklin Lakes, USA
petridishes 100mm	Greiner Bio One	Frickenhausen, DE
96-well-micro-plates black, flat bottom, fluotrac200	Greiner Bio One	Frickenhausen, DE
96-well-micro-plates clear, flat bottom	Greiner Bio One	Frickenhausen, DE
96-well-micro-plates white , flat bottom	Greiner Bio One	Frickenhausen, DE
Falcons 15ml	Greiner Bio One	Frickenhausen, DE
Falcons 50ml	Greiner Bio One	Frickenhausen, DE
NuPage 3-8% Tris-Acetate Gel	Novex	California, USA
NuPage 7% Tris-Acetate Gel	Novex	California, USA
Gloves, nitril powder free	Ansell	Tamworth, UK
Nitrocellulose Membrane Ammersham Hybond-P	GE Healthcare	Buckinghamshire, UK
Parafilm “M”	Bemis	Wisconsin, USA
Pipette filter tip	Star Lab	Hamburg,DE
Pipette tip TipOne 10µl	Star Lab	Hamburg,DE
Pipette tip TipOne 100µl	Star Lab	Hamburg,DE
Pipette tip TipOne 1000µl	Star Lab	Hamburg,DE
Safe-Lock tubes 2,0ml	Eppendorf	Hamburg,DE
Single-use Syringes 10ml	B. Braun AG	Melsungen, DE
Single-use Syringes 20ml	B. Braun AG	Melsungen, DE
Single-use Syringes 5ml	B. Braun AG	Melsungen, DE
Stripetten Costar® 10ml	Corning	NY, USA
Stripetten Costar® 25ml	Corning	NY, USA
Stripetten Costar® 5ml	Corning	NY, USA

3.6 Gadgets

Autoclave Systec DX-90	Systec	Linden, DE
Centrifuge 5417 R	Eppendorf	Hamburg, DE
Centrifuge Mikro 200R	Hettich	Kirchlengern,DE
Centrifuge Rotina 380R	Hettich	Kirchlengern,DE
Centrifuge Rotina 35R	Hettich	Kirchlengern,DE
Incubator Heraeus BBD 6220	Thermo Scientific	Massachusetts, USA
Incubator Heracell 240	Thermo Scientific	Massachusetts, USA
Laminar air flow	Clean Air	Illinois, USA

FACSVerse	BD biosciences	New Jersey, USA
Fluostar Omega	BMG Labtech GmbH	Ortenberg, DE
Millipore Filter	Merck Millipore	Darmstadt, DE
Micro pippets	Eppendorf	Hamburg, DE
Vortex	scientific industries	New York, USA
Heating cabinet TH15	Edmund Bühler	Tübingen, DE
Heating oven	Memmert GmbH & CO. KG	Schwabach, DE
ImageQuant LAS400	GE Healthcare	Buckinghamshire, UK
pH Meter Lab870	Schott Instruments	Mainz, DE
Pipet boy comfort	Integra Bioscience	Biebertal, DE
Testtube heater	Stuart Scientific	Staffordshire, UK
Thermo Cycler		
ABI Prism 7900HT Sequence Detection System	applied biosystems	Carlsbad, USA

3.7 Kits

Glutathione (GSH) Colorimetric Detection Kit	arbor assays	Michigan, USA
cGMP	arbor assays	Michigan, USA
DC Protein Assay	BioRad	Munich, Germany

3.8 Software

MS excel	microsoft	Albuquerque, USA
MS word	microsoft	Albuquerque, USA
MS powerpoint	microsoft	Albuquerque, USA
EndNote	Thomson Reuters (Scientific) LLC	New York City, USA
FlowJo	FlowJo Enterprise LLC	Ashland, Oregon, USA
GraphPad Prism	GraphPad Software, Inc.	La Jolla, CA, USA

4 Methods

4.1 Static Cell Culture

The human umbilical vein endothelial cells (HUVEC-c pooled, Promocell, Lot No. 1110701 and 1122701) were maintained in endothelial cell growth medium (PromoCell, C-22210) and endothel cell growth medium supplement mix (PromoCell, C-39215) with 1% of the antibiotics penicillin and streptomycin. For culturing we used 100 mm^2 petri dishes coated with a fibronectin layer. Therefore a 10 µg/ml Fibronectin/PBS solution was added to the dishes and aspirated at once. The dishes were dried 30 minutes. The HUVECs were passaged two times for the experiments (P2), because there were nearly no effects of the treatments in higher passages. The dishes were washed once with PBS and incubated at 37 °C and 5% CO_2 with 3 ml trypsin. After 3 minutes of incubation the progress of dissolving of the endothelial cells was checked under a light microscope. If the cells detached from the dish layer, 5 ml of DMEM with 2% FCS (inactivated by incubating 10 minutes at 60°C) and 1 % antibiotics (penicillin and streptomycin) were put on the trypsin/cell solution and were centrifugated for 10 minutes with 300 g. The pellets were mixed with 1 ml growth medium per new dish and were proliferated 1:3. For the experiments the cells were seeded into 6well or 12well plates. Therefore, they were trypsinated another time and counted in a *Neubauer Chamber*. Hence, 10 µL of the cell suspension were pipetted in the chamber. Then, the cells in the 16 squares were counted under a light microscope. The result was then divided with four. This number was multiplicated by 10^4, giving the number of cells per ml. For each experiment, we used the same cell count (e.g. 6well plates 1.5 x 10^5 cells/ml).

4.2 Summary of the main methods

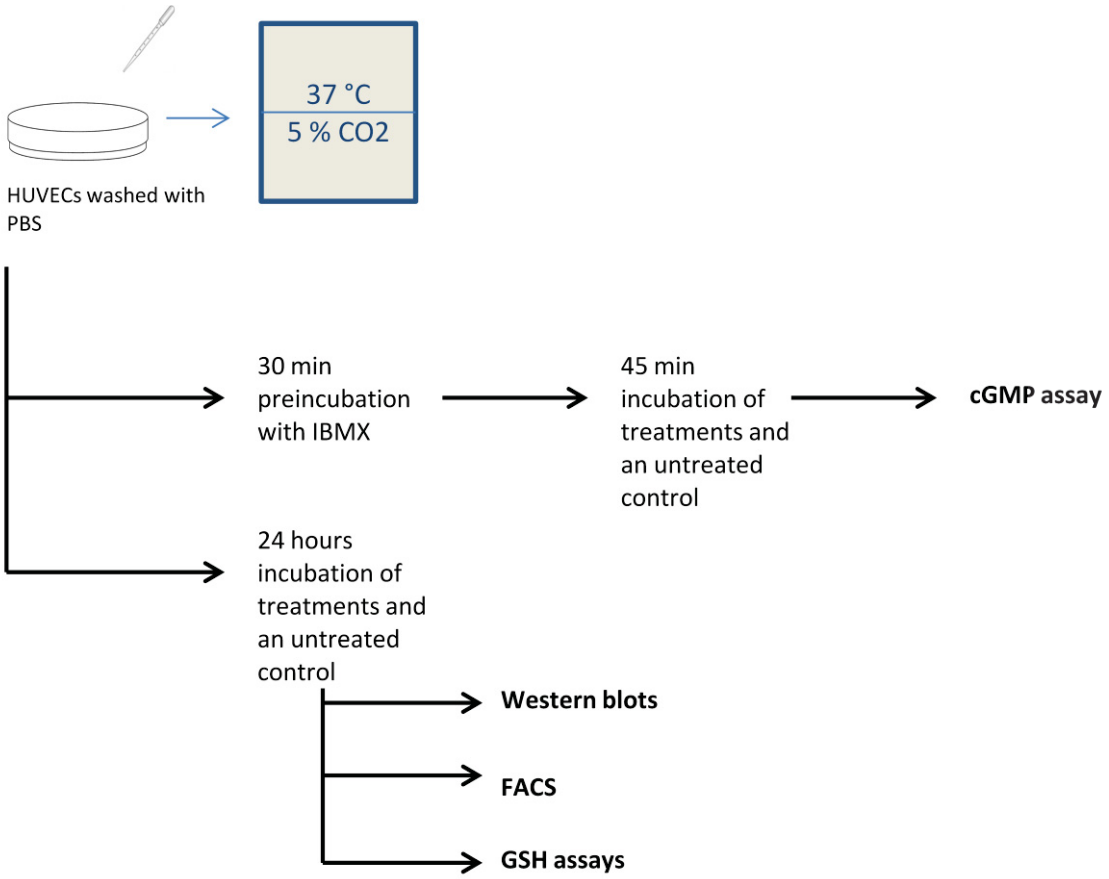


Fig. 8: Summary of the main methods in cell culture and their incubation times.

4.3 Neutral red toxicity test

For toxicity tests we used the neutral red toxicity test to estimate the cell viability. HUVECs were seeded in 12well plates (1 x 10^5 cells/ml) and were checked under the light microscope. When they were 80 % confluent, medium was aspirated and washed one time with PBS. Then the EC were maintained in medium incubated with different treatments for 24 hours.

4.3.1 Treatments

1. (-) – Epicatechin: 0.1 µM; 1 µM; 10µM; 100µM; 1µM tert butylhydrochinon (tBHQ) and a control without treatment.
2. GYY: 10 µM, 50 µM, 100 µM and a control without treatment.
3. A control without treatment, 100 µM SNAP, double incubation of 10 µM, 50 µM, 100 µM and 1000µM GYY with 100 µM SNAP.

4. A control without treatment, 10 µM SNAP, double incubation of 10 µM, 50 µM, 100 µM and 1000µM GYY with 100 µM SNAP.
5. L – Name – double incubation: 10 µM, 50 µM, 100 µM and 1000µM GYY with 1mg L- Name (L-N^G-Nitroarginine methyl ester) and a control without treatment

After the 24 hours of incubation medium was aspirated and the HUVECs were washed three times with PBS.

20 µl of neutral red were pipetted in 2 ml of medium (1:100 dilution) and put on each well. After 60 minutes of incubation at 37°C and 5% CO_2 the cells were washed 3 times with PBS, then were dried under the extractor hood. To measure the absorption at 450 nm, 500 µl isopropanolol with 1% of 1M HCl were added to each well. Those were transferred in 96well plates to measure the absorption at 450 nm using a photometer.

4.4 Western blots

HUVECs seeded in 6well plates (1.5 x 10^5 cells/ml) were checked under the light microscope. When they were totally confluent medium was aspirated and washed one time with PBS. Then the EC were maintained in medium with different treatments and were incubated for 24 hours at 37°C and 5% CO_2.

4.4.1 Treatments

1. (-) – Epicatechin: 0.1 µM; 1 µM; 10µM; 100µM; 1µM tert butylhydrochinon (tBHQ) as positive control and a control without treatment.
2. GYY: 10 µM, 50 µM, 100 µM, a control without treatment, 10 µM SNAP as a positive control, double incubation of 10 µM, 50 µM, 100 µM GYY with 10 µM SNAP.
3. GYY: 10 µM, 50 µM, 100 µM, a control without treatment, 100 µM SNAP as a positive control, double incubation of 10 µM, 50 µM, 100 µM GYY with 100 µM SNAP.
4. L – Name: 10 µM, 50 µM, 100 µM, 1mg L- Name (L-N^G-Nitroarginine methyl ester), a control without treatment, 100 µM SNAP as a positive control, double incubation of 10 µM, 50 µM, 100 µM GYY with 1mg L - Name.

After the 24 hours of incubation medium was aspirated and the HUVECs were washed three times with PBS.

PBS was aspirated completely and 50 µl of RIPA Buffer (ThermoFischerScientific) and protease/phosphatase-inhibitor (BioRad) were added. The cells were solved from the wells using a cell scraper. Then the solution of RIPA Buffer, protease/phosphatase-inhibitor and cells were put in one Eppendorf tube per well. These were put directly on ice. The cold Eppendorf tubes were taken in an ultrasonic bath at 4°C for 1 minute and then centrifugated for 10 minutes at 4°C by 13 000 g. The supernatant of the Eppendorf tubes was aspirated and each was put into another fresh tube.
To determine the concentration of proteins in each Eppendorf tube, we used the BioRad protein assay, based on the method of Bradford.

