Activity of endothelial nitric oxide synthase: substrates, modulators and products

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Activiteit van endotheliaal stikstofoxide synthase:
substraten, modulators en producten

(Met een samenvatting in het Nederlands)

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Neem je ervaringen van gisteren altijd mee naar morgen!
In elk moment schuilt de kiem van geluk.
Elk moment draagt bij aan je ontwikkeling.
Teleurstellingen en frustraties horen daar bij.

Vorm!
Evolueer!
En geniet!

Leef.

Jeroen

Contents

Abbreviations

A23187	Calcium ionophore
ACh	Acetylcholine
ADMA	Asymmetric dimethylarginine
Akt	Protein kinase B
BH2	Dihydrobiopterin
BH4	Tetrahydrobiopterin
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CaM	Calmodulin
Cav-1	Caveolin-1
cGMP	Cyclic guanosine monophosphate
CKD	Chronic kidney disease
DCF	Dichlorodihydrofluorescein
DAF	Diaminofluorescein
DETA/NO	Diethylenetriamine NONOate
DPI	Diphenyleneiodonium
EDHF	Endothelial-derived hyperpolarising factor
EDRF	Encothelial-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
EPR	Electron paramagnetic resonance
FCS	Foetal calf serum
Fe-DETC	Iron-diethyl dithiocarbamate
FFA	Free fatty acids
H_2O_2	Hydrogen peroxide
HFS	Hyperfine structure
HMEC	Human microvascular endothelial cells
Hsp90	Heat shock protein 90
HUVEC	Humand umbilical vein endothelial cells
iNOS	Inducible nitric oxide synthase
L-NAME	Nω-nitro-L-arginine methyl ester
L-NNA	Nω-nitro-L-arginine
MNIC	Mono-nitrosyl iron-dithiocarbamate
mtNOS	Mitochondrial nitric oxide synthase
NADPH	Nicotinamide-adenine dinucleotide phosphate
NAR	Nagase analbuminemic rats
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
$O_2^{\cdot-}$	Superoxide
OA	Oleic acid
$ONOO^-$	Peroxynitrite
PA	Palmitic acid
PECAM-1	Platelet-endothelial cell adhesion molecule
PGI_2	Prostacyclin
ROS	Reactive oxygen species
SDR	Sprague-Dawley rats
sGC	Soluble guanylate cyclase
SHR	Spontaneously hypertensive rats
TTFA	Thenoyltrifluoroacetone
VEGF	Vascular endothelial growth factor

Chapter 1

General introduction

Endothelium

The endothelium, a monolayer of endothelial cells, forms the lining of the vascular system, thereby separating circulating blood in the lumen from the remainder of the vessel wall. The entire circulatory system, which consists of an estimated 96000 km of blood vessels, is lined with endothelial cells. The endothelium controls many key processes in physiology but also in pathophysiology, e.g., in atherogenesis (for review see: [2]). It functions as a barrier, controlling the transmigration of white blood cells and the passage of several factors in and out of the bloodstream. In some organs endothelial cells perform specialised filtering functions, i.e. in the blood-brain barrier and the renal glomerulus. Endothelial cells are involved in angiogenesis, which is the formation of new blood vessels, and play a role in inflammation. The endothelium releases a variety of vasoactive substances, such as nitric oxide (NO), prostacyclin (PGI_2) and prostaglandins, endothelial-derived hyperpolarizing factor (EDHF), platelet-activating factor, endothelin-1 and reactive oxygen species (ROS).
In agreement with the essential role of endothelial biology in (prevention of) atherogenesis, clinical studies have shown that measurements of endothelial function can predict the occurrence of cardiovascular events in patients[3]. Several factors are known to increase the risk of cardiovascular disease, including smoking, dyslipidaemia, diabetes mellitus and obesity, and all these factors appear to be associated with endothelial malfunction.
NO and ROS are two pivotal players in the regulation of endothelial function and dysfunction. NO, which is generated by endothelial nitric oxide synthase (eNOS), is involved in vessel dilation, inhibition of platelet and leukocyte adhesion, and inhibition of proliferation and migration of vascular smooth muscle cells (for review see: [4]). NO plays a positive role in maintaining endothelial function and contributes to prevention of endothelial dysfunction, which will be discussed below. ROS, which is in the vasculature mainly produced by NADPH oxidase[5], play an important role in signal transduction, gene expression and proliferation and are involved in the regulation of the biologically effective concentration of NO[6]. However, in vascular disease states, excessive production of ROS may overwhelm the anti-oxidant defence mechanisms of cells, resulting in oxidative stress, damage to the arterial wall and, ultimately, development of atherosclerotic plaques[7].

Nitric oxide synthase

Introduction

In 1980, Furchgott discovered the existence of the endothelium-derived relaxing factor (EDRF)[8], which was later found to be identical to NO[9, 10]. In 1998, Furchgott, Murad and Ignarro received the Nobel Prize in Medicine for their discoveries concerning nitric

oxide as a signalling molecule in the cardiovascular system.
NO is generated during the conversion of L-arginine into L-citrulline, a reaction that is catalysed by nitric oxide synthase (NOS). It acts as a signalling molecule in the nervous system, is involved in protection of the body against infections, and regulates blood pressure and controls the amount of blood flowing to different organs. In the nervous system, NO modulates many functions, ranging from behaviour to gastrointestinal motility. White blood cells, such as macrophages, can produce large amounts of NO, which is used to kill invading bacteria and parasites. NO produced in mitochondria is involved in the energy regulation of the body[11]. NO produced in endothelial cells exerts its action on smooth muscle cells, resulting is dilatation of the vessels. In addition, NO inhibits platelet and leukocyte adhesion and the proliferation and migration of vascular smooth muscle cells in the vascular system (for review see: [4]). There are four NOS isoforms: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), endothelial NOS (eNOS or NOS III) and mitochondrial NOS (mtNOS)[12], which are highly homologous[4].

Structure
All NOS isoforms have a common bidomain structure existing of a reductase and an oxygenase domain[14] (figure 1a; modified from [13]). The reductase domain, which is closely homologous with cytochrome P-450 reductase, contains binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and NADPH. The oxygenase domain consists of a haem centre and binding sites for L-arginine and tetrahydrobiopterin (BH4). Between these two regions lies the binding domain for calmodulin (CaM), which regulates the transfer of electrons from the reductase domain to the oxygenase domain[15] and plays an important role in the structure as well as the function of the enzyme (figure 1a). The main difference between the four isoforms lies in their calcium (Ca^{2+}) dependence: nNOS, mtNOS and eNOS are Ca^{2+}-dependent isoforms[16] and require elevated levels of intracellular calcium to become activated. In contrast, CaM binding to iNOS is so tight that the activity of this isoform is independent of intracellular calciumfluxes[17].
Another difference between eNOS and the other isoforms is the maximal enzymatic activity: nNOS[16] and iNOS[18] exert much higher overall activities than eNOS[16, 19]. The lower activity of eNOS is caused by a lower ability of its flavoprotein reductase domain to transfer electrons to the catalytic haem domain[20]. However, the structural features that impair the reductase activity in eNOS, and hence lower the overall catalytic activity, remain unknown.
NOS is only active as a homodimer (figure 1b; modified from [13]). The haem group, bound via a proximal cysteine ligand[21], plays an essential role in the dimerisation of the enzyme. In the absence of haem, NOS exists as a monomer, which is unable to bind BH4 or L-arginine and is thus unable to generate NO[19].

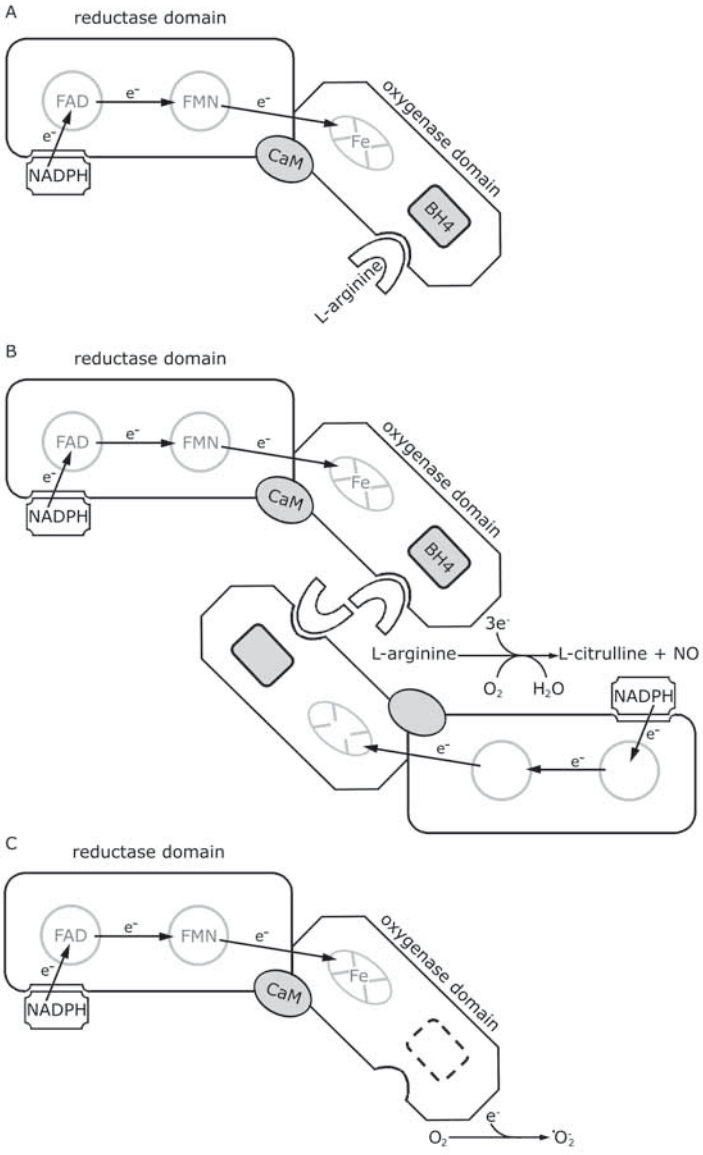

Figure 1. Schematic representation of the eNOS enzyme (modified from [13]).
The eNOS dimer (A), producing NO in the presence of L-arginine and BH4 (B), and producing $O_2^{\cdot-}$ in the absence of L-arginine and BH4 (C). BH4, tetrahydrobiopterin; CaM, calmodulin; e^-, electron; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; Fe, haem group; NADPH, nicotinamide adenine dinucleotide phosphate

Under certain conditions, such as suboptimal concentrations of BH4, eNOS becomes dysfunctional and produces $O_2^{\cdot-}$ rather than NO, a process referred to as eNOS uncoupling[22, 23] (figure 1c; modified from [13]) which will be discussed more extensively in the section 'eNOS uncoupling'.

Localisation of eNOS

Localisation of eNOS within the endothelial cell determines its activity. The eNOS activation/deactivation cycle is shown in figure 2 (modified from [4]). eNOS resides in the Golgi complex, where it is anchored to the membrane with one myristoyl and two palmitoyl groups (1)[4, 24]. Via vesicular transport, eNOS is translocated to the plasma membrane (2), where eNOS associates with caveolae (3), flask-shaped invaginations in the plasma membrane which are rich in cholesterol and sphingolipids[25]. In caveolae, eNOS binds to caveolin-1 (cav-1) via its so-called scaffolding domain[26]. This interaction inhibits eNOS catalytic activity, because caveolin-1 interferes with the binding of CaM at low concentrations of cytosolic calcium[27]. Upon calcium influx in the cell, CaM binds to eNOS (4), thereby disrupting the eNOS-cav-1 interaction, leading to activation of the enzyme. At the same time, eNOS is depalmitoylated and the enzyme translocates to the cytosol (5)[28]. Protein kinase B (Akt) phosphorylates eNOS at a serine group at position 1177 (human S1177; murine S1175), leading to further activation of the enzyme[29, 30]. Association of eNOS with heat-shock protein 90 (hsp90), previously termed endothelial nitric oxide synthase-associated protein 1 (ENAP-1)[31], facilitates the binding of CaM[32] and mediates the interaction between eNOS and Akt[33]. During the activation cycle of eNOS, efficient supply of the substrate L-arginine is essential for NO production. The supply of L-arginine is ensured by the localisation of the arginine transporter cationic amino acid transporter in caveolae[34]. Dephosphorylation of eNOS leads to relocation of the enzyme to the Golgi complex (6), where the enzyme is repalmitoylated, which enables eNOS to be transported to the caveolae again.

Localisation of eNOS at cell-cell junctions is established by an interaction with platelet-endothelial cell adhesion molecule (PECAM-1; CD31)[35, 36] (7). This co-localisation seems to be important for eNOS activity[35-38]. Besides the active pool of eNOS in the cytosol and at cell-cell junctions, it has also been shown that the enzyme can produce NO when located at the Golgi complex[39].

In addition, localisation and activity of eNOS is influenced by interactions of eNOS with several other proteins. Some interactions lead to activation of the enzyme. Increased calcium levels or stimulation with bradykinin promotes the interaction between eNOS and the voltage-dependent anion/cation channel (porin), which augments eNOS activity[40]. Although, the function of eNOS-porin interactions is not entirely known, it may play an important role in trafficking and regulation of enzymatic activity, either directly or via the regulation of calcium concentrations in the vicinity of eNOS. Interaction of

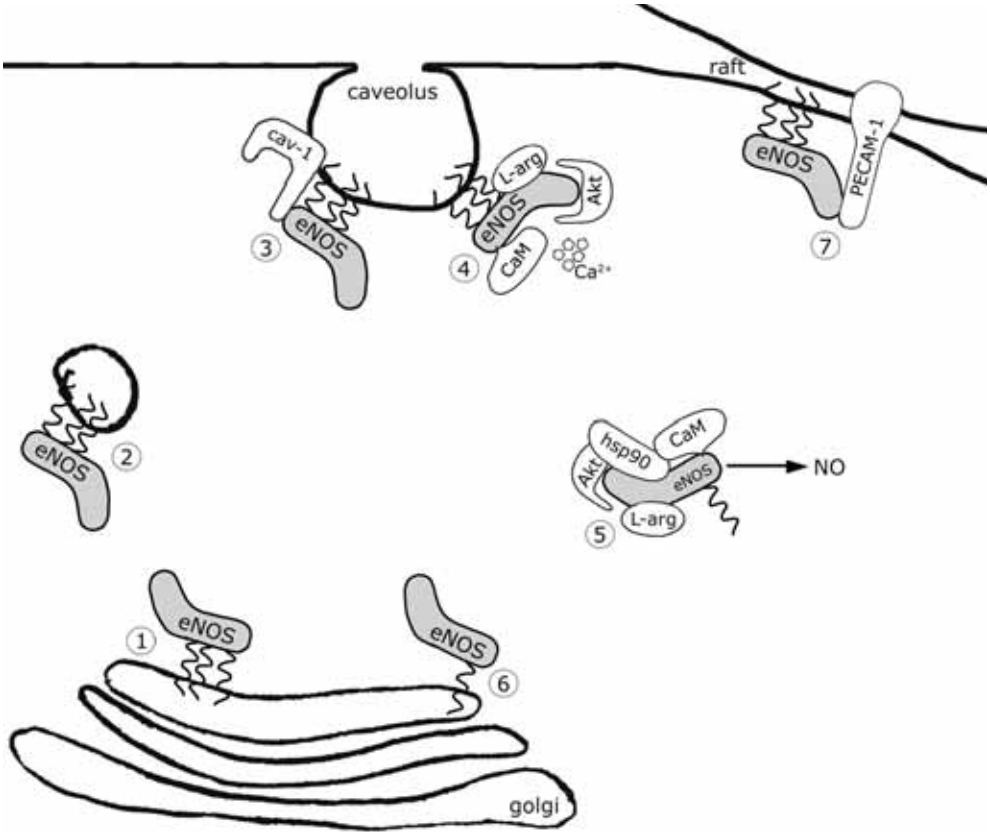

Figure 2. The eNOS activation/deactivation cycle (modified from [4]).
eNOS is resident in the Golgi complex, where it is anchored in the membrane with one myristoyl and two palmitoyl groups (1). Via vesicular transport, eNOS is translocated to the plasma membrane (2), where eNOS is associated with caveolae, bound to cav-1 (3). Upon Ca^{2+} influx in the cell, CaM binds to eNOS (4). eNOS translocates to the cytosol (5). Akt, hsp90, L-arg and CaM are all involved in activation of eNOS. Eventually eNOS is relocated to the Golgi (6). At cell-cell junctions, eNOS co-localises with PECAM-1 (7). Akt, protein kinase B; Ca^{2+}, calcium; CaM, calmodulin; cav-1, caveolin-1; eNOS, endothelial nitric oxide synthase; hsp90, heat-shock protein 90; L-arg, L-arginine; PECAM-1, platelet-endothelial cell adhesion molecule

eNOS with protein kinase A (PKA)[41], which colocalise at cell-cell junctions, in caveolae, at the Golgi complex and in endosomes, leads to phosphorylation and activation of the enzyme. Dynamin-2, which is involved in the translocation of eNOS from the Golgi compartment to the plasma membrane, has been shown to enhance eNOS activity[42].
Other protein interactions with eNOS lead to inhibition of the enzyme. Association

of eNOS with the bradykinin B2 receptor inhibits eNOS activity[43]. eNOS interacting protein (NOSIP) induces eNOS translocation into the proximity of the Golgi apparatus and cytoskeletal structures[44], whereas eNOS traffic inducer (NOSTRIN) is involved in translocation of eNOS in vesicle-like structures spread all over the cytosol[45]. Both proteins inhibit eNOS activity.

Functional eNOS

NO acts on vascular smooth muscle cells (VSMC) by activating soluble guanylyl cyclase (sGC), which results in the formation of 3',5'-cyclic guanosine monophosphate (cGMP) and subsequent vasodilatation[46]. Stimulation of sGC by NO leads to a 200-fold increase in catalytic rate, i.e., the conversion of GTP to cGMP.

NO production from endothelial cells is stimulated by a variety of humoral factors and mechanical forces, such as shear stress and cyclic strain. Phosphorylation is the main regulatory mechanism controlling eNOS activation. eNOS has several phosphorylation sites which play a role in activation and inhibition of the enzyme.

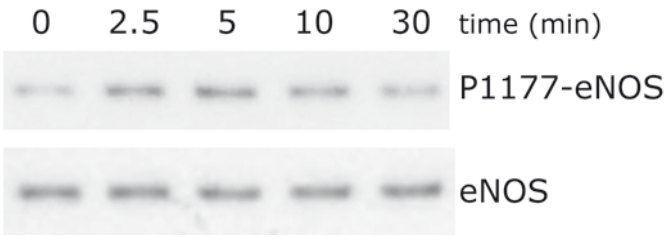

Figure 3. Representative western blot depicting VEGF-induced eNOS phosphorylation at Serine 1175 (S1175).

Murine bEnd.3 cells were incubated with VEGF for the indicated time. Western blots were incubated with antibodies against phosphorylated eNOS (Cell Signaling) and eNOS (Transduction Laboratories). Incubation with VEGF results in a time-dependent increase in eNOS phosphorylation. After 30 minutes phosphorylation levels of eNOS are back to basal.

The main phosphorylation site leading to activation of eNOS is Serine 1177 (human S1177; murine S1175). Stimulation with agonists, like VEGF, acetylcholine (ACh) and insulin, or shear stress leads to the activation of different pathways involving protein kinases, all resulting in increased NO production (figure 3). Akt[29, 30], PKA[47, 48], protein kinase G (PKG)[48], AMP-activated protein kinase (AMPK)[49] and CaM-dependent kinase II (CaMKII)[50] are all involved in the phosphorylation of eNOS at S1177. Dephosphorylation of S1177 is regulated by protein phosphatase 2A (PP2A), which results in a decrease in NO production[51].

In response to VEGF, ATP and bradykinin, serine 633 (S633) and serine 615 (S615) are

phosphorylated. S633 is thought to play a role in maintaining basal, but not stimulated NO production in a Ca^{2+}-independent manner[52, 53]. S615 may not be important for directly regulating NO release, but is important as a modulator of phosphorylation at other sites and of protein-protein interactions[52].
Threonine 495 (T495) is constitutively phosphorylated, resulting in inhibition of eNOS. Phosphorylation of T495 may interfere with CaM binding to the enzyme at low Ca^{2+} concentrations[54]. T495 can be phosphorylated by protein kinase C (PKC). In the absence of Ca^{2+}/CaM, stimulation with AMPK[49] can also lead to phosphorylation of T495 and subsequent inhibition of NO production. Note that in the presence of Ca^{2+}/CaM, stimulation with AMPK leads to activation of the enzyme[49].
Dephosphorylation of T495 is controlled by protein phosphatase 1 (PP1)[54]. Like T495, serine 114 (S114) is also phosphorylated in a resting state of the cell and is dephosphorylated in response to VEGF[55]. In proliferating mesenchymal stem cells eNOS phosphorylated at S114 is heavily enriched in the nucleus and is associated with the generation of O_2^{-} anions[56].

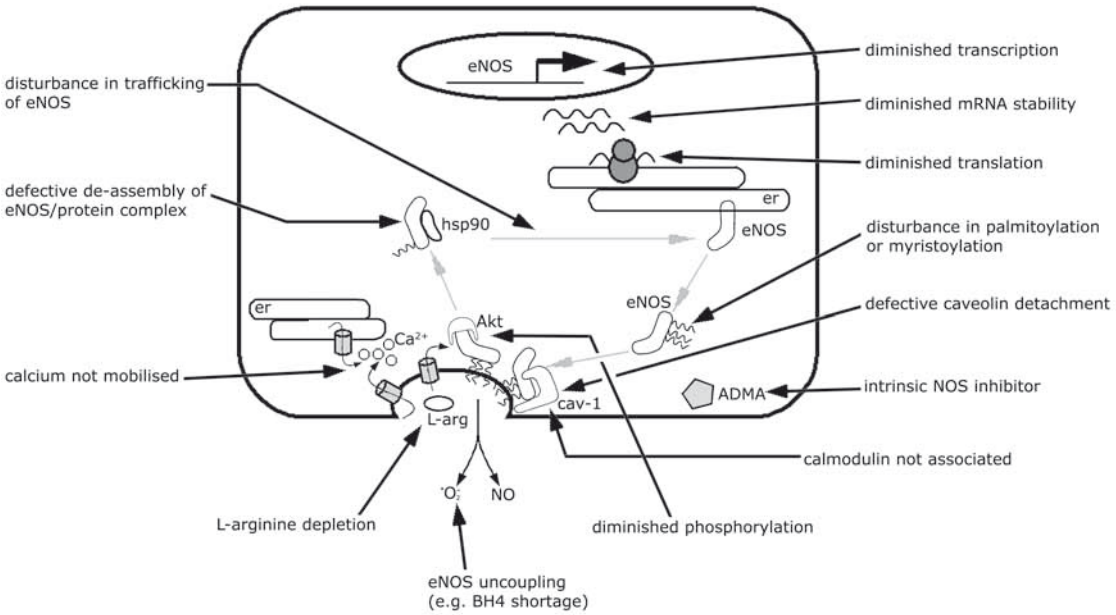

Figure 4. Two potential sources of problems of diminished NO bio-activity (adapted from [57]).
ADMA, asymmetric dimethylarginine; Akt, protein kinase B; BH4, tetrahydrobiopterin; Ca^{2+}, calcium; cav-1, caveolin-1; eNOS, endothelial nitric oxide synthase; er, endoplasmic reticulum; hsp90, heat-shock protein 90; L-arg, L-arginine; NO, nitric oxide; O_2^{-}, superoxide

In summary, eNOS activity and NO release is closely regulated by post-translational control mechanisms, such as phosphorylation, its localisation in the cell and protein-protein interactions. This modulation of eNOS has a direct effect on the duration and magnitude of NO release.

Dysfunctional eNOS

Dysfunctional behaviour of eNOS can lead to a decrease in NO bioavailability, which is associated with pathologies, such as cardiovascular disease. There are 4 principle causes of diminished NO bio-activity: decreased activity and/or expression of the eNOS enzyme, eNOS uncoupling (figure 4; adapted from [57]), enhanced breakdown or scavenging of NO, and impaired transmission of NO-mediated signalling events (failure of the effector mechanisms).

Posttranslational eNOS modification

Under hyperglycemic conditions or during high free fatty acid fluxes, which occur for example in diabetes mellitus type II, mitochondria can produce $O_2^{\cdot-}$, which is discussed later in this chapter. Mitochondrial-derived $O_2^{\cdot-}$ inhibits glyceraldehyde phosphate dehydrogenase (GAPDH), the enzyme responsible for shuttling glucose into the glycolytic pathway. Inhibition of GAPDH leads to accumulation of fructose-6-phosphate, which results in the activation of the hexosamine biosynthesis pathway (HBP). The enzyme glutamine:fructose-6-phosphate aminotransferase (GFAT) converts fructose-6-phosphate via glucosamine-6-phosphate to UDP-N-acetylglucosamine (UDPGlcNAc). UDPGlcNAc is subsequently coupled to proteins by action of O-linked N-acetylglucosamine transferase (OGT)[58] (figure 5).

In 2000, Du et al. showed that eNOS can be glycosylated at S1177[58], resulting in a reduction in eNOS activity because this glycosylation prevents phosphorylation. In 2002, Federici et al. showed increased endothelial O-GlcNAcylation in carotid plaques from type II diabetic patients compared to carotid plaques from nondiabetics[59]. Furthermore, they showed that hyperglycemia, through the HBP, impairs activation of the PI3-K/Akt pathway, resulting in dysregulation of eNOS activity[59]. It is known that the activity of the HBP can be severalfold increased by incubating endothelial cells with glucosamine with subsequent inactivation of eNOS.

eNOS uncoupling

L-arginine and BH4 are both essential factors for adequate eNOS function. Reduced levels of the substrate or cofactor lead to uncoupling of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidation and NO synthesis, with oxygen as terminal electron acceptor instead of L-arginine, resulting in the generation of $O_2^{\cdot-}$ by eNOS. This process is referred to as eNOS uncoupling[22, 23, 59].

Arginine interacts with eNOS with a Km of approximately 2.9 μM, and as the

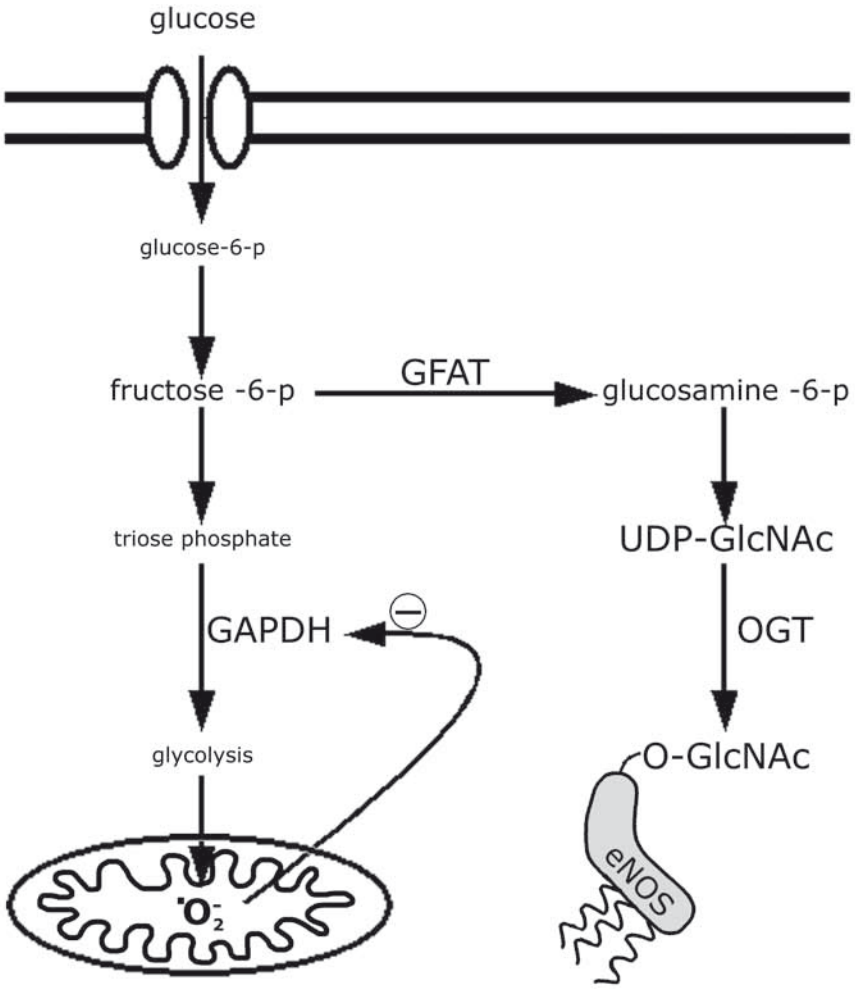

Figure 5. Schematic representation of the hexosamine biosynthesis pathway.
High influx of glucose into the cell, eventually leads to an increase in glycolysis. This results in superoxide production in the mitochondria. Superoxide inhibits glyceraldehyde phosphate dehydrogenase (GAPDH), which results in accumulation of fructose-6-phosphate. Increased fructose-6-phosphate activates the hexosamine biosynthesis pathway. Glutamine: fructose-6-phosphate aminotransferase (GFAT) converts fructose-6-phosphate into UDP-N-acetylglucosamine (UDP-GlcNAc), which is subsequently coupled to proteins (eg. eNOS) by action of O-linked N-acetylglucosamine transferase (OGT). (-), inhibition; GAPDH, phosphate dehydrogenase; GFAT, glutamine:fructose-6-phosphate aminotransferase; GlcNAc, N-acetylglucosamine; eNOS, endothelial nitric oxide synthase; $O_2^{\cdot-}$, superoxide; O-GlcNAc, O-linked N-acetylglucosamine; OGT, O-linked N-acetylglucosamine transferase

cytoplasmic levels of L-arginine vary between 600 and 900 μM[60], L-arginine supply is not likely to be insufficient. In addition, the plasma concentration of arginine is 100 μM[61], making it even more unlikely that shortage of L-arginine will lead to a decrease in NO production *in vivo*. However, supplementation of L-arginine in conditions associated with endothelial dysfunction improved NO-availability[62]. This 'L-arginine paradox' may be explained by competing levels of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA). The formation and degradation of ADMA are both affected by oxidative stress, resulting in enhanced ADMA concentrations. Increased concentrations of ADMA may lead to decreased substrate availability for eNOS and may even cause eNOS uncoupling[63]. Although several studies showed beneficial effects of L-arginine, the role of exogenous L-arginine supplementation for improvement of endothelial function is still controversial. L-arginine supplementation may paradoxically contribute to, rather than reduce atherosclerotic lesion formation by mechanisms that involve peroxynitrite ($ONOO^-$) formation, BH4 depletion and eNOS uncoupling, as will be described in this thesis. In line with this, Chen et al[64] found that chronic L-arginine supplementation negated the protective effect of iNOS deficiency in apoE/iNOS knockout mice and Satoh et al[65] demonstrated that L-arginine supplementation increased ROS production in diabetic rats.

Reduced BH4 levels can also result in eNOS uncoupling. In animal models as well as in patients with cardiovascular risk factors, BH4 supplementation enhanced NO activity, suggesting that reduced BH4 contributes to endothelial dysfunction[66-68]. In addition, decreased levels of BH4 were observed in the vessel wall of diabetic[69], hypercholesterolemic[70] and hypertensive animals[71]. However, other studies observed no decrease in BH4 levels in atherosclerotic models[72].

Decreased levels of BH4 can be caused by direct oxidation of BH4, which leads to the formation of 7,8-dihydrobiopterin (BH2)[71], or can be caused by a decrease in GTP cyclohydrolase 1 (GTPCH1) activity, the rate-limiting enzyme in the synthesis of BH4[73]. It has been shown that high glucose levels, with actions on endothelial cells and consequences comparable to elevated levels of free fatty acid (FFA), decrease GTPCH1 mRNA expression, GTPCH1 activity and intracellular BH4 levels in cultured endothelial cells[74].