4.4.2 SDS-Page

Samples were diluted in double distilled water to a final concentration of 50 µg protein in 16.25 µl of H2Odd. 6.25 µl of NuPage 4x LDS sample buffer and 2.5 µl of NuPage 10 x sample reducing agent were added and all sample mixtures were incubated at 70°C for 10 minutes. In the mean time the 1x running buffer (5 % tris acetate) for the outer chamber was prepared. 500 µl of NuPage Antioxidant was mixed with 200 ml of the running buffer to prepare the buffer for the inner chamber. The gel (10 % tris acetate, fitted to the size of the protein of interest) was put into the chamber and after the buffers were added, the comb was removed and the slots were washed one time with buffer.
10µl of the molecular weight marker (Magic Mark XP by life technologies) was put into the first slot and the prepared samples were inserted into the other ones. To start the SDS-Page the running module was connected to a continuous current of 200 mV for one hour.

4.4.3 Transfer

After the one hour run the gel was removed from the chamber and placed on a whatman paper saturated in transfer buffer (Aqua bidest with 10 % of MeOH, 5 % of 20 x NuPage transfer buffer and 0.1% NuPage antioxidant). A membrane was positioned onto the gel and a second saturated whatman paper was put above. This element was rolled out with an empty falcon tube to avoid any bubbles inside and put between 4 sponges into the transfer module. The inner chamber was filled with transfer buffer, the outer chamber just with double distilled water. The whole blot module was connected to a continuous current of 30 mV for one hour.

4.4.4 Blocking

After taking the membrane out of the module, it was incubated with Ponceau S for 5 minutes to detect if the transfer was successful and to consolidate the proteins. Then the membrane was washed with H_2Odd several times. Afterwards it was incubated with blocking solution (5% skim milk/T-TBS) at room temperature for 2 hours.

4.4.5 Primary antibody incubation

Gene symbol	term	#ID	
NQO1	NADPH dehydrokinase quinone 1.	2346	Abcam
GCLC	glutamate cysteine ligase catalytic subunit	41463	Abcam
HO-1	hemeoxigenase I	13248	Abcam
αTub	Alpha Tubulin	4074	Abcam

Tbl. 1: Antibodies for western blot

After 15 minutes washing in TBS solution the membrane was incubated at 4°Cover night with different concentrations of primary antibodies (see Tbl. 1) diluted in 5% BSA/T-TBS solution.

4.4.6 Secondary antibody incubation

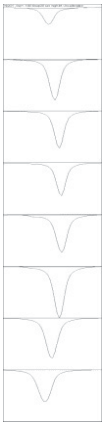
Membranes were washed 5 x 5 minutes in T-TBS. Then they were incubated in 1:5000 different secondary antibody solutions (referred to the primary antibodies) also diluting of 5% BSA/T-TBS.

4.4.7 Chemiluminescence

Membranes were washed again 5 times for 5 minutes in T-TBS and then put on a T-TBS saturated whatman paper. To detect the protein we used the detection kit and measured the chemiluminescence signal with ImageQuant by GE.

4.4.8 Stripping

To strip the membranes, they were put in stripping buffer 49 µl betaMercaptoethanol diluted in 7 ml of stripping buffer at 70°C for 30 minutes and then washed one time for 15 minutes in TBS on the shaker.



4.4.9 Densitometric analysis

The densitometric analysis was made using ImageJ software. Bands were quantified through this program to determine the colour strength of each band. Then the area under the curve (see Fig. 9) was calculated and compared using Excel. The graph was developed using graph prism 5.0.

Fig. 9: Densitometric analysis using ImageJ software. The colour strength of each band was quantified. This figure shows some representative areas under the curve. These were calculated to get a densitometric analysis

4.5 Flow cytometry

For flow cytometry analysis HUVECs seeded in 12well plates (1 x 10^5 cells/ml) were checked under the light microscope. When they were 80 % confluent medium was aspirated and washed one time with PBS. Then the EC were maintained in medium with different treatments for 24 hours at 37°C and 5% CO_2.

1. (-) – Epicatechin: 0.1 µM; 1 µM; 10µM; 100µM; 1µM tBHQ as positive control, double incubation of all used concentrations of (-)-epicatechin with 1 mM of L-Name and a control only with L-Name and a control without treatment.
2. GYY: 10 µM, 50 µM, 100 µM, a control without treatment, 10 µM SNAP as a positive control, double incubation of 100 µM, 500 µM, 1000 µM GYY with 10 µM SNAP.
3. GYY: 10 µM, 50 µM, 100 µM, a control without treatment, 100 µM SNAP as a positive control, double incubation of 100 µM, 500 µM, 1000 µM GYY with 100 µM SNAP.

After the incubation medium was aspirated and the cells were washed one time with PBS, then the cells were maintained in medium. 5 µM of Thiol Tracker Violet (Thermo Fisher Scientific, diluted in DMSO) as a GSH detection reagent was pipetted on the ECs. After 30 minutes incubation at 37°C, the medium diluted with thiol tracker was aspirated and the cells were washed again one time with PBS. Then 500µl accutase was put on each well and incubated again for 10 minutes at 37°C and 5 % CO_2. The mixture was transferred in falcon tubes for performing the FACS analysis by BD FACSVerse.

4.6 Glutathione Assay

HUVECs seeded into 6well plates (1.5 x 10^5 cells/ml) were checked under the light microscope. When they reached a confluence of 80 %, differences in cellular glutathione levels were detected by incubating 24 hours with following treatments:

1. GYY: 10 µM, 50 µM, 100 µM, a control without treatment, 10 µM SNAP as a positive control, double incubation of 10 µM, 50 µM, 100 µM GYY with 10 µM SNAP.
2. GYY: 10 µM, 50 µM, 100 µM, a control without treatment, 100 µM SNAP as a positive control, double incubation of 10 µM, 50 µM, 100 µM GYY with 100 µM SNAP.
3. L – Name: 10 µM, 50 µM, 100 µM, 1mg L- Name (L-N^G-Nitroarginine methyl ester), a control without treatment, 100 µM SNAP as a positive control, double incubation of 10 µM, 50 µM, 100 µM GYY with 1mg L - Name.

After aspirating the medium and washing one time with PBS, cells were lysed in 200 µl of 0.1 M HCl per well. The cells were solved from the wells using a cell scraper. Then the solution was transferred to one Eppendorf tube per well. These were put directly on ice. The iced Eppendorf tubes were taken in an ultrasonic bath at 4°C for 1 minute and then centrifugated for 10 minutes at 4°C and 13 000 G. The supernatant of the Eppendorf tubes was aspirated and each pellet was transferred into another fresh tube.

To detect cellular protein levels the NanoQuant protein assay was chosen. Samples were diluted 1:20 and buffered in a 100 mM TRIS buffer.

Total GSH and GSSG levels were determined by GSH and total GSH detection kit from Arbor Assays.

4.7 cGMP Assay

HUVECs seeded into 6well plates (1.5 x 10^5 cells/ml) were checked under the light microscope. When they were confluent, medium was aspirated and the endothelial cells were incubated for 30 minutes in 1 µl 1-Methyl-3-Isobutylxanthine (IBMX)/ml Medium to inhibit the cyclic GMP phosphodiesterase. By inhibiting PDE, IBMX increases cellular cAMP and cGMP levels.

After 30 minutes of incubation the following treatments were added (2 wells for each treatment):

1. GYY: 10 µM, 50 µM, 100 µM, a control without treatment, 10 µM SNAP as a positive control, double incubation of 10 µM, 50 µM, 100 µM GYY with 10 µM SNAP.
2. GYY: 10 µM, 50 µM, 100 µM, a control without treatment, 100 µM SNAP as a positive control, double incubation of 10 µM, 50 µM, 100 µM GYY with 100 µM SNAP.
3. L – Name: : 10 µM, 50 µM, 100 µM, 1mg L- Name (L-N^G-Nitroarginine methyl ester), a control without treatment, 100 µM SNAP as a positive control, double incubation of 10 µM, 50 µM, 100 µM GYY with 1mg L - Name.

The cells were incubated for 45 minutes and then, the medium was aspirated and the cells were washed one time with PBS. For lysis of the cells 50 µl of 0.1 M HCl was added to each well and those wells with the same treatment were pooled in one Eppendorf tube. These were put directly on ice. The iced Eppendorf tubes were taken in an ultrasonic bath at 4°C for 1 minute and then centrifugated for 10 minutes at 4°C by 13 000 G. The supernatant of the Eppendorf tubes was aspirated and each pellet was transferred into another fresh tube.

For the detection of cellular protein levels NanoQuant protein assay was chosen. Samples were diluted 1:20 in a 100 mM TRIS buffer.

To determine the cGMP concentrations Direct Cyclic GMP Chemiluminescent Kit from Arbor Assays was used.

4.8 Statistic analysis + Software

All values are reported as means with standard errors of the means (SEM). Comparisons were performed via two tailed T-test or ANOVA with Bonferroni post hoc tests for multiple comparisons. Differences were considered significant with $p<0.05$. Assay analysis and descriptive statistics was performed with Microsoft Excel.

Major parts of descriptive and all inference statistics were performed with GraphPad Prism 5. For reference managing EndNote X7 was used.

5 Results

5.1 Plan of the study and experimental setup

To address the aims of this study we modulated the redox state of the EC through exogenous and endogenous stimuli.

The first step was to determine whether (-)-epicatechin as exogenous stimuli and H_2S and NO as endogenous stimuli affect the Nrf2 pathway by increasing the phase II detoxifying enzymes. Another possibility to modulate the redox state of the EC we used was the regulation of the GSH/GSSG system. Furthermore, the affection of the cGMP levels in EC was tested.

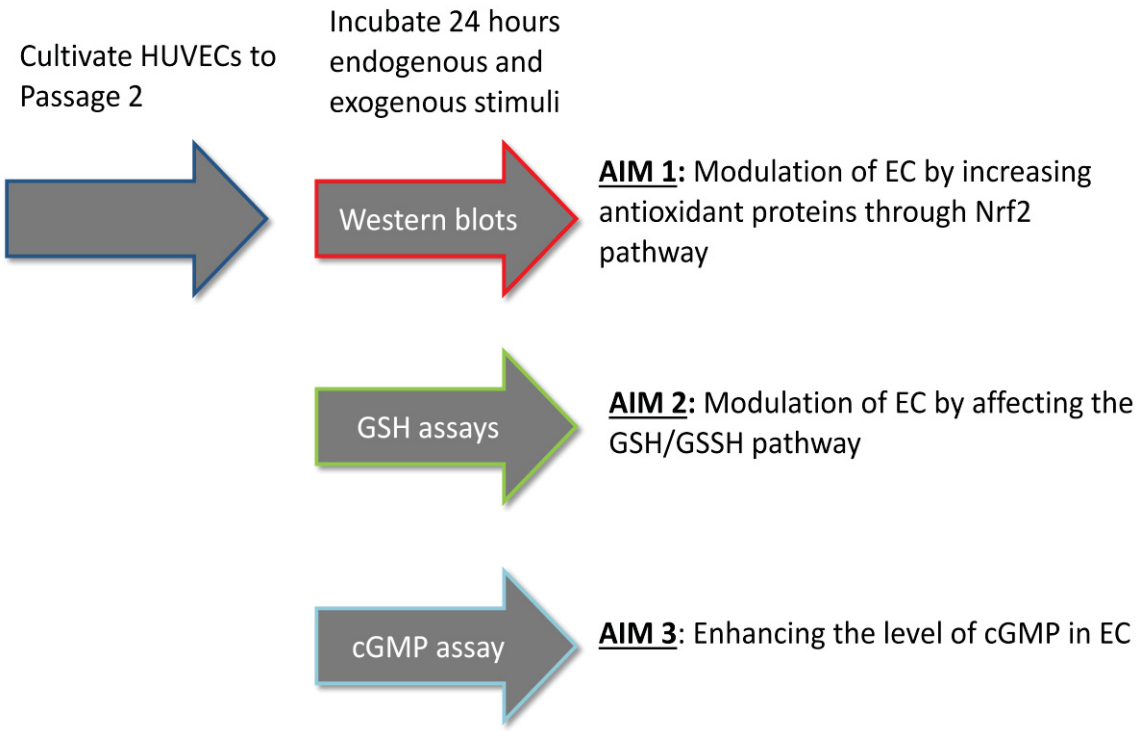


Fig. 10: Experimental setup. The experimental setup was based on western blots, GSH assays and cGMP assays of EC.

5.2 Neutral red staining experiments

To ensure that the compounds and their different concentrations which were used in this study have no toxic effects on HUVECs neutral red staining experiments were performed. For this reason, the cells were treated for 24 h with all compounds used in this work and stained with neutral red afterwards.

1. (-) – Epicatechin: 0.1 µM; 1 µM; 10µM; 100µM; 1µM tert butylhydrochinon (tBHQ) and a control without treatment.
2. GYY: 10 µM, 50 µM, 100 µM and a control without treatment.
3. A control without treatment, 100 µM SNAP, double incubation of 10 µM, 50 µM, 100 µM and 1000µM GYY with 100 µM SNAP.
4. A control without treatment, 10 µM SNAP, double incubation of 10 µM, 50 µM, 100 µM and 1000µM GYY with 100 µM SNAP.
5. L – Name – double incubation: 10 µM, 50 µM, 100 µM and 1000µM GYY with 1mg L- Name (L-N^G-Nitroarginine methyl ester) and a control without treatment

Fig. 11 and Fig. 12 show the absorbance of 450 nm correspondent to HUVECs viability after treatment.

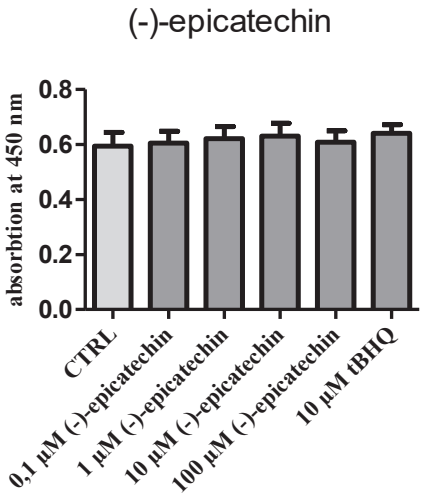


Fig. 11: Neutral red assay shows no toxic effects of (-)-epicatechin and tBHQ. EC were incubated for 24 h in the presence of the treatment indicated. Viability was determined by neutral red staining. The Graph shows absorbance at 450 nm equivalent to cell viability. (-)-epicatechin exerted no toxic effects on EC. Values represent the mean±SEM (n=6).

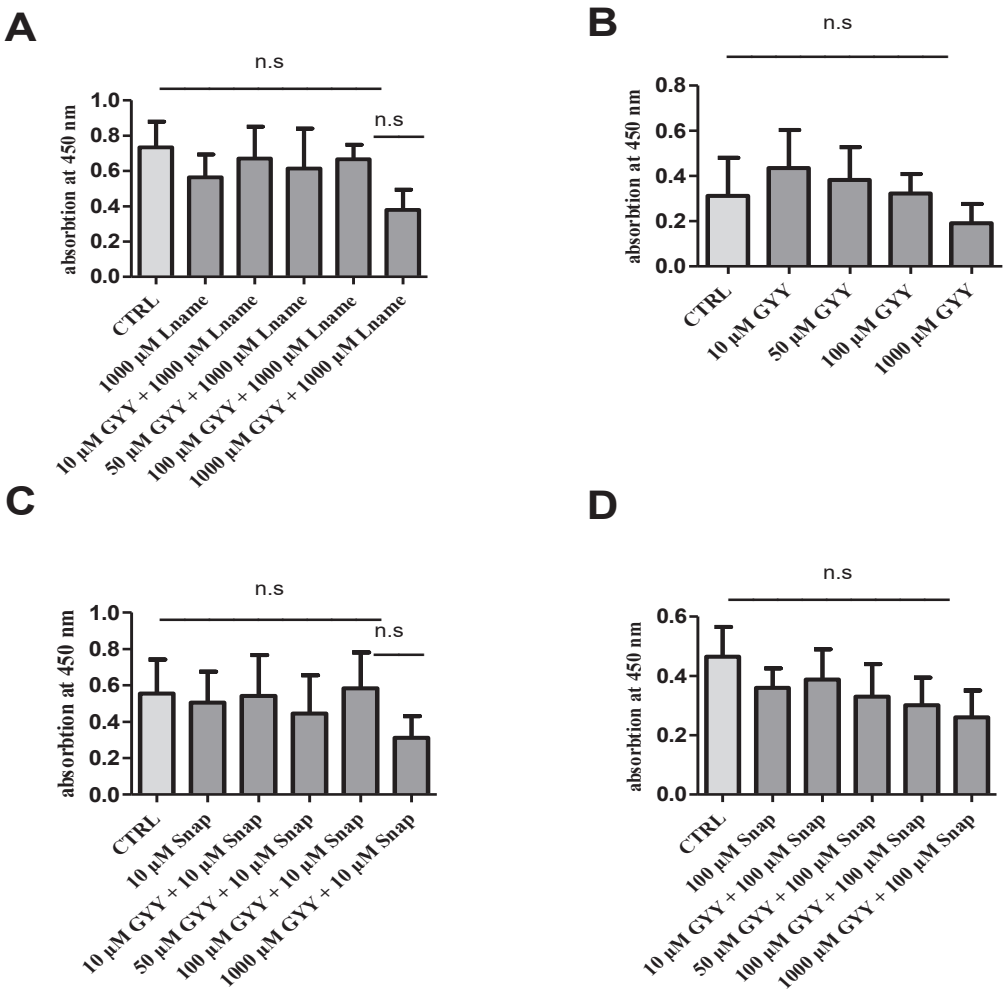


Fig. 12: EC were incubated for 24 h in the presence of the treatment indicated. Viability was determined by neutral red staining. The graph shows absorbance at 450 nm equivalent to cell viability. The treatment with 1000 µM of GYY released possible toxic effects on EC. Values represent the mean ± SEM (n=3, * = p < 0,005, n.s. = no statistical difference).

Neutral red staining experiments showed that for the treatments A, C and D no significant effects of the viability of ECs at the concentrations selected. However, GYY 4137 showed decreased cell viability at concentrations of 1000 µM (see Fig. 12 B), but not statistically significant from control. These findings were confirmed with observations via microscopy, which are not displayed. Cellular deformations were seen after administration of 1000 µM GYY 4137 to the medium.

Still, the other treatments did not affect cell viability while treatments with 1000 µM GYY 4137 reduced it considerably.

5.3 Western blots

5.3.1 (-)-epicatechin did not increase phase II detoxifying enzymes significantly

To analyze if (-)-epicatechin increases the protein levels of antioxidants as NQO1, HO-1 and GCLC western blots were used. Therefore, HUVECs were incubated 24 hours at 37°C, 5 % CO_2 with 0.1 µM, 1 µM, 10 µM and 100 µM (-)-epicatechin (solved in DMSO) and with 1 mM of tBHQ as positive control.

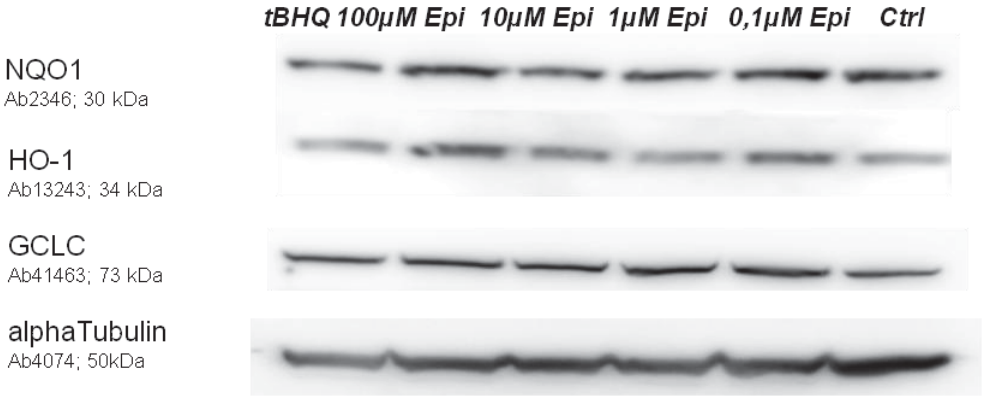


Fig. 13: Effects of Epicatechin on the protein levels of NQO1, HO1 and GCLC in HUVECs. EC were incubated for 24 h in the presence of the treatment indicated. tBHQ was used as positive control. Total protein extract (50 µg) was separated and analyzed by western blot. This figure shows representative bands from the same membrane.

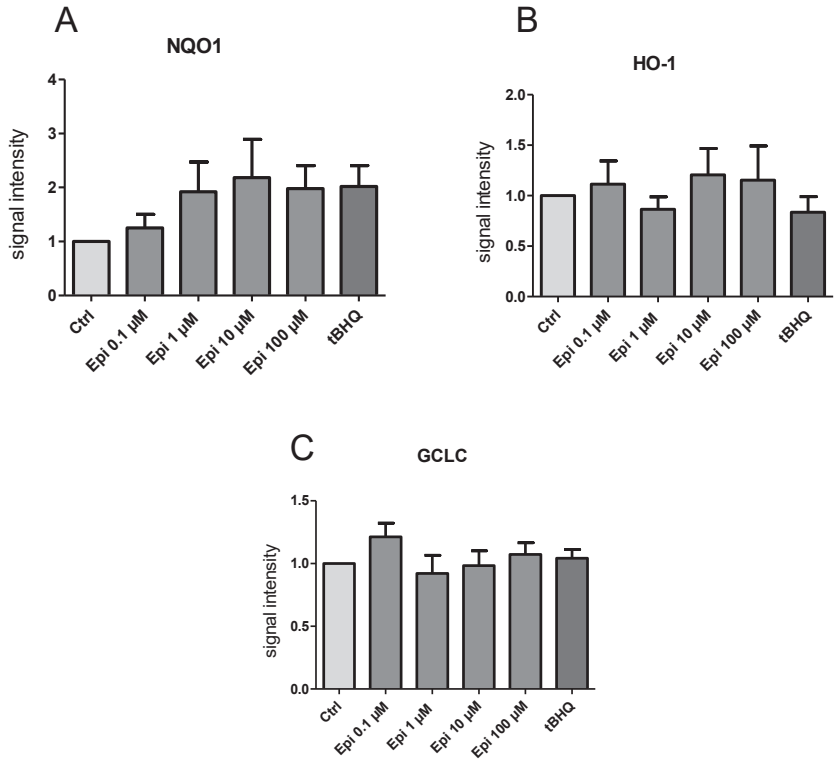


Fig. 14: Densitometric analysis of western blots. A) NQO1 (n=6) interacted with (-)-epicatechin depending on its concentration (error bars show SEM). B) HO1 (n=4) expression changes especially with 0.1 and 100µM (-)-epicatechin. C) The effect of (-)-epicatechin to GCLC (n=5) was enhanced of the treatment with 0.1 µM (-)-epicatechin 1,218 fold [95% CI 0.86; 1.59].

As shown in Fig. 13 and Fig. 14 (-)-epicatechin increased the protein levels of NQO1, GCLC and HO1 in a concentration-dependent manner, but the results showed no statistical significant difference, so there are no significant effects on the protein levels of phase II enzymes by incubation with (-)-epicatechin.