The formation of $O_2^{\cdot-}$ by eNOS can enhance further uncoupling of eNOS. For instance, oxidation of BH4 by ROS leads to a decrease in BH4 availability and thus an increase in eNOS uncoupling[75]. NO and $O_2^{\cdot-}$ can react rapidly to form $ONOO^-$, a highly active radical that has been shown to have deleterious effects with respect to vascular function. $ONOO^-$ can directly influence the zinc-thiolate cluster in eNOS, thereby disrupting eNOS dimers, which leads to uncoupling[76]. Furthermore, $ONOO^-$ can also lead to oxidation of BH4 and subsequent eNOS uncoupling[76].

Reactive oxygen species

As mentioned before, ROS fulfil a myriad of functions in physiology including a variety of signalling processes as well as elimination of pathogens by monocytes and neutophils. The major sources of ROS in the human body will be discussed in this section.

Mitochondrial uncoupling

Mitochondria are sites of respiration in eukaryotic cells. A cell may contain as much as 100 to more than 1000 mitochondria which differ in size, shape and structure depending on the tissue and the physiological state of the cell. Under normal conditions, mitochondria provide energy to the cell by regenerating ATP. The driving force for ATP synthesis is the mitochondrial proton gradient that is generated as electrons are running down the respiratory chain. The mitochondrial respiratory chain is formed by five complexes. The main electron donor is NADH, which provides electrons for complex I. The other electron donor is $FADH_2$, which donates electrons to complex II. Electrons from both these complexes are transferred to coenzyme Q and subsequently to complex III, cytochrome c and complex IV. This electron transfer along the complexes is accompanied by the transfer of protons from the mitochondrial matrix to the inner membrane compartment. This proton gradient dissipates, which is partly used to drive ATP synthesis[77]. In addition to their central bioenergetic task of ATP regeneration, mitochondria produce both ROS and anti-oxidants[78] and are involved in the regulation of intracellular calcium levels[79].

Respiration is associated with production of ROS, because the oxygen molecule is capable of accepting an additional electron to create $O_2^{\cdot-}$ [78]. Complex I and complex III were shown to be responsible for much of the $O_2^{\cdot-}$ generated by mitochondria[80, 81]. It has been calculated that 1-4% of oxygen reacting with the respiratory chain is incompletely reduced to form ROS[82, 83]. However, under hyperlipidaemic and hyperglycaemic conditions, the increased electron flow through the respiratory chain leads to an extensive increase in mitochondrial formation of $O_2^{\cdot-}$ [58, 84] (figure 6; adapted from [85]). This results in an increase in the voltage gradient across the mitochondrial membrane until a critical threshold is reached. Subsequently, electron transfer inside complex III is blocked[86], causing the electrons to back up to coenzyme Q, which donates the electrons one at a time to molecular oxygen, thereby generating $O_2^{\cdot-}$. The mitochondrial isoform of the enzyme superoxide dismutase (MnSOD) degrades this oxygen free radical to hydrogen peroxide (H_2O_2), which is then converted to H_2O and O_2 by catalase.

NADPH oxidase

NADPH oxidase is a major source of ROS production in endothelial cells, consisting of membrane-associated subunits and several cytosolic subunits, termed Nox proteins (figure 7; modified from [87]). Upon activation by stimuli such as angiotensin II, stretch,

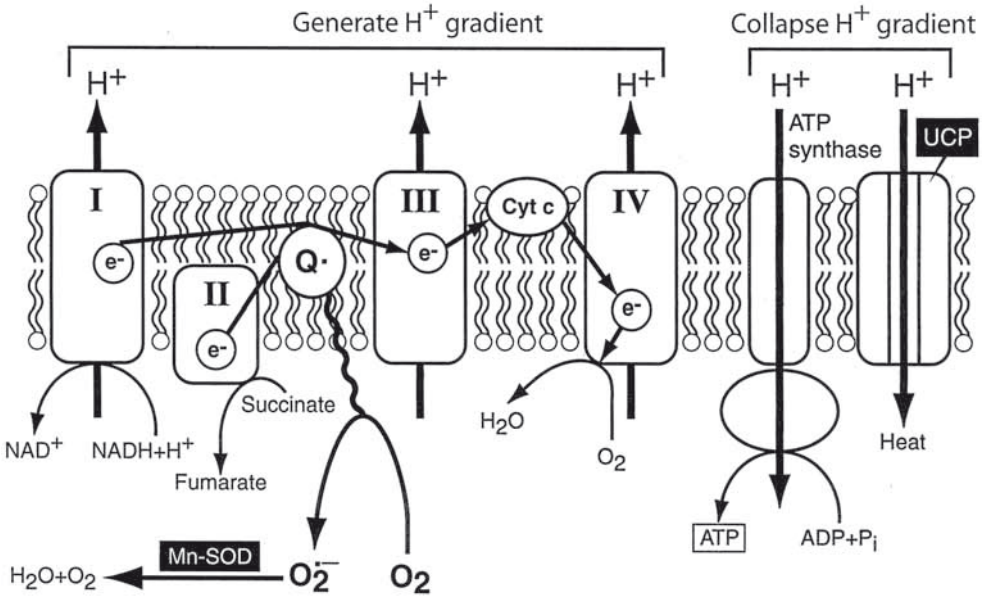

Figure 6. Hyperglycaemia-induced production of superoxide by the mitochondrial electron transport chain (adapted from [85]).

In normal cells, as electrons are transported from left to right in this figure, some of the energy of those electrons is used to pump protons across the membrane at complexes I, III and IV. This generates a pH gradient across the mitochondrial membrane. The energy from this voltage gradient drives the synthesis of ATP by ATP synthase. Uncoupling proteins (UCP) can bleed down the voltage gradient to generate heat as a way of keeping the rate of ATP generation constant. However, in diabetes, with high intracellular glucose, more glucose is being oxidised in the tricarboxylic acid cycle, which in effect pushes more electron donors (NADH and $FADH_2$) into the electron transport chain. As a result, the pH gradient across the mitochondrial membrane increases until a critical threshold is reached. At this point, electron transfer inside complex III is blocked, causing the electrons to back up to coenzyme Q, which donates the electrons to molecular oxygen, thereby generating $O_2^{\cdot-}$ [85]. ADP, adenosinediphosphate; ATP, adenosinetriphosphate; Cyt c, cytochrome c; e^-, electron; $FADH_2$, reduced form of flavin adenine dinucleotide; H+, proton; NAD+/NADH, nicotinamide adenine dinucleotide; O_2, oxygen; $O_2^{\cdot-}$, superoxide; Pi, phosphate; Q, coenzyme Q; UCP, uncoupling protein

endothelin-1, thrombin and catecholamies, the subunits assemble at the plasma membrane and form the functional enzyme, leading to $O_2^{\cdot-}$ production, which involves NADPH as a cofactor and requires electron transfer to molecular oxygen[88]. Protein

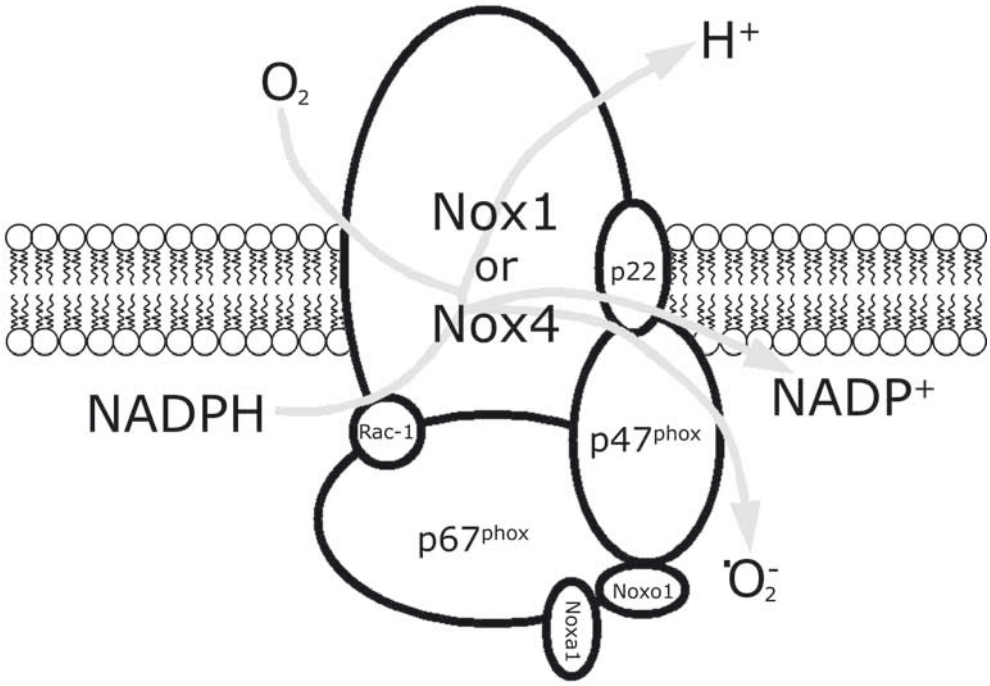

Figure 7. A schematic representation of the vascular NADPH oxidase complex (modified from [87]).

The Nox subunit (Nox1 in vascular cells; Nox4 in endothelial cells) is bound to $p22^{phox}$ in the plasma membrane, resulting in a stabilised complex. The subunits $p47^{phox}$ and $p67^{phox}$ are essential for superoxide production. For a complete activation of NADPH oxidase, the translocation of the small g protein Rac-1 and the subunits Noxo1 and Noxa1 is also necessary. H^+, proton; NADPH, nicotinamide adenine dinucleotide phosphate; Noxa1, NADPH oxidase activator 1; Noxo1, NADPH oxidase organizer 1; O_2, oxygen; $O_2^{\cdot-}$, superoxide

levels of Nox1 are low in vascular cells, but can be induced by several stimuli, such as angiotensin II, platelet-derived growth factor and serum[89]. Nox4 is constitutively expressed and constitutively active in endothelial cells and is the major catalytic component of endothelial NADPH oxidase[90]. The subunit $p22^{phox}$ serves as a docking protein for all the other subunits and stabilises the Nox proteins[91]. Finally, for a complete activation of NADPH oxidase, the translocation of the small g protein Rac-1 and the subunits NADPH oxidase organizer 1 (Noxo1) and NADPH oxidase activator 1 (Noxa1) is also necessary[92].

Elevated levels of FFA can lead to upregulation of diacyl glycerol (DAG), which in turn leads to activation of PKC. Activation of PKC can lead to activation of NADPH oxidase and thus ROS production[93].

Xanthine oxidase

Xanthine oxidoreductase is another important source of ROS production in endothelial cells. Xanthine oxidoreductase exists in two forms, as xanthine dehydrogenase (XDH) and as xanthine oxidase (XO)[94]. XDH produces NADH and uric acid by transferring electrons from hypoxanthine and xanthine to NAD^+. XO uses oxygen as an acceptor of electrons from xanthine and hypoxanthine to form $O_2^{\cdot-}$ and H_2O_2. Therefore, the ratio of XO and XDH in a cell is critical and determines the amount of ROS produced by these enzymes. Inflammatory cytokines, like tumor necrosis factor- , and oxidants, such as $ONOO^-$, can stimulate conversion of XDH to XO[95, 96]. The levels of XO are influenced by the presence of a functioning NADPH oxidase. In the absence of NADPH oxidase, the levels of XO are extremely low[97]. XO is capable of producing large amounts of ROS under pathophysiological conditions[98].

NO and ROS in disease

Dysbalanced production of NO and ROS may contribute to development or progression of a variety of disease states. Diseases potentially associated with deranged NO and/or ROS production which will be topics of this thesis will be discussed below.

Hyperlipidaemia

The term hyperlipidaemia refers to the raised serum levels of lipids or FFA. Elevated levels of FFA may lead to the narrowing and blocking of blood vessels, thereby increasing the risk for cardiovascular disease. Diets rich in fat play a major role in the development of increased levels of FFA. Dietary fatty acids are involved in cardiovascular-related mortality[99, 100]. In addition, increased levels of FFA are associated with insulin resistance and type 2 diabetes[101, 102].

Fatty acids are utilised by mitochondria for the formation of ATP and thus the generation of energy. Elevated levels of FFA can lead to overload of mitochondria and subsequent mitochondrial $O_2^{\cdot-}$ leakage[58, 84], which has been described above, thereby activating pro-atherogenic signals and inhibiting anti-atherogenic enzymes (figure 8; modified from [103]). Increased FFA oxidation can lead to activation of PKC resulting in a decrease in eNOS activity. In addition, mitochondrial $O_2^{\cdot-}$ production leads to the activation of the hexosamine biosynthesis pathway, resulting in increased GlcNAc levels and subsequent eNOS inhibition, as discussed previously in this chapter. Oxidation of FFA can directly interfere with eNOS activity[104]. In addition, the expression of PGI_2, a potent vasodilator, is decreased by increased FFA levels[105]. Thus elevated levels of FFA and subsequent increased FFA oxidation results in a shift towards the activation of proinflammatory pathways and inactivation of anti-atherogenic factors.

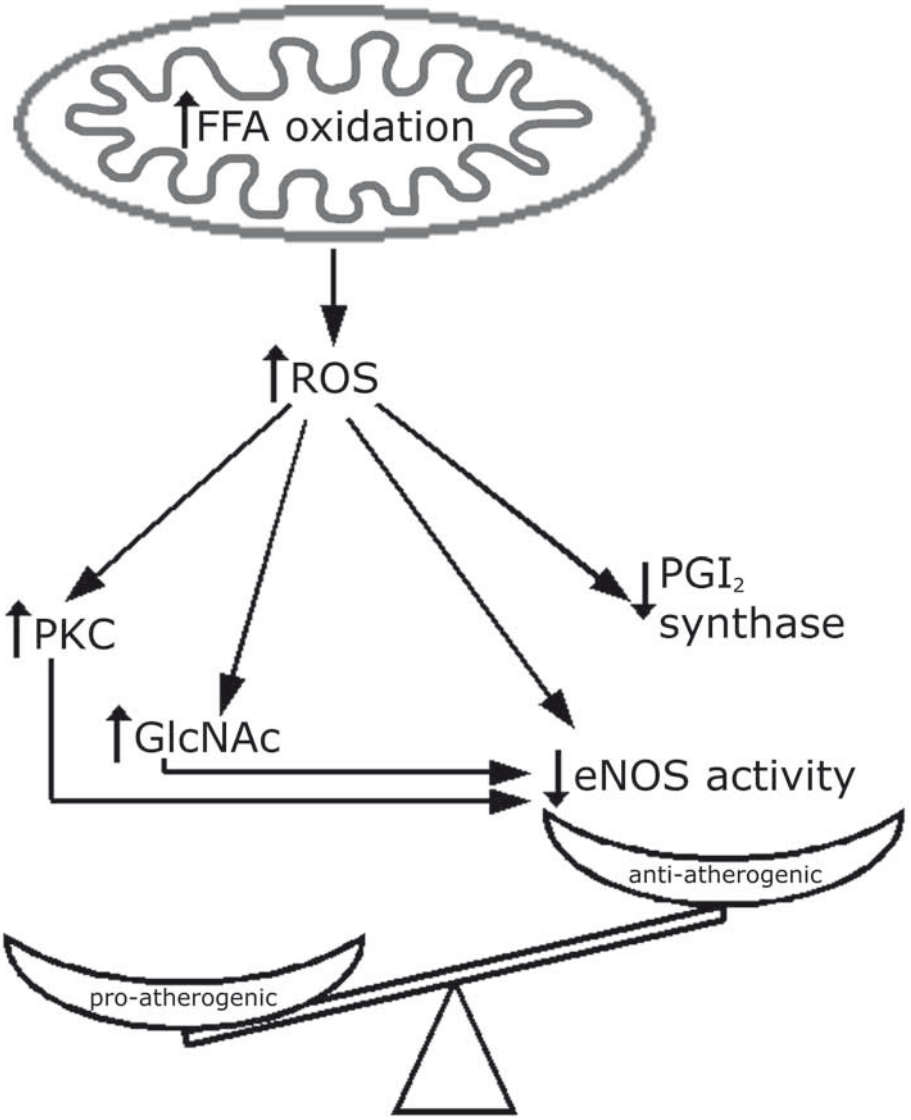

Figure 8. Effect of elevated levels of FFA on pro-atherogenic and anti-atherogenic factors (modified from [103]).

Increased oxidation of FFA leads to overload of mitochondria and subsequent mitochondrial $O_2^{\cdot-}$ leakage and subsequent activation of pro-atherogenic signals and a deactivation of anti-atherogenic enzymes. eNOS, endothelial nitric oxide synthase; FFA, free fatty acids; GlcNAc, N-acetylglucosamine; PGI_2, prostacyclin; PKC, protein kinase C; ROS, reactive oxygen species

Hypoalbuminaemia

Albumin is the most abundant circulating protein in humans[106] and is exclusively synthesized in the liver and degraded by the vascular endothelium. The homeostasis of the plasma albumin concentration is maintained by a negative feedback mechanism[107]: increases in the albumin concentration induce increased endothelial albumin degradation. Conversely, low levels of albumin reduce the degradation of the protein[108]. Hypoalbuminaemia, a clinical condition where levels of albumin in blood serum are abnormally low, is caused by an imbalance between the synthesis on the one hand and loss and degradation of albumin on the other. Loss of albumin can be due to glomerulopathies, resulting in severe proteinuria and nephrotic syndrome, or by protein-losing enteropathy. Acute disease, systemic malnutrition, infection and severe injury can all be associated with a decrease in serum albumin levels[109, 110], caused by alterations in the catabolism or synthesis of albumin, loss of albumin, or redistribution between various fluid compartments in the body[111].

Hypoalbuminemia is associated with endothelial dysfunction[112, 113], all-cause mortality in patients with chronic kidney disease (CKD)[114] and cardiovascular mortality in patients with end-stage renal disease[115] (figure 9; modified from [116]). However, it is unclear whether endothelial dysfunction is a direct result of the decreased levels of albumin or whether it is caused by factors such as chronic inflammation[117] and dyslipidemia[112], present in most conditions associated with hypoalbuminemia.

Since NO is highly reactive, the effects of NO are restricted to the vicinity of its production site. Because of rapid oxidation, the major fraction of NO will be destroyed before it reaches a target cell. However, it has been suggested that the bioactivity of NO can be conserved, allowing more distant and sustained effects. For example, NO can be transported in blood and tissues in the form of S-nitrosothiols (SNOs), which are formed when thiol groups react with NO[118]. Albumin nitrosylation, the formation of S-nitrosoalbumin, serves as a major shuttle for NO transport from sites of production to remote tissues[119]. It is conceivable that decreased albumin levels, e.g. in hypoalbuminaemic subjects, results in diminished formation of S-nitrosoalbumin. This will result in diminished removal of eNOS-derived NO and thus increase vascular tissue NO, giving rise to increased peroxynitrite through reaction with oxygen radicals. Formation of S-nitrosoalbumin may be a pathway through which albumin prevents peroxynitrite formation, and thus albumin may serve as extracellular antioxidant[120].

Hypoxia

As discussed in the previous paragraph NO is rapidly oxidised and therefore exerts its actions in the vicinity of its production site. However, in addition to the formation of SNOs, another pathway has been suggested to increase the range of action of NO: the reduction of nitrite (NO_2^-) to form NO. NO_2^- is an oxidation product of NO[121].

Under conditions where oxygen levels are low and vasodilatation is most required,

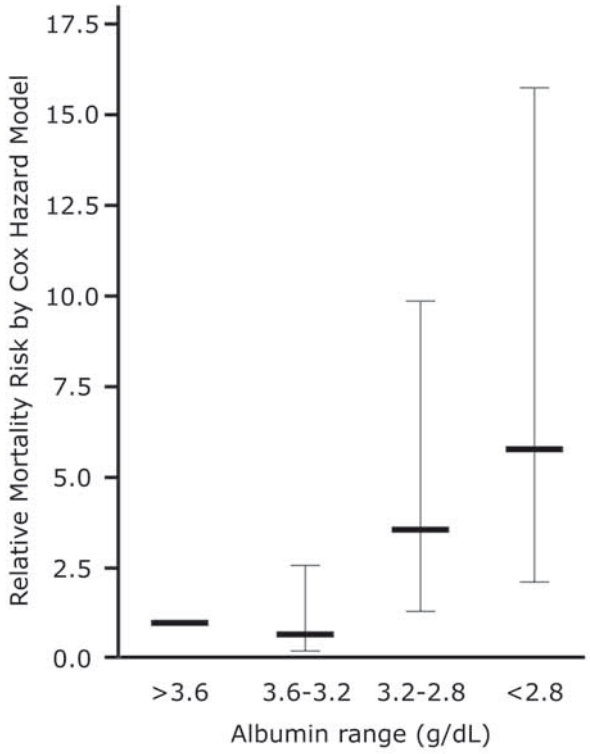

Figure 9. Relation between albumin levels and risk for mortality.
Graph adapted from table 5 in [116]. P<0.01

enzymatic activity of eNOS is blocked, since the conversion of L-arginine into L-citrulline and NO requires oxygen[122]. This condition, in which the body as a whole (generalized hypoxia) or region of the body (tissue hypoxia) is deprived of adequate oxygen supply, is referred to as hypoxia. Hypoxia in which there is complete deprivation of oxygen supply is called anoxia. Hypoxia plays a role at sites of ischaemia-reperfusion, during microangiopathy, stenosis or other pathophysiological conditions resulting in decreased levels of oxygen. Under hypoxic conditions, circulating NO_2^- is reduced to NO by glutathione-S-transferases, XO, protons (H^+), deoxy-Hb or cytochrome P-450 enzymes[123, 124] (figure 10a; modified from [124]).
The reduction of NO_2^- to form NO is a three step reaction (figure 10b; adapted from [124]) First, NO_2^- is acidified, yielding nitrous acid (HNO_2, reaction 1). HNO_2 spontaneously decomposes to NO and other nitrogen oxides (reactions 2 and 3). The amounts of NO generated from NO_2^- is dependent on NO_2^- concentrations and pH. The reaction is most significant in the stomach, where there is an extremely low pH in combination

with a very high NO_2^- content[125]. In addition, the conversion of NO_2^- into NO is also dependent on several other factors amongst which the presence of reducing agents, such as vitamin C, and the oxygen tension[126].
However, it has been shown that acid-catalysed NO_2^- reduction to NO can also take place in blood vessels and tissues with moderately low pH and NO_2^- concentrations normally present *in vivo*[127]. Another remarkable observation is that eNOS itself can reduce NO_2^- to NO under anoxic conditions[123]. The magnitude of the NO release suggests that the NO_2^- reductase activity of eNOS has relevance for maintaining vasodilatation and a fast NO delivery in tissues under acute hypoxia. Conceptually, this anoxic nitrite reductase pathway of eNOS could provide a significant alternative source of NO for tissues under acute hypoxia.

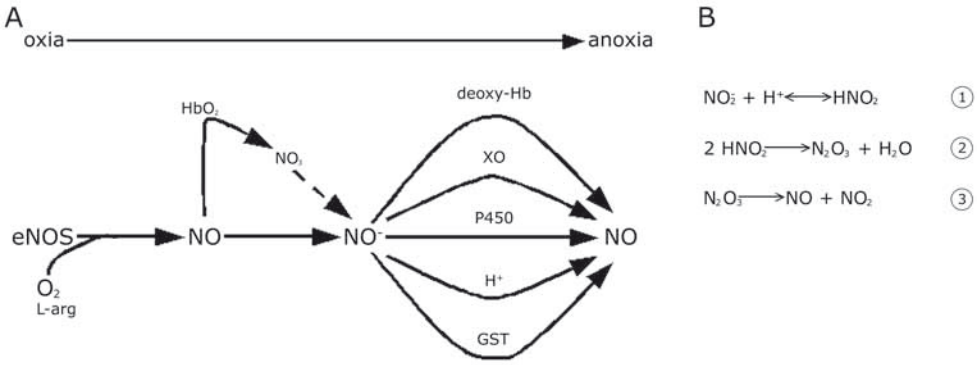

Figure 10. Formation of NO by reduction of nitrite (modified from [124]).
A. In the presence of oxygen (oxia) eNOS produces NO. NO is oxidised into nitrate (NO_3^-) by oxyhaemoglobin (HbO_2) and nitrite (NO_2^-). In the absence of oxygen (anoxia), nitrite is reduced to NO by deoxy-haemoglobin (deoxy-Hb), xanthine oxidase (XO), cytochrome P450 enzymes (P450), protons (H^+) and glutathione-S-transferases (GST). B. The reduction of NO_2^- to form NO. deoxy-Hb, deoxy-Haemoglobin; eNOS, endothelial nitric oxide synthase; GST, glutathione-S-transferase; H^+, proton; HbO_2, oxyhaemoglobin; L-arg, L-arginine; NO, nitric oxide, O_2, oxygen; NO_2^-, nitrite; NO_3^-, nitrate; P450, cytochrome P450 enzymes; XO, xanthine oxidase

Aim of this thesis

In this thesis, the activity of eNOS and the effects of its substrates, modulators and products will be described. In all studies, a microvascular endothelial cell line (bEnd.3)[128] is used. These cells express high levels of eNOS but lack neuronal or inducible NOS[129]. In addition, bEnd.3 cells produce relatively large amounts of NO, which facilitates the

detection of subtle differences in NO production as a consequence of treatment with agonists or antagonists.
In chapter 2, different methods to measure NO and ROS in a cell system are described. In chapter 3, the quantification of NO production in bEnd.3 cells is described. Electron paramagnetic resonance (EPR) is used to measure the concentrations NO produced by stimulated and unstimulated bEnd.3 cells. The mechanism of NO trapping by iron-dithiocarbamate complexes, an essential step in the measurement of NO by EPR, has been investigated. The redox state of the iron complex which traps the NO radical and the pathways involved in the transition of the monotyrosyl complexes to a ferrous paramagnetic state are discussed. In chapter 4 the focus is on the localisation of eNOS in endothelial cells in relation to eNOS activity. In chapter 5, the effects of the substrate L-arginine and the cofactor BH4 on eNOS activity and uncoupling are described. In chapter 6 and 7 the effects of elevated levels of free fatty acids and hypoalbuminaemia, respectively, on eNOS activity, NO production and down-stream effectors are discussed. Chapter 8 deals with the effect of hypoxia on eNOS function and activity. In chapter 9, the results presented in the previous chapters are discussed in a broader context. The importance of eNOS and its possible therapeutic benefits in the struggle against endothelial dysfunction and cardiovascular disease are discussed.

Questions of the study

NO measurements (chapter 3)
How can NO production be quantified in biological systems?
What is the mechanism of NO trapping by iron-dithiocarbamate complexes?
What is the redox state of the iron complex which traps the NO radical?
Which redox pathways are involved in the transition of the mononitrosyl complexes to a ferrous paramagnetic state?

eNOS localisation (chapter 4)
How does cellular eNOS localisation affect eNOS activity?
What is the cellular localisation of eNOS?
How is eNOS activity influenced by cellular localisation of the enzyme?

Substrates and cofactors (chapter 5)
Does deficiency of L-arginine or tetrahydrobiopterin lead to eNOS uncoupling?
Does eNOS uncoupling occur in bEnd.3 cells?
Does stimulation of bEnd.3 cells with VEGF enhance eNOS uncoupling?
If eNOS uncoupling occurs, is it due to a shortage of L-arginine, BH4 or both?

Hyperlipidaemia (chapter 6)
How do fatty acids influence eNOS activity and NO production?
Does OA affect ROS production and/or NO production?
Does OA induce eNOS uncoupling and/or inhibit eNOS activity?
If eNOS uncoupling occurs, is this due to a shortage of BH4?

Hypoalbuminaemia - model: analbuminaemia (chapter 7)
Does hypoalbuminaemia lead to endothelial dysfunction?
Is eNOS activity affected by low levels of albumin?
Is NO production affected by low levels of albumin?
If eNOS activity and NO production are affected, does that lead to endothelial dysfunction *in vivo*?

Hypoxia - model: anoxia (chapter 8)
Does hypoxia lead to altered eNOS activity and function?
Is eNOS activity affected by hypoxia?
Is eNOS function affected by hypoxia?
Does eNOS provide an alternative source of NO for tissues under acute hypoxia (anoxic nitrite reductase pathway)?

Chapter 2

Detection of nitric oxide and reactive oxygen species

Introduction

Nitric oxide (NO) is one of the key players in the vasculature. It is involved in vessel dilation, inhibition of platelet and leukocyte adhesion, and inhibition of proliferation and migration of vascular smooth muscle cells[4]. The actual NO bioavailability is dependent on the amount of NO produced by NOS and the level of degradation by reactive oxygen species (ROS), predominantly superoxide (O_2^{-}). ROS play an important role in signal transduction, gene expression and proliferation and are involved in the regulation of the biologically effective concentration of NO[6]. There is a delicate balance between the amount of NO and ROS produced in a cellular system. In vascular disease states, excessive production of ROS may overwhelm the anti-oxidant defence mechanisms of cells, resulting in oxidative stress, damage to the artery wall and, ultimately, development of atherosclerotic plaques[7]. In order to determine the effects of NO and ROS on cardiovascular disease mechanisms, accurate techniques to measure the production and concentration of the radicals are required. In this chapter, different methods for NO and ROS detection and their limitations will be discussed.

bEnd.3 cells

The murine microvascular endothelial cell line bEnd.3 is an ideal cell line to detect and quantify subtle differences in NO production as a consequence of treatment with agonists and antagonists. bEnd.3 cells express relatively high levels of eNOS and produce large amounts of NO as compared to primary endothelial cells, such as human umbilical vein endothelial cells (HUVEC) or human microvascular endothelial cell (CDC.HMEC).
During cell growth, cells go through the four distinct phases of the cell cycle. Although the various stages are not usually morphologically distinguishable, each phase of the cell cycle has a distinct set of specialized biochemical processes that prepare the cell for entry into the next stage. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G_0 phase. This G_0 phase can be obtained by serum deprivation of cells. Studying cells in the G_0 phase reduces interexperimental variability.
Although in some cells removal of serum may lead to apoptosis, in bEnd.3 cells it has been shown that prolonged serum deprivation does not lead to substantial apoptosis (Shi (2006) *Chin Med J* 119, 725-30). Moreover, our own experiments have shown that serum deprivation between 0 and 24 hours does affect basal as well as stimulated NO and ROS production (data not shown). Recently several studies have been reported in which bEnd.3 cells are serum deprived for 12 to 24 hours in order to synchronize all cells in the G_0 phase (Seok (2006) *Toxicol Lett* 165, 212-20; Ning (2006) *Biochem Biophys Res Commun* 342,1249-56; Zhang (2006) *Life Sci* 78, 2983-8). Similarly all

experiments with bEnd.3 cells described in this thesis were performed after 16 to 18 hours of serum deprivation.

Detection techniques

Nitric oxide (NO)

Detection techniques for NO can be divided into thee major classes: 1) direct detection; 2) indirect detection; and 3) secondary detection. The first group, direct detection of NO, includes the oxidation of haemoglobin[130], fluorimetric trapping with 2,3-diaminonaphthalene (DAN)[131], diaminofluorescein (DAF)[132] or fluorescent cheletropic nitric oxide traps (FNOCT)[133], chemiluminescence, both in the gaseous phase by reaction with ozone[134] and in the liquid phase using luminol[135], electrochemical detection by means of electrodes[136] and spin trapping of NO[137]. The second class of NO detection techniques, indirect detection of NO, is based on analytical methods that identify reaction products of NO with other agents. This group consists of spectrophotometric detection of NO_2^- and NO_3^- (Griess reaction)[138], L-arginine to L-citrulline conversion[139], the conversion of GTP into cGMP and pyrophosphate[140] and measurement of nitrosylated proteins[141]. Secondary detection of NO is based on the detection via systemic response. In this third class, NO itself is not detected. Instead the radicals are determined via their effect on the systemic response of a complete and fully functional organism. This class includes the detection of NO dependent vasorelaxation in extracted tissues[8], flow mediated dilatation[142], venous occlusion plethysmography[143] and measurement of platelet adhesion to endothelium[144].