5.3.2 GYY Crosstalk

5.3.2.1 The co – incubation of H_2S and NO caused a higher enhancement of phase II detoxifying enzymes than single incubation of H_2S

5.3.2.1.1 Co – incubation with 100 µM of NO-donor SNAP

To analyze if GYY and the GYY/SNAP crosstalk increases the protein levels of antioxidants as NQO1, HO-1 and GCLC western blots were used. Therefore, HUVECs were incubated for 24 hours with 10 µM, 50 µM and 100 µM of GYY 4137 (solved in H_2O_{dd}) and with 100 µM of SNAP (solved in 5% DMSO). Furthermore, to analyze the crosstalk of GYY and SNAP, HUVECs were incubated with both substances in the same dish.

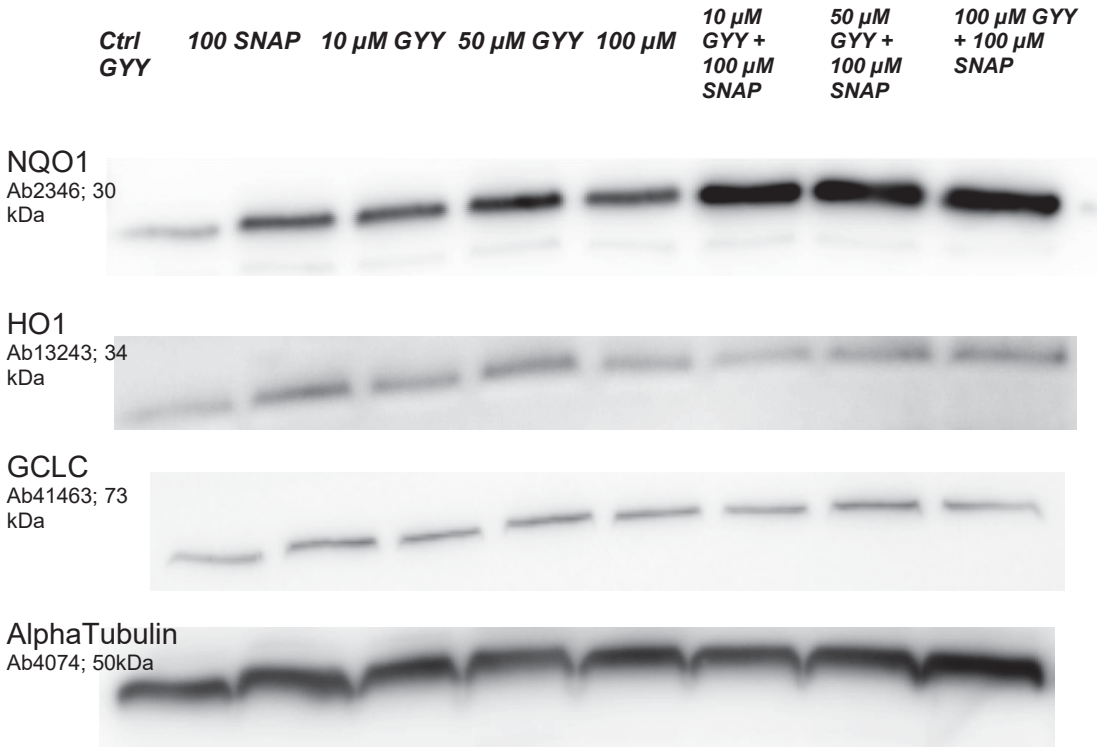


Fig. 15: GYY increases protein levels of NQO1, GCLC and HO1. EC were incubated 24 hours in the presence of the treatment indicated. 100 µM of S-Nitrosopenicillamin (SNAP) were used as a positive control. Total protein extract (50 µg) was separated and analyzed by western blot. This figure shows representative bands from the same membrane.

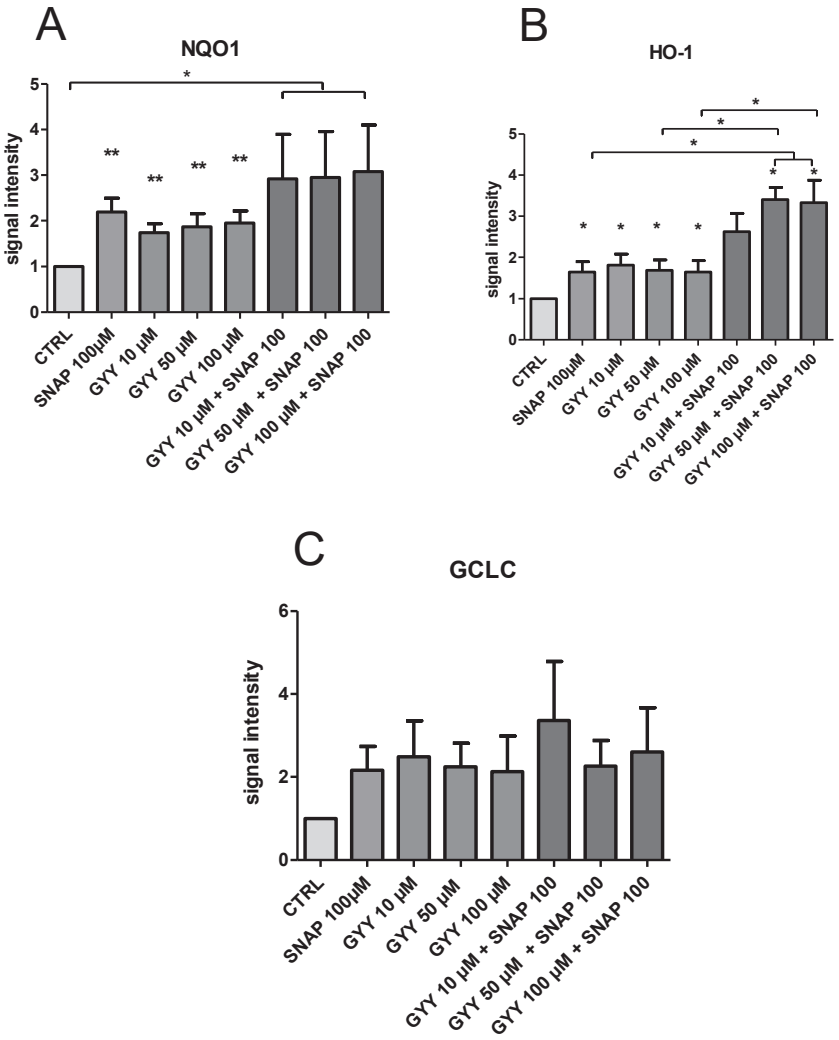


Fig. 16: GYY increases protein levels of NQO1 and HO-1. Densitometric anaylsis of western blots. GYY increased the protein levels of A) NQO1 (n=6), B) HO-1 (n=3) and C) GCLC (n=4). Co-incubation of SNAP and GYY increased the protein levels considerably (error bars show SEM, * P<0.05, ** P<0.01). GYY does not increase the protein levels of GCLC significantly.

Fig. 15 and Fig. 16 show that GYY itself increased the protein levels of HO1 and NQO1. Moreover, the crosstalk of the hydrogen sulfide donors GYY and 100 µM SNAP as a NO donor increased the protein levels of these antioxidant proteins considerably in a concentration-dependent manner. Though, the co-incubation of GYY and SNAP increased the protein levels of HO-1 significantly in comparison to 100 µM SNAP or 50 µM and 100 µM GYY in single incubation. The protein levels of NQO1 were enhanced depending on the GYY concentrations.

5.3.2.1.2 Co – incubation with 10 µM of NO-donor SNAP

To determine in which concentrations of SNAP the crosstalk of SNAP and GYY is more potent, western blots with 10 µM SNAP as a positive control were made as well as double incubation of 10 µM SNAP with the same concentrations of GYY.

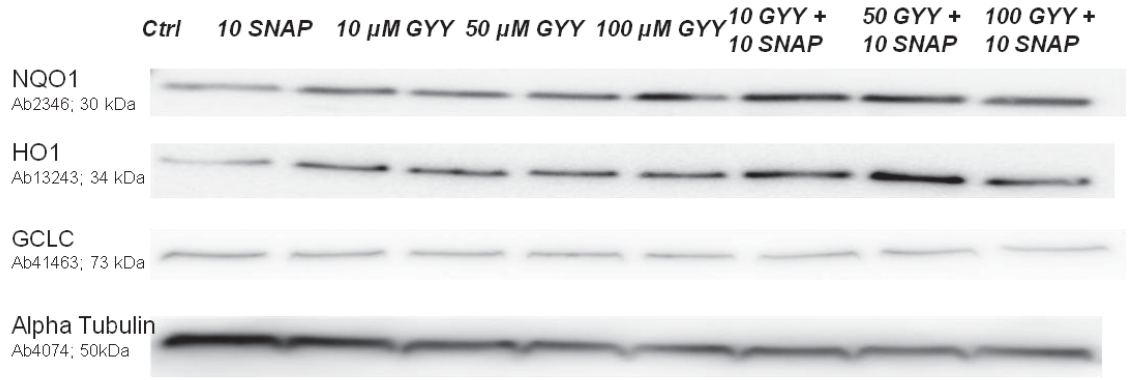


Fig. 17: GYY increases protein levels of NQO1 and HO1. EC were incubated 24 hours in the presence of the treatment indicated. 10 µM of SNAP was used as a positive control. Total protein extract (50 µg) was separated and analysed by western blot. This figure shows representative bands from the same membrane.

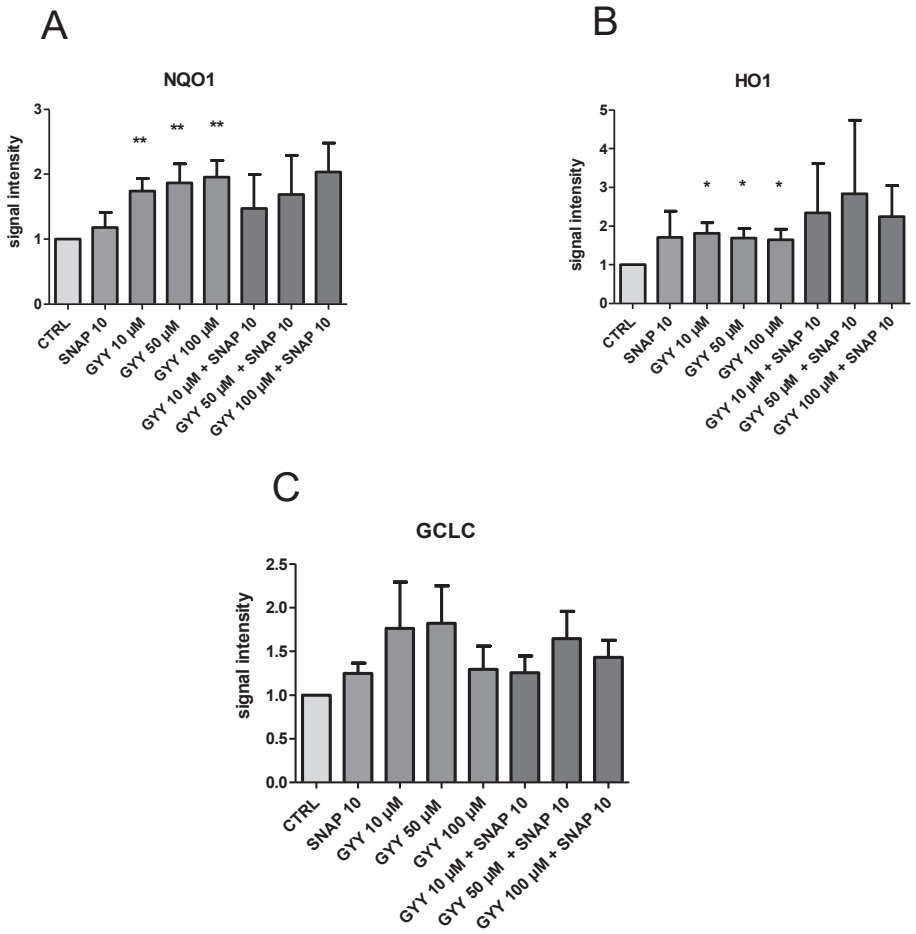


Fig. 18: GYY increases protein levels of NQO1 and HO1. Densitometric anaylsis of western blots. GYY increases the protein levels of NQO1 and HO1. Double incubation with 10 µM SNAP also increases the protein levels. GYY does not increase the protein levels of GCLC significantly (error bars show SEM, * P<0.05, ** P<0.01).

Fig. 17 and Fig. 18 show that 10 µM SNAP in single incubation increases the protein levels although not in the same dimension as single incubation of 100 µM SNAP. Nevertheless, co – incubation of 10 µM SNAP and GYY does not increase the protein levels of HO1 and NQO1 significantly. GYY as well as SNAP do not enhance the protein levels of GCLC significantly.

5.3.2.2 The NOS-Inhibitor L-NAME reduced the H_2S triggered enhancement of NQO1, HO1 and GCLC in double incubation with H_2S

To analyze if L – NAME as an NOS-inhibitor decreases NO production and affects the rise of NQO1, HO-1 and GCLC through GYY, western blots were used. HUVECs were incubated 24 hours with 10 µM, 50 µM and 100 µM of GYY 4137 (solved in clean water), 100 µM SNAP as positive control and with 1 mM L –Name in double incubation with the different concentrations of GYY.

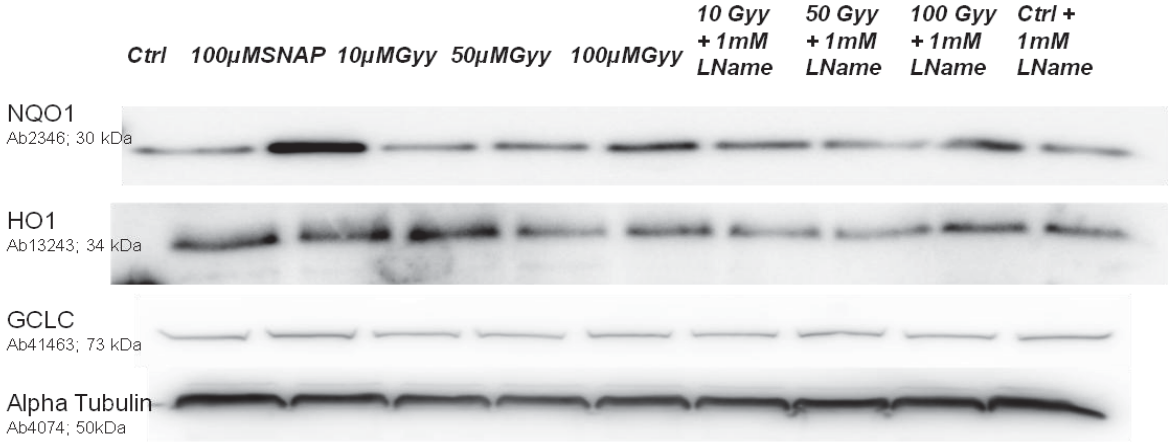


Fig. 19: GYY increases the protein levels of NQO1 and HO1 in HUVECs. L-Name however affects the effect of GYY as a sulfide donor and reduces the mentioned protein levels. EC were incubated for 24 h in the presence of the treatment indicated. 100 µM of SNAP was used as positive control. Total protein extract (50 µg) was separated and analysed by western blots. This figure shows representative bands from the same membrane.

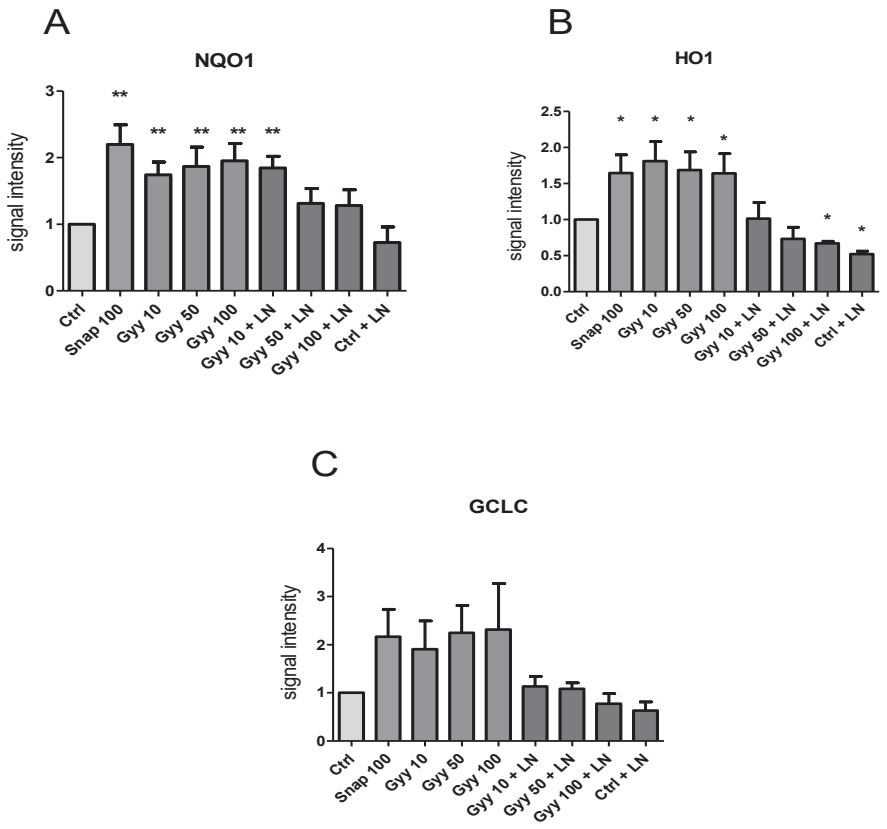


Fig. 20: GYY increased the protein levels of NQO1 and HO-1 in HUVECs. L-Name however affected the effect of GYY as a sulfide donor and reduced the mentioned protein levels. Densitometric analysis of western blots. HO-1 reacts especially with 10 and 50 µM GYY. L- Name inhibits the effect of 10, 50 and 100 µM GYY. NQO1 (n=6) interacts with GYY depending to its concentration. GYY does not increase the protein levels of GCLC significantly (errorbars show SEM, * P<0.05, ** P<0.01).

As seen in the figures before, GYY increased the protein levels of NQO1 and HO1. HO1 interacted especially with 10 and 50 µM GYY. The effect of GYY on NQO1 depended on the concentration of GYY. L – NAME as NOS inhibitor inhibited those gains, but only on HO-1 in a significant manner.

5.4 cGMP levels in EC were especially enhanced through co – incubation of H_2S and NO

5.4.1 Co – incubation of H_2S with 10 µM SNAP

To show how GYY and the co – incubation with SNAP and L – NAME effects the cGMP accumulation, a cGMP assay from Arbor Assays was used. HUVECs were preincubated 30 minutes with 1 µl Methyl-3-Isobutylxanthine (IBMX)unit?/ml Medium and then incubated 45 minutes with 10 µM, 50 µM and 100 µM of GYY 4137 (solved in sterile water).The HUVECs were incubated with 100 µM SNAP, 10 µM SNAP and 1 mM L –NAME in co – incubation with the different concentrations of GYY.

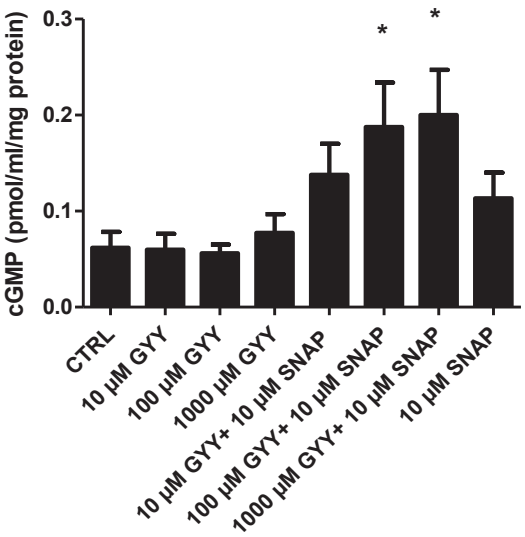


Fig. 21: Effects of co – incubation of GYY and 10 µM SNAP on the cGMP levels in HUVECs. EC were incubated for 45 minutes in the presence of the treatment indicated. cGMP assay was used to determine the cGMP concentration in each sample depending to its protein concentration. The co-incubation of 10 µM of SNAP and 100 and 1000 µM of GYY increases the cGMP levels significantly. Values represent the mean±SEM (n=4, *P<0.05, all means are significantly different (P<0.01)).

Fig. 21 shows that GYY in different concentrations did not enhance the cGMP levels in ECs significantly. SNAP in single incubation increased the cGMP levels as well, but not in a significant manner. However, the co – incubation of GYY with 10 µM of the NO donor SNAP enhanced the concentration of cGMP in ECs even more considerable due to the concentration of GYY significantly in comparison to the control.

5.4.2 Co – incubation of H_2S with 100 µM SNAP

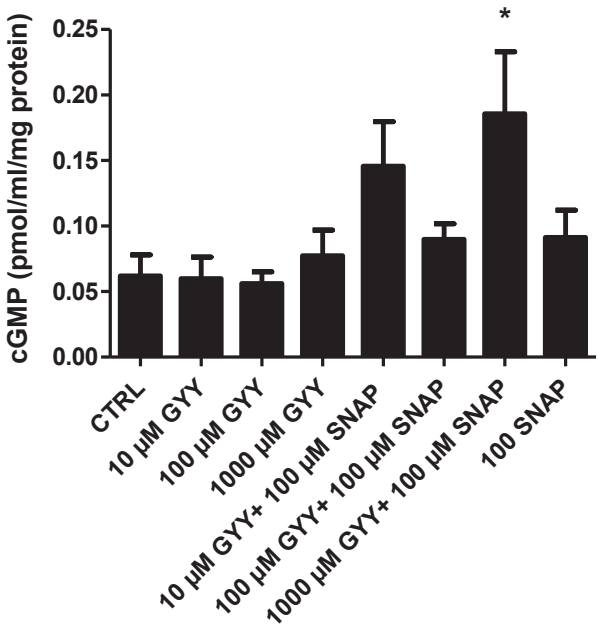


Fig. 22: Effects of the cGMP levels in HUVECs by incubation with GYY and 100 µM SNAP in different concentrations. EC were incubated for 45 minutes in the presence of the treatment indicated. cGMP assay was used to determine the cGMP concentration in each sample depending to its protein concentration. The double incubation of 100 µM of SNAP and 10, 100 and 1000 µM of GYY enhanced the cGMP levels in contrast to the single incubation, but not in a significant manner. Only the combination of 100 µM SNAP and 1000 µM GYY had a significant effect on the cGMP levels. Values represent the mean±SEM (n=3, *P<0.05, all means are significantly different (P<0.01)).

100 µM SNAP incubated in the presence of GYY increased the cGMP levels in a high intensity depending on the mixed concentrations like Fig. 22 shows. 100 µM of SNAP incubated with 100 µM of GYY didn´t rise the cGMP levels significantly as well as 10 µM of GYY. 1000 µM of GYY though, incubated together with 100 µM SNAP did enhance the cGMP levels significantly in comparison to the untreated control.

Taken together, these results show that only the co-presence of GYY and SNAP increased the cGMP concentrations in ECs significantly.