Reactive oxygen species (ROS)

Detection methods for ROS can be divided into two classes: direct and indirect detection techniques. The direct detection of ROS includes spin trapping[145], electrochemical detection[146], chemiluminescent detection of $O_2^{\cdot-}$ or $ONOO^-$ with coelenterazine[147], luminol or lucigenin[148], oxidation of hydroethidine to ethidium by $O_2^{\cdot-}$ [149], fluorimetric trapping of H_2O_2 with dichlorofluorescein (DCF)[150] or of $ONOO^-$ with dihydrorhodamine oxidation (DHR)[151] and horse-radish peroxidase-linked[152] and catalase-based assays[153] for the detection of H_2O_2. Indirect detection techniques include the reduction of nitroblue tetrazolium[154] or cytochrome c[155] by $O_2^{\cdot-}$, the oxidation of epinephrine to adrenochrome[156], inhibition of aconitase by $O_2^{\cdot-}$ thereby preventing the conversion of isocitrate to cis-aconitate[157], detection of H_2O_2 with ferrithiocyanate[158] and the determination of nitrotyrosine formation, which is a measure for $ONOO^-$ [159].
Within the context of my thesis the rest of this chapter will only focus on the detection methods used therein.

Measurement of NO - Electron Paramagnetic Resonance
Electron paramagnetic resonance (EPR) spectroscopy is a technique which detects molecules with unpaired electrons, such as NO. NO binds to the iron of a diamagnetic iron(II)-carbamate complex. The resulting complex is a paramagnetic mononotrosyl-iron complex (MNIC) which can be detected using EPR. In contrast, diamagnetic complexes can not be detected with EPR. For detection of NO in cell cultures, generally the lipophilic and cell permeable Fe-diethyl dithiocarbamate (Fe-DETC) is used. The major advantage of EPR is that it can be used to detect NO within cells and tissues. The detection limit for EPR is approximately 1 μM.

Measurement of NO - DAF-2 DA assay
The cell-permeable 4,5-diaminofluorescein diacetate (DAF-2 DA)[132] is probably the most widely-used indicator for NO. The two acetate groups make the DAF-2 DA lipophilic, which allows it to cross the cell membrane. Once inside the cell, intracellular esterases hydrolyse the acetate groups resulting in the hydrophilic 4,5-diaminofluorescein (DAF-2) which is trapped in the cell. DAF-2 remains non-fluorescent until it reacts with NO, resulting in a fluorescence signal with an excitation maximum of 495 nm and an emission maximum of 515 nm. Early studies with DAF-2 reported absolute specificity for NO[160, 161], however, more recent publications indicate that DAF-2 might preferably react with intermediate products formed during the oxidation of NO[162, 163]. The detection limit of DAF-2 is approximately 10 nM.

Measurement of eNOS activity - arginine-citrulline conversion assay
NO is produced during the conversion of L-arginine to L-citrulline by eNOS. The arginine-citrulline conversion assay makes use of radio-labelled L-^{3}H-arginine. Since conversion of L-arginine, in the presence of sufficient amounts of eNOS cofactors FAD, FMD, NADPH, Ca^{2+}, CaM and BH4, leads to equimolar amounts of NO and L-citrulline, the amount of L-^{3}H-citrulline formed, is a measure of NO production. L-^{3}H-citrulline is harvested via column separation and the yield of can be measured in a liquid scintillation counter. To correct for non-specific activity an additional measurement is performed in which NADPH is substituted by a NOS inhibitor. This technique can be used to determine NO production *in vitro* in cultured cells and tissue. The detection limit of the arginine-citrulline conversion assay is in the picomolar range.

Measurement of nitrite - Griess assay
Under physiological conditions, NO is readily oxidised to nitrite (NO_2^-) and nitrate (NO_3^-). The Griess reagent reacts with nitrites and nitrates that have been reduced to nitrites. The assay is based on the reaction of NO_2^- with sulfanilic acid in acidic solution to form an intermediate diazonium salt which couples to N-(1-naphthyl)ethylenediami ne, resulting in a purple azo derivate that can be measured by spectrophotometry at 548

nm. A drawback of the Griess assay is that the method detects nitrite rather than NO itself. Total NO levels is determined indirectly from the yield of oxidation products like NO_2^- or NO_3^-. The detection limit of the Griess assay is about 100 nM[164].

Measurement of NO dependent vasorelaxation in extracted tissues - Organ chamber experiments

Detection of NO dependent vasorelaxation is one of the first methods described to measure NO production[8]. Pre-contracted endothelium produces NO in response to eNOS agonists, such as VEGF and ACh. In addition to NO, the endothelium produces other relaxing substances, such as endothelium derived hyperpolarizing factor (EDHF) and PGI_2[165]. But with the appropriate controls, this method is quite specific for NO. However, with this technique one can not detect NO directly and quantification of NO is not possible. A detectable response has been reported for NO release in the nanomolar range.

Measurement of ROS - CM-H_2DCFDA assay

Oxidation of non-fluorescent 2'-7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) to the fluorescent 2'-7'-dichlorofluorescein (DCF) is a common method to detect oxidative stress[166]. The chloromethyl derivative of H_2DCFDA, 5-chloromethyl-2'-7'-dichlorodihy drofluorescein diacetate (CM-H_2DCFDA), is very useful to measure intracellular ROS. The probe passively diffuses into cells, where intracellular esterases hydrolyse the acetate groups. In addition, the chloromethyl group reacts with intracellular glutathione and other thiols, to produce membrane-impermeant glutathione-fluorescent dye adducts. CM-H_2DCFDA does not react directly with O_2^-, but with a series of ROS, such as H_2O_2, OH^-, HOO^- and $ONOO^-$ [167]. When oxidised, DCF becomes fluorescent and can be detected with fluorescence spectroscopy at an excitation maximum of 488 nm and an emission maximum of 525 nm. The detection limit of DCF is in the nanomolar range.

Conclusion

There are several different methods available for the detection of NO and ROS. However, since the chemical reactivity of the reactive oxygen and nitrogen species towards the detector molecules is often not specific and sometimes even overlapping, none of the abovementioned techniques will accurately reflect quantitative rates of the formation of one particular reactive species under all circumstances. Usually, more than one detection method is needed in order to form a robust conclusion.

Chapter 3

Reduction enhances yields of nitric oxide trapping by iron-diethyldithiocarbamate complex in biological systems

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Chapter 3

Abstract

Electron paramagnetic resonance (EPR) was used to quantify nitric oxide (NO) production in bEnd.3 cells. In addition, the mechanism of NO trapping by iron-diethylthiocarbamate complexes was investigated. Basal levels of NO produced by bEnd.3 cells can be measured by EPR. Stimulation with calcium ionophore increases the NO trapping yield. NO radicals are trapped by iron-diethylthiocarbamates both in the ferrous and ferric state. After trapping NO, the nitrosylated Fe-DETC adducts are predominantly in a diamagnetic ferric state. Only a minor fraction is reduced to a paramagnetic ferrous state by endogenous biological reductants. Treatment with dithionite, an exogenous reductant, considerably increases the formation of the paramagnetic ferrous DETC complex, which extensively increases NO trapping yields.

Introduction

There are several techniques to detect nitric oxide (NO). However, most of these techniques are not accurate to quantify the concentrations of NO produced in biological systems. In addition, chemical reactivity of nitrogen species towards the detector molecules is often not specific. In the present study, we used electron paramagnetic resonance (EPR) to quantify basal and stimulated NO production in a microvascular endothelial cell line (bEnd.3)[128]. These cells produce large amounts of NO in comparison with primary endothelial cells, e.g., human umbilical vein endothelial cells (HUVEC) or a human microvascular endothelial cell line (CDC.HMEC-1)[129]. These cells are very useful to detect subtle differences in NO production.

EPR spin trapping with iron dithiocarbamate complexes is a proven technique for detection of NO in cultured cells and tissues. In this study, we use EPR to quantify the total NO production in cultured bEnd.3 cells. For reliable quantification of NO production in biological systems, two criteria must be satisfied: 1. all NO needs to be trapped and 2. the resulting spin trap adduct needs to be in a paramagnetic state in order to be detectable with EPR. The second criterion is nontrivial since NO adducts of iron-dithiocarbamates can exist in a ferrous (EPR silent) as well as in a ferric (paramagnetic) state. Iron-diethyldithiocarbamate (Fe-DETC) complexes are widely used as *in vivo* traps for NO[168-172]. It is generally assumed that NO is trapped by diamagnetic ferrous Fe^{2+}-dithiocarbamate complexes. The resulting adduct is a paramagnetic ferrous mononitrosyl-iron complex (MNIC) that can be detected by EPR spectroscopy. However, this assumption is difficult to reconcile, since in biological material, where micromolar levels of oxygen are present, it is conceivable that ferrous (Fe^{2+}) iron is oxidised to ferric (Fe^{3+}) iron. In addition, it has been reported that the experimental MNIC yields in animal tissues are not dependent on the original oxidation state of iron

added to initialise the formation of Fe-DETC complexes[173]. The relevant pathways that are involved in NO spin trapping are shown in scheme 1.

Fe^{3+}-DETC (I) $\rightarrow$ NO-Fe^{3+}-DETC (III)
$\updownarrow$
Fe^{2+}-DETC (II) $\rightarrow$ NO-Fe^{2+}-DETC (IV)

Scheme 1: The four possible Fe-DETC complexes considered in this paper.
The ferric mononitrosyl-iron complex III is diamagnetic, whereas the ferrous complex IV is paramagnetic (S = 1/2). Complex IV is observed with EPR. The vertical arrows represent reversible redox reactions.

In the present study, we investigated lipophilic iron complexes with diethyldithiocarbamate ligands (Fe-DETC complexes). These complexes favourably locate in lipid and protein compartments of cells, which are low polarity compartments preferred by NO radicals. We found that initial trapping of NO resulted in the formation of a diamagnetic NO-Fe^{3+}-DETC complex, which could be reduced to paramagnetic NO-Fe^{2+}-DETC by sodium dithionite.

Materials and methods

Materials

Na-DETC, sodium dithionite, HEPES and LPS were purchased from Sigma. Ferrous sulphate was from Fluka. Gaseous NO was obtained by acidic reduction of nitrite in the reaction of FeSO4 and NaNO2 in 0.1 M HCl. The escaping NO was subsequently purified by low-temperature sublimation.

Optical and EPR assays

Optical spectra of iron-DETC complexes and their nitroso adducts were recorded at ambient temperature in 150 mM HEPES buffer (pH 7.4) using UV-2501PC spectrometer (Shimadzu Europe GmbH, Duisburg, Germany) in open or closed cuvettes with d = 10 mm optical pathlength.
EPR spectra were recorded at 77 K on a modified X-band ESP 300 radiospectrometer (Bruker BioSpin, Karlsruhe, Germany) operating near 9.5 GHz with 0.5 mT field modulation and 10 mW power. Time constant and ADC conversion time were set to 82 ms. Four scans were accumulated to reduce instrumental noise. The receiver gain was $2 \cdot 10^5$. From the reaction mixtures 250 µl aliquots were drawn by syringe (id 4.8 mm), snap frozen in liquid nitrogen and placed in a quartz liquid finger dewar at the centre of a Bruker ER4103TM cavity. The spin densities were calibrated with frozen reference solutions of NO-Fe^{2+}-$(MGD)_2$ in PBS buffer.

NO trapping in cultured cells

An immortalized bEnd.3 cell line[128] was generously provided by Dr. Alan Schwartz (University of Washington, St Louis, MO). Cells were cultured to confluency in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 10 IU/ml penicillin and 100 µg/l streptomycin and penicillin/streptomycin (Life Technologies, Burlington, ON, Canada) at 37º C under a controlled atmosphere containing 5% CO_2 and 20% O_2. At confluence, each flask contains ca. $7.5 \cdot 10^6$ endothelial cells. NO trapping was initiated by replacing DMEM by PBS (15 mM, pH 7.4) containing 2.5 mM Na-DETC and 100 µM L-arginine. Subsequently, ferrous sulphate (20 µM) was added to initialise the formation of hydrophobic Fe-DETC complexes in the lipid membrane compartment. The cells were kept at 37º C under a controlled atmosphere (5% CO_2 / 20% O_2) throughout the experiment. NO production was stimulated by 5 µM Ca-ionophore A23187. In some experiments NOS activity was blocked by pre-incubation with 1 mM of NOS inhibitor N-nitro-L-arginine (L-NNA) for 30 min. After 15 min the flask was placed on ice and the cells were harvested in 3 ml of fresh PBS buffer. The lipid fraction with the Fe-DETC complexes was isolated by centrifugation (1000 g for 5 min) and resuspended in 0.5 ml HEPES buffer (150 mM, pH 7.4). Of this suspension 250 µl was drawn into a syringe (id 4.8 mm) and snap frozen in liquid nitrogen. If applicable, the samples were thawed, reduced by supplementation of solid sodium dithionite (10 mM final) for 30 min and snap frozen.

Results

The oxidation state of iron-DETC complexes

In organisms or cell cultures, lipophilic Fe-DETC complexes will tend to accumulate in the membrane compartment. To investigate the redox state of iron in the Fe-DETC complex during trapping of NO, the organic solvent dimethylsulfoxide (DMSO) was used. The accompanying spectra can be found in [174].

Within several seconds after addition of $FeSO_4$ (0.1 mM) and DETC (1 mM) to DMSO, the solution turned dark[174] due to rapid oxidation of the ferrous complexes by dioxygen, indicating the formation of complex I (scheme 1). Addition of 1 mM citrate resulted in a rapid (3 min) bleaching of the solution[174], implying the formation of complex II (scheme 1). This replacement was reversible, as the original absorption spectrum could be restored by addition of 10 mM DETC (data not shown).

Treatment of complex I, dissolved in DMSO, with gaseous NO changed the colour of the solution from dark to yellow[174], which is a characteristic of complex III (scheme 1). Under anaerobic conditions the colour of the solution changed (10 min) to green[174], implicating the formation of complex IV (scheme 1). EPR and UV/VIS spectra confirmed that the yield of complex IV reached at least 90% of the available iron. The

reduction of complex III to complex IV was significantly increased by addition of 1 mM of sodium dithionite (data not shown).
In unreduced samples, EPR spectra appear as the superposition of the signal of complex IV with triplet hyperfine structure (HFS; g = 2.035) and the signal from Cu^{2+}-DETC with quartet HFS[169, 175] (data not shown). Reduction of the sample with dithionite not only leads to an increased yield of complex IV, but at the same time results in a considerable decrease in the overlapping Cu^{2+}-DETC signal, thereby facilitating the quantification of complex IV.

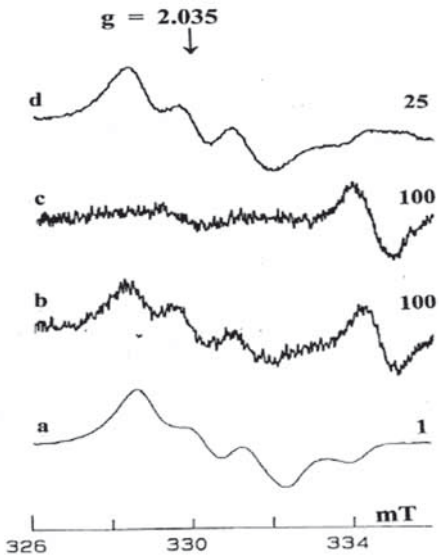

Figure 1. EPR spectra at 77 K of complex IV in bEnd.3 cells.
(a) reference spectrum from 250 µl of 0.1 mM paramagnetic NO-Fe^{2+}-MGD in PBS buffer. (b) yield 15 minutes after stimulation with Ca-ionophore, unreduced. (c) sample (b), pre-treated with 1 mM NOS-inhibitor L-NNA for 30 minutes prior to trapping. (d) sample (b) after reduction with 10 mM dithionite for 30 minutes. The 250 µl aliquots contain about half of the lipid cell fraction of the cell culture. Scaling factors are indicated on the right.

Quantification of NO in bEnd.3 cells
Non-stimulated bEnd.3 produced 0.055 ± 0.003 nmol NO per $7.5 \cdot 10^6$ cells. When stimulated by calcium-ionophore, NO production increased to a total of 0.42 ± 0.10 nmol NO per $7.5 \cdot 10^6$ bEnd.3 cells. Reduction with dithionite raised the detection of NO fivefold to 2.0 ± 0.3 nmol per $7.5 \cdot 10^6$ cells (figure 1) Pre-incubation with the nitric oxide synthase (NOS) inhibitor L-NNA reduced the MNIC yield to below the detection limit of 10 pmol at the given spectrometer settings.

Nitrite does not induce formation of complex IV in strong buffer

It has been reported that elevated levels of ferrous dithiocarbamate complexes can lead to the reduction of nitrite to form NO at physiological pH[176]. To investigate the possibility of artificial NO release from nitrite, the formation of complex IV in the presence of 1.5 mM $NaNO_2$ was studied. Addition of 10 mM dithionite to distilled water containing $NaNO_2$, DETC and $FeSO_4$ resulted in the formation of 40 µM of complex IV (scheme 1). In PBS, addition of dithionite yielded 10 µM of complex IV. In addition, dithionite induced acidification of PBS, caused by the consumption of OH^- upon electron donation by dithionite. The quantities of complex IV formed were proportional to the initial nitrite concentration.

In contrast, in a strong buffer reduction lead to the generation of negligible amounts of NO. Levels of complex IV remained below 10 nM when 10 mM dithionite was added to HEPES (150 mM, pH 7.4). The yield increased to a marginal 80 nM after addition of 100 mM dithionite.

Discussion

In the present study, we used EPR to quantify the amount of NO produced in stimulated and non-stimulated endothelial cells. In addition, we determined the oxidation state of iron during trapping of NO. The main results may be summarized as follows: (i) both basal and stimulated NO production can be quantified using EPR. (ii) NO binds to ferrous as well as ferric Fe-DETC. Accordingly, NO trapping forms MNICs as a mixture of ferric and ferrous charge state. In cultured endothelial cells, the redox equilibrium lies firmly on the ferric side. Therefore, the majority of mononitrosyl complexes was diamagnetic and remained undetectable by EPR. (iii) The yield of complex IV is enhanced considerably by subsequent *ex vivo* reduction with dithionite. Concomitantly, the overlapping EPR signal from Cu^{2+}-DETC complexes was abolished by the reduction of Cu^{2+} to EPR silent Cu^+. This facilitated the quantification of complex IV, which is highly relevant for practical NO trapping. (iv) Reduction of biological samples with dithionite may induce an artificial release of NO by reduction of endogenous nitrite. This artefact may be avoided by adequate buffering of the samples prior to reduction.

Ferric DETC complexes are easily soluble in organic solvents, however, the stability of Fe^{3+}-DETC is low. Addition of 1 mM citrate resulted in the replacement of DETC, leading to the formation of Fe-citrate. Addition of excess DETC reversed the ligand replacement. No significant amounts of ferrous complexes were detected. These results demonstrate that the observed bleaching of ferric-DETC complexes was not caused by reduction. Instead, the bleaching was caused by replacement of the DETC ligands.

In the hydrophilic compartment of cells and tissues, various endogenous ligands

like citrate could compromise the formation of Fe-DETC complexes, regardless of whether the iron has endogenous or exogenous origin. Excess levels of DETC can prevent the interference of endogenous ligands[174]. This is easily accomplished in NO trapping experiments in cultured (endothelial) cells. Addition of 2.5 mM DETC to the incubation medium results in a clear excess of DETC over ligands such as citrate. Therefore, endogenous and exogenous iron is likely to bind to DETC, thereby forming complexes that readily oxidise to Fe^{2+}-DETC.

Stimulation of bEnd.3 cells increased the production of NO almost 7-fold. Reduction with dithionite further increased the yield in stimulated bEnd.3 cells. In the presence of the NOS inhibitor L-NNA the MNIC yield remained below the detection limit, clearly identifying NOS as the most significant source of NO in this experiment.

Under acidic conditions, nitrite is reduced to nitric oxide according to the following reaction[177]:

$$NO_2^- + 2\ H^+ + e^- \rightarrow NO + H_2O$$

It has been reported that elevated levels of ferrous dithiocarbamate complexes can lead to the reduction of nitrite to form NO at physiological pH[176]. In addition, dithionite induced acidification of PBS, caused by the consumption of OH^- upon electron donation by dithionite:

$$S_2O_4^{2-} + 4\ OH^- \rightarrow 2\ SO_3^{2-} + 2\ H_2O + 2\ e^-$$

It should be noted that the beneficial effect of dithionite on adduct yield has been observed before[178, 179] both *in vitro* as well as in tissue preparations. However, inadequate buffering in these cases may have influenced the results by artificial release of NO from nitrite, nitrate or other metabolites of NO. Endogenous nitrite levels in biological systems are in the range of a few micromolar[180 and references therein], i.e. 2-3 orders of magnitude below the nitrite concentration used in this study. Our data show that, in an adequate buffering environment, dithionite can be safely used for the reduction of complex III to IV, without artificial NO release from reduction of nitrite.

In addition to the reduction of nitrite, several other candidates have been proposed as endogenous NO donors. Dinitrosyl-iron complexes (DNIC) may form endogenously in tissues[181] and are capable of donating their NO moiety to iron-dithiocarbamate complexes in a transnitrosation reaction. In the present study, no signals from paramagnetic DNIC were observed in EPR, indicating that their concentration is lower than the detection limit of 10 pmol/250 µl = 40 nM. Compared to the yields of complex IV in our

experiments, these DNIC concentrations are negligible.
S-nitrosothiols may also act as NO donors. By photolysis or by a reaction catalysed by Fe^{2+} or Cu^{+}, S-nitrosothiols decay into NO and a thiyl radical. Alternatively, they may transfer their NO moiety directly to Fe-DETC, however, this reaction is very slow. It has been reported that the tissue levels of S-nitrosothiols are in the 30-40 nanomolar range in healthy animals[182]. These amounts of S-nitrosothiols will produce levels of NO that are negligible as compared to the quantities of NO produced by NOS.
A third endogenous source of NO is the nitroxyl anion NO^{-}, which can be formed in the cellular milieu by several routes[183]. NO^{-} can react directly with complex I (Fe^{3+}-DETC) to form complex IV (NO-Fe^{2+}-DECT)[184]. During this reaction, complex III would be bypassed as a reaction intermediate and only appear at a later stage via the very slow re-oxidation of complex IV by oxygen. However, complex IV is very stable and not readily re-oxidised. In addition, our results show that mononitrosyl complexes in biological materials are a mixture of ferric and ferrous forms. Therefore, it is inconceivable that NO^{-} contributes to the amount of complex IV formed.

The goal of the present study was to quantify the amount of NO produced by bEnd.3 cells. The yields of NO trapping with dithiocarbamate complexes allow us to estimate this cellular NO production. In unstimulated bEnd.3 cells 55 pmol NO was trapped by the Fe-DETC complexes during 15 minutes. Reduction with dithionite revealed that the total amount of trapped NO was 200 pmol per flask during 15 min, corresponding to 15 pmol NO trapped per minute. However, it is conceivable that the total NO production was higher since a fraction of the NO produced will be bound in the form of EPR-silent diamagnetic NO-Fe^{3+}-DETC complexes and some will be lost via other reaction pathways. Given that the trap concentration is 20 µM, the trapping reaction is likely to be the dominant reaction pathway for NO. Therefore, we estimate the total NO release in a confluent layer of unstimulated bEnd.3 cells to be 15-20 pmol NO per min. A confluent layer of bEnd.3 cells in a 75 cm^2 flask contains $7.5 \cdot 10^6$ cells. When reaching confluency, approximately 70% of the flask area is covered. The diameter of bEnd.3 cells is approximately 10 µm, therefore the total intracellular volume will be 10 µm · 75 cm^2 · 70% = 50 µl. Since most of the cell volume exists of cytosol, with the same density as water, 50 µl corresponds to 50 mg endothelial cells. This results in an estimate of 0.3-0.4 pmol NO · min^{-1} · (mg endothelial cells)$^{-1}$, which is in reasonable agreement with 0.8 pmol NO · min^{-1} · (mg endothelial cells)$^{-1}$ as reported for human endothelial cells[185, 186].
Stimulation with calcium ionophore increased the NO yield: After reduction, a total of 2000 pmol MNIC complexes were detected after 15 minutes. This corresponds with 130 pmol NO/min. This is equivalent to 3-4 pmol NO · min^{-1} · (mg endothelial cells)$^{-1}$, nearly an order of magnitude larger as compared to unstimulated bEnd.3 cells. These results are comparable to the calcium ionophore-induced increase in DAF signal (data not shown).

bEnd.3 cells express high levels of eNOS[129]. The present study confirms that, as a result of high eNOS expression levels, bEnd.3 cells produce large quantities of NO, especially after activation of eNOS. These results prove the usefulness of bEnd.3 cells for detecting subtle differences in NO production.

Chapter 4

Endothelial nitric oxide synthase activity is linked to its presence at cell-cell contacts

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Chapter 4

Abstract

The enzyme endothelial nitric oxide synthase (eNOS) is essential for vascular integrity. Many studies have demonstrated a link between the localization and activity of eNOS. Here, we studied the influence of cell-cell contact on this link in the microvascular endothelial bEnd.3 cell line. By immunofluorescence microscopy, eNOS localization at the plasma membrane was found to be dependent on cell-cell contact. In particular, eNOS was highly enriched at the intercellular contact sites. Further analysis showed that the pattern of eNOS localization at the plasma membrane resembled that of platelet endothelial cell adhesion molecule 1 (PECAM-1), but not that of the adherens junction proteins vascular endothelial-cadherin and plakoglobin. eNOS that was localized at the contact sites was, in part, Triton X-100-insoluble, in contrast with eNOS at the Golgi complex, which may indicate an association of eNOS with the actin cytoskeleton. Interestingly, eNOS activity was up-regulated in confluent monolayers compared with subconfluent cells, while there was no difference in eNOS expression. This correlation between cell confluence and eNOS activity was also found when primary bovine aortic endothelial cells were studied. These data imply that cell-cell contact induces the localization of eNOS at intercellular junctions, which is required for agonist-induced eNOS activation.

Introduction

The integrity of the endothelial lining of the vascular system is important for vessel function[187]. It protects the vessel wall against thrombus formation and atherogenesis. Moreover, changes in the integrity of the endothelium might facilitate transendothelial migration of inflammatory cells and intravasation of metastases[188-190]. The integrity depends on the adhesion of the endothelial cells to the underlying basement membrane, as well as on the adhesion of the cells to each other. The intercellular adhesion is accomplished predominantly by the calcium-dependent adherens junctions and, to a lesser extent, by tight junctions and gap junctions[189]. In addition to its protective properties, the adherens junctions also play an important role in cell motility, proliferation and differentiation[191]. The proteins that are mostly involved in these junctions are members of the cadherin family. Vascular endothelial cadherin (VE-cadherin) mediates the extracellular contact between the cells[192]. While its luminal part binds to the extracellular domain of a VE-cadherin molecule of a neighbouring endothelial cell, its cytosolic part is involved in the intracellular organization of the junction. This domain binds ß-catenin, plakoglobin and p120, which are linked to the actin cytoskeleton via a-catenin, resulting in stabilization of the junction.
Platelet endothelial cell adhesion molecule 1 (PECAM-1; also known as CD31) is another

molecule that plays a role in the intercellular adhesion of the endothelium[193]. As for the cadherins, PECAM-1 is also involved in proliferation and migration of endothelial cells[194] and in angiogenesis[195]. In contrast with cadherins, its role in intercellular adhesion is calcium-independent[196]. When endothelial cells are forming a confluent monolayer, PECAM-1 becomes localized at cell-cell contacts much later than VE-cadherin[196]. PECAM-1 does not become associated with the adherens junctions, but may bind the adherens junction component ß-catenin[195], which indicates that cadherins and PECAM-1 might be linked to the actin cytoskeleton via the same intermediate protein. Whether PECAM-1 is associated with another specific subdomain of the contact sites is currently unknown.

An important characteristic of the endothelial junction is its dynamic organization. The junctions can be rapidly modulated to allow the inter-endothelial passage of macromolecules and circulating cells from the blood. Changes in the endothelial junctions can be induced by hypoxia, leucocyte adhesion, and by agents such as thrombin, bradykinin, histamine, vascular endothelial growth factor (VEGF) and inflammatory cytokines, and might occur within minutes[197, 198]. Increases in endothelial permeability are often associated with increases in tyrosine phosphorylation of components of the intercellular junction, including VE-cadherin, ß-catenin, plakoglobin, p120 and PECAM-1[199, 200], and their subsequent disappearance from the cell surface[201, 202]. In agreement with this observation, tyrosine phosphatase inhibitors increase endothelial permeability[203]. One of the key players that regulates acute changes in the organization of the intercellular junctions is NO. For instance, VEGF-induced increases in endothelial permeability require NO. This is evident from studies in which endothelial cells that were pre-treated with nitric oxide synthase (NOS) inhibitors had lost their ability to respond to VEGF[204]. Since NO plays a role in the regulation of endothelial junctions, we studied whether endothelial (e)NOS is localized at intercellular contact sites. In microvascular as well as in macrovascular endothelial cells, eNOS was enriched at contact sites. Our data indicate that eNOS, like PECAM-1, is probably not present in adherens junctions, but at other parts of the contact sites. Inter-endothelial cell-cell contact was required for proper eNOS activation. Therefore our findings suggest that eNOS needs to be localized at contact sites in order to respond to agonists.

Materials and methods

Materials

The bEnd.3 cell line was generously given by Alan Schwartz (University of Washington, St Louis, MO, U.S.A.). Primary bovine aortic endothelial cells were obtained from Clonetics (Walkersville, MD, U.S.A.). A23187 and 4,5-diaminofluorescein-2 diacetate (DAF-2 DA) were purchased from Calbiochem (San Diego, CA, U.S.A.). Acetylcholine

and Nω-nitro-L-arginine methyl ester (L-NAME) were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Antibodies against eNOS, caveolin-1 and GM130 were obtained from Transduction Laboratories (San Diego, CA, U.S.A.). Polyclonal anti-caveolin antibody (#C13630) labelled caveolin-1 in caveolae, whereas monoclonal anti-(caveolin-1) antibody (#C37120) labelled caveolin-1 in the Golgi. Anti-(GOS-28) antibody was generously given by Peter van der Sluijs (UMC Utrecht, Utrecht, The Netherlands)[205]. Horseradish-peroxidase-conjugated anti-mouse antibody, as well as antibodies against VE-cadherin, plakoglobin and PECAM-1, were from Santa Cruz (Santa Cruz, CA, U.S.A.). Fluorescent secondary antibodies were purchased from Jackson Immunochemicals (West Grove, PA, U.S.A.).

Cell culture

Immortalized murine microvascular endothelial bEnd.3 cells[128] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin, and split 1:4 to 1:8 upon reaching confluence. bEnd.3 cells expressed high levels of eNOS, a phenomenon that was probably caused by the polyoma virus middle T oncogene, which was used to immortalize the primary cells[206]. Western blot analysis demonstrated that these cells did not express a detectable amount of inducible NOS (results not shown).