5.4.3 L-NAME did not affect the cGMP levels

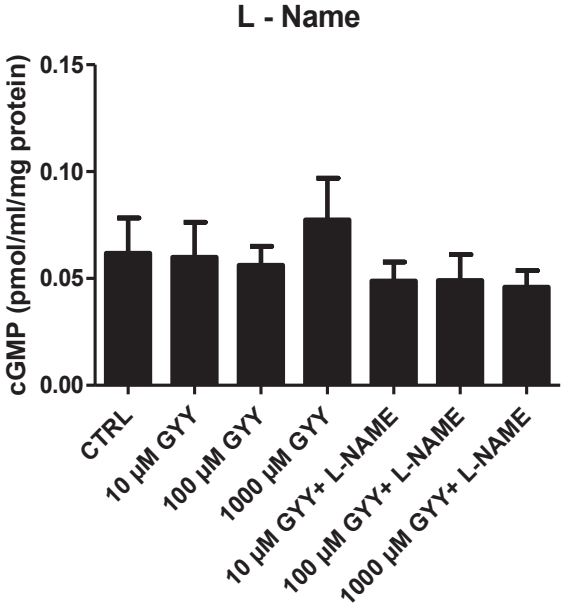


Fig. 23: L-Name did not affect the cGMP levels significantly. EC were incubated for 45 minutes in the presence of the treatment indicated. cGMP assay was used to determine the cGMP concentration in each sample depending to its protein concentration. GYY did not increase the cGMP levels and L-Name was not influencing cGMP synthesis in EC significantly. Values represent the mean±SEM.

GYY as well as the co-incubation with 1 mM of the NOS Inhibitor L-Name showed no significant effect on cGMP synthesis. These results show that L-Name as an eNOS inhibitor did not influence the cGMP synthesis.

5.5 Flow cytometry

5.5.1 0.1 µM and 100 µM of (-)-epicatechin enhanced the GSH levels in EC

To analyze if (-) epicatechin can affect the GSH levels in HUVECs, FACS analysis was used. Therefore EC were incubated 24 hours with 0.1 µM, 1 µM, 10 µM and 100 µM of (-)-epicatechin and in double incubation of these concentrations of (-)-epicatechin and 1 mM L-Name. The incubation with 10 µM tBHQ was used as a control as a known antioxidant. After 30 minutes incubation with thiol tracker, the EC were washed with PBS and 500 µl accutase was added to each well, incubated 10 minutes and the mixture was transferred in falcon tubes to measure the GSH levels by flow cytometry.

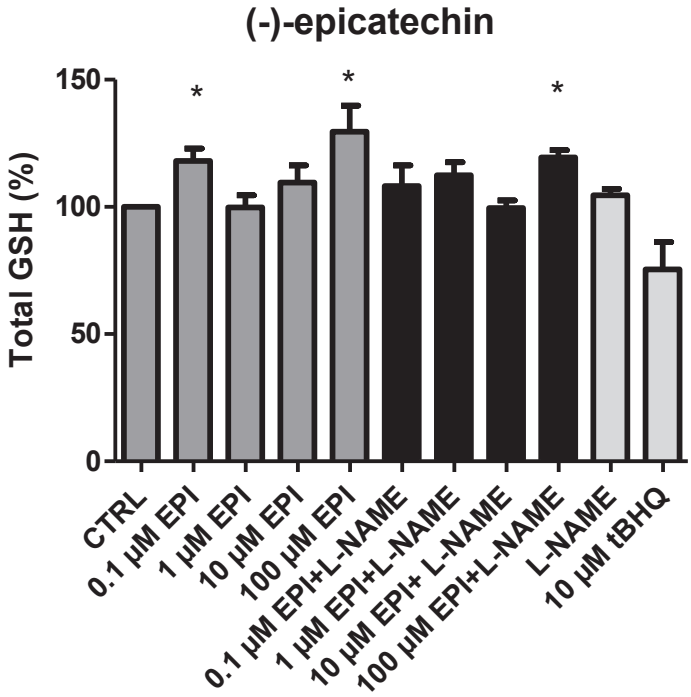


Fig. 24: (-)-epicatechin elevated the production of GSH in HUVECs. The NOS inhibitor L-NAME reduced this enhancement. EC were incubated in medium with the treatments indicated for 24 hours at 37°C and 5% CO_2. FACS analysis was used to determine the total GSH in EC (n=5). 0.1 µM and 100 µM (-)-epicatechin enhanced the GSH production in HUVECs significantly. 1 µM and 10 µM of (-)-epicatechin did not influence the total GSH concentration considerably. Double incubation with L-NAME reduced the enhancement of the GSH levels in HUVECs. tBHQ had no significant effet on the GSH levels. Values represent the mean±SEM (n=4, *P<0.05).

Fig. 24 shows that (-)-epicatechin increased the GSH levels in EC depending on its concentration. 0.1 µM and 100 µM of (-)-epicatechin released the strongest enhancement of total GSH in comparison to the untreated control. 1µM and 10 µM of (-)-epicatechin did not affect the GSH level considerably. The double incubation with (-)-epicatechin and L-NAME however reduced the increase of the GSH levels of 100 µM of (-)-epicatechin significantly. tBHQ however had no significant effect on the GSH levels.

5.5.2 H_2S and NO did not increase the GSH levels in EC in FACS analysis

To determine if GYY has an influence on the GSH levels in HUVECs, FACS analysis was used. Therefore EC were incubated 24 hours with 100 µM, 500 µM and 1000 µM of GYY and in double incubation with 10 µM SNAP as well as 100 µM SNAP. The single incubation with 10 µM and 100 µM SNAP was used as a control. After 30 minutes incubation with thiol tracker, the EC were washed with PBS and 500 µl accutase was put on each well, incubated 10 minutes and the mixture was transferred in falcon tubes to measure the GSH levels in ECs by flow cytometry.

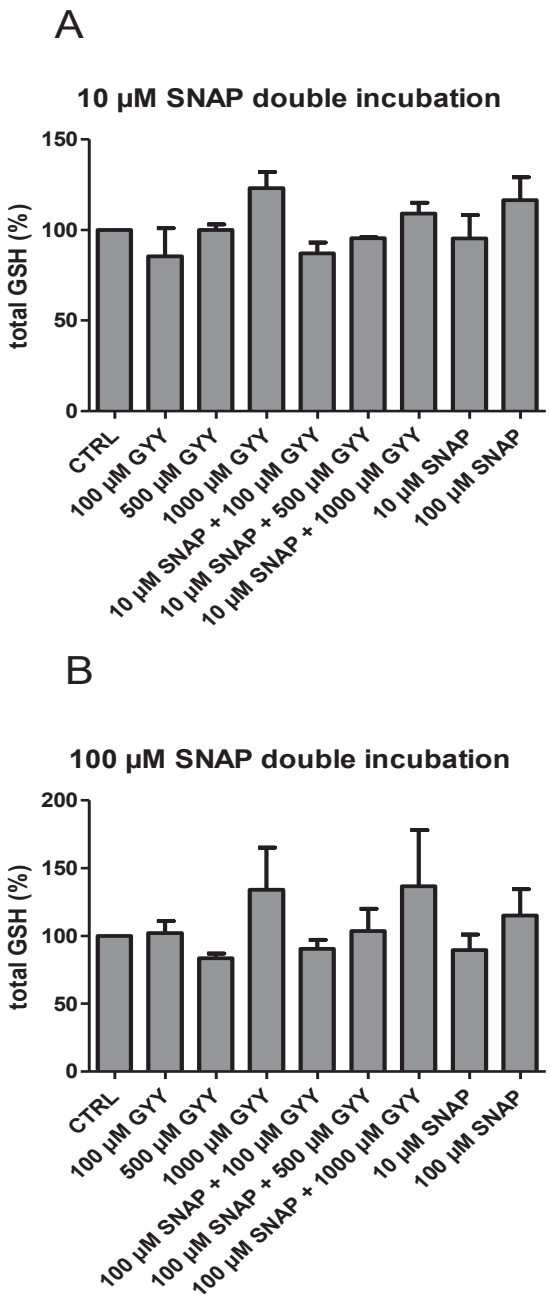


Fig. 25: Single incubation of GYY or SNAP showed no significant effect on the production of GSH in HUVECs. The co – incubation with GYY and SNAP as well had no significant influence on the enhancement of total GSH in EC. EC were incubated in medium with the treatments indicated for 24 hours at 37°C and 5% CO_2. FACS analysis was used to determine the total GSH in EC. 1000 µM GYY enhanced the GSH production in HUVECs, but not in a significant manner. 100 µM and 500 µM of GYY did not influence the total GSH concentration considerably. Double incubation with SNAP had no effect on the enhancement of the GSH levels in HUVECs. Values represent the mean±SEM (n=2).

Relating to Fig. 25 the 24 hours incubation with GYY and SNAP showed no significant rise of the GSH levels in EC. GYY did not release an enhancement of total GSH in HUVECs as well as the co – incubation of GYY and SNAP in different concentrations.

5.6 Glutathione assay

5.6.1 Co – incubation of GYY and SNAP increased the GSH levels only in distinct concentrations

To get a more exact insight if GYY and the double incubation with SNAP influence the GSH levels, GSH assay from Arbor Assays were used. HUVECs were incubated for 24 hours at 37°C and 5% CO_2 with 10 µM, 100 µM and 1000 µM of GYY 4137 (solved in DMSO). Besides, ECs were incubated with 10 µM of SNAP, 100 µM of SNAP and in double incubation with SNAP and the different concentrations of GYY.

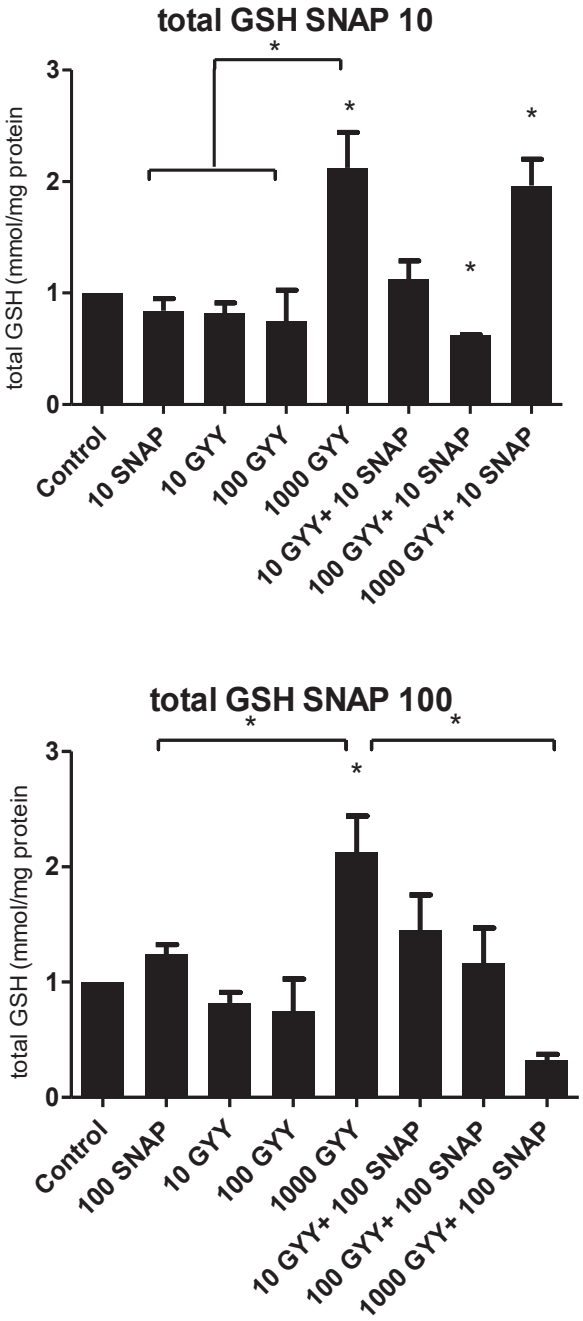


Fig. 26: 1000 µM GYY enhanced the total GSH in comparison to the untreated control. The crosstalk however, showed that the augmentation of GSH levels depended on the concentration of NO and H_2S donor. HUVECs were incubated 24 hours with the treatment indicated. Then, GSH assays were performed. One of them in low concentration and the other one in high concentration showed a higher outcome. Both donors in high concentrations reduced the GSH levels. Values represent the mean±SEM (n=4, *P<0.05).