Primary bovine aortic endothelial cells (BAEC) were cultured according to the manufacturer's instructions. BAEC were cultured in EGM BulletKit medium (Clonetics), split 1:6 upon reaching confluence, and used at passage 4. For immunofluorescence microscopy, cells were grown on non-coated glass coverslips. For nitrite measurements and for Western blotting, cells were grown in six-well culture dishes. For DAF-2 DA experiments, cells were grown in black clear-bottom 96-well plates (Costar; Acton, MA, U.S.A.). bEnd.3 cells were grown for 18 h in FCS-free medium supplemented with 0.1% (w/v) BSA before initiating the experiments. BAEC were not put on FCS-free medium. For experiments in which the correlation between cell confluence and NO synthesis was studied, cells were treated with trypsin at the day they reached confluence, and split as indicated in the figures.

Immunofluorescence microscopy

Endothelial cells were immunostained by indirect fluorescent labelling. To analyse Triton X-100 solubility, cells were washed three times with ice-cold PBS, incubated with 0.2% (v/v) Triton X-100 in PBS for 10 min on ice, and then washed twice with PBS before fixation. Cells were fixed with 3% (w/v) paraformaldehyde, permeabilized with 0.1% saponin, blocked with normal serum, incubated with primary antibodies for 60 min, washed three times with PBS, incubated with FITC- and Texas Red-labelled secondary antibodies for 30 min, and then washed three times with PBS and embedded in Mowiol. Confocal laser scanning microscopy was performed using a Leica TCS 4D

system. FITC- and Texas Red-labelling were examined by scanning sequentially and overlaid images, using Adobe Photoshop software. All experiments were repeated at least three times. Representative images are shown in the figures.

Analysis of eNOS activity by nitrite measurement

The accumulation of nitrite in the medium has been used to assess eNOS activity[30, 207]. bEnd.3 cells were washed with Hepes buffer [20 mM Hepes/133 mM NaCl/6.5 mM KCl/1 mM $CaCl_2$/1 mM $MgCl_2$/5.5 mM glucose/50 µM L-arginine/0.1% (w/v) BSA (pH 7.4)], and incubated in Hepes buffer with or without eNOS agonists in the absence or presence of 1 mM L-NAME for 1 h at 37° C. The nitrite that had been released in the Hepes buffer was measured fluorimetrically (excitation wavelength 355 nm; emission wavelength 460 nm), according to the manufacturer's instructions (Cayman, Ann Arbor, MI, U.S.A.), except that all nitrite samples were mixed with nitrite assay buffer in a 1:1 ratio to increase sensitivity. eNOS activity was expressed as the L-NAME-dependent release of nitrite·mg of cellular protein^{-1}. To determine their protein content, cells were incubated with 1% (v/v) Triton X-100 in PBS for 30 min on ice, scraped and centrifuged for 15 min at 16000 g. Protein concentration in the supernatant was measured using bicinchoninic acid reagent (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's instructions. Experiments were repeated three times. Within each experiment, all cell incubations were performed in duplicate. Nitrite concentration in each sample was measured in triplicate.

Analysis of eNOS activity by DAF-2 fluorescence

Intracellular NO was measured in real time using the NO-specific fluorescence probe DAF-2 DA. DAF-2 DA is able to diffuse freely across the membrane, and is hydrolysed by intracellular esterases, resulting in the formation of DAF-2. Intracellular DAF-2 reacts with the NO oxidation product N2O3, which generates the stable highly fluorescent derivative DAF-2 triazole[132, 208]. Cells were washed with Hepes buffer, incubated with 5 µM DAF-2 DA in Hepes buffer for 30 min at room temperature, washed again with Hepes buffer and then incubated with Hepes buffer for 30 min at 37° C in the absence or presence of 1 mM L-NAME, after which calcium ionophore A23187, acetylcholine or Hepes buffer was added to the wells. Fluorescence (emission wavelength, 485 nm; excitation wavelength, 538 nm) was measured temporally at 37° C from 10 to 70 min after the addition of A23187, acetylcholine or Hepes buffer using the bottom-reading mode in a fluorescence microtitre plate reader (type Fluoroskan Ascent; Labsystems, Helsinki, Finland). eNOS activity was expressed as the L-NAME-dependent increase in fluorescence in 60 min · µg of cellular protein^{-1}. To determine the cellular protein content, parallel cell cultures were grown in 60mm dishes, lysed in 1% (v/v) Triton X-100 and analysed for protein content as described above. DAF-2 DA experiments were repeated three times. Within each experiment, four wells were used for every NO measurement.

Western blotting
Aliquots of bEnd.3 and BAEC lysates were subjected to SDS/PAGE and transferred to PVDF membranes. Blots were incubated with anti-eNOS and anti-(caveolin-1) antibodies, and subsequently with horseradish-peroxidase-conjugated anti-mouse antibody. Membrane-bound antibodies were visualized using chemiluminescence reagent (Roche, Mannheim, Germany).

Results

To study the localization of eNOS at endothelial junctions, the murine microvascular endothelial cell line bEnd.3 was used[128]. To characterize this cell line in regard to eNOS activity, bEnd.3 cells were incubated with various eNOS agonists. eNOS activity was determined by measuring the L-NAME-dependent release of nitrite into the cell medium (Table 1). Calcium ionophore A23187 was used as a positive control. This agonist clearly activated eNOS in bEnd.3 cells. Acetylcholine, 5-hydroxytryptamine (serotonin), and, to a lesser extent, VEGF, also induced eNOS activation. Bradykinin and histamine had no effect.

Agonist	Nitrite (nmol·mg of protein^{-1})		
	- L-NAME	+ L-NAME	D
None	0.31 ± 0.05	0.08 ± 0.05	0.23 ± 0.07
A23187 (5 µM)	1.44 ± 0.06	0.35 ± 0.06*	1.09 ± 0.08†
Acetylcholine (1 µM)	0.83 ± 0.12	0.06 ± 0.09	0.77 ± 0.15†
5-Hydroxytryptamine (1 µM)	0.94 ± 0.07	0.05 ± 0.05	0.88 ± 0.08†
VEGF (20 ng/ml)	0.60 ± 0.03	0.10 ± 0.03	0.50 ± 0.05†
Bradykinin (1 µM)	0.28 ± 0.06	0.04 ± 0.05	0.24 ± 0.07
Histamine (10 µM)	0.36 ± 0.04	0.08 ± 0.05	0.28 ± 0.06

* A23187 autofluorescence.

† Statistically significant effect compared with basal nitrite formation, as analysed by Student's t test ($P < 0.05$).

Table 1 eNOS activation in microvascular endothelial bEnd.3 cells.
Cells were incubated for 1 h at 37° C with or without agonist in the absence or presence of 1mM l-NAME. The amount of nitrite in the medium was determined and normalized for the amount of cellular protein. Data from three experiments are presented as mean ± S.D. 'D' represents L-NAME-dependent (i.e. NOS-mediated) nitrite formation.

Endothelial nitric oxide synthase activity is linked to its presence at cell-cell contacts

The presence of eNOS at cell-cell contacts was studied by indirect immunofluorescence microscopy. Confluent and subconfluent bEnd.3 cells were immunolabelled with anti-eNOS and anti-(caveolin-1) antibodies and subjected to confocal laser scanning analysis (figure 1a). In 'subconfluent' cells (i.e. cells having a limited number of cell-cell contacts), eNOS was present at a perinuclear region, determined previously as being the Golgi complex[24]. eNOS was hardly detectable at the plasma membrane. In contrast, caveolin-1 was mostly present at the plasma membrane, presumably at caveolae. Caveolin-1 staining was similar in confluent cells: it still remained at the plasma membrane. eNOS staining was clearly different in these cells compared with subconfluent cells. Its localization in the Golgi complex was unaffected, but now eNOS was also clearly visible at the plasma membrane, where it co-localized to a large extent with caveolin-1. Such an effect of cell confluence on eNOS localization was also found in primary BAEC (results not shown). To determine whether the cell-cell contact induced a general redistribution of eNOS towards the plasma membrane, or whether eNOS was specifically enriched at these cell-cell contacts, confluent bEnd.3 cells were immunolabelled with eNOS and PECAM-1 antibodies, and a top-to-bottom confocal scanning analysis was performed (figure 1b). At the plasma membrane, PECAM-1 and eNOS overlapped to a large extent and were specifically enriched at the contact sites. In contrast with eNOS, PECAM-1 was not found at the Golgi complex.

Since eNOS was enriched at cell-cell contacts, it was likely that eNOS was present at adherens junctions. Therefore we determined by immunofluorescence microscopy whether eNOS co-localized with VE-cadherin in early confluent as well as in late confluent cell layers (figure 2a and 2b). Surprisingly, eNOS and VE-cadherin co-localized at the plasma membrane in late confluent cells, but not in early confluent cells. Because plakoglobin associates with adherens junctions at a later stage of junction maturation than VE-cadherin[209], the overlap in the localization of eNOS and plakoglobin was studied (figures 2c and 2d). As for VE-cadherin, plakoglobin co-localized with eNOS at the plasma membrane in late confluent cell layers, but not in early confluent cells, indicating that eNOS is not present in adherens junctions. Therefore the localization of eNOS at cell-cell contacts was compared with that of PECAM-1, which is known to be present at contact sites, but not at adherens junctions (fgures 2e and 2f). As for eNOS, PECAM-1 localized at the contact sites of late confluent cells only. PECAM-1 co-localized with eNOS at these sites to a large extent.

Contact sites are known to be enriched in proteins that anchor the actin cytoskeleton to these sites, making them insoluble in detergents such as Triton X-100. VE-cadherin and, to a lesser extent, PECAM-1 are indeed partially insoluble in Triton X-100[196]. We then determined whether the eNOS that is present at the contact sites is insoluble in Triton X-100. Immunofluorescence microscopy was performed on cells that were extracted with

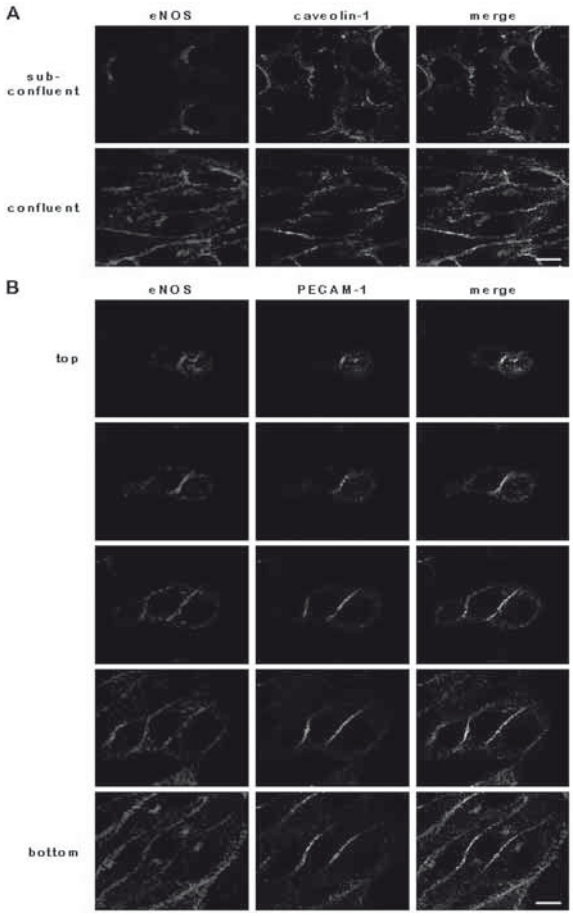

Figure 1 Localisation of eNOS at cell-cell contacts.
(A) Subconfluent and confluent bEnd.3 cells were fixed, permeabilised and immunolabelled with monoclonal anti-eNOS and polyclonal anti-(caveolin-1) antibodies, and with Texas Red-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit antibodies. Fluorescent secondary antibodies were visualized by confocal microscopy. The bar represents 10 µm. (B) Confluent bEnd.3 cells were fixed, permeabilised and immunolabelled with monoclonal anti-eNOS and polyclonal anti-(PECAM-1) antibodies, and with TRITC-conjugated donkey anti-mouse and FITC-conjugated donkey anti-goat antibodies. Labelled cells were subjected to confocal microscopy. Images were collected from the top to the bottom of the cells. The bar represents 10 µm. A colour version of this figure can be found in [36].

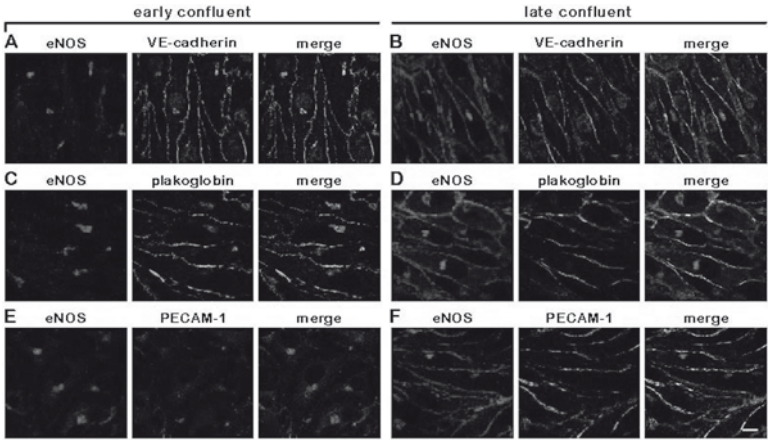

Figure 2 Co-localisation of eNOS with junction proteins at the plasma membrane.
Early (A, C and E) and late (B, D and F) confluent bEnd.3 monolayers were fixed, permeabilised and immunolabelled with monoclonal anti-eNOS (A-F) and polyclonal anti-(VE-cadherin) (A and B), anti-plakoglobin (C and D) and anti-(PECAM-1) (E and F) antibodies, and subsequently with Texas Red-conjugated donkey anti-mouse and FITC-conjugated donkey anti-goat antibodies. Fluorescent secondary antibodies were visualized by confocal microscopy. The bar represents 10 µm. A colour version of this figure can be found in [36].

Triton X-100 before fixation (figure 3). eNOS, caveolin-1, as well as PECAM-1 were present in Triton X-100-insoluble membrane patches at the plasma membrane (figures 3a-3d). Caveolin-1 is Triton X-100-insoluble because of the detergent insolubility of caveolae. In Triton X-100-treated cells, eNOS co-localized to a small extent with caveolin-1, and to a larger extent with PECAM-1. The use of a monoclonal antibody that specifically recognizes caveolin-1 at the Golgi enabled us to show that the caveolin-1 that is present at caveolae is detergent-insoluble, whereas that which is present at the Golgi complex is Triton X-100-soluble (figures 3e and 3f). The loss of Golgi-associated eNOS and caveolin-1 upon Triton X-100 treatment might be explained by a complete wash-out of the Golgi complex by the detergent. Therefore detergent-treated cells were double-labelled for the Golgi 'SNARE' (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) GOS-28 and either eNOS or GM130 (figures 3g-3j). While labelling of eNOS at the Golgi complex was lost upon Triton X-100 treatment, the labelling of GOS-28 as well as GM130 remained, indicating that eNOS and caveolin-1 at the Golgi were specifically solubilised by the detergent.
So far, our data have indicated that eNOS is present and enriched at cell-cell contacts, where it co-localizes with PECAM-1 in detergent-insoluble membrane domains that are different from adherens junctions. In contrast, in endothelial cells that do not make contact with neighbouring cells, eNOS is hardly found at the plasma membrane. To determine whether the presence of eNOS at these contact sites is involved in agonist-

induced NO generation, confluent as well as 'sparse' bEnd.3 cells (i.e. cells having no cell-cell contacts) were incubated in the absence or presence of A23187 or acetylcholine. eNOS activity was assessed by measuring the L-NAME-dependent release of nitrite in the medium (figure 4a). Nitrite accumulation was normalized for cellular protein content. Basal and stimulated eNOS activity was present in confluent cells, but hardly in sparse cells. Western blots showed that this difference in eNOS activity was not due to a difference in eNOS or caveolin-1 expression. Since these results are in contrast with data obtained by other research groups[210-212], we also evaluated eNOS activity in cell cultures of varying densities by means of DAF-2 fluorescence (figure 4b). bEnd.3

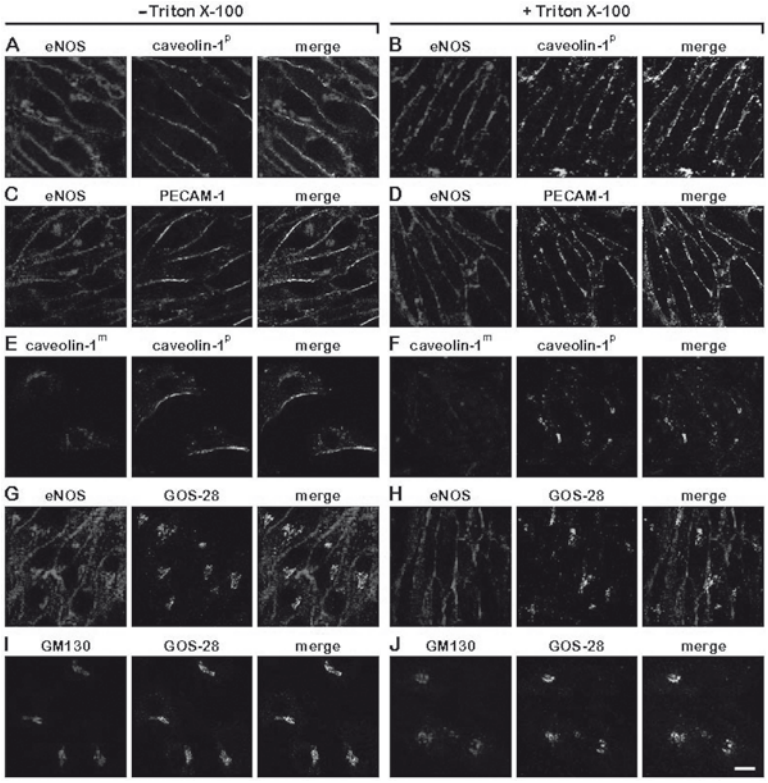

Figure 3 Effect of Triton X-100 extraction on eNOS localization.
bEnd.3 cells were either fixed directly or incubated with 0.2% Triton X-100 in PBS for 10 min at 4° C before fixation, as indicated. Cells were labelled with the indicated antibodies and with fluorescent secondary antibodies, and analysed by confocal microscopy. Caveolin-1p is polyclonal anti-(caveolin-1) antibody, which recognizes caveolin-1 in caveolae; caveolin-1m is monoclonal anti-(caveolin-1) antibody, which recognizes caveolin-1 in the Golgi complex. The bar represents 10 μm. A colour version of this figure can be found in [36].

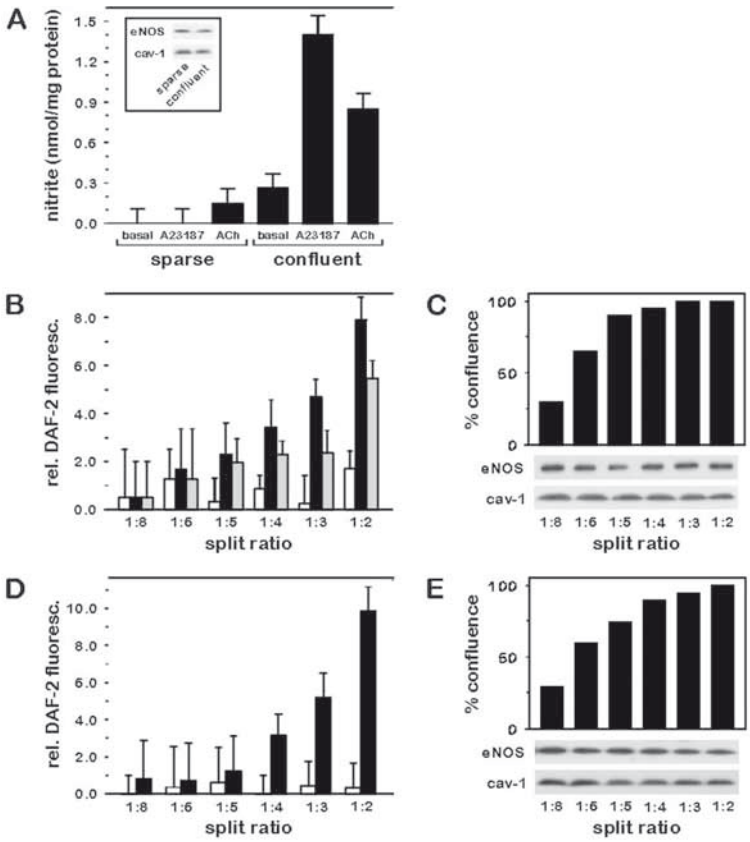

Figure 4. Influence of cell confluence on eNOS activity.
(A) Sparse and confluent bEnd.3 cells were incubated for 1 h at 37° C in the absence (basal) or presence of 5 µM A23187 (A23187) or 1 µM acetylcholine (ACh). The amount of nitrite in the medium was determined, and NOS activity was assessed as the amount of L-NAME-dependent release of nitrite in the medium · mg of cellular protein^{-1}. Lysates were prepared from both cell cultures, and from these lysates 3.5 µg of protein was subjected to SDS/PAGE, transferred to PVDF and analysed using the antibodies indicated in the inset. (B-E) bEnd.3 cells (B) and BAEC (D) were treated with trypsin, split in ratios from 1:8 to 1:2 (resulting in initial confluencies ranging from 12.5-50%), cultured for 48 h, loaded with DAF-2 DA and incubated without agonist (white bars) or with 2.5 µM A23187 (black bars) or 1 µM acetylcholine (grey bars). eNOS activity was assessed as the L-NAME-dependent increase in fluorescence · µg cellular protein^{-1}. Lysates were prepared from bEnd.3 (C) and BAEC (E) cultures, and from these lysates 4.0 µg (bEnd.3) and 2.4 µg (BAEC) of protein was subjected to SDS/PAGE, transferred to PVDF and analysed using the relevant antibodies. Confluence of bEnd.3 (C) and BAEC (E) cultures was estimated by light microscopy.

cells were treated with trypsin and split 1:8, 1:6, 1:5, 1:4, 1:3 and 1:2. After 2 days, cells were loaded with DAF-2 DA and incubated with or without calcium ionophore A23187 or acetylcholine. NO generation was monitored by the increase in fluorescence. eNOS activity was expressed as L-NAME-dependent DAF-2 fluorescence · µg of cellular protein^{-1}. Both A23187- and acetylcholine-induced eNOS activity were markedly reduced in cells that had not yet reached confluence. It is noteworthy that the decrease in eNOS activity was most dramatic in early confluent bEnd.3 cells (split 1:3) compared with late confluent cells (split 1:2). The effect of cell confluence on basal eNOS activity was less clear in these experiments. The reduced eNOS activity in subconfluent cells was not caused by differences in either eNOS or caveolin-1 expression (figure 4c). To ascertain that the effect of cell confluence on eNOS activity was not specific for bEnd.3 cells, similar experiments were performed with BAEC. Since BAEC did not respond to acetylcholine, only A23187 was used to stimulate eNOS activity. As for the bEnd.3 cells, eNOS activation was markedly reduced in subconfluent BAEC (figure 4d), while neither eNOS nor caveolin-1 expression was affected by the differences in cell density (figure 4e), thereby implying a general endothelial regulatory mechanism in which eNOS is directed towards the inter-endothelial contact sites, where it might be activated by agonists.

Discussion

NO plays an important role in maintaining inter-endothelial junctions. By regulating these junctions, NO determines the relative permeability of the endothelial lining of the vessel wall[204, 213]. In the present study, we have shown that most of the eNOS that is localized at the plasma membrane is present at cell-cell contacts. At these contact sites, eNOS is probably not localized at adherens junctions, but at membrane domains which also contain PECAM-1, and which are at least in part insoluble in solutions containing low concentrations of the detergent Triton X-100. Furthermore, our data indicate that, in subconfluent proliferating cells that do not exhibit cell-cell contacts, eNOS is hardly activated by agonists, while in confluent quiescent cell layers eNOS is activated to a large extent by calcium-mobilizing agents. In conclusion, our data show a clear correlation between eNOS activity and its presence at cell-cell contacts, indicating that eNOS needs to be present at cell-cell contacts to become activated.

To date, eNOS localization studies have focused mainly on the presence of eNOS at the Golgi[24] and at cell-surface caveolae[214, 215]. In addition, it has been reported that eNOS might be present at intercellular contact sites[216]. Nevertheless, it was proposed that the apparent presence of eNOS at these sites could have been a visual artefact, and that the presence of eNOS at the plasma membrane was accentuated by the close proximity of the membranes at these spots. Our data exclude such an explanation, since we found

a high concentration of eNOS at these sites and virtually no eNOS at the remaining part of the plasma membrane (figure 1). The presence of eNOS at these contact sites in detergent-insoluble membrane domains, also called rafts or detergent-insoluble glycolipid-enriched complexes (DIGs), might implicate a link between eNOS and the actin cytoskeleton[217,218]. Involvement of the actin cytoskeleton in regulation of eNOS has already been suggested[219]. The detergent insolubility of eNOS at the contact sites closely resembles that of the junctional proteins VE-cadherin and PECAM-1[196], indicating that eNOS is indeed part of a specific membrane subdomain at the cell-cell contacts. In addition, it is possible that eNOS at the contact sites is localized in caveolae, since caveolae are also Triton X-100-insoluble[220]. This might be reflected by the localization of caveolin-1 at the Triton X-100-insoluble lateral membranes of adherent cells (figure 3). Previously, eNOS was found to be present in detergent-insoluble membranes, especially after bradykinin treatment[221]. However, we have not identified any change in solubility of eNOS in Triton X-100 upon incubation of the cells with agonists.

Our data show that, in both microvascular and macrovascular endothelial cells, eNOS and caveolin-1 expression are not affected by changes in cell confluence. In contrast with our results, Arnal and co-workers[210] have shown that, in BAEC, basal and calcium-ionophore-induced NO production is highest in subconfluent cells and falls rapidly when cells become confluent. This was accompanied by decreases in the amount of eNOS mRNA and protein. Zöllner and co-workers[222] also identified similar results for BAEC. In our experiments, BAEC, as well as microvascular endothelial cells, produced the highest amount of NO at a late confluent state. This apparent controversy might be explained by the different methods with which these experiments were performed. After treatment with trypsin, Arnal's and Zöllner's groups split the cells in an equal ratio for all experimental conditions, and assayed the cells in consecutive days. We have split the cells in different ratios, cultured them for 3 days and performed all cell assays on the same day, so that changes in eNOS activity could not be attributed to differences in time of cell culture after trypsin treatment of the cells, but only to differences in cell confluence. There has been a report on endothelial cells that also shows an increase in eNOS activity in confluent cells compared with subconfluent cells[223]; this increase was restricted to pulmonary endothelial cells. Furthermore, it was accompanied by a huge increase in eNOS protein levels, which is in contrast with our findings, since we have detected an increase in eNOS activity without any change in eNOS protein levels.

In conclusion, our experimental findings may be different from those of other groups because of slight differences in cell-culture methods, experimental conditions or the procedures by which the cells were isolated. In addition, the expression and regulation of eNOS have been suggested to depend on the vascular bed from which the cells are derived[224-226]. This could also account for the contrasting data. Nevertheless, the link between eNOS localization at cell-cell contacts and eNOS activity in both aortic macrovascular and brain microvascular endothelial cells suggests that our findings

represent a general mechanism for eNOS regulation in endothelial cells. Previously, it has been shown that eNOS activity is regulated by phosphorylation[29, 227]. Western blot analysis of eNOS using a phospho-eNOS-specific antibody demonstrated that, in bEnd.3 cells and in BAEC, eNOS phosphorylation is not changed when cells reach confluence (results not shown), which implies that eNOS phosphorylation is not the key mediator in the linkage between eNOS activity and cell confluence.

Apparently, eNOS needs to be localized at cell-cell contacts in order to become activated. Why should eNOS be activated at cell-cell contacts? One of the functions of NO within the vessel wall is to regulate vascular permeability. The eNOS agonists VEGF, histamine, ionomycin and ATP increase vascular permeability in an NO-dependent manner[228-230]. On the other hand, NO is also required for maintaining the integrity of the endothelium (i.e. decreasing permeability)[213, 231]. This paradox may be explained either by the different conditions in which NO-dependent endothelial permeability was studied or by differences in NO levels. Moderate NO concentrations may decrease permeability, whereas low (in the presence of NOS inhibitor) or high (in the presence of eNOS agonist) levels of NO may increase permeability[232, 233]. Interestingly, the role of NO in endothelial permeability has been linked with NO-mediated changes in the cytoskeleton[213, 231]. This implies a functional role for the eNOS that is present in the Triton X-100-insoluble, cytoskeleton-associated intercellular junctions. In this regard, it is noteworthy that, in brain capillary endothelial cells, NO induces phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a protein present at cell-cell contacts[234]. This might provide a mechanism by which eNOS locally regulates intercellular junctions.

In summary, NO plays an important role in the dynamic regulation of the intercellular junctions of the endothelium. We have shown that eNOS is enriched at these junctions, which is a prerequisite for its activation by agonists. At the junctions, eNOS co-localizes with PECAM-1, but not with VE-cadherin and plakoglobin. The nature of the molecular mechanisms that lead to the enrichment of eNOS at intercellular junctions, and which allow these junctions to be regulated by NO, remains to be determined.

Aknowledgements

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CHAPTER 5

Tetrahydrobiopterin, but not L-arginine, decreases NO synthase uncoupling in cells expressing high levels of endothelial NO synthase

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Abstract

Endothelial NO synthase (eNOS) produces superoxide when depleted of (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4) and L-arginine by uncoupling the electron flow from NO production. High expression of eNOS has been reported to have beneficial effects in atherosclerotic arteries after relatively short periods of time. However, sustained high expression of eNOS may have disadvantageous vascular effects because of uncoupling. We investigated NO and reactive oxygen species (ROS) production in a microvascular endothelial cell line (bEnd.3) with sustained high eNOS expression and absent inducible NOS and neuronal NOS expression using 4,5-diaminofluorescein diacetate and diace tyldichlorofluorescein as probes, respectively. Unstimulated cells produced both NO and ROS. After stimulation with vascular endothelial growth factor (VEGF), NO and ROS production increased. VEGF-induced ROS production was even further increased by the addition of extra L-arginine. Nω-nitro-L-arginine methyl ester decreased ROS production. These findings strongly suggest that eNOS is a source of ROS in these cells. Although BH4 levels were increased as compared with another endothelial cell line, eNOS levels were >2 orders of magnitude higher. The addition of BH4 resulted in increased NO production and decreased generation of ROS, indicating that bEnd.3 cells produce ROS through eNOS uncoupling because of relative BH4 deficiency. Nevertheless, eNOS-dependent ROS production was not completely abolished by the addition of BH4, suggesting intrinsic superoxide production by eNOS. This study indicates that potentially beneficial sustained increases in eNOS expression and activity could lead to eNOS uncoupling and superoxide production as a consequence. Therefore, sustained increases of eNOS or VEGF activity should be accompanied by concomitant supplementation of BH4.

Introduction

In the vasculature, nitric oxide (NO) is generated by endothelial NO synthase (eNOS), where it regulates vascular tone (reviewed in reference [4]) and affects endothelial transcription[235]. Reactive oxygen species (ROS) play a role in signal transduction and are involved in the regulation of the biologically effective concentration of NO[6]. In vascular disease states, excessive production of ROS may overwhelm the antioxidant defense mechanisms of cells, resulting in oxidative stress[7]. Interestingly, eNOS itself can produce superoxide, a process referred to as 'eNOS uncoupling'[236]. Reduced levels of BH4 or L-arginine lead to uncoupling of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidation and NO synthesis, with oxygen as terminal electron acceptor instead of L-arginine, resulting in the generation of superoxide ($O_2^{\cdot-}$) by eNOS[4, 22, 23].