Relating to Fig. 25 and Fig. 26 only a high concentration of GYY 4137 showed an increase of the total GSH levels in HUVECs. 1000 µM GYY showed a considerable rise of the GSH levels in EC. 100 µM and 500 µM did not enhance total GSH. The double incubation with the 10 µM and 100 µM SNAP and GYY however seemed to depend on the concentration mixed together. A low concentration of an NO-donor and a high concentration of the hydrogen sulfide donor enhanced the GSH levels considerably as well as a high concentration of an NO-donor and a low concentration of the hydrogen sulfide donor.

5.6.2 Simultaneous incubation of GYY and L-Name had no significant effects on the total GSH levels

To show the influence of L-NAME on the GSH levels in HUVECs, GSH assay from Arbor Assays was used. Therefore HUVECs were incubated 24 hours with 10 µM, 100 µM and 1000 µM of GYY 4137. Besides, ECs were double incubated with 1mM L-NAME and the different concentrations of GYY. The incubation with 100 µM SNAP was used as a positive control.

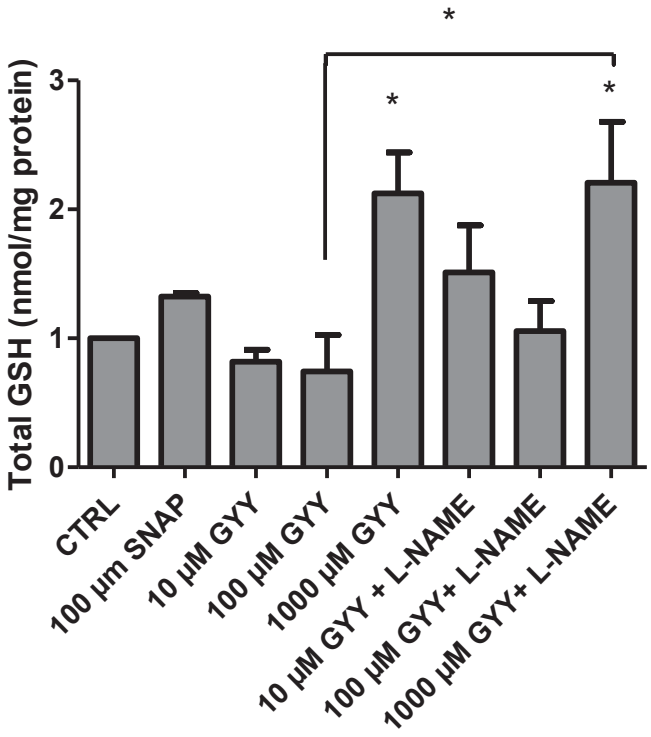


Fig. 27: A high concentration of GYY increased the total GSH levels in HUVECs significantly in comparison to the untreated control. In double incubation with L-NAME however, lower concentrations GYY released to enhance the total GSH in comparison to single incubation. HUVECs were incubated 24 hours with the treatment indicated. Then, GSH assays were performed (n=4). In single incubation, only 1000 µM GYY rose the GSH levels in EC. In double incubation with L-NAME however, 10 µM especially but also 100 µM increased the total GSH as well. Values represent the mean±SEM (n=4, *P<0.05).

Fig. 27 demonstrated that only a high concentration of GYY enhanced the GSH levels in HUVECs significantly. L-NAME co-incubation had no significant effects on the GSH levels. The enhancement of the total GSH levels through incubation with 1000 µM GYY was not decreased through co – incubation with L-NAME and GYY.

5.6.3 Angeli´s Salt increased GSH levels due to its concentration

To test if Angeli's salt as a nitroxyl donor influences the GSH levels in HUVECs like the NO donor SNAP does, GSH assay from Arbor Assays was used. Therefore HUVECs were incubated 24 hours with 1 µM, 10 µM and 100 µM of Angeli's salt. The incubation with 100 µM SNAP was used as a positive control.

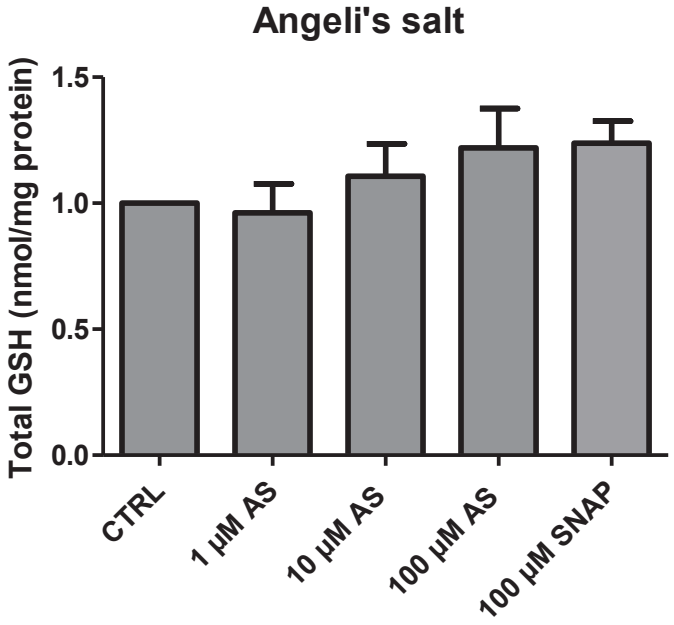


Fig. 28: AS had no significant influence on the total GSH levels. HUVECs were incubated 24 hours with the treatment indicated. Then, GSH assays were performed. AS enhanced the total GSH in EC on a similar level as SNAP, but not in a significant manner. Values represent the mean±SEM (n=4).

Fig. 28 demonstrates that the incubation with AS for 24 hours did not influence the GSH levels in HUVECs significantly.

6 Major findings

The aim of this study was to modulate the redox state in favor of antioxidants of the EC through exogenous and endogenous stimuli by inducing the Nrf2 pathway. Major finding of this study are

I) (-)-epicatechin had an influence on the direct antioxidative response of HUVECs by increasing protein levels of the phase II detoxifying enzymes of the Nrf2 pathway by 24 hours incubation. It also enhanced the total GSH levels in HUVECs dependent on the concentration of (-)-epicatechin.

II) Incubation of endothelial cells with the H_2S donor GYY4137 increased the protein levels of the antioxidant proteins NQO1 and HO-1. Only a high concentration of 1mM of GYY augments the cellular cGMP levels as well as the total GSH concentration in endothelial cells.

III) Although it is generally assumed that NO and H_2S use unique signaling pathways, the current results demonstrate a considerable level of indirect interaction between these signaling pathways in ECs. The combined treatments augmented the protein levels of the mentioned detoxifying enzymes even more. The cGMP production was also considerably increased by co – incubation of SNAP and GYY4137. L-NAME as a NOS inhibitor which reduces the endogenous NO production decreased the through GYY triggered enhancement of the detoxifying enzymes. The GSH levels did show a similar rise, but depended more on the concentration of the combined treatments. L-NAME did not inhibit the effects of GYY on the total GSH.

Therefore we could conclude that there is a possible modulation of the redox state of endothelial cells through exogenous stimuli such as (-)-epicatechin and endogenous stimuli such as NO and H_2S.

7 Discussion

7.1 Influence of (-)-Epicatechin on production of antioxidant proteins of the Nrf2- pathway

Schroeter, Heiss et al showed 2006 that (-)-epicatechin and its metabolites mediate beneficial effects on vascular function in humans [1]. Since then there were several studies investigating on the molecular short [44] and long-time mechanisms. Redox-homeostasis was considered as one auspicious cause of these long-term effects.

(-)-epicatechin is known to have ROS scavenging- as well as RNS scavenging activities [45-47], which prevent oxidative stress through direct antioxidative effects.

In this study we investigated the indirect effect on antioxidant response by (-)-epicatechin on HUVECs via the expression of antioxidant proteins that are known to be controlled by Nrf2. Nehlig et al. and Chang et al. could show that (-)-epicatechin influences the antioxidant effects of neurons via the same pathway by reducing apoptosis through the decrease of oxygen radicals [89, 90]. Also in vivo effects of (-) epicatechin could be shown. To proof the involvement of the Nrf2 pathway in vivo, Shat et al used Nrf2 -/-mice and also HO1 -/-mice [91]. However, the indirect antioxidative response of (-)-epicatechin was not analyzed for human primary endothelial cells.

Therefore, we incubated the HUVECs for 24 hours with different concentrations of (-)-epicatechin to see if in comparison to an untreated control, (-)-epicatechin incubation is able to enhance the cellular protein levels of phase II detoxifying HO-1, NQO1 and GCLC. The phase II detoxifying enzymes are produced via the Nrf2 pathway [30, 31].

Considerably, different concentrations of (-)-epicatechin did not provoke another maximum of the three different phase II protein levels significantly. 10 µM (-)-epicatechin showed the highest gains of protein levels on HO-1 and NQO1. (-)-epicatechin triggered NQO1 into a considerably high protein level, but we could not show a significant result. After all, the number of experiments could be too low, so that further experiments are necessary to show a significant effect of (-)-epicatechin and maybe an enhancement of the phase II enzymes.

Effects on a GSH enhancement of (-)-epicatechin as another antioxidant modulation of EC were shown in vitro for primary cultures of neurons and astrocytes [92]. We also incubated our endothelial cells for 24 hours and then measured the total GSH levels via FACS. 0.1 µM and 100 µM of (-)-epicatechin enhanced the total GSH in HUVECs significantly. L-Name decreased this enhancement in these concentrations. So it can be assumed, that (-)-epicatechin increases GSH in an eNOS dependent manner. It could be possible that it activates eNOS and the de novo synthesis of GSH. But still, there seem other mechanisms to be involved, since L-NAME was incapable to completely abolish the epicatechin effects.

These findings give an important new insight to the possible mechanisms of cellular signaling and antioxidant activity of (-)-epicatechin in an in vitro model of HUVECs and could also be considered as a potential codetermining part of the long-term flavanol effect, which could not be described so far [54].

7.2 Modulation of the redox state of EC by endogenous stimuli – Is there an increase of antioxidant response by simultaneous incubation with NO and H_2S?

As a gaseous signaling molecule, the ability of freely diffusing across cell membrane independent from a receptor makes H_2S an attractive pharmacological agent for the treatment of cardiovascular disease [93]. It has been shown that H_2S has the potential to protect against ischemic injury amongst others by up regulating cellular antioxidants in the heart [93]. These effects could be explained by the phosphorylation of eNOS through the influence of H_2S [83, 87]. The phosphorylation activates eNOS, so that these effects were able to arise from the enhanced production of NO. Altaany et al. showed that H_2S is able to induce the phosphorylation of eNOS and to inhibit its S-nitrosylation, which consequently promotes eNOS dimerization [94].

eNOS has a self inhibiting regulation via S-nitrosation [67, 95]. H_2S itself is capable of reducing S-nitrosyls to S-nitrosothiols [96] and thereby inhibits this negative regulation of eNOS. Hence, Teng et al defined that H_2S is able to release nitric oxide itself from S-nitrosothiols [97]. Nevertheless, H_2S also increases the nuclear localization of Nrf2 [93].

In this study we used GYY4137 as a slow-releasing H_2S compound with vasodilator and antihypertensive activity. GYY4137 was confirmed to be useful in the study of the many and varied biological effects of H_2S [98-101]. It also proved its effects of

therapeutic value in cardiovascular disease [98]. The H_2S donor decomposes slowly to generate small amounts of H_2S in vitro and in vivo [98]. This characteristic was used in this study to incubate endothelial cells for several hours and to acquire related conditions as in vivo.