The antiatherogenic actions of NO on the vessel wall suggest that increasing eNOS expression may inhibit the development of atherosclerosis. Indeed, short-term studies on upregulation of eNOS by gene transfer of recombinant eNOS demonstrated beneficial effects in atherosclerotic arteries[237-239]. However, longer-term high expression of eNOS may not be as beneficial. In prehypertensive spontaneously hypertensive rats (SHR), increased eNOS expression has been observed[240], but this was associated with decreased NO release and increased superoxide production in aortic tissue, suggesting the presence of eNOS uncoupling, which may contribute to the development of hypertension and its vascular complications in the SHR[66]. Moreover, studies in apolipoprotein (apoE)-deficient mice showed that chronic overexpression of eNOS accelerated atherosclerosis, which was associated with lower NO production relative to eNOS expression and enhanced superoxide production in the endothelium[241]. Supplementation of BH4 in these mice reduced atherosclerotic lesion size, suggesting that in these hypercholesterolemic mice, reduced BH4 availability is involved in eNOS dysfunction during chronic eNOS overexpression.

We hypothesized that under conditions of sustained high expression of eNOS, acute stimulation of eNOS induces uncoupling of the eNOS enzyme because of a relative shortage of substrate and/or cofactors with superoxide production as a consequence. bEnd.3 cells[128] chronically express high levels of eNOS protein and produce large amounts of NO in comparison with primary endothelial cells [eg, human umbilical vein endothelial cells (HUVECs)] or a human microvascular endothelial cell line (CDC. HMEC-1) while retaining the functional properties of endothelial cells. Use of bEnd.3 cells facilitates the detection of subtle differences in NO production as a consequence of treatment with agonists or antagonists. In this study, we addressed the following questions: (1) Does eNOS uncoupling occur in bEnd.3 cells?; (2) Does stimulation of bEnd.3 cells with vascular endothelial growth factor (VEGF) enhance eNOS uncoupling?; and (3) If this uncoupling occurs, is the uncoupling of eNOS because of a shortage of L-arginine, BH4, or both?

Materials and methods

Materials

All of the drugs were purchased from Sigma, except recombinant human VEGF165 (Peprotech), diethylenetriamine NONOate (DETA/NO; Cayman Chemical), H_2O_2 (Merck) and BH4 (Schircks Laboratories).

Cell culture

An immortalized bEnd.3 cell line[128] was generously provided by Dr. Alan Schwartz

(University of Washington, St Louis, MO). Cells were cultured in DMEM supplemented with 10% FCS and penicillin/streptomycin (Life Technologies).

HUVECs were harvested from freshly obtained umbilical cords by use of the method described by Jaffe et al[242] The cells were cultured in fibronectin-coated T-flasks with EBM-2 (Bio-Whittaker) supplemented with 0.4% human fibroblast growth factor B, 0.1% human endothelial growth factor, 0.1% ascorbic acid, 0.1% gentamicin sulfate-amphotericin-B, 0.1% VEGF, 0.1% recombinant long R insulin-like growth factor, 0.1% heparin, 0.04% hydrocortisone (all supplements from Bio-Whittaker), 2% FCS (Life Technologies), 100 IU/mL penicillin, and 100 IU/mL streptomycin.

A human microvascular endothelial cell line (CDC.HMEC-1)[243] was generously provided by Dr Edwin Ades, Francisco J. Candal (Centers for Disease Control and Prevention/National Center for infectious Diseases, Atlanta, GA), and Dr Thomas Lawley (Emory University, Atlanta, GA). CDC.HMEC-1 were cultured at 37° C in humidified 95% air-5% CO_2 in MCDB 131 (Life Technologies) supplemented with 10% FCS, 10 ng/mL human endothelial growth factor, 0.05 µM hydrocortisone, 10 mM glutamine, 100 IU/mL penicillin, and 100 IU/mL streptomycin.

Measurement of NO - DAF-2 DA assay

To measure intracellular NO production, the cell-permeable fluorescent NO indicator 4,5-diaminofluorescein diacetate (DAF-2 DA; Calbiochem) was used. bEnd.3 cells were grown to confluence in a black clear-bottom 96-well plate and serum deprived for 16 hours in DMEM containing penicillin/streptomycin and 0.1% BSA. BH4, tetrahydroneopterin (NH4), and apocynin were incubated for 16 hours during starvation of the cells. All of the solutions were prepared in Tris buffer [200 mM Tris-HCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 1.33 mM NaCl, 65 mM KCl, 1% (w/v) D-glucose, 0.1% (w/v) BSA, and 50 µM L-arginine (pH 7.4)]. Cells were washed and incubated with 5 µM DAF-2 DA for 40 minutes at room temperature in the dark. After incubation, cells were washed twice and incubated for 20 minutes at 37° C in the presence or absence of inhibitors or scavengers. VEGF was added, after which fluorescence was measured every 2 minutes for 70 minutes (excitation wavelength, 485 nm; emission wavelength, 538 nm; Fluoroskan Ascent, Labsystems).

To determine the reactivity of DAF-2 DA toward NO, DAF-loaded bEnd.3 cells were exposed to the exogenous NO donor DETA/NO (1 to 10 µM), and fluorescence was measured. At the highest concentrations of DETA/NO, the cells were still viable as determined by Trypan blue staining (data not shown). The fluorescent signal increased linearly over time (figure 1a). DETA/NO concentration dependently increased the DAF signal as compared with basal NO production in bEnd.3 cells (figure 1b), demonstrating time- and concentration-dependent linearity for the DAF assay of NO production.

Treating bEnd.3 cells with H_2O_2 (1-10 µM) increased fluorescence (figure 1c), however, Nω-nitro-L-arginine methyl ester (L-NAME) attenuated the increase in DAF signal, implying H_2O_2-induced eNOS activation, rather than direct oxidation of DAF-2 by H_2O_2.

Measurement of ROS - CM-H_2DCFDA assay

Intracellular ROS were measured using diacetyldichlorofluorescein (CM-H_2DCFDA; Molecular Probes), a nonfluorescent cell-permeable indicator for ROS. Cells were seeded in a clear 96-wells plate and treated as in the DAF-2 DA assay. All solutions were made in phosphate buffered saline with additions (1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% (w/v) D-glucose, 50 µM L-arginine). Cells were washed and incubated with 10 µM CM-H_2DCFDA for 30 min at 37° C in the dark. Interventions and measurements were done in a fashion comparable to the DAF-2 DA assay.

Reactivity of CM-H_2DCF towards H_2O_2 was determined by exposing bEnd.3 cells to 1-10µM H_2O_2. H_2O_2 induced time- and concentration-dependent increase in the CM-H_2DCF assay (figure 1d). Treating bEnd.3 cells with DETA/NO (0.1-10 µM) did not affect the DCF signal (figure 1e), indicating that DCFDA is not reactive towards NO.

Measurement of biopterin levels in cell lysates

Biopterin levels in lysates of bEnd.3 cells or CDC.HMEC-1 were determined as described previously[244]. Briefly, cell pellets from T-flasks were lysed in cold extract buffer [50 mM Tris-HCl, 1 mM DTT, 1 mM EDTA, and 0.4 µM 6,7-dimethylpterine (pH 7.4)]. Protein concentration was measured using the Pierce BCA protein assay. The whole procedure was performed in the dark. Proteins were removed by adding 10 µL of a 1:1 mixture of 1.5 M HClO4 and 2 M H3PO4 to 90 µL of extracts followed by centrifugation. Total biopterins [BH4, 7,8-dihydro-L-biopterin (BH2), and biopterin] were determined by acid oxidation. Therefore, 10 µL of 1% iodine in 2% KI solution was added to 90 µL protein-free supernatant. BH2 and biopterin were determined by alkali oxidation by adding 10 µL of 1 M NaOH to 80 µL of extract followed by 10 µL of iodine/KI solution. Samples were incubated at room temperature for 1 hour. Alkaline-oxidation samples were acidified with 20 µL of 1 M H3PO4. Iodine was reduced by adding 5 µL of fresh ascorbic acid (20 mg/mL).

Pterines were measured by high-performance liquid chromatography (HPLC) on a Waters 600E HPLC (Etten-Leur). The HPLC was equipped with a Pontisil ODS 10 µm column (Alltech Associates Inc). A linear gradient was used for elution of the pterines [80% A [50% MeOH in H_2O (v/v)]] and 20% B (H_2O) in 10 minutes. After 10 minutes the column was washed with H_2O for 20 minutes. Fluorescence detection (360 nm excitation and 435 nm emission) was performed using a Waters 2475 Multi Lambda Fluorescence Detector. BH4 concentration, expressed as picomoles per milligram of protein, was calculated by subtracting BH2 + biopterin from total biopterins.

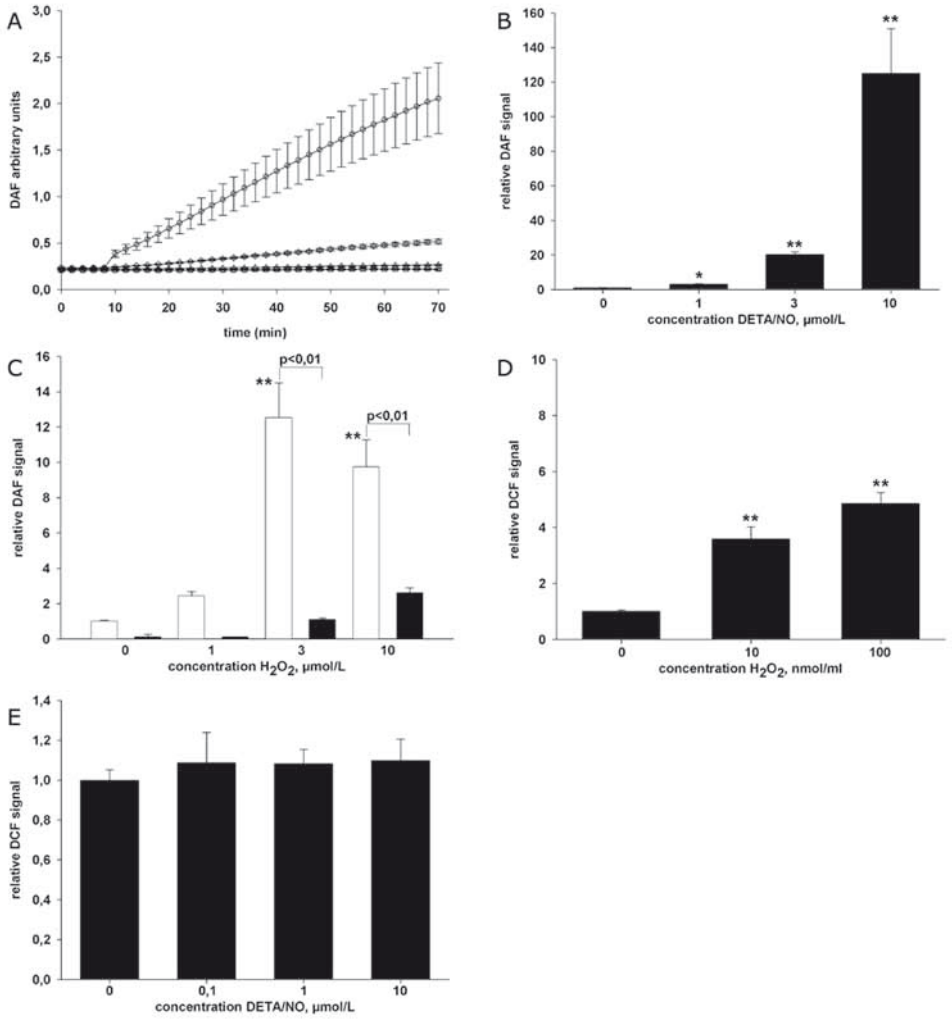

Figure 1. Effect of NO and H_2O_2 on DAF and DCF.
bEnd.3 cells were loaded with DAF-2 DA or CM-H_2DCFDA and exposed to DETA/NO or H_2O_2. (A) Time-dependent effects of DETA/NO on the DAF signal. □, 0 µM DETA/NO; △, 1 µM DETA/NO; ◊, 3 µM DETA/NO; ○, 10 µM DETA/NO. (B) Concentration-dependent effects of DETA/NO on the DAF signal. (C) Concentration-dependent effects of H_2O_2 on the DAF signal. □, H_2O_2; ■, H_2O_2 + L-NAME (30 µM). (D) Concentration-dependent effects of H_2O_2 on the DCF signal. (E) Effect of DETA/NO on the DCF signal. *P<0.05 vs 0 µM; **P<0.01 vs 0 µM

Protein measurements

To confirm high expression of eNOS and rule out expression of inducible NO synthase (iNOS) or neuronal NOS (nNOS), bEnd.3 cells, HUVECs, and CDC.HMEC-1 were lysed [20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100, and protease inhibitors (MiniComplete, Roche Diagnostics Corporation); pH 7.4]. Cell extracts and positive control protein supplied with antibodies were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membrane. Blots were incubated with monoclonal antibodies against eNOS, nNOS (Transduction Laboratories), and iNOS (Alexis) and subsequently with a horseradish peroxidase-conjugated rabbit anti-mouse antibody (Jackson Immunochemicals). Membrane-bound antibodies were visualized using chemiluminescence reagent (Roche).

Expression of eNOS was also determined using an ELISA. A polyclonal rabbit antibody raised against a recombinant fragment spanning residues 1 to 67 of bovine eNOS (unpublished data) was bound to microtiter plates (NUNC-Immuno Plate Maxisorb Surface, NUNC) by incubation at 4° C. Plates were washed 3 times in Tris buffer [50 mM Tris-HCl and 150 mM NaCl (pH 7.4)] with 0.1% Tween 20 and then incubated with 3% BSA in Tris buffer with 0.05% Tween 20 for 2 hours at room temperature. Samples were incubated in a total volume of 50 µL for 2 hours at room temperature. Plates were thoroughly washed 3 times and incubated for 2 hours at room temperature with 100 µL of 125 ng/mL eNOS antibody (Transduction Laboratories) in Tris buffer with 0.1% BSA and 0.05% Tween 20. Subsequently, plates were incubated with 650 µg/mL horseradish peroxidase-conjugated rabbit anti-mouse antibody (Jackson Immunochemicals) in Tris buffer with 0.1% BSA and 0.05% Tween 20 for 2 hours at room temperature. A color reaction was observed by incorporating 3,3',5,5'-tetramethyl-benzidine into the reaction, which was stopped by adding 50 µL of 2 M H_2SO_4. The optical density was measured at 450 nm in a microplate reader (Multiskan Ascent, Labsystems). The assay was validated using a dilution series of bEnd.3 cells. The interassay variability was 5 ± 2%, whereas the intraassay variability was 2.0 ± 0.8%.

Calculations and statistical analysis

NO and ROS production was calculated by determining the slopes of each line with linear regression. Results were expressed relative to control. Statistical comparisons were made by 1-way or 2-way ANOVA, as required. Subsequent post hoc testing was done with the Student-Newman-Keuls test. P values <0.05 were considered statistically significant.

Results

eNOS in bEnd.3 cells is - in part - in an uncoupled state

To identify the source of NO production in bEnd.3 cells, expression levels of eNOS, nNOS, and iNOS were determined. High expression of eNOS and the absence of iNOS or nNOS protein were confirmed by Western blot (figure 2a). Nonstimulated bEnd.3 cells produced detectable levels of NO (figure 2b). Incubation with L-NAME showed that 30 µM was the minimal concentration to inhibit NO production (data not shown). Similar results were obtained with NG-methyl-L-arginine acetate (minimal concentration, 100 µM) and Nω-nitro-L-arginine (minimal concentration 30 µM; data not shown).

Incubation of bEnd.3 cells with the DCF probe resulted in a fluorescent signal, which was decreased 18% ($P<0.05$) by the addition of L-NAME. Because CM-H_2DCFDA is not reactive toward NO (figure 1e), these data show that, other than NO production, there is constitutive eNOS-dependent formation of ROS, that is, uncoupling, in the basal state.

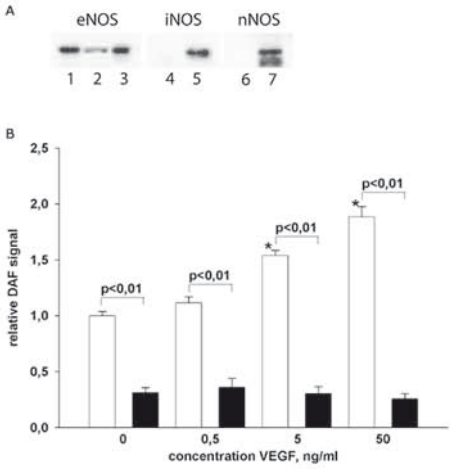

Figure 2. Effect of VEGF on eNOS-dependent NO production.

(A) eNOS, iNOS, and nNOS expression levels in bEnd.3 cells. Lanes 1, 4, 6: bEnd.3 (15 µg protein/lane); lane 2: HUVEC (150 µg protein/lane); lane 3: human aortic endothelial cell (50 µg protein/lane); lane 5: macrophage + IFN-γ/lipopolysaccharide (LPS; 15 µg protein/lane); lane 7: rat pituitary (15 µg protein/lane). (B) bEnd.3 cells loaded with DAF-2 DA were stimulated with VEGF (1 to 50 ng/mL) in the presence and absence of L-NAME. Open bars: control; closed bars: 30 µM L-NAME. *$P<0.01$ vs 0 ng/mL VEGF.

VEGF enhances eNOS-dependent NO production but also ROS production in bEnd.3 cells

VEGF induced a concentration-dependent increase in eNOS-dependent NO production (figure 2b). VEGF also significantly increased eNOS-dependent ROS production (figure 3). In VEGF-stimulated cells, the addition of L-NAME resulted in a 35% (P<0.05) decrease in the DCF signal versus an 18% (P<0.05) decrease in DCF signal in nonstimulated cells.

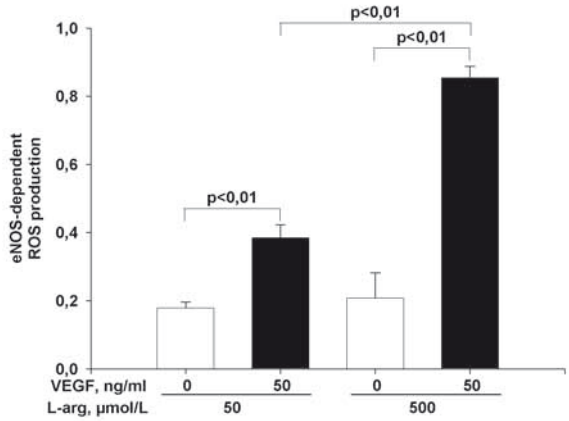

Figure 3. Effect of VEGF and L-arginine on eNOS-dependent ROS production.
bEnd.3 cells were loaded with CM-H_2DCFDA and stimulated with VEGF in the presence and absence of L-NAME. Results plotted as eNOS-dependent ROS production (difference between total ROS production and eNOS-independent ROS production).

eNOS uncoupling is because of relative shortage of BH4 but not because of shortage of L-arginine

Shortage of BH4 can lead to eNOS uncoupling. Determination of biopterin levels in bEnd.3 cells and CDC.HMEC-1 revealed that BH4 levels are >8 times increased in bEnd.3 cells as compared with CDC.HMEC-1 (figure 4a). The BH4/BH2 + biopterin ratio in bEnd.3 cells and HMECs was not significantly different. In addition, eNOS expression in bEnd.3 cells was 250-fold increased in comparison with CDC.HMEC-1 as determined with Western blot (figure 4b) and ELISA (OD450 bEnd.3 cells, 2.162 ± 0.3; CDC.HMEC-1, 0.007 ± 0.001). The addition of BH4 (1 to 10 µM) to bEnd.3 cells resulted in a significant (P<0.01) increase in NO production at 3 and 10 µM (figure 5a). A similar result was obtained with VEGF. ROS levels showed a tendency to decrease in the presence of BH4 (1 to 10 µM; figure 5b). A similar result was found in the presence of VEGF. To exclude any antioxidant effects of BH4, NH4 was used as a negative control. The addition of 10 µM NH4 had no effects on NO production and decreased ROS production in bEnd.3 cells (figure 6).

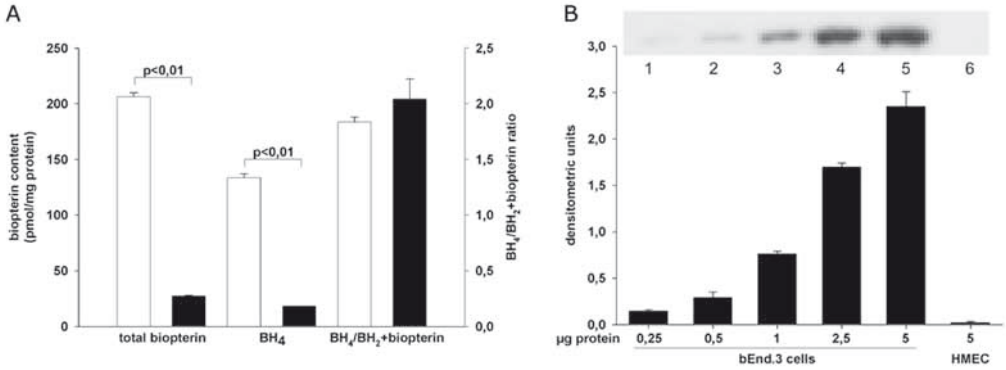

Figure 4. Biopterin and eNOS content in bEnd.3 cells and CDC.HMEC-1.
(A) BH4 concentration was calculated by subtracting BH2+biopterin from total biopterins. Open bars: bEnd.3 cells; closed bars: CDC.HMEC-1. (B) Representative Western blot and densitometric analysis of the bands (n=6) on eNOS expression in bEnd.3 cells and CDC. HMEC-1. Lanes 1 to 5: bEnd.3 cells (0.25, 0.5, 1, 2.5, and 5 µg protein/lane, respectively); lane 6: CDC.HMEC-1 (5 µg protein/lane).

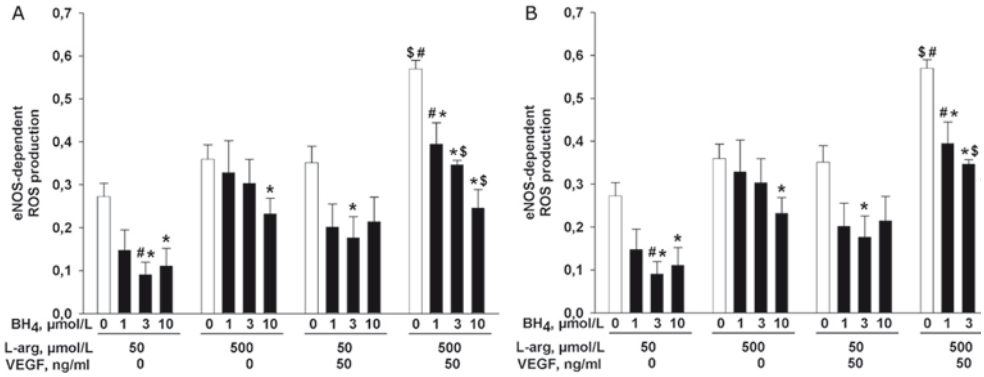

Figure 5. Effect of BH4 on NO and ROS production.
bEnd.3 cells were incubated for 16 hours with BH4. Results were plotted as eNOS-dependent NO or ROS production (as in figure 3). (A) Effect of BH4 on NO production. All VEGF-stimulated bEnd.3 cells had significantly higher NO production than nonstimulated cells. (B) Effect of BH4 on ROS production. *P<0.05 vs 0 µM BH4; #P<0.05 vs 50 µM L-arginine; $P<0.05 vs 0 ng/mL VEGF.

Because eNOS uncoupling can also be caused by a shortage of the substrate L-arginine, the effect of additional L-arginine on NO and ROS production was determined. In the presence of high L-arginine levels (500 µM), the addition of BH4 did not significantly increase basal NO production and also had no effect on VEGF-induced NO production

(figure 5a). Increasing L-arginine concentrations up to 500 µM did not affect the basal ROS production but did lead to a significant increase in eNOS-dependent ROS production in the presence of VEGF (fgure 3). In the presence of high L-arginine levels, ROS production was significantly decreased after the addition of 3 and 10 µM BH4, both in the absence and presence of VEGF (fgure 5B).

Other potential ROS sources

Diphenyleneiodonium (DPI) was used to identify flavin-containing enzymes as possible ROS sources. Incubation of bEnd.3 cells with DPI lead to a significant 20% ($P<0.05$) decrease in basal ROS production (figure 7) and a 25% ($P<0.05$) decrease in the presence of VEGF (data not shown). To determine whether NADPH oxidase, xanthine oxidase, and mitochondria are possible ROS sources, bEnd.3 cells were incubated with apocynin, oxypurinol, and thenoyltrifluoroacetone (TTFA), respective inhibitors of these oxidase systems. Apocynin and TTFA had no effect on the DCF signal (figure 7). Oxypurinol decreased ROS production slightly in the absence (figure 7) but not in the presence of VEGF (data not shown). DPI and oxypurinol were also combined with L-NAME, resulting in an additional decrease in ROS production as compared with the effect of the separate inhibitors (fgure 7).

To exclude the involvement of peroxynitrite ($ONOO^-$) in eNOS uncoupling, cells were treated with the $ONOO^-$ scavenger Ebselen. Incubation with 10 µM Ebselen did not significantly affect the DCF signal (control, 1.00 ± 0.02; 10 µM Ebselen, 1.05 ± 0.05; 50 ng/mL VEGF, 1.12 ± 0.06; 50 ng/mL VEGF +10 µM Ebselen, 0.96 ± 0.05).

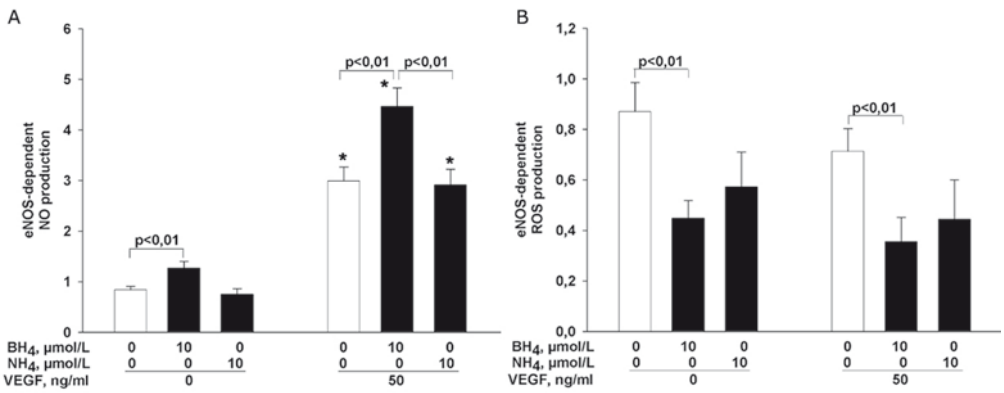

Figure 6. Effect of NH4 on NO and ROS production.

bEnd.3 cells were incubated for 16 hours with 10 µM BH4 or 10 µM NH4. Results were plotted as eNOS-dependent NO or ROS production (as in figure 3). (A) Effect of BH4 and NH4 on NO production. (B) Effect of BH4 and NH4 on ROS production. *P<0.01 vs 0 ng/mL VEGF.

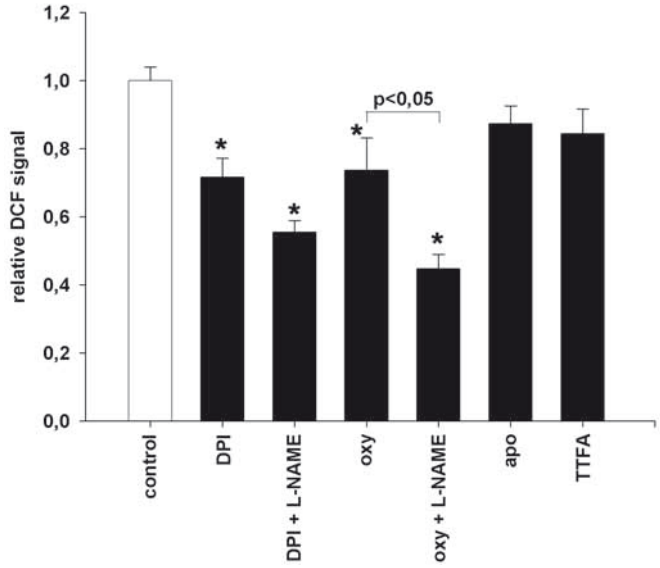

Figure 7. Sources of ROS in bEnd.3 cells.
ROS production was measured with the CM-H_2DCFDA assay in the presence or absence of 10 µM DPI, 100 µM apocynin (apo), 100 µM oxypurinol (oxy), 1 µM TTFA, or a combination of 30 µM L-NAME and 100 µM oxypurinol or 10 µM DPI. *P<0.01 vs control.

Discussion

It has been proposed that long-term elevation of eNOS expression may require adjustments in tetrahydrobiopterin metabolism[22], which could explain the controversial results between studies on prolonged[66, 241] and short-term eNOS overexpression[237-239]. The present study demonstrates that bEnd.3 cells chronically express high eNOS levels but do not express the other NOS isoforms. With respect to eNOS, bEnd.3 cells are relatively deficient in BH4 as compared with other endothelial cells. As a consequence, eNOS is partially in the uncoupled state. Stimulation of eNOS activity in these cells by VEGF enhanced ROS production, particularly at high L-arginine concentrations, which was corrected to a substantial degree by BH4. Our data suggest that under conditions of chronically increased eNOS expression and particularly if eNOS activity is stimulated, a relative shortage of BH4 may lead to eNOS uncoupling, resulting in superoxide production. Our results are in line with observations by Ozaki et al[241], who demonstrated that overexpression of eNOS in the endothelium promoted atherosclerosis in apoE-knockout mice. Dysfunctional eNOS and subsequent increased superoxide production seems to be responsible for the progression of atherosclerosis in these animals, mainly

caused by a deficiency of the cofactor BH4. In agreement with this study, Cosentino et al[66] showed that increased eNOS expression in SHR is also associated with dysfunctional eNOS, which may contribute to the development of hypertension and its vascular complications in these rats.

bEnd.3 cells are particularly useful for studying eNOS uncoupling, because NADPH oxidase, which is a major source of ROS in endothelial cells[245], does not contribute to ROS production in these cells. The combined results of this study show that the ROS production in bEnd.3 cells is eNOS dependent, implying that eNOS in bEnd.3 cells is partly in an uncoupled state.

We hypothesized that activation of the enzyme would enhance ROS production. Indeed, stimulation with VEGF increased eNOS-dependent ROS production. This is in line with studies in other endothelial cells that show an eNOS-dependent increase in ROS production after VEGF stimulation[246, 247].
We and others found a decrease in eNOS-dependent ROS production after addition of L-NAME, whereas Colavitti et al[246] reported the opposite. The latter observation is consistent with the common perspective that inhibition of functional NOS and, thus, blockage of NO production, results in the loss of the NO-scavenging effect and, thus, increased $O_2^{\cdot-}$ levels. On the other hand, inhibition of dysfunctional NOS will lead to a decrease in ROS production because of the coupling state of eNOS[65, 248-250].
eNOS dysfunction and subsequent $O_2^{\cdot-}$ production is commonly thought to result from a shortage of BH4[22]. In the present study, the addition of BH4 resulted in a significant increase in NO production in bEnd.3 cells, whereas ROS levels showed a tendency to decrease. NH4 was used as a negative control, because it exerts antioxidant effects but has no influence on eNOS uncoupling. The addition of NH4 had no effect on NO production and decreased ROS production in bEnd.3 cells. From these results, it can be concluded that the decreased ROS production in the presence of BH4 is because of a decrease in eNOS uncoupling and not caused by antioxidant effects of BH4. In line with these results, it has been shown that overexpression of GTP-cyclohydrolase I, the rate-limiting enzyme in BH4 synthesis, reduced endothelial dysfunction in apoE-knockout mice[251]. In addition, GTP-cyclohydrolase I-knockout mice, which are deficient in BH4, showed hypertension and increased vascular superoxide production. The latter was inhibited by Nω-methyl-L-arginine acetate, indicating that ROS was generated by uncoupled eNOS[248].