By incubating HUVECs for 24 hours with GYY we demonstrated that H_2S itself enhanced significantly the production of phase II detoxifying enzymes HO-1 and NQO1 in a concentration – dependent manner. GCLC also was increased by incubation with GYY.

However, Yoon, P.J. et al showed that a NO donor and a sulfide donor mixed together have a cumulative effect to NOS activation in interstitial cells of Cajal [86]. The results of the performed co – incubation of GYY and SNAP in two different concentrations in this study showed comparable effects in human endothelial cells. When the two donors were incubating simultaneously, cross-talk occurred causing the augmentation of the protein levels of NQO1 and HO-1. The effect of double incubation seemed to depend on the concentrations mixed together. 10 µM of SNAP enhanced the protein levels of the phase II detoxifying enzymes not as high as 100 µM SNAP. In the case of GCLC incubation with GYY and SNAP did not enhance protein expression significantly. The toxicity tests via the neutral red assay could exclude toxic effects on the treated EC. Only the treatment with 1000 µM of GYY showed slightly toxic effects on HUVECs. For this reason this concentration was excluded from western blotting.

To provide another modulation of the redox state of EC the influence of NO and H_2S on the GSH levels was investigated. GSH is able to scavenge ROS and free radicals directly or indirectly by being oxidized to GSSG [40]. Therefore EC were incubated 24 hours with different concentrations of GYY and in simultaneous incubation with 10 µM SNAP as well as 100 µM SNAP. Initially FACS analysis was used, but there was no significant enhancement of the GSH levels. To reach more sensitivity on the GSH measurement, we then used a GSH assay [102]. There we could show that GYY in high concentrations increased the GSH levels in EC significantly, but there was no enhancement through co – incubation with GYY and SNAP.

Furthermore, Angeli's salt as a known nitroxyl donor did not influence the GSH levels in HUVECs significantly.

In summary, we could see a considerable enhancement of the protein levels of HO-1 and NQO1 by co – incubation of both gasotransmitters which turned up higher than in single incubation with GYY. The enhancement of the protein levels of NQO1 and HO-1

was dependent on the concentration of SNAP and GYY we used. 100 µM SNAP in co-incubation with 10µM, 50µM or 100µM of GYY made no difference on the enhancement of protein levels of NQO1, where as the protein levels of HO-1 showed the highest enhancement with 50µM GYY incubated together with 100µM of SNAP. Besides, the GSH levels were only enhanced by a high concentration (1000µM) of GYY.

7.3 Double incubation with L-NAME - Is it all about the activation of NOS?

Wang et al. suggested that the angiogenic effect of NO might be, amongst others, mediated by H_2S biosynthesis [69]. In this study, the treatment with L-NAME on endothelial cells was used. L-NAME is a known NOS inhibitor, which also inhibits the Nrf2 translocation and ARE indirectly [103]. These characteristics were used to see if the actions of NO and H_2S converge.

As described above, eNOS is phosphorylated directly by the protein kinase Akt and this phosphorylation activates eNOS [104]. It could be assumed, that the Nrf2 pathway and phase II enzymes production could also depend on the phosphorylation of NOS. H_2S – induces however, the phosphorylation of Akt [87].

To determine if the effect on phase II proteins of H_2S depends on induction of the eNOS, we incubated HUVECs with different concentrations of GYY4137 in the presence of 1 mM L-NAME.

The results of the developed western blots showed that L-Name inhibited the enhancement of protein levels of HO-1 and NQO1 by H_2S. Nevertheless, L-NAME reduced the protein levels in co – incubation with GYY in subject to the concentrations we used. 100 µM of GYY enhanced the protein levels of HO-1, but reduced the protein levels in comparison to the control in co- incubation with L-NAME.

This showed comparable effects to the studies of Altaany et al from 2013 leading to the assumption that the production of phase II proteins depends directly on the phosphorylation of eNOS by the protein kinase Akt and H_2S can induce this pathway in the presence of eNOS [87].

However, the co – incubation of GYY with L-NAME did not show a significant effect on the GCLC protein levels as well as the GSH levels. It seems like it could even increase the GSH production. It could be assumed, that the activation of eNOS does not

play a major role in the increasing of the GSH levels, but there are further experiments required to amplify this pathway.

7.4 Are the cGMP levels in EC dependent on H_2S and NO?

The NO-induced vasorelaxation is mainly mediated by the cGMP pathway in smooth muscle cells [105-107]. In EC, cGMP is able to enhance angiogenesis [78]. Thus, in this study the influence of H_2S and NO on the cGMP level in ECs was surveyed.

The H_2S donor GYY however showed according to Li et al. no direct effect on smooth muscle cells [98].

To consider if there is a direct effect on the cGMP levels in HUVECs by H_2S and if the NO/H_2S double incubation affects them even more, cGMP assays were conducted. To increase the cellular cGMP level and inhibit the degradation of cGMP by phosphodiesterases, the HUVECs were preincubated 30 minutes with IBMX as a non-specific inhibitor of cAMP and cGMP phosphodiesterases. Afterwards HUVECs were incubated for 45 minutes with the different concentrations of the sulfide donor GYY as well as in co – incubation with the NO donor SNAP as used for the western blotting as well (see Fig. 21, Fig. 22, Fig. 23).

The results of this experiment showed that the single incubation of H_2S did not enhance the endothelial cGMP levels, similar to the results of Li et al in 2008. Only a very high concentration of 1 mM of GYY showed a little increase of the cGMP levels in ECs, but without statistical significance.

Though, GYY incubated together with the NO donor SNAP demonstrated that the cross talk of both gasotransmitters augmented the cGMP considerably in distinct concentrations significantly. Especially the high concentrations of the donors SNAP and GYY incubated together displayed a significant enhancement of the cGMP levels (1000µM of GYY and 100 µM of SNAP as well as 100µM of GYY and 10 µM of SNAP).

Thereby, we assume that H_2S stimulates Akt in endothelial cells, and induces eNOS activation through phosphorylation of Ser1177 and dephosphorylation at the inhibitory site Thr495 [83, 108]. H_2S also delays the degradation of cGMP by inhibiting PDE5. It is also considered that H_2S- and NO-induced vasorelaxation is cooperative and converges on the cGMP pathway. Both, H_2S and NO activate PKG and its cellular signalling [83].

Thus, the activation of eNOS and the Nrf2 pathway is multiplicated by the concomitant incubation of NO- and H_2S donors. This could indicate a possible co-dependency of the gasotransmitters.

The finding that L-NAME as a NOS inhibitor abolished the ability of H_2S to increase cGMP levels in human endothelial cells suggests that the biosynthesis of NO is necessary for H_2S to exert its vascular effects [83], but further studies are required amongst others to amplify the exact pathyway and to prove a clear co – dependency of NO and H_2S.

7.5 Conclusion

In summary, this study could show the following findings:

1) It is possible to modulate the redox state of EC by increasing antioxidant proteins of the Nrf2 pathway through incubation with H_2S and NO.

   (-) – epicatechin had no influence on the direct antioxidative response as it is not increasing the protein levels of the phase II detoxifying enzymes of the Nrf2.

   Incubation of endothelial cells with the hydrogen sulfide donor GYY4137 increased the protein levels of the antioxidant proteins NQO1 and HO-1. The combined treatments augmented the protein levels of the mentioned detoxifying enzymes even more than the effect of both single incubation experiments. L-Name inhibited the effects of GYY in co-incubation with GYY, so that we can assume therefore we assume that the production of phase II proteins depends directly on the eNOS activity.

2) (-) – epicatechin enhanced the total GSH levels in HUVECs in a concentration – dependent manner. These findings provide new insights to the possible mechanisms of cellular signaling and antioxidant activity of (-)-epicatechin in an in vitro model of HUVECs.

   Incubating EC with H_2S did show a similar effect, which was statistically significant at higher concentrations. However, concomitant incubation of H_2S and NO did not show enhanced effects. Co – incubation with L-NAME also did not have any significant effects on the total GSH levels.

   Hence, (-) – epicatechin and H_2S can modulate the redox state of EC through the GSH/GSSH pathway.

3) Cellular cGMP levels are only affected by high concentrations of GYY demonstrating that H_2S influences the antioxidative response in HUVECs.

   The cGMP production showed a considerably rise through the simultaneous incubation of SNAP and GYY4137, indicating synergistic effects.

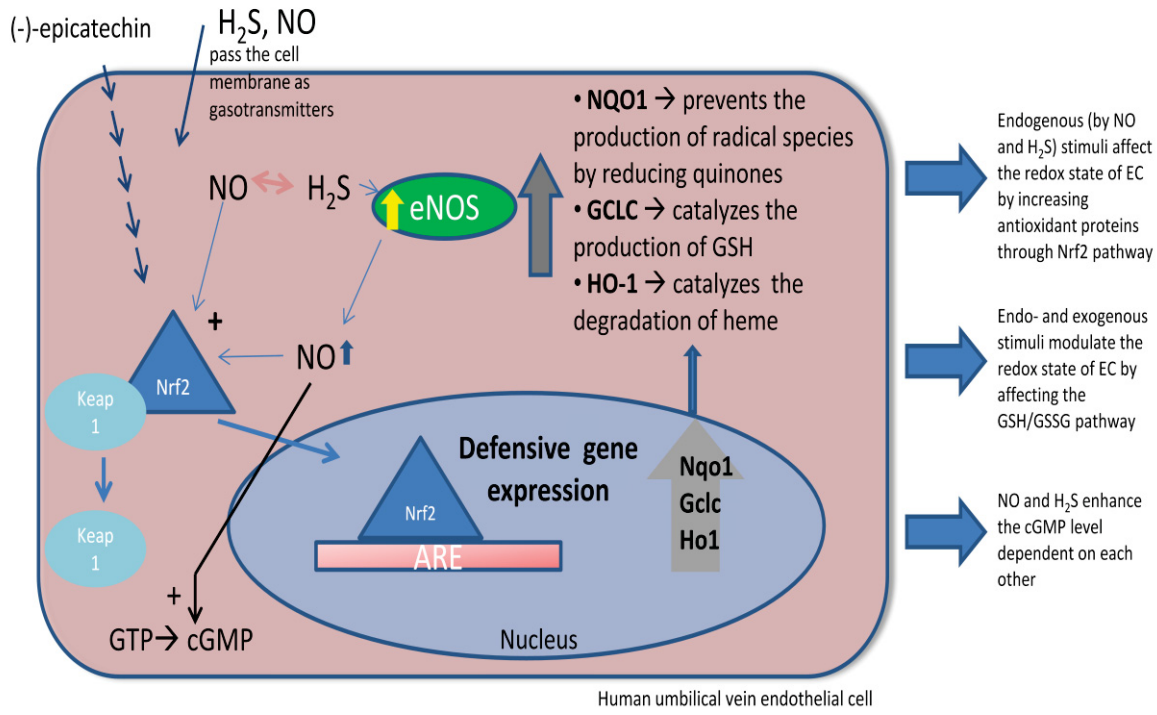


Fig. 29: Major finding of the influence on phase II enzymes of the Nrf2 pathway and the cGMP levels in endothelial cells of nitric oxide, hydrogen sulfide and their cross talk.

So the up-regulation of antioxidant enzyme levels and GSH showed a possible way of modulating the redox state of the EC in favor of an antioxidative response, which can protect the cell from further oxidative cell damage. Furthermore, the indirect interaction of the ubiquitously present gasotransmitters became apparent through a potentiation of especially the levels of phase II enzymes.

This study demonstrated, that H_2S and NO as well as (-)-epicatechin are able to gain a protection against oxidative cell damage in ECs. This could reach to prevention of atherosclerosis through endo – and exogenous stimuli by investigating these stimuli as possible treatments against oxidative stress in the human body.

8 Literature

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