Other than BH4 deficiency, shortage of the substrate L-arginine could theoretically lead to uncoupling of eNOS[4]. However, there is limited evidence on the role of L-arginine in eNOS dysfunction[252, 253]. Moreover, there is no consensus on whether supplementation of L-arginine is beneficial under conditions of endothelial dysfunction. Some studies

in animals and humans show restored endothelial function[254, 255], whereas others fail to show a beneficial effect of L-arginine[256-258]. Chen et al[64] found that chronic treatment with L-arginine negated the positive effect of iNOS deficiency in apoE/iNOS double-knockout mice. Furthermore, diabetic rats showed increased ROS production after the addition of L-arginine, which was partly reduced by the addition of L-NAME and DPI and the addition of BH4[65]. Our results indicate that a shortage of L-arginine is not causing eNOS-dependent ROS production in bEnd.3 cells. In fact, increasing the L-arginine concentration enhanced VEGF-induced ROS production. There are 2 possible explanations for these findings. First, increased L-arginine levels have been associated with an increase in total biopterin levels, but unaltered BH4 levels, suggesting that L-arginine supplementation leads to oxidation of biopterin[64]. Second, increased O2·- production in the presence of L;arginine might be because of increased calmodulin binding and a resulting increased electron flow through the enzyme[122, 259], leading to a relatively augmented shortage of BH4. The mechanism through which increasing levels of L-arginine enhances ROS production by increasing L-arginine levels in bEnd.3 cells is unclear.

Although the addition of BH4 led to a reduction of ROS production in bEnd.3 cells, it did not completely abolish ROS production. These results correspond with the *in vitro* study with the purified enzyme[23], in which BH4 only partly inhibited uncoupling of the purified eNOS enzyme. We identified xanthine oxidase as an additional source of ROS in bEnd.3 cells under basal conditions. $ONOO^-$, NADPH oxidase, and mitochondria were excluded as contributors to ROS production in bEnd.3 cells.

Several reports have been published on intrinsic superoxide production by NO synthases. For nNOS, it has been reported that ROS are produced by the haem group[260]. For iNOS and eNOS, however, the source of ROS production is unclear. Some reports suggest that both the haem and the flavin domain in eNOS produce superoxide[252, 261]. In bEnd.3 cells, the addition of DPI led to a decrease in but not complete inhibition of ROS production, indicating that flavins are not involved in the intrinsic ROS production by eNOS in these cells. These data suggest that the source of the remaining eNOS-dependent ROS production in bEnd.3 cells, even in the presence of additional BH4, is probably the haem group in eNOS[23, 252, 261].

Perspectives

Although eNOS gene transfer has been proposed to be useful in the treatment of endothelial dysfunction[237-239], we now show that sustained high expression of eNOS protein results in ROS production because of uncoupling. eNOS-dependent ROS production in bEnd.3 cells is partly corrected by BH4 supplementation. These findings suggest that attempts to improve endothelial function, by sustained increases of eNOS

expression and activity, for instance by gene transfer of eNOS or VEGF, should be accompanied by concomitant increases of BH4 to prevent eNOS uncoupling.

Acknowledgements

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Chapter 6

Oleic acid increases mitochondrial ROS production and decreases eNOS activity in cultured endothelial cells

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submitted

Abstract

Elevated plasma levels of free fatty acids (FFA) are associated with increased cardiovascular risk. This may be related to FFA-induced elevation of oxidative stress in endothelial cells. We hypothesized that, in addition to mitochondrial production of reactive oxygen species (ROS), endothelial nitric oxide synthase (eNOS)-mediated ROS production contributes to oleic acid (OA)-induced oxidative stress in endothelial cells, due to eNOS uncoupling. Here, we measured ROS production and eNOS activity in cultured endothelial cells (bEnd.3) in the presence of OA bound to bovine serum albumin, using the DCF assay and the L-arginine/citrulline conversion assay, respectively. OA induced a concentration-dependent increase in ROS production, which was inhibited by the mitochondrial complex II inhibitor TTFA. OA had little effect on eNOS activity when stimulated by a calcium-ionophore, but decreased both basal and insulin-induced eNOS activity, which was restored by TTFA. Pretreatment of bEnd.3 cells with tetrahydrobiopterin (BH4) prevented OA-induced ROS production and restored inhibition of eNOS activity by OA. In conclusion, elevation of OA levels leads to both impairment in receptor-mediated stimulation of eNOS and to production of mitochondrial-derived ROS and hence endothelial dysfunction.

Introduction

Plasma free fatty acid (FFA) levels are elevated in conditions associated with insulin resistance including obesity, type 2 diabetes and hypertension. Our group[262-264] and others[265-267] have shown that FFA elevation impairs NO availability, assessed as endothelium-dependent vasodilatation, which may contribute to the accelerated atherosclerosis in patients with insulin resistance. *In vitro* it has been shown that elevated levels of FFA, in particular palmitic acid (PA) and OA, can cause oxidative stress due to increased mitochondrial uncoupling[268, 269]. During mitochondrial uncoupling, the electron transfer through the respiratory chain is blocked, which results in an increase in the voltage gradient across the mitochondrial membrane and eventually electron transfer to molecular oxygen, thereby generating superoxide ($O_2^{\cdot-}$)[85]. Another potential source of $O_2^{\cdot-}$ in the vascular wall is endothelial nitric oxide synthase (eNOS)[236]. Reduced levels of the cofactor tetrahydrobiopterin (BH4) or the substrate L-arginine lead to uncoupling of NADPH oxidation and NO synthesis, with oxygen instead of L-arginine as terminal electron acceptor, resulting in the generation of $O_2^{\cdot-}$ by eNOS, a process referred to as 'eNOS uncoupling' [4, 22, 23].

We hypothesized that, in addition to mitochondrial ROS production, eNOS-mediated ROS production contributes to oleic acid (OA)-induced oxidative stress in endothelial

cells, due to eNOS uncoupling. The uncoupling of eNOS may be caused by a relative shortage of BH4, as a consequence of BH4 oxidation by mitochondrial-derived ROS[67, 249], and may be aggravated upon stimulation of eNOS activity by insulin. We have used a microvascular endothelial cell line (bEnd.3)[128], expressing high levels of eNOS but lacking neuronal or inducible NOS[129] to assess this hypothesis. These cells produce relatively large amounts of NO, which facilitates the detection of subtle differences in NO production as a consequence of treatment with agonists or antagonists.

In order to determine the mechanisms that account for the endothelial dysfunction found in patients with increased plasma FFA levels, we used PA and OA bound to albumin, since albumin is the major carrier of fatty acids in the circulation[270]. We assessed basal and insulin-stimulated eNOS activity by measuring L-arginine conversion. The effect of BH4 supplementation on ROS production was measured and the contributions of mitochondria, NADPH oxidase and xanthine oxidase of ROS to OA-induced ROS production were determined.

Materials and methods

Materials

Culture media were obtained from Life Technologies (Burlington, ON, Canada). CM-H_2DCFDA was from Molecular Probes (Leiden, The Netherlands). BH4 was purchased from Schircks Laboratories (Iona, Switzerland) and H_2O_2 was obtained from Merck (Hohenbrunn, Germany). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture

An immortalized microvascular endothelial cell line (bEnd.3)[128] was generously provided by Dr. Alan Schwartz (University of Washington, St Louis, MO). These cells, that only contain the eNOS isoform, produce much higher levels of NO as compared to other endothelial cells such as HUVEC or HMEC[129]. bEnd.3 cells are an excellent model to study subtle differences in NO production after treatment with agonists or antagonists. Cells were cultured at 37° C in humidified 95% air-5% CO_2 in DMEM containing 1000 mg/L glucose, supplemented with 10% FCS, 2 mM glutamine, 100 IU/mL penicillin and 100 IU/mL streptomycin. Prior to each experiment, cells were starved for 16 h in DMEM containing 0.1% bovine serum albumin (BSA).

Preparation of fatty acid-complex

In humans, albumin is the major carrier of fatty acids in the circulation[270]. Normally, the fatty acid:albumin molar ratio is 0.7:1, but this ratio can be markedly raised up to

6:1 or 7:1 in disease states such as diabetes mellitus[271] or the nephrotic syndrome[272]. To investigate the effect of increased fatty acids on endothelial cells, PA and OA were bound to fatty acid free BSA in different molar ratios as previously described[273]. The solution was sterilized through a 0.45 µm filter, stored in 1 mL aliquots at -20° C and used within 3 weeks after preparation.

Protein measurements

The uptake of fatty-acid free BSA and OA-loaded BSA in confluent bEnd.3 cells was determined by rocket electrophoresis (Laurell technique) using rabbit-anti-bovine albumin (Nordic Immunological Laboratories, Tilburg, The Netherlands). Electrophoresis and development of the gel was performed as described previously[274]. A standard curve was used to convert peak heights to concentration units. The inter-assay variability was 3.4 ± 1.2%, and the intra-assay variability was 2.4 ± 1.5%.

The effect of OA on eNOS protein mass was determined as described previously[129]. The monoclonal antibody against eNOS was purchased from Transduction Laboratories and the horseradish peroxidase-conjugated rabbit anti-mouse antibody was from Jackson Immunochemicals.

Measurement of endothelial NO synthase activity - arginine-citrulline conversion assay

Cells were grown to confluency in 75-cm^2 flasks, starved for 16 h and incubated for 20 min with 0.5 mM fatty-acid free BSA or 4 mM OA-loaded BSA at 37° C. Cells were (pre-)treated with tetrahydrobiopterin (BH4; 3 µM, 16 h), calcium ionophore (A23187; 10 µM 20 min), thenoyltrifluoroacetone (TTFA; 10 µM, 40 min), insulin (human; recombinant from *Saccharomyces cerevisiae*; 300 µM (43 IU/mL), 10 min), or a combination of TTFA and insulin. After incubation, cells were washed with ice-cold PBS and homogenized in a buffer consisting of 50 mM Tris buffer, pH7.4, 320 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol (DTT), 2 mg/L aprotinin and 100 mg/L phenylmethylsulfonylfluoride. Samples were stored at -80° C for determination of NOS activity, which was determined as the conversion of L-^{3}H-arginine into L-^{3}H-citrulline. Homogenate (50 µL) was incubated in a final volume of 100 µl at 37° C for 30 min with 1 mM L-citrulline, 300 µM tetrahydrobiopterin, 3000 U/mL calmodulin, 0.5 mM NADPH, and 1 mM $CaCI_2$, all dissolved in a buffer consisting of 50 mM KH_2PO_4 containing 1 mM DTT and 10 µM L-^{3}H-arginine. Prior to incubation, L-^{14}C-citrulline was added to correct for procedural losses. Correction for nonspecific activity was made by substituting NADPH by 100 mM L-Nω-nitro-arginine methyl ester (L-NAME). The incubation was stopped by placing the tubes on ice and adding 20 mM ice-cold Hepes buffer, pH 5.5, followed by separation of arginine and citrulline on Dowex 50X8-200 ion exchanger resin and liquid scintillation counting of the citrulline fraction. All measurements were performed in triplicate. Results are expressed as pmol

per min per milligram protein. The inter-assay variability was 2.2 ± 0.8%, and the intra-assay variability was 2.2 ± 0.3%.

Measurement of ROS - CM-H_2DCFDA assay

Intracellular ROS were measured using diacetyldichlorofluorescein (CM-H_2DCFDA) as described previously[129]. Briefly, bEnd.3 cells were loaded with 10 µM CM-H_2DCFDA and loaded with several inhibitors to determine the contribution of different sources to the total OA-induced ROS production. Apocynin and BH4 were incubated for 16 h during starvation of the cells[129]. Diphenyleneiodonium (DPI), apocynin, oxypurinol, rotenone, L-NAME or TTFA were incubated for 20 min at 37° C[129]. OA was added and fluorescence was measured every 2 min during 20 min (excitation wavelength, 485 nm; emission wavelength, 538 nm; Fluoroskan Ascent; Labsystems, Helsinki, Finland).
ROS production was calculated by determining the slopes of each line by linear regression analysis and the reactivity of CM-H_2DCF towards H_2O_2 was determined by exposing bEnd.3 cells to 1-10 µM H_2O_2, as described previously[129]. The inter-assay variability was 3 ± 1%, and the intra-assay variability was 0.72 ± 0.09%.

Statistical analysis

Group values are expressed as mean ± SEM. Statistical comparisons were made by one-way or two-way ANOVA with the studentized Newman-Keuls test as posthoc test. P values <0.05 were considered to be statistically significant.

Results

Efficient uptake of BSA and OA-loaded BSA in bEnd3 cells

Addition of fatty acid free BSA and OA-loaded BSA led to efficient and not significantly different uptake of these compounds after 20 min of incubation (n=6). The uptake-efficiency was 95 ± 3% for 3 mM and 86 ± 4% for 4 mM OA-loaded BSA, respectively, as compared to fatty acid free BSA (0.5 mM; 100 ± 2%).
A physiological concentration of the vehicle (BSA) was used throughout the study (0.5 mmol/L, about 33 g/L). The concentration OA was increased to a fatty acid:albumin molar ratio of 8:1[271, 272], corresponding to 4 mmol/L OA. This concentration had no toxic effect for up to 60 min (LDH activity at 0 min: 0.14 ± 0.02 and LDH activity at 60 min: 0.12 ± 0.03). However, longer exposure times were associated with toxicity (LDH activity at 120 min: 0.05 ± 0.01 and LDH activity at 180 min: 0.01 ± 0.005).

OA-induced ROS production in bEnd.3 cells

Incubation of bEnd.3 cells with different concentrations of OA or PA lead to a dose-dependent increase in ROS production (figure 1). Since 4 mM OA resulted in a

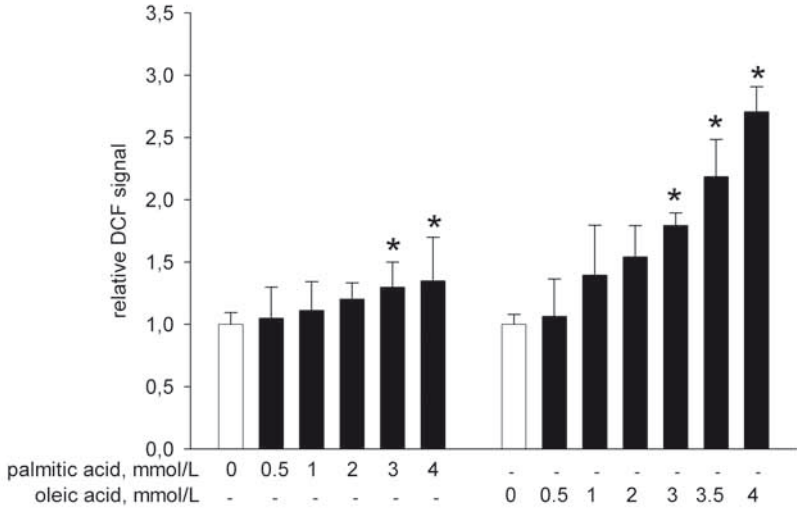

Figure 1. Dose-dependent increase in OA- and PA-induced ROS production.
Mean ± S.E.M. * P<0.05 compared to control without PA or OA, respectively. N=20

significant, almost 3-fold increase in ROS production, we used this concentration to determine the effects of OA on ROS production and eNOS activity in bEnd.3 cells.

Origin of ROS in response to OA
To determine the origin of the OA-induced ROS production in bEnd.3 cells, inhibitors of different ROS sources were used. In the presence of OA, TTFA (10 μM) significantly inhibited ROS production, which was further decreased by addition of the eNOS inhibitor L-NAME (300 μM). DPI (10 μM), an inhibitor of flavin-containing enzymes, apocynin (100 μM), a NADPH oxidase inhibitor, oxypurinol (100 μM), an inhibitor of xanthine oxidase, rotenone (3 μM), an inhibitor of complex I of the mitochondrial respiratory chain, and L-NAME alone had no significant effect (figure 2). Although the OA-induced ROS production is numerically lower in the presence of TTFA and L-NAME than with TTFA alone, this difference was not significant.

Diminished ROS production by BH4
Pre-incubation of bEnd.3 cells with 3 μM BH4 or 10 μM TTFA significantly inhibited ROS production in the presence of OA (figure 3; n=12). Combined incubation with BH4 and TTFA did not further decrease OA-induced ROS production (figure 3; n=6).

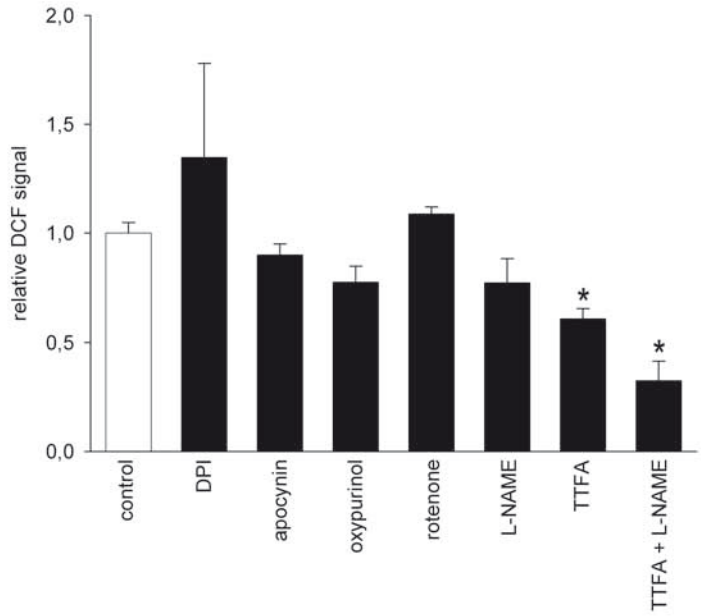

Figure 2. Mitochondria and eNOS are sources of ROS production induced by OA in bEnd.3 cells.

ROS production was measured in the presence of 4 mM OA and in the presence or absence of 10 μM DPI, 100 μM apocynin, 100 μM oxypurinol, 3 μM rotenone, 10 μM TTFA, 300 μM L-NAME, or a combination of 10 μM TTFA and 300 μM L-NAME. Mean ± S.E.M. * P<0.05 compared to control (OA only). N=16

Diminished basal and induced eNOS activity in the presence of OA

Incubation with OA had no effect on eNOS protein mass (n=12, figure 4). In the presence of OA, the remaining eNOS activity was 3% of the basal eNOS activity (figure 5). Calcium ionophore (A23187) was used to define the potential signaling defect. The relative increase of eNOS activity after incubation with A23187 was much stronger in the presence of OA than without OA (BSA only) (figure 5). Although the A23187-induced eNOS activity is numerically lower in the presence of OA as compared to control, this difference was not significant.

Addition of TTFA, a mitochondrial complex II inhibitor[275], almost completely restored eNOS activity in the presence of 4 mM OA. In the presence of 0.5 mM BSA, 300 μM insulin (figure 6a), used to determine the effect of OA on insulin-induced eNOS activity, induced an approximate twofold increase in eNOS activity, whereas in the presence of OA, the insulin-induced increase in eNOS activity was completely abolished (figure 6b). However, when insulin and TTFA were combined, the eNOS activity in the presence of OA was restored to basal levels. Incubation with BH4 restored OA-induced decrease in eNOS activity back to basal activity levels (figure 6).

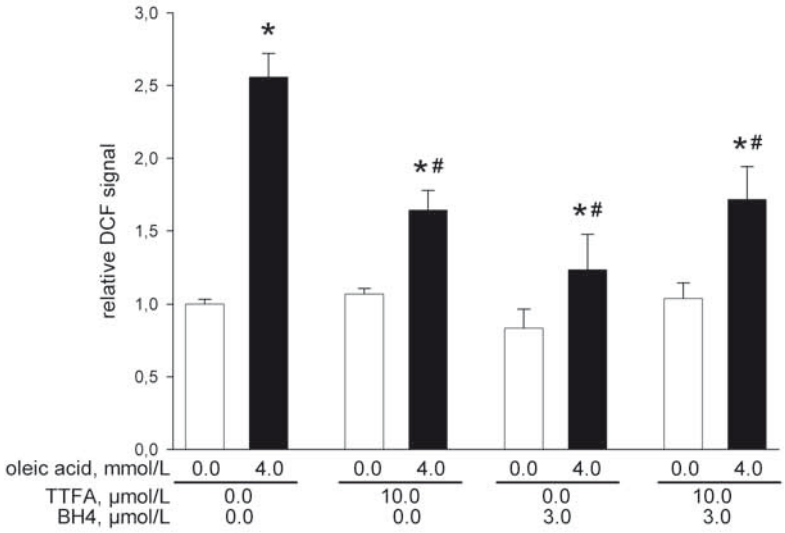

Figure 3. BH4 prevents OA-induced ROS production in bEnd.3 cells.
ROS production was measured in the presence or absence of 3 µM BH4, 10 µM TTFA, or both. Mean ± S.E.M. * P<0.05 compared to control without OA; # P<0.05 compared to 0 µM TTFA, 0 µM BH4. N=16

Discussion

Elevated plasma FFA levels have been associated with endothelial dysfunction and increased oxidative stress. We have shown previously that, in healthy volunteers, an oral fat load containing primarily OA significantly impaired endothelial function, as measured by flow-dependent vasodilatation in the fore-arm[276], a process previously shown to be dependent on nitric oxide[277]. The cellular mechanisms responsible for fatty acid-induced endothelial dysfunction have not been studied in detail, although it is evident that OA decreases NO production[278, 279] and increases the generation of ROS[268, 269]. High glucose levels and free fatty acids have been reported to increase ROS production in endothelial cells through protein kinase C-dependent activation of NADPH oxidase[93]. In addition, it was recently shown that insulin resistance reduces eNOS activity due to increased fatty acid oxidation[103]. Here we show that mitochondrial uncoupling predominantly generates OA-induced ROS. TTFA, an inhibitor of the mitochondrial respiratory chain, significantly decreases OA-induced ROS production. Incubation with L-NAME in the presence of OA resulted in a small, non-significant decrease in ROS production, indicating that uncoupling of eNOS does only to a small extent contribute to OA-induced ROS production. Oxidation of BH4 by mitochondrial-derived ROS,

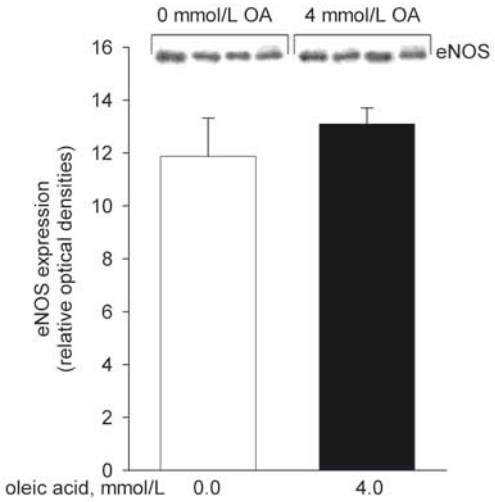

Figure 4. eNOS expression is not affected by OA in bEnd.3 cells.
Representative western blots and group data depicting eNOS protein abundance in the presence and absence of 4 mM OA.

leading to a shortage of the cofactor, is the most likely cause of partial uncoupling of eNOS. The decrease in eNOS activity is most likely caused by interference of ROS with the signalling pathway of eNOS activation.
In this study, bEnd.3 cells were used, since these cells express high levels of eNOS protein and produce large amounts of NO in comparison with primary endothelial cells [e.g., human umbilical vein endothelial cells (HUVEC)] or a human microvascular endothelial cell line (CDC.HMEC-1) while retaining the functional properties of endothelial cells[129]. Use of bEnd.3 cells facilitates the detection of subtle differences in NO production as a consequence of treatment with agonists or antagonists[129].

In accordance with previous studies[268, 269], we observed a concentration-dependent increase in production of ROS on addition of OA or PA. OA was chosen for further studies, because it resulted in the most prominent increase in ROS production. Furthermore, *in vivo* studies performed by our group[264, 280] showed that an oral fat load, consisting of predominantly OA, lead to endothelial dysfunction. Interestingly, 4 mM OA resulted in a 2-fold increase in ROS 3 mM OA, despite slightly less efficient uptake. Thus a relatively small increase in the saturation level of albumin (from 3 to 4 mM oleic acid) leads to a considerable increase in ROS production.
The plasma concentration of oleic acid in type 2 diabetic patients is approximately 500 µM[281]. However, in the present study we used approximately 10-fold higher dose of oleic acid. We chose to use these high concentrations, because we wanted to use a

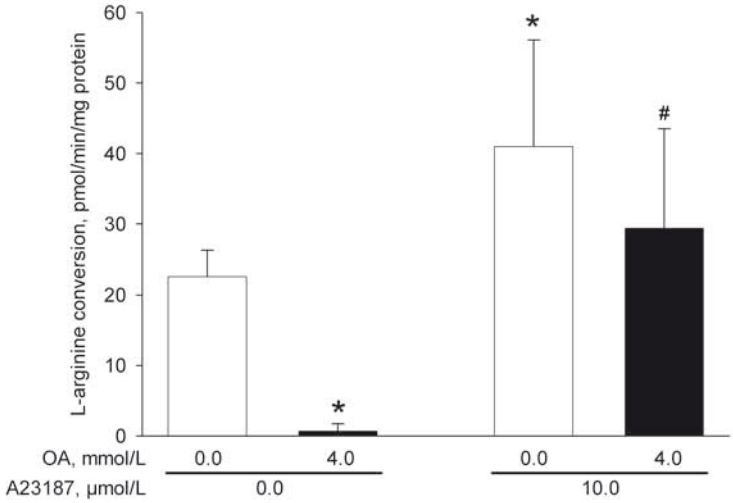

Figure 5. eNOS activity is impaired in the presence of OA and increased by calcium ionophore.
The results are corrected for nonspecific activity in the presence of L-NAME. Mean ± S.E.M. * P<0.05 compared to 0 mM OA; # P<0.05 compared to 4 mM OA. N=4

physiological concentration of albumin (0.5 mM, about 33 g/L). In disease states, such as diabetes mellitus or the nephrotic syndrome, the fatty acid:albumin molar ratio can be markedly raised up to 8:1[271, 272], which, at a fixed concentration of 0.5 mM albumin, corresponds to 4 mM OA. This concentration had no toxic effect for up to 60 min. However, longer exposure times were associated with toxicity.

In accordance with our *in vivo* data[264] where NO bioavailability was decreased as a response to OA, in the present *in vitro* study OA led to a substantial decrease in both basal and insulin-mediated endothelial NO production. The decreased eNOS activity could be due to eNOS uncoupling, since this phenomenon leads to decreased NO and increased ROS production[4, 22, 23]. Oxidation of BH4 to BH2 leads to a shortage of the cofactor, which results in uncoupled eNOS, even without stimulation of the enzyme, as was previously shown by us[129]. Stimulation with insulin may enhance the uncoupling of eNOS. Stimulation of endothelial cells with calcium ionophore leads to direct activation of eNOS without the 'intervention' of receptors. Calcium ionophore increased eNOS activity much more in the presence of OA than in the presence of vehicle (fatty-acid free BSA). These results indicate a potential defect at the insulin receptor, rather than a defect in eNOS phosphorylation or calcium signalling.
Insulin has direct, eNOS-dependent, vasodilator effects on the endothelium[282, 283],

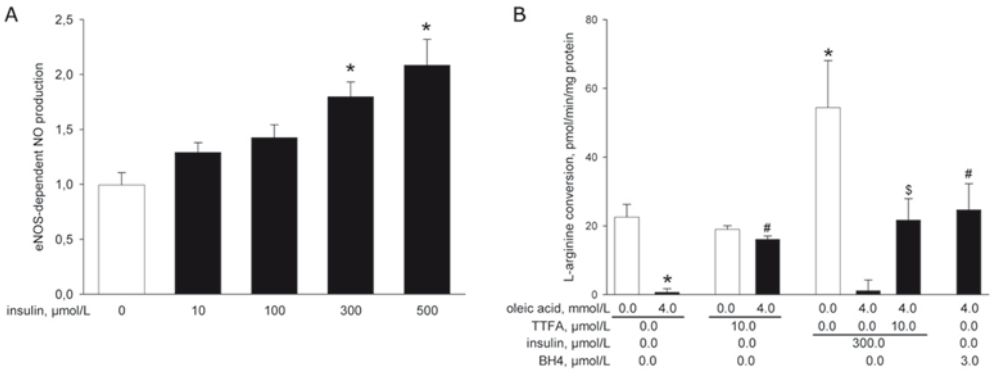

Figure 6. TTFA and BH4 restore OA-impaired eNOS activity.
A. Insulin dose-dependently increases NO production in bEnd.3 cells, as measured with DAF-2 DA[129]. B. The results are corrected for nonspecific activity in the presence of L-NAME. Mean ± S.E.M. * P<0.05 compared to 0 mM OA; # P<0.05 compared to 4 mM OA; $ P<0.05 compared to 4 mM OA, 300 µM insulin. N=4

which are reduced in subjects with impaired glucose tolerance and diabetes[101, 282, 284]. ROS have been shown to impair insulin signalling by inhibiting activation of eNOS[285-287]. In addition, peroxynitrite, formed during the reaction of NO with $O_2^{\cdot-}$, has also been shown to reduce insulin-induced activation of eNOS[288]. In the present study, OA not only decreased basal eNOS activity, but also abolished insulin-induced eNOS activity in the presence of OA, as compared to 0.5 mM BSA without OA. The high levels of ROS production induced by OA may account for the impaired insulin-induced eNOS activity. Possibly incorporation of OA-enriched phospholipid into the cell membrane affects the conformation of caveolae, 'flask shaped' invaginations of the plasma membrane. Many receptors are localized in caveolae of endothelial cells, including the insulin receptor[289] and other receptors involved in eNOS signaling[290, 291]. A change in conformation of caveolae may causes a general interference with receptor-mediated stimulation of eNOS[292]. However, this remains to be shown for insulin.

Oxidation of the eNOS cofactor BH4 by ROS leads to diminished levels of BH4, resulting in eNOS uncoupling[4, 22, 23]. To determine whether OA-induced mitochondrial-derived ROS leads to oxidation of BH4 and subsequent eNOS uncoupling, bEnd.3 cells were incubated with TTFA, an inhibitor of complex II of the mitochondrial respiratory chain[275]. TTFA prevented the decrease in eNOS activity in the presence of 4 mM OA, indeed indicating that mitochondrial-derived ROS are directly linked to decreased NO production. In addition, TTFA restored insulin-induced eNOS activity in the presence of OA back to basal activity levels. However, insulin-induced eNOS activity in the

presence of OA and TTFA was not increased up to the level of insulin-induced activity in the presence of vehicle. This supports the notion that OA impairs insulin-mediated signalling. In addition, it may be possible that a low concentration of OA-induced mitochondrial-derived ROS is produced, leading to oxidation of a small amount of BH4. This may result in a relative deficiency of the cofactor, eventually leading to eNOS uncoupling and diminished NO production. Accordingly, we found that addition of BH4 also prevents OA-induced inhibition of eNOS activity.

In addition to the protective effects of TTFA on OA-induced decrease in eNOS activity, OA-induced ROS production was inhibited by TTFA suggesting the involvement of uncoupling of the mitochondria, in agreement with the mechanism proposed by Brownlee[85]. Increased oxidative stress inducers lead to increased oxidation of FFA by mitochondria, eventually leading to the generation of $O_2^{\cdot -}$ [293]. Although complex I and complex III were shown to be responsible for much of the $O_2^{\cdot -}$ generated by mitochondria[80, 81], complex II has also been shown to be involved in ROS production by mitochondria[294, 295]. In the present study, inhibition of mitochondrial complex I had no effect, but inhibition of mitochondrial complex II prevented OA-induced ROS production.

It has been reported that increased $O_2^{\cdot -}$ production in platelets of patients with coexisting hypertension and diabetes is due to uncoupling of eNOS[296] which is most likely a result of diminished BH4 levels due to oxidation by ROS[67, 249]. In line with these observations, a recent study showed increased oxidation of BH4 in the aorta of Goto-Kakizaki rats, an animal model for non-obese type II diabetes[297]. Consistently, in the present study, supplementation of bEnd.3 cells with BH4, at least in part, restored the inhibition of eNOS activity in the presence of OA and prevented OA-induced ROS production. On the other hand, it is also possible that the decrease in ROS production is caused by the direct antioxidant properties of BH4[22, 298] independent of its interaction with eNOS. Alternatively, BH4 may act directly on mitochondrial ROS production. It has been reported that BH4 inhibits complex I and IV of the mitochondrial respiratory chain[299]. This would explain why co-treatment of bEnd.3 cells with TTFA and BH4 in the presence of OA did not lead to a further decrease in ROS production, since both inhibitors exert their actions at the level of mitochondria.

Thus addition of BH4 may lead to a decrease in ROS production due to the prevention of eNOS uncoupling or by its antioxidant activities. In order to distinguish between these options, we examined the effect of L-NAME on OA-induced ROS production. From a previous study we know that incubation of bEnd.3 cells with L-NAME for 20 min completely abolishes eNOS activity[129]. Treatment with L-NAME resulted in a slight, but non-significant decrease in OA-induced ROS production. Co-treatment of

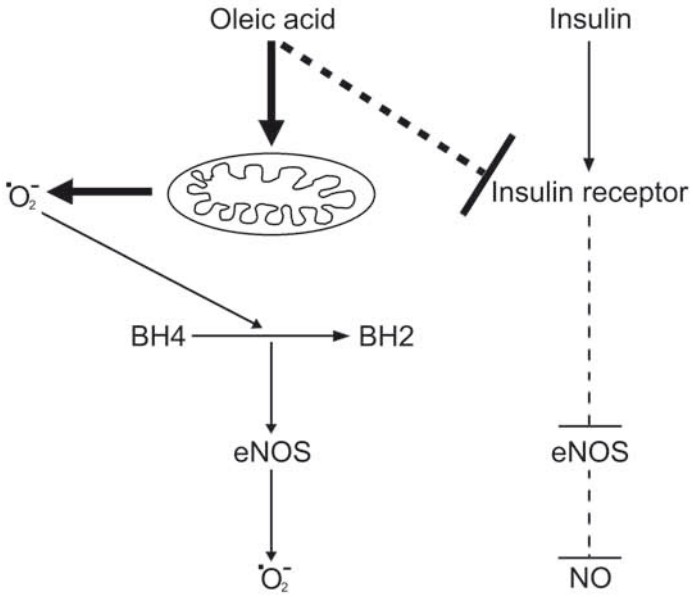

Figure 7. Effects of oleic acid on superoxide and nitric oxide production.
Continuous lines represent stimulation and dashed lines inhibition. Pathways predominantly affected by oleic acid are indicated with thick lines and secondary pathways with thin lines. Abbreviations: O_2^-, superoxide anion; BH4, tetrahydrobiopterin; BH2, dihydrobiopterin (oxidized form of BH4); eNOS, endothelial nitric oxide synthase; NO, nitric oxide

bEnd.3 cells with TTFA and L-NAME, lead to enhanced inhibition of ROS production as compared to TTFA alone, but this difference was not significant indicating that eNOS may not be primarily involved in OA-induced ROS production in bEnd.3 cells, and thus excluding eNOS uncoupling as the dominant source of OA-induced ROS production. However, mitochondrial uncoupling leads to the formation of considerable amounts of ROS[85]. The small but non-significant decrease in ROS production in the presence of L-NAME could imply that uncoupling of eNOS is involved in OA-induced ROS production, but that the contribution is so small that it is not possible to discern it in the excess of mitochondrial ROS production.

The effects of OA on eNOS activity and ROS production in bEnd.3 are not comparable to the effects of eNOS inhibitors like aminoguanidine and Nω-monomethyl-L-arginine. As shown in the present study, incubation of bEnd.3 wells with OA leads to a decrease in eNOS activity and an increase in ROS production. In contrast, in a recent study we found that incubation with eNOS inhibitors results in a small decrease in ROS production, but complete inhibition of eNOS activity[129].

In conclusion, our original hypothesis that eNOS uncoupling contributes to OA-induced oxidative stress in endothelial cells in addition to mitochondrial ROS production has been proven to be incorrect. This study shows that increased levels of OA predominantly lead to mitochondrial-derived ROS production, accompanied by defects in receptor mediated eNOS stimulation (figure 7). The increased levels of ROS can lead to oxidation of BH4, leading to a shortage of the cofactor and subsequent increased eNOS uncoupling and diminished eNOS activity. The ROS-induced decrease in BH4 results in a vicious cycle: superoxide impairs BH4 leading to more superoxide, due to eNOS uncoupling, These mechanisms together may, at least in part, provide a possible mechanism for the relationship between postprandial endothelial dysfunction observed in patients with type 2 diabetes or renal disease.

Acknowledgments

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CHAPTER 7

Low albumin levels increase endothelial NO production and decrease vascular NO sensitivity

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in press

Abstract

Hypoalbuminemia is associated with increased risk of cardiovascular disease. It is unclear whether endothelial dysfunction is a direct result of low albumin or whether it is caused by factors like chronic inflammation or dyslipidemia. In this study, the effect of low albumin concentrations on endothelial NO synthase (eNOS)-dependent NO production was determined *in vitro* and *ex vivo*. eNOS activity, assessed by arginine-citrulline conversion, and NO production, determined by 4,5-diaminofluorescein diacetate, electron paramagnetic resonance and Griess colorimetry, were measured in cultured endothelial cells expressing high levels of eNOS (bEnd.3) after exposure to albumin concentrations ranging from 0.5 mM (33 g/L) to 0 mM. Analbuminemic and control rat plasma NO metabolites and aortic eNOS protein mass were determined and aortic endothelium-independent and endothelium-dependent vasodilator tone were measured *ex vivo* under albumin-free conditions. *In vitro*, eNOS activity was significantly increased in the absence of albumin (75 ± 2 vs. 26 ± 6 pmol/min/mg protein; $p<0.01$). Low albumin levels consistently increased NO production in endothelial cells. Plasma NO metabolites were increased (18.2 ± 1.9 vs. 12.5 ± 0.8 µM; $p<0.05$) and endothelium-independent relaxation was markedly blunted in analbuminemic rats, resulting in a considerably higher ED50 (80 ± 2 vs. 1.1 ± 0.2 nM, $P<0.01$), while endothelium-dependent dilatation was slightly, but significantly, increased. Aortic eNOS protein mass was not affected. This implies that *in vivo* hypoalbuminemia reduces vascular NO sensitivity. We show that low albumin as such seems to enhance, rather than diminish, eNOS-mediated endothelial NO production.

Introduction

Hypoalbuminemia is associated with endothelial dysfunction[112, 113], all-cause mortality in patients with chronic kidney disease (CKD)[114] and cardiovascular mortality in patients with end-stage renal disease[115]. However, it is unclear whether endothelial dysfunction is a direct result of the decreased levels of albumin or whether it is caused by factors such as chronic inflammation[117] and dyslipidemia[112], present in most conditions associated with hypoalbuminemia. Albumin may be viewed as a negative acute phase protein[300] and hypoalbuminemia may well be a marker for chronic inflammation rather than a direct cause of endothelial dysfunction.

In the present study, we hypothesized that acute and chronic exposure to low albumin levels as such, in the absence of other systemic factors, does not decrease eNOS activity, diminish NO production, or cause endothelial dysfunction. Vasodilatation is a response involving the interaction of endothelium with vascular smooth muscle. Therefore, possible effects of hypoalbuminemia on endothelium and vascular smooth muscle should be considered separately.

A microvascular endothelial cell line (bEnd.3), expressing high levels of eNOS but lacking neuronal or inducible NOS[129], was used to determine the effect of acute exposure to albumin levels, ranging from a physiological concentration of 0.5 mM (33 g/L) to 0 mM, on eNOS activity and NO production *in vitro*. Consistent with our hypothesis this experiment showed that acute exposure to low albumin levels does not decrease eNOS activity or diminish NO production. On the contrary eNOS activity and NO production were consistently enhanced. Thus we tested our hypothesis under conditions of chronic exposure to low albumin in analbuminemic rats[301] by measuring plasma NO metabolites and eNOS protein mass *in vivo*, and endothelium-independent and endothelium-dependent aortic vascular function *ex vivo* under albumin-free conditions.

Materials and methods

Cell culture

An immortalized bEnd.3 cell line was provided by Dr. Alan Schwartz (University of Washington, St Louis, MO). These cells, that only contain eNOS, show substantially higher NO production than other endothelial cells such as HUVEC or HMEC[129]. Cells were cultured as described previously[129]. Prior to each experiment, cells were starved for 16 h in DMEM containing only 0.1% bovine serum albumin (BSA). Culture media were obtained from Life Technologies (Burlington, ON, Canada).

Measurement of osmolality and colloid osmotic pressure (COP)

Osmolality and colloid osmotic pressure (COP) in PBS containing different concentrations of BSA were measured as previously described[301]. No differences were found in osmolality, but there were substantial differences in COP (table 1).

	Osmolality mosm/kg	COP mm Hg
0 mM BSA	288	0
0.2 mM BSA	288	3.0
0.5 mM BSA	289	10.2

Table 1. Osmolality and colloid osmotic pressure (COP) of PBS without and with BSA.

Measurement of NO - 4,5-diaminofluorescein diacetate assay

To measure intracellular NO production, the cell-permeable fluorescent NO indicator 4,5-diaminofluorescein diacetate (DAF-2 DA; Calbiochem) was used as described previously[129]. Briefly, bEnd.3 cells were loaded with 5 µM DAF-2 DA and incubated for 20 min at 37° C in the presence or absence of the NOS inhibitor L-Nω-nitro-arginine

methyl ester (L-NAME; 300 µM; Sigma, St. Louis, MO). Different concentrations of fatty acid free BSA (Sigma) were added, after which fluorescence was measured every 2 min for 20 min (excitation wavelength, 485 nm; emission wavelength, 538 nm; Fluoroskan Ascent, Labsystems, Helsinki, Finland). Note that no stimulus for NO production was given to the cell cultures.
Endogenous NO production was calculated by determining slopes of each line by linear regression analysis. Reactivity of DAF-2 DA towards NO was calibrated by exposing bEnd.3 cells to 10-100 µM diethylenetriamine NONOate (DETA/NO; Sigma), as described previously[129]. Results were expressed, relative to 0.5 mM BSA (33 g/L), as eNOS-dependent NO production (total NO production minus NO production in the presence of L-NAME). Inter-assay variability was 3.2 ± 1.3%, and intra-assay variability was 4.0 ± 1.2%.

Measurement of nitrite - Griess assay
Both intra- and extracellular nitrite levels were determined using the Nitrate/Nitrite Colorimetric Assay Kit of Cayman Chemical (ITK Diagnostics, Uithoorn, The Netherlands). Cells were incubated for 20 min with different concentrations of BSA in the presence or absence of 50 ng/mL vascular endothelial growth factor (VEGF; Sigma) or 300 µM L-NAME at 37° C. Absorbance was measured at 540 nm using a plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland). Results were expressed, relative to control, as eNOS-dependent NO production (total NO production minus NO production in the presence of L-NAME).

Measurement of NO - Electron Spin Resonance
NO trapping with Fe-DETC complexes was initiated by replacing the medium with 10 ml fresh DMEM containing 2.5 mM diethyl dithiocarbamate (DETC). Subsequently 10 µM ferrous sulfate (Fluka Buchs, Switzerland) was added. After 10 min, medium was replaced with medium containing either no BSA or 0.5 mM BSA in the presence or absence of 300 µM L-NAME. After 20 min of NO trapping at 37° C, the flask was placed on ice for 2 min to terminate enzymatic activity. Note that no stimulus for NO production was given to the cell cultures. Cells were harvested in 10 mL incubation medium. The cell fraction with Fe-DETC complexes was separated by centrifugation (1000 g for 10 min at 4º C), resuspended in 350 µL incubation medium, drawn into a syringe (id 4.8 mm) and snap frozen in liquid nitrogen until EPR assay.
EPR spectra were recorded at 77 K on a modified X-band ESP 300 radiospectrometer (Bruker BioSpin, Karlsruhe, Germany) operating near 9.54 GHz with 20 mW power. Frozen samples were placed in a quartz liquid finger dewar at the center of a Bruker ER4103TM cavity. Field modulation was 0.5 mT, gain 2x105, time constant and ADC conversion time 82 ms. Nine scans were accumulated to reduce instrumental noise. Spin densities were calibrated with frozen reference solutions of $NO\text{-}Fe^{2+}\text{-}(MGD)_2$ in PBS buffer.

Measurement of eNOS activity - Arginine-citrulline conversion assay

NOS activity was measured by determining the formation of L-^{3}H-citrulline from L-^{3}H-arginine. Cells were incubated for 20 min with either no or 0.5 mM BSA and homogenized in a buffer consisting of 50 mM Tris buffer, pH7.4, 320 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol (DTT), 2 mg/L aprotinin and 100 mg/L phenylmet hylsulfonylfluoride. Homogenate (50 µL) was incubated in a final volume of 100 µl at 37° C for 30 min with 1 mM L-citrulline, 300 µM tetrahydrobiopterin, 3000 U/mL calmodulin, 0.5 mM NADPH, and 1 mM $CaCl_2$, all dissolved in a buffer consisting of 50 mM KH_2PO_4 containing 1 mM DTT and 10 µM L-^{3}H-arginine. Prior to incubation, L-^{14}C-citrulline was added to correct for procedural losses. Substituting NADPH by 100 mM L-NAME corrected for non-specific activity. The incubation was stopped by placing the tubes on ice and adding 20 mM ice-cold Hepes buffer, pH 5.5, followed by separation of arginine and citrulline on Dowex 50X8-200 ion exchanger resin and liquid scintillation counting of the citrulline fraction. All measurements were performed in triplicate. Results are expressed as pmol per min per milligram protein. Inter-assay variability was 2.2 ± 0.8%, and intra-assay variability was 2.2 ± 0.3%.

Animals

Adult (3-4 month old) male Sprague-Dawley (SDR; 200-350 g; Harlan-Olac, The Netherlands) and Nagase analbuminemic rats (NAR; 200-350 g; Central Laboratory Animal Institute, Utrecht, The Netherlands) were housed in a temperature- and light-controlled room. Rats had free access to standard rat chow (Special Diet Services, Witham, Essex, UK) and tap water. Sentinel animals were monitored regularly for infection by nematodes and pathogenic bacteria, as well as antibodies for a large number of rodent viral pathogens, and were consistently negative throughout the course of the experiments. The Utrecht University board for studies in experimental animals approved the studies.

Organ chamber experiments

Under general barbiturate anaesthesia rats were euthanized by exsanguination. Plasma nitrite plus nitrate levels were measured with the Griess assay as described above. Thoracic aortas were dissected free and immersed in a carbogenized Krebs-Ringer buffer, pH 7.4. Indomethacin (10 µM) was added to the buffer to prevent the formation of endogenous prostaglandins. Aortas were carefully cleaned of blood clots and peri-aortic tissue and cut into rings of similar weight and dimensions (2-4 mm long). Care was taken not to damage the endothelium. Rings were mounted horizontally between two stainless steel hooks in organ chambers filled with 10 mL of Krebs-Ringer buffer at 37° C plus indomethacin and gassed with 95% O_2 / 5% CO_2. One hook was anchored in the organ chamber and the other was connected to a strain gauge transducer for the measurement of isometric tension. Note that these *ex vivo* measurements were done under albumin-free

conditions. Aortic rings were progressively stretched to an optimal basal tension of 1 g, and the contraction ability of the rings to a saturated solution of KCl was checked. Only rings that could generate a 2 g contraction force were studied. All drugs were dissolved and diluted in saline (0.9% NaCl), except KCl that was dissolved in demineralised water. Relaxation to sodium nitroprusside (SNP; 0.01 nM to 100 µM; Sigma) or acetylcholine (ACh; 0.01 to 100 µM; Sigma) was examined and compared between SDR and NAR. Relaxation was studied after preconstriction by sufficient phenylephrine (PE; Sigma) to generate a 2 g contraction force. ACh measurements were also performed after pre-incubation for 90 min with the arginine-analogue Nω-nitro-L-arginine (L-NNA; 1 mM Krebs buffer). L-NNA was also added to the organ chamber. At least four rats and three rings per rat were used in each condition. The delay between killing the rats and measurement of relaxation was standardized at 90 min.

eNOS expression in aorta

Frozen tissue was processed for determination of eNOS protein abundance. eNOS monoclonal antibody, and monoclonal eNOS, as well as peroxidase-conjugated goat anti-mouse IgG antibody were purchased from Transduction Laboratories. Total protein was determined with a kit (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

Results are expressed as mean ± SEM. In preconstricted rings, ACh and SNP responses are expressed as percent relaxation. Dose-response data were compared with two-way analysis of variance. If a variance ratio reached statistical significance, the studentized Newman-Keuls test was performed as posthoc test. Unpaired data were compared with a t-test. $P<0.05$ was considered significant.

Results

Acute effects of low albumin on eNOS activity and NO production in endothelial cells

Exposure time to BSA in the cell experiments was based on time course studies. No significant differences in DAF signal were found between 20, 60 and 180 min of incubation (1.00 ± 0.04, 0.99 ± 0.11 and 1.14 ± 0.13, respectively). As longer incubations had no effect, we chose to incubate for 20 min.

Arginine-citrulline conversion was used to assess the acute *in vitro* effect of low albumin on eNOS activity. The activity of eNOS was significantly increased in the absence of bovine serum albumin (BSA; 75 ± 2 vs. 26 ± 6 pmol/min/mg protein; $p<0.01$; figure 1). Measurement of NO production was critical for our question. Thus to measure the effects of low albumin on NO production, three different methods were used. The

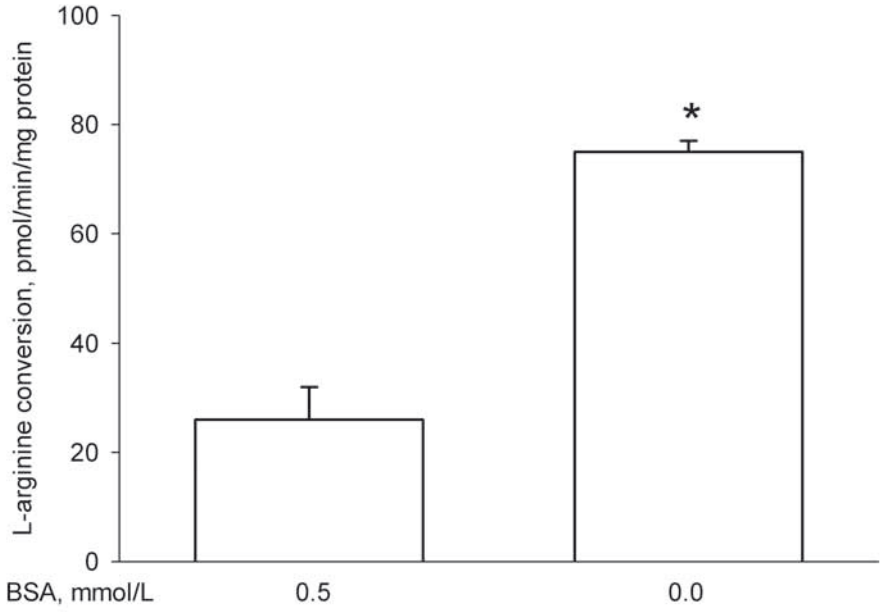

Figure 1. eNOS activity measured by arginine-citrulline conversion in bEnd.3 cells.
* P<0.05 vs. 0.5 mM BSA

NO-induced DAF signal (figure 2a) and both basal as well as VEGF-induced nitrite levels (figure 2b) were all inversely related to BSA concentration. Since these two techniques are indirect ways to measure NO production, EPR was used to determine NO production directly. A typical EPR spectrum (figure 2c) showed a clear triplet hyperfine structure (HFS) centred at g = 2.035 as expected for ferrous mono-nitrosyl iron-dithiocarbamate complexes (MNIC). As expected for biological samples[175], a small contribution from paramagnetic Cu^{2+}-DETC complexes was superposed. The intense central hyperfine line of these Cu^{2+}-DETC complexes is visible near g = 2.01 (figure 2c). Pre-incubation for 20 min with 300 μM L-NAME reduced the MNIC yield in a single 75 cm^2 flask to below the detection limit of 10 pmol at the given spectrometer settings. NO production was significantly increased in the absence of BSA (55.3 ± 3.3 pmol per flask) as compared to 0.5 mM BSA (35.4 ± 5.0 pmol per flask; p=0.034). Note that after incubation with or without BSA cells were always washed before the *in vitro* measurements were performed.

Plasma NO metabolites, vascular function and eNOS mass in aorta from NAR
To assess the chronic effect of hypoalbuminemia we used hereditary analbuminemic rats (NAR, n=6)[301] and compared them to control SDR (n=6). Plasma NO metabolites were

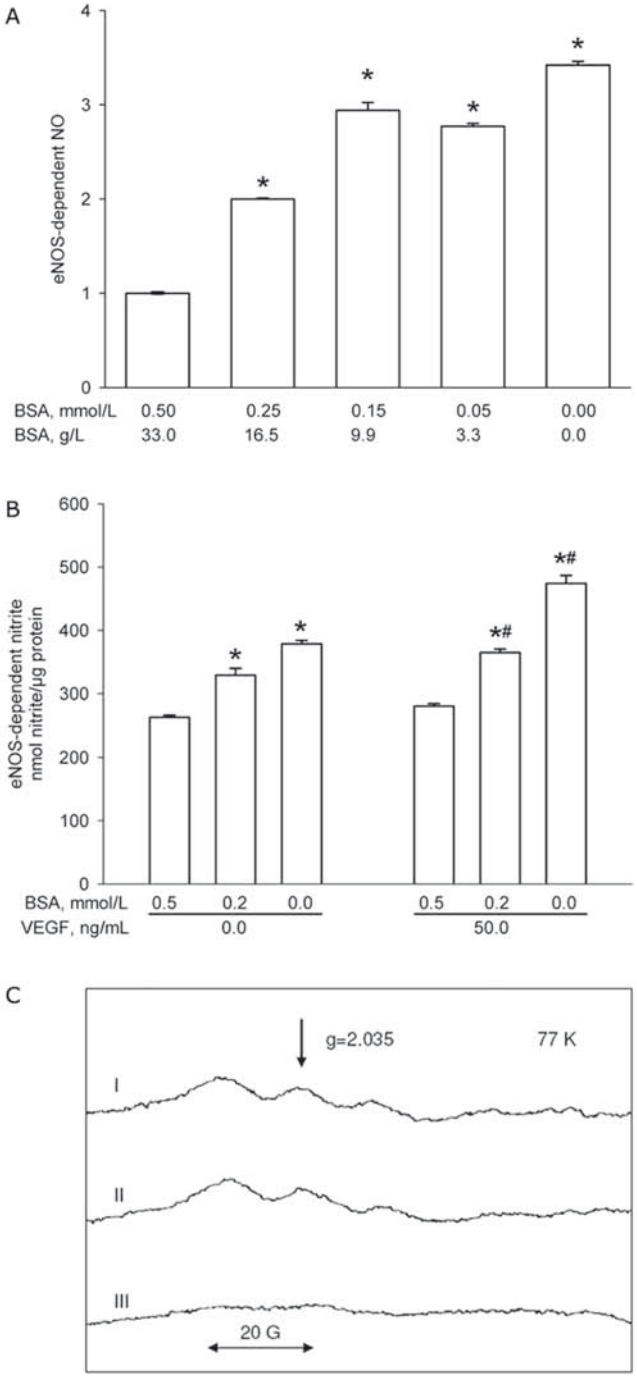

Figure 2. Intracellular nitric oxide (NO) levels in bEnd.3 cells.

A. DAF-2 DA assay. B. Griess assay. C. Representative electron paramagnetic resonance (EPR) spectra. I: yield of 60 ± 5 pmol complexes in the absence of bovine serum albumin (BSA); II: yield of 50 ± 5 pmol complexes in the presence of 0.5 mM BSA; III: absence of complexes in the presence of 0.5 mM BSA and 300 µM L-NAME. * P<0.05 vs. 0.5 mM BSA; # P<0.05 vs. 0 ng/mL VEGF

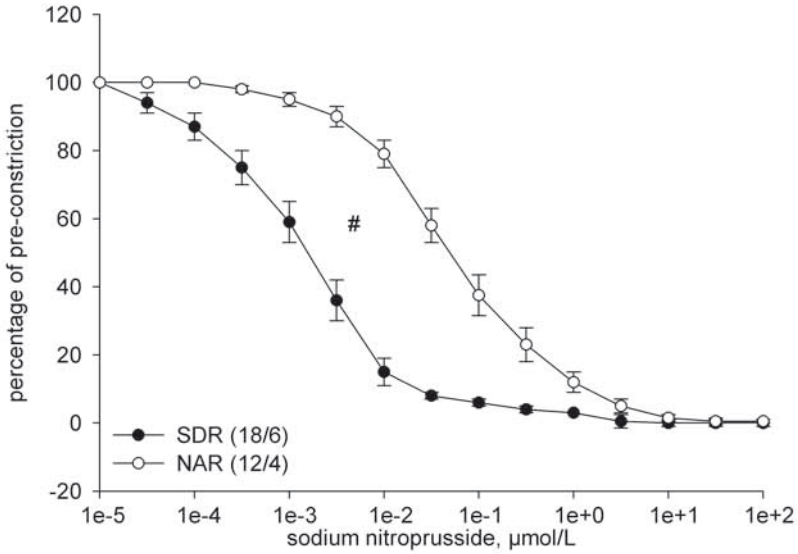

Figure 3. Relaxation to sodium nitroprusside in aortic rings of SDR and NAR preconstricted with phenylephrine.

Number of experiments is indicated as rings/rats. # ED50; NAR: 80 ± 2 nM vs. 1.1 ± 0.2 nM, P<0.01

increased *in vivo* (18.2 ± 1.9 vs. 12.5 ± 0.8 µM; p<0.05). Endothelium-independent vasodilatation induced by SNP is a measure of the sensitivity of the vascular smooth muscle to NO. The SNP response measured under albumin-free conditions *ex vivo* was significantly blunted in NAR aortic rings (figure 3). This resulted in a considerably higher ED50 in NAR vs. SDR (80 ± 2 vs. 1.1 ± 0.2 nM, P<0.01). Endothelium-dependent vasodilatation as induced by acetylcholine (ACh) is a measure of NO bioavailability and, indirectly, eNOS activity. *Ex vivo* ACh-induced relaxation was slightly, but significantly (P<0.05), stronger in NAR than in SDR (figure 4). Pre-incubation with L-NNA completely prevented ACh-mediated relaxation in both strains. Protein abundance of eNOS in the aorta was similar in NAR and SDR (figure 5).

Discussion

In this study, we demonstrated that low albumin levels, often viewed as causal factor in the pathogenesis of endothelial dysfunction and cardiovascular disease in CKD and proteinuric conditions, does not reduce eNOS activity and NO production in the absence of other systemic factors. Albumin concentrations ranging from a physiological

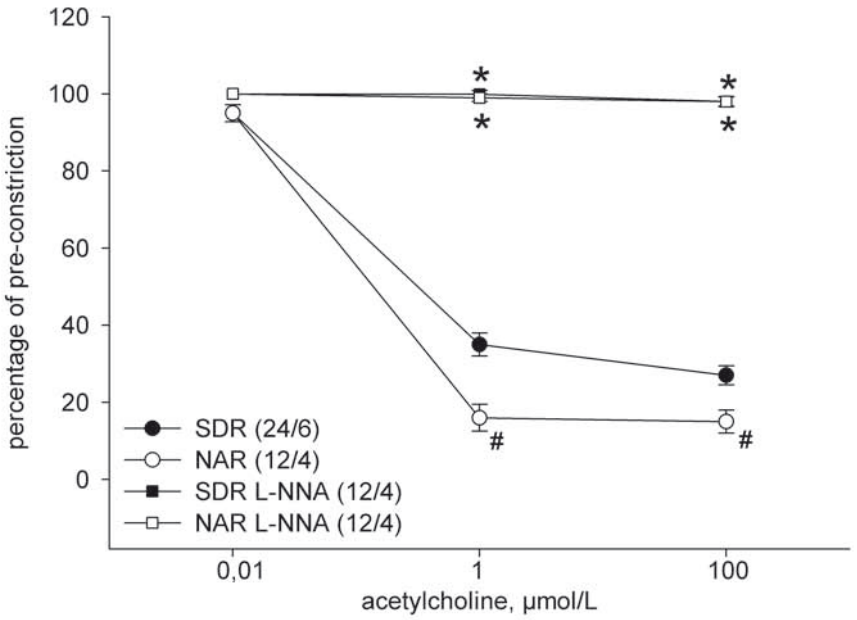

Figure 4. Relaxation to acetylcholine in aortic rings of SDR and NAR preconstricted with phenylephrine.

Number of experiments is indicated as rings/rats. * P<0.05 vs. baseline; # P<0.05 vs. SDR

concentration of 0.5 mM (33 g/L) to 0 mM were used. Using several different experimental methods for the critical measurement of NO production, we consistently found that NO production *in vitro* was not decreased, but in fact increased in the absence of albumin. Our findings are in line with observations by others that the causes of hypoalbuminemia, such as malnutrition, dyslipidemia[112, 113], insulin resistance[112] and inflammation[300], rather than the reduced albumin levels itself explain the relation with endothelial dysfunction in CKD and proteinuric conditions[115].

The NAR, a mutant Sprague-Dawley rat[301], has extremely low albumin levels in the absence of chronic inflammation. Little is known on regulation of vascular function as such in NAR. In NAR the half-life of nitric oxide (NO) from an exogenous donor was reduced by 50% and plasma S-nitrosothiols were much lower than in control rats and barely increased in response to a NO-donor[302]. However, arterial pressure is at control levels[303], suggesting that a systemic increase in oxidative stress due to lack of albumin[120] may be adequately compensated despite a deficiency of nitrosylated albumin. Such compensation could involve constitutive upregulation of vascular NO synthase activity. Indeed we found an increased plasma level of NO metabolites and blunted endothelial-

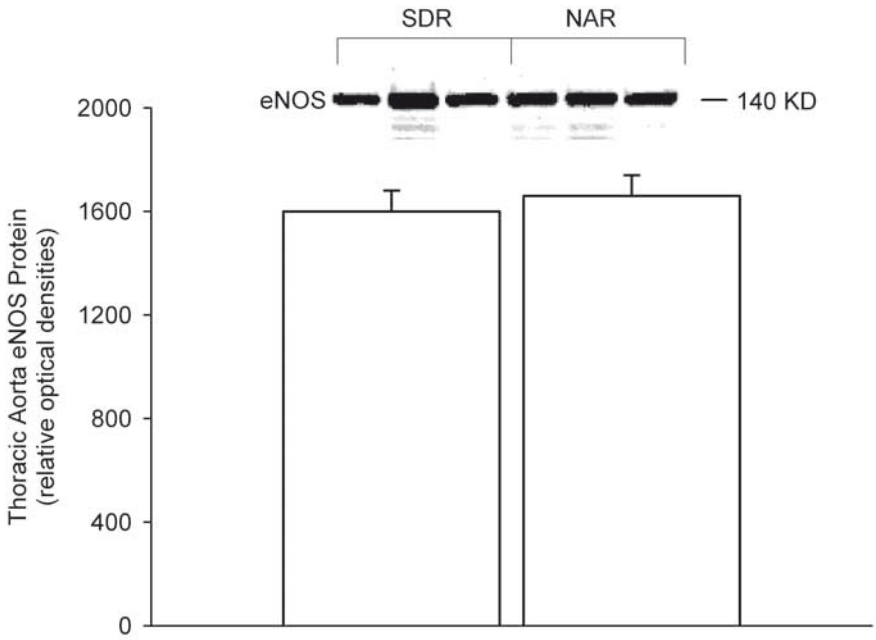

Figure 5. Representative Western blots and group data (average of western blots; 6 rats/ strain) depicting thoracic aorta eNOS protein abundance in SDR and NAR.

independent relaxation. Furthermore ACh-dependent relaxation was enhanced, also suggesting high levels of NO production, even though eNOS protein mass was not increased. All these observations support the notion that hypoalbuminemia as such does not impede endothelial function. In fact, the stimulatory effects of acute exposure to low albumin levels on eNOS *in vitro* appear to persist under chronic conditions *in vivo* judging by plasma NO metabolites. Evidence to support this can be gathered when studying isolated vascular tissue *ex vivo* under albumin-free conditions.

In order to exclude osmotic or oncotic influences, osmolality and COP were measured. As expected we found no differences in osmolality, but we did find substantial differences in COP. However, additional experiments with an oncotic control (e.g. ficoll) have not been performed for two reasons. First, it was not the specific aim of the study to differentiate between hypoalbuminemia and low COP because this is not clinically relevant. Secondly, the reduction in COP in adult male NAR is only about 4 mm Hg because of an increase of other plasma proteins[301]. One would expect that the reduced sensitivity of NAR aorta to SNP should also imply reduced sensitivity to endogenous NO induced by ACh. Another dilatory factor, apart from NO, such as endothelial-derived hyperpolarizing factor (EDHF), might play a role in the ACh-induced

relaxation. However, EDHF-mediated relaxation is defined as relaxation induced by a circulating non-NO, nonprostanoid factor, upon stimulation by a transmitter such as ACh or bradykinin. As can be seen in figure 4, relaxation to ACh within the dose-range studied was absent in both rat strains in the presence of L-NNA (plus indomethacin). Thus within this dose range, ACh does not induce appreciable EDHF release in the rat aorta. Although, we cannot exclude that a different endothelium-dependent relaxing agent and/or higher concentrations of ACh would have stimulated EDHF release, it is doubtful whether this is relevant *in vivo*.

The mechanism of the adaptation of vascular NO synthase activity to low albumin levels *in vivo* is unclear. In the present study eNOS localisation, essential for proper eNOS activity[36], was not studied. To date, no studies have been published concerning the effect of low albumin on eNOS localization. Another option is altered calcium partitioning. Since albumin binds Ca^{2+}, its absence can increase the available pool of Ca^{2+} for uptake by endothelial cells. We accounted for this problem in the *in vitro* experiments by providing excess Ca^{2+} in the experimental buffers. To our knowledge, no studies have been published concerning the cytosolic calcium concentration in NAR, although it has been reported that serum ionised calcium is slightly decreased in NAR[304]. Hence, it is conceivable that in NAR, the absence of albumin results in higher cytosolic calcium concentration, leading to an increased cellular concentration of Ca^{2+}-calmodulin complexes which are essential for eNOS translocation and activity. By the same token, the greater cytosolic calcium concentration in vascular smooth muscle cells in NAR can raise vascular tone and confer some degree of NO resistance. This may, in turn, contribute to maintenance of normal blood pressure[303], despite profound hypoalbuminemia in NAR *in vivo*.

SNP-mediated relaxation was blunted in the NAR aorta suggesting reduced NO sensitivity of guanylate cyclase[305], possibly by increased basal NO production, or altered distribution of intracellular calcium towards the sarco(endo)plasmic reticulum[306]. The sensitivity of target tissues for increased NO is determined by three factors. First, the expression and function of NO-sensitive guanylate cyclase may be depressed[307]. Second, activity of cGMP-dependent kinases, which mediate the effects of cGMP, may be reduced. Finally, presence and activity of phosphodiesterases responsible for cGMP breakdown may be enhanced[308]. Reducing ambient NO level, by de-endothelialisation or by pharmaceutical means, sensitises the vasodilator response to NO itself, and conversely NO desensitises the effector pathway, also designated as 'nitrate tolerance'[307]. Previously, we reported normal arterial pressure, renal blood flow and glomerular filtration rate in NAR[303]. The absence of hypotension and renal hyperperfusion despite upregulation of NO production in vascular tissue of NAR can be due to decreased NO export by albumin, leading to a local increase in NO which hypothetically can

either lower NO production by eNOS[309] or desensitise the effector pathway, hence creating endogenous resistance to local enhanced NO-production[305]. However, when stimulated by acetylcholine the magnitude of relaxation was enhanced rather than depressed, suggesting that the sustained response to exogenous NO from a source such as nitroprusside (this study) or NOC7[302] is impeded. This combination is suggestive of enhanced cGMP breakdown, rather than reduced NO production, guanylate cyclase expression or down-stream signalling. Nitrosylated albumin derivatives have been advanced as pharmacologically active NO transporters[309]. The present study suggests that, although possibly useful in an acute setting, such compounds may induce adaptive down-regulation of endogenous NO effector sensitivity.

In the clinical setting of hypoalbuminemia it is not easy to isolate the effect of low albumin from other factors that have well established effects on endothelial function: chronic renal disease, inflammation, dyslipidemia, and the sympathetic nervous system. By choosing an *in vitro* model to examine the effects of hypoalbuminemia on NO production, we purposely excluded such factors. This allowed us to determine the direct effect of albumin on eNOS activity and NO production. Similar considerations motivated us to study vascular rings isolated from male analbuminemic rats. Although this rat is obviously not the ideal model for hypoalbuminemia, it is devoid of inflammation and renal disease, and in the males dyslipidemia is mild[310]. To our knowledge, this is the best available 'clean' model of hypoalbuminemia.

In conclusion, *in vitro* low albumin enhances eNOS activity and NO production from the endothelium rather than the reverse. In agreement, *in vivo* plasma NO metabolites were increased, and *ex vivo*, the relaxation of vascular smooth muscle in response to the NO donor SNP was blunted. Both observations suggest that adaptation to chronic hypoalbuminemia raised the local NO levels in vascular tissues. The enhanced ACh response and normal eNOS protein expression disproves that isolated hypoalbuminemia causes endothelial dysfunction. Thus as postulated previously[115] hypoalbuminemia is largely a surrogate marker as opposed to inflammation and dyslipidemia which may well be the real culprits in the pathogenesis of endothelial dysfunction associated with hypoalbuminemia.

Chapter 8

Nitric oxide synthase acts as nitrite reductase under anoxia

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Abstract

Cultured bEnd.3 cells show a marked increase in nitric oxide (NO) production when subjected to anoxia, even though the normal arginine pathway of NO formation is blocked due to absence of oxygen. NO is released with a constant rate that is sustained for over 30 minutes. The rate of anoxic NO production is intermediate between basal, unstimulated NO synthesis and synthesis as stimulated with calcium ionophore. After 40 minutes, the rate sharply decelerates and NO production ceases. The anoxic release of NO is mediated by endothelial nitric oxide synthase and can be abolished by specific inhibitors of NO synthase (NOS). The anoxic NO release is unaffected by the xanthine oxidase inhibitor oxypurinol. The phenomenon is attributed to anoxic reduction of intracellular nitrite by endothelial NOS (eNOS). Its magnitude and duration suggests that nitrite reductase activity of eNOS is relevant for fast NO delivery in hypoxic vascular tissues.

Introduction

Nitrite has been shown to have beneficial effects when living tissue is subjected to conditions of low oxygen tension as may arise in ischemia, hypoxia or anoxia[311-313], but the mechanism of this phenomenon remains controversial. Nitrite anions (NO_2^-) are metastable intermediates in the oxidation cascade[314] of nitric oxide (NO) radicals to the stable metabolite nitrate (NO_3^-). Typical nitrite levels[311, 314, 315] are 0.5-1.0 µM in plasma and 5-20 µM in normoxic tissues. Nitrite levels in the vascular system are positively correlated with endothelial nitric oxide synthase (eNOS) activity[314, 316-318]. It was verified that almost all circulating nitrite in the blood of fasting humans originates from the oxidation of L-arginine by NOS enzymes[319]. Studies of human vascular flow have suggested that a significant fraction of infused NO is rapidly oxidized to nitrite and transported in this form for considerable distances along the vascular tree[320]. Such convective transport is facilitated by its fairly long lifetime in human blood[314]. Interestingly, nitrite concentrations in oxygen-rich arterial blood were found to be higher than in venous blood[186, 315]. This arterial-venous gradient was interpreted as a manifestation of nitrite delivery to perivascular tissues and suggested that nitrite actually plays an active physiological role in the control of vascular flow. However, infusion studies in humans did not show vasodilatory capacity of nitrite under normoxia[318]. Rather, low oxygen tension seemed crucial for vasodilation induced by direct infusion of nitrite in *in vivo* studies[316, 321].

The beneficial effects of nitrite under ischemia are attributed to the reduction of nitrite to NO, but the dominant mechanism for this reduction is controversial. Direct, uncatalysed reduction of nitrite is very slow[177] except at extreme acidic conditions as may arise in

the stomach[322, 323], urine[324] or ischemic tissue[127, 325]. Therefore enzymatic mediators for nitrite reduction have been proposed. Dedicated nitrite reductases are known in bacteria, but are lacking in mammals. However, some mammalian enzymes show nitrite reductase capacity in addition to their normal physiological function. Examples are glutathion-S-transferase[326], xanthine reductase (XOR)[327-329], deoxy-hemoglobin[311, 330, 331] and cytochrome P-450[332]. Recently, we reported that eNOS has the capacity to reduce nitrite to NO under anoxic conditions *in vitro*[123]. eNOS catalyses the synthesis of NO from L-arginine via an oxygen-consuming pathway. Since the conventional arginine pathway is blocked under conditions of low oxygen tension[122], we speculated that the newly discovered anoxic nitrite reductase pathway of eNOS might provide a significant alternative source of NO for tissues under acute hypoxia. We demonstrate that the absence of oxygen causes a considerable enhancement of the NO release from endothelial cells. The reaction mechanism is enzymatically catalysed by nitric oxide synthase and may be modulated by exogenous membrane-penetrating agents.

Materials and methods

Diethyldithiocarbamate (DETC) was purchased from Sigma, ferrous sulphate from Fluka and cell culture materials from Life Technologies. Oxypurinol, the haem inhibitor imidazole and the NOS inhibitors Nω-nitro-L-arginine (NLA) and Nω-nitro-L-arginine-methylester (L-NAME) were from Sigma. Argon was purchased as compressed gas from Hoekloos and used without further processing.

An immortalized murine microvascular brain endothelial (bEnd.3)[128] cells was kindly provided by Dr. Alan Schwartz (University of Washington, St. Louis). Cells were cultured in DMEM containing 10% FCS, 2 mM L-glutamine, 10 IU/mL penicillin and 100 µg/L streptomycin, at 37° C under a controlled atmosphere containing 5% CO_2 and 20% O_2. L-arginine concentrations were sufficient, avoiding depletion even at high levels of NOS activity. A confluent monolayer of cells consisted of $7.5 \pm 0.5 \cdot 10^6$ bEnd.3 cells. Previous to each experiment, confluency was verified by optical inspection via a stereomicroscope and cells were counted.

Prior to NO trapping, the culture medium was replaced with 10 ml fresh DMEM of 37° C containing 2.5 mM DETC. Trapping was initiated by adding ferrous sulphate (10 µM final). It should be noted that no stimulus for NO production was given to the cells. Trapping proceeded either in a normoxic atmosphere (5% CO_2 / 20% O_2) or in an anoxic argon atmosphere at 37° C.

Anoxia was induced by replacing the medium with argon-bubbled DMEM containing 2.5 mM DETC and flushing the culture flask with argon before closing it with an airtight top. During flushing with argon temperature of the medium was kept at 37° C. No chemical stimulus for NO production was administered. After 20 min, cells

were placed on ice to terminate enzymatic activity. Cells were harvested and the cellular fraction containing the Fe-DETC complexes was separated by centrifugation (1000 g for 10 min at 4º C), resuspended in 350 mL incubation medium, pipetted into a syringe (id 4.8 mm), snap frozen and stored in liquid nitrogen until assayed with electron paramagnetic resonance (EPR). If applicable, inhibitors like NLA, L-NAME and oxypurinol were administered 20 min prior to initialisation of NO trapping. During the subsequent 20 min of NO trapping the inhibitors were also present. The general haem inhibitor imidazole was administered 1 min prior to initialisation of NO trapping. In one experiment, L-NAME was administered by preincubation for 2 min instead of the usual 20 min. Alternatively, 250 µM exogenous sodium nitrite was administered 20 min prior to NO trapping.
EPR spectra were recorded at 77 K on a modified X-band ESP 300 radiospectrometer (Bruker BioSpin, Karlsruhe, Germany) operating near 9.54 GHz with 20 mW power. The frozen samples were placed in a quartz liquid finger dewar at the center of a Bruker ER4103TM cavity. The field modulation was 0.5 mT, gain $2 \cdot 10^5$, time constant and ADC conversion time 82 ms. Four scans were accumulated to reduce instrumental noise. The spin densities were calibrated with frozen reference solutions of paramagnetic NO-Fe^{2+}-$(MGD)_2$ in PBS buffer.
Intracellular nitrite concentrations were determined using the nitrite colorimetric Griess reagent (Cayman Chemical, ITK diagnostics, Uithoorn). For $7.5 \cdot 10^6$ endothelial cells in 0.5 ml lysis buffer, the detection limit is 0.2 µM. This corresponds to a total quantity of 100 pmol intracellular nitrite.
Cell viability after anoxia was verified by trypan-blue staining. Staining and counting were performed within 15 minutes after readmission of ambient air.

Results

NO trapping in $7.5 \pm 0.5 \cdot 10^6$ bEnd.3 cells under normoxic conditions during 20 min yielded 110 ± 8 pmol paramagnetic NO-Fe^{2+}-DETC mononitrosyl-iron complexes (MNIC) as detected by EPR (figure 1a). This basal yield was obtained without any stimulus. The yield was reproducible within 10% from flask to flask and day to day. A typical EPR spectrum (figure 1, spectrum a-d) showed a clear triplet hyperfine structure (HFS) centered at g = 2.035. As expected for biological samples[175], a small contribution from paramagnetic Cu^{2+}-DETC complexes was superposed, with its most intense central hyperfine line visible near g = 2.01 (figure 1). Preincubation for 20 min with 57 µM NLA reduced the MNIC yield to below the detection limit of 10 pmol at the given spectrometer settings. At 5 µM NLA, a small yield of 25 ± 2 pmol MNIC was detected (figure 1b). Stimulation of bEnd.3 cells with calcium ionophore (A23187; 5 µM) considerably increased NO production, resulting in a MNIC yield of 400 ± 40 pmol (data not shown).

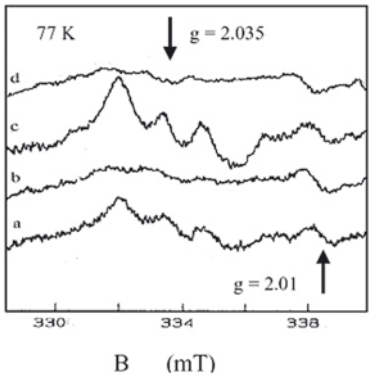

Figure 1 EPR spectra at 77 K from the cellular fraction of 7.5 ± 0.5 · 10^6 endothelial cells after NO trapping at 37° C with iron-dithiocarbamate complexes.
The triplet near g=2.035 is identified as the nitrosylated MNIC adduct. The absorption line near g=2.0 is the most intense hyperfine line of paramagnetic Cu^{2+}-DETC complexes[175]. (a) 110 pmol MNIC formed during 20 min under a controlled atmosphere (5% CO_2 / 20% O_2). (b) 25 pmol MNIC formed during 20 min after preincubation with 5 µM NLA (5% CO_2 / 20% O_2). (c) 160 pmol MNIC formed after 20 min anoxia. (d) 33 pmol MNIC formed during 20 min anoxia after preincubation with 5 µM NLA.

Argon-induced anoxia resulted in a major increase in MNIC yields as compared to basal normoxic yields. In the absence of inhibitors, the anoxic yield from 7.5 ± 0.5 · 10^6 bEnd.3 cells increased to 160 ± 10 pmol MNIC (figure 1c). Preincubation with 57 µM NLA completely abolished MNIC yield under anoxia. An anoxic yield of 33 ± 2 pmol MNIC was detected in the presence of 5 µM NLA (figure 1d), slightly higher than observed in the presence of oxygen. The intensity of the EPR absorption from paramagnetic Cu^{2+}-DETC complexes was not affected by anoxia.
Preincubation with 5 µM L-NAME for 20 min decreased the anoxic MNIC yield to 85 ± 8 pmol. The same yield was obtained when cells were preincubated with L-NAME for only 2 min prior to anoxia. Addition of 50 µM L-NAME further reduced the anoxic yields to 40 ± 5 pmol. Preincubation with 10 mM of the haem inhibitor imidazole diminished both normoxic and anoxic yields to 80 ± 10 pmol. In contrast, the xanthine oxidase inhibitor oxypurinol (100 µM) did not affect the anoxic or normoxic MNIC yields.
Addition of extracellular nitrite did not affect the MNIC yields, neither in the presence nor absence of oxygen. However, reduction with 20 mM sodium dithionite during 10 min enhanced the MNIC yields more than an order of magnitude to 4 nmol. In all cases considered, the supernatant liquids were free of any EPR signals from MNIC or Cu^{2+}-DETC, attesting to the complete recovery of the paramagnetic complexes by separation of the cellular fraction via centrifugation.

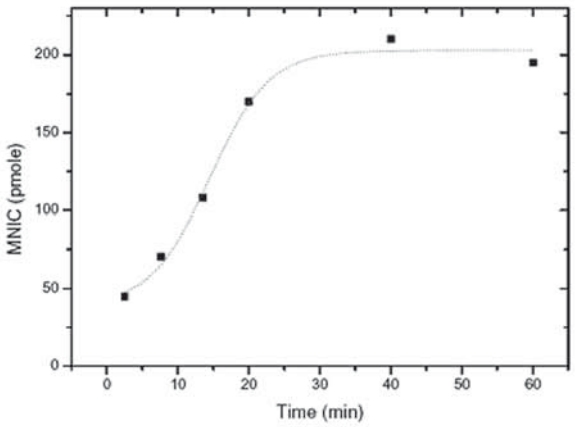

Figure 2 Kinetics of the formation of MNIC adducts in 7.5 ± 0.5 · 10^6 endothelial cells. Anoxia is applied at 0 min. The detection limit is 10 pmol MNIC, and the experimental error 10%.

The kinetics of MNIC formation under anoxia was studied as a function of time (figure 2). The MNIC yield linearly increased in time up to 30 min after induction of anoxia. The signal intensity saturated at an asymptotic value of 200 ± 20 pmol MNIC.
The pH of the DMEM was affected by the imposition of anoxia: Immediately after removal from the controlled atmosphere (5% CO_2 / 20% O_2), the pH was 7.5 ± 0.1. Equilibration with ambient air increased the pH to 8.4 ± 0.1, attesting to the small buffering capacity of DMEM medium with respect to the presence of carbon dioxide in the ambient atmosphere. Exposure to anoxia for 20 min enhanced the pH of the DMEM to 9.4 ± 0.2. Under anoxic conditions, the pH increased in time for up to 30 min (data not shown). The rise in pH after anoxia was unaffected by addition of NLA, L-NAME, oxypurinol or imidazole.

Discussion

The main and most prominent result from our experiments is the increased MNIC yield in the bEnd.3 cells upon introduction of anoxia. The magnitude of the anoxic NO production is surprising, as the regular enzymatic pathway for NO production from arginine is blocked due to lack of oxygen. In view of our earlier *in vitro* experiments[123], we attribute the anoxic NO production to the reduction of intracellular nitrite by NOS. The significant suppression of the MNIC yields by imidazole suggests that a haemprotein is involved in the observed NO production. The dose dependent inhibition by NLA and L-NAME inhibitors is specific for NOS. We did not attempt to estimate the IC50

dosages as these depend on the (unknown) intracellular L-arginine concentration, but we noted that the inhibitory doses for the anoxic pathway were comparable with those found for the regular arginine pathway. This suggests that, in absence of oxygen, nitro-arginine-inhibitors act via direct competition with arginine for the arginine binding site of NOS, analogous to the inhibition of the arginine pathway under normoxia. The complete abolishment of the MNIC yield by NOS inhibitors shows that NOS is the dominant mediator in bEnd.3 cells. Although anoxia may stimulate acidification of the intracellular compartment[325], the inhibition of NO release by NLA and L-NAME excludes acidic reduction of intracellular nitrite. In addition, XOR could be ruled out explicitly as a significant source of NO in bEnd.3 cells under hypoxia. Although this flavoenzyme is expressed in endothelial cells and has proven nitrite reductase activity under anoxic conditions[327-329], the anoxic NO yields in bEnd.3 cells were not affected by addition of the XOR inhibitor oxypurinol.

In addition to enzymatic NO production, chemical sources of NO should also be considered. There are five possible chemical NO sources: Extracellular nitrite, intracellular nitrite, nitrate, arginine and endogenous nitrosothiols. Since the MNIC yield did not increase upon administration of extracellular nitrite prior to anoxia, extracellular nitrite can be ruled out as the source of anoxic NO. It is conceivable that the timescale for equilibration of intra- and extracellular nitrite is long compared to our experiments, since nitrite anions do not spontaneously cross the membrane barrier and the degree of protonation is very small at physiological pH (pKa = 3.2). Nitrate can be excluded since its reduction requires either extreme reductive conditions far beyond those found in cell cultures, or the presence of specific catalysing metal ions that are lacking in our assay. Arginine can also be ruled out as a direct source since arginine oxidation requires the presence of dioxygen. Although some residual spurious oxygen may still be present in our system just after imposition of anoxia, it is inconceivable that the arginine pathway remains active for up to 30 min, let alone enhances its activity by 50%. It is known that the throughput of the arginine pathway collapses when dioxygen concentrations drop below 20 μM[175]. In contrast, arginine presumably is the indirect source of NO and its oxidised downstream metabolites found in bEnd.3 cells. Therefore, isotopic labeling of arginine with ^{15}N isotopes will not discriminate between the various potential sources of NO since all NO metabolites in the system would ultimately end up being labeled. Endogenous nitrosothiols can be excluded considering that their NO release is known to be catalysed by reduced transition metal ions. In addition, NO formation from nitrosothiols should remain unaffected in the presence of NOS inhibitors, contrary to our observations. Having ruled out extracellular nitrite, nitrate, arginine and nitrosothiols, intracellular nitrite remains as a plausible source of NO released under hypoxia. In line, our previous data have shown that eNOS is capable of nitrite reduction under hypoxia *in vitro*[123].

The kinetics of anoxic NO release showed that the MNIC yield increased linearly with

time for up to about 30 min (figure 2). This behaviour demonstrates a steady rate of formation of 200 pmol/20 min ~ 10 pmol MNIC/min in the early stages of anoxia. The total NO production is likely to be higher, since a fraction of the produced NO will be bound in the form of EPR-silent diamagnetic NO-Fe^{3+}-DETC complexes and a part will be lost via other reaction pathways. The total NO release in a confluent monolayer of bEnd.3 cells (75 cm^2) should be around 20-50 pmol NO per min. With an estimated intracellular volume of 75 µl (see below), this is equivalent to 0.3-0.7 pmol NO · min^{-1}· (mg endothelial cells)$^{-1}$. This rough estimate corresponds remarkably well with the estimate of 0.8 pmol NO · min^{-1} · (mg endothelial cells)$^{-1}$ as basal yield in humans[185, 186].

After 30 min, the MNIC yield is stationary. Addition of exogenous nitrite and subsequent reduction with dithionite resulted in the formation of 4 nmol Fe-DETC traps, which is at least an order of magnitude larger than the asymptotic yield of 200 pmol MNIC and proves that only a small fraction of traps actually binds NO under the conditions used. These results indicate that the saturation in the formation of MNIC is not due to an artefact caused by depletion of Fe-DETC traps, but that the formation of NO under anoxia ceases after 30 min. Several explanations for this observation seem plausible. First, sustained anoxia can cause irreversible damage to the cells and change the chemical composition of the intracelular compartment. Second, haemproteins, including eNOS, are potential targets for inhibition by NO through nitrosylation of the haem moiety[175]. We have previously reported such self-poisoning of eNOS in experiments with anoxic nitrite reduction[123]. In the present study, however, self-poisoning of the eNOS is inconceivable since the abundance of Fe-DETC traps acts as an efficient NO sink, diminishing the concentration of free NO radicals. The third, and in our opinion the most plausible explanation for the ceasing of NO formation after 30 min is the depletion of intracellular nitrite. With the cellular monolayer being approximately 10 µm in height, we estimate the total intracellular volume in a confluent monolayer of bEnd.3 cells (75 cm^2 flask) as 75 cm^2 · 10 µm = 75 µL. With a reasonable intracellular nitrite concentration of 10 ± 1.5 µM, the intracellular nitrite pool is 750 ± 150 pmol in total. This estimate suggests that nitrite depletion might well be the reason for the MNIC yields to become stationary after 30 min. The reason for the saturation behaviour was not further investigated, since 30 min of anoxia is likely to cause many changes inside the cells, thereby making their physiology less and less representative for actual *in vivo* tissue endothelium as time proceeds. Instead, the magnitude of the anoxic NO release and, in particular, its extended duration, indicate that the nitrite reductase capacity of eNOS is a remarkably robust reaction mechanism.

The strong and sustained NO release from the anoxic endothelial cell cultures suggests that eNOS mediated nitrite reduction is physiologically relevant for NO levels near endothelium under acute hypoxia.

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Chapter 9

Summary & Perspectives

In the vasculature, the simple gas nitric oxide (NO) is generated by endothelial nitric oxide synthase (eNOS), where it regulates vascular tone (reviewed in [4]) and affects endothelial transcription[235]. Reactive oxygen species (ROS) play a role in signal transduction and are involved in the regulation of the biologically effective concentration of NO[6]. In vascular disease states, excessive production of ROS may overwhelm the antioxidant defence mechanisms of cells, resulting in oxidative stress[7]. In this thesis the activity of eNOS is discussed with specific attention to the effects of its substrates, modulators and products. An overview of the principal results is shown in figure 1-6.

Throughout the studies, a microvascular endothelial cell line (bEnd.3)[128] is used. These cells express high levels of eNOS protein and produce large amounts of NO in comparison with primary endothelial cells, e.g., human umbilical vein endothelial cells (HUVEC) or a human microvascular endothelial cell line (CDC.HMEC-1) while retaining the functional properties of endothelial cells[129]. Use of bEnd.3 cells facilitates the detection of subtle differences in NO production as a consequence of treatment with agonists or antagonists. In addition, bEnd.3 cells lack neuronal or inducible NOS and NADPH oxidase does not contribute to ROS production in these cells[129], therefore this cell type is very useful to study eNOS activity and uncoupling. In HUVECs or CDC.HMEC-1 other NOS isoforms or NADPH oxidase will most likely contribute to the effects on NO and ROS production, which will make it very hard to distinguish between effects caused by eNOS itself or by other NO or ROS sources.

Detection of NO

As described in chapter 2, detection of NO in biological systems is very difficult. NO is a highly reactive radical with a short half life. Systemic levels of NO usually remain in the nanomolar range. In addition, reactivity of reactive oxygen and nitrogen species towards detector molecules is often not specific.
In chapter 3 we used electron paramagnetic resonance (EPR) to quantify basal NO production in bEnd.3 cells. In addition, we aimed to determine the mechanism of NO trapping by iron-dithiocarbamate complexes. We were especially interested in the redox state of the iron complex which traps the NO radical and the redox pathways involved in the transition of the mononitrosyl complexes to a ferrous paramagnetic state.
We found that EPR is an excellent technique to quantify subtle changes in NO production in endothelial cells. During spin trapping experiments in bEnd.3 cells, NO binds to the haem centre of a Fe^{3+}-DETC complex, resulting in the formation of diamagnetic NO-Fe^{3+}-DETC, which is undetectable by EPR (figure 1). A fraction of the formed NO-Fe^{3+}-DETC complexes spontaneously reduced to the paramagnetic NO-Fe^{2+}-DETC complex, which is detectable by EPR, however, this reaction was very

slow. Addition of exogenous dithionite significantly augmented the rate of the reduction reaction, resulting in a considerable increased yield of the paramagnetic NO-Fe^{2+}-DETC complex. Thus dithionite proved to be an effective tool to ameliorate NO detection by EPR. Nevertheless, dithionite should be used with care, since reduction of endogenous nitrite may induce artificial release of NO. The pH should be maintained at 7.4 by means of a sufficiently strong buffer in order to avoid this artefact.

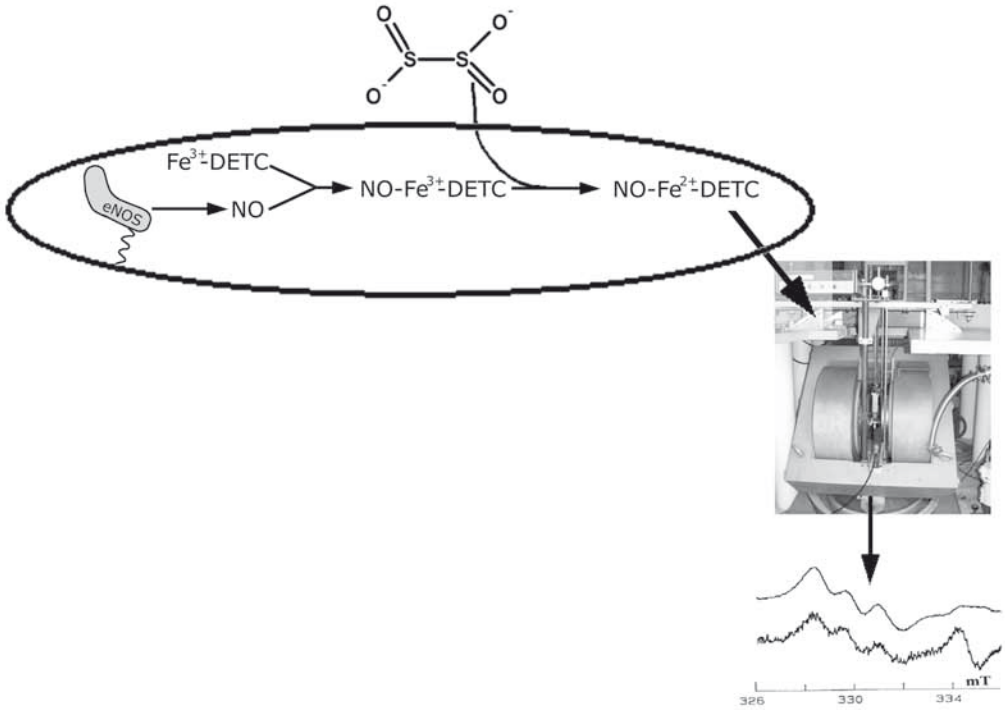


Figure 1. Chapter 3: NO measurements.
NO binds to ferric Fe^{3+}-DETC to form diamagnetic NO-Fe^{3+}-DETC. Dithionite reduces NO-Fe^{3+}-DETC to the paramagnetic NO-Fe^{2+}-DETC complex, which is detectable by EPR. eNOS, endothelial nitric oxide synthase; Fe-DETC, iron diethyldithiocarbamate; NO, nitric oxide; SO_4^{2-}, dithionite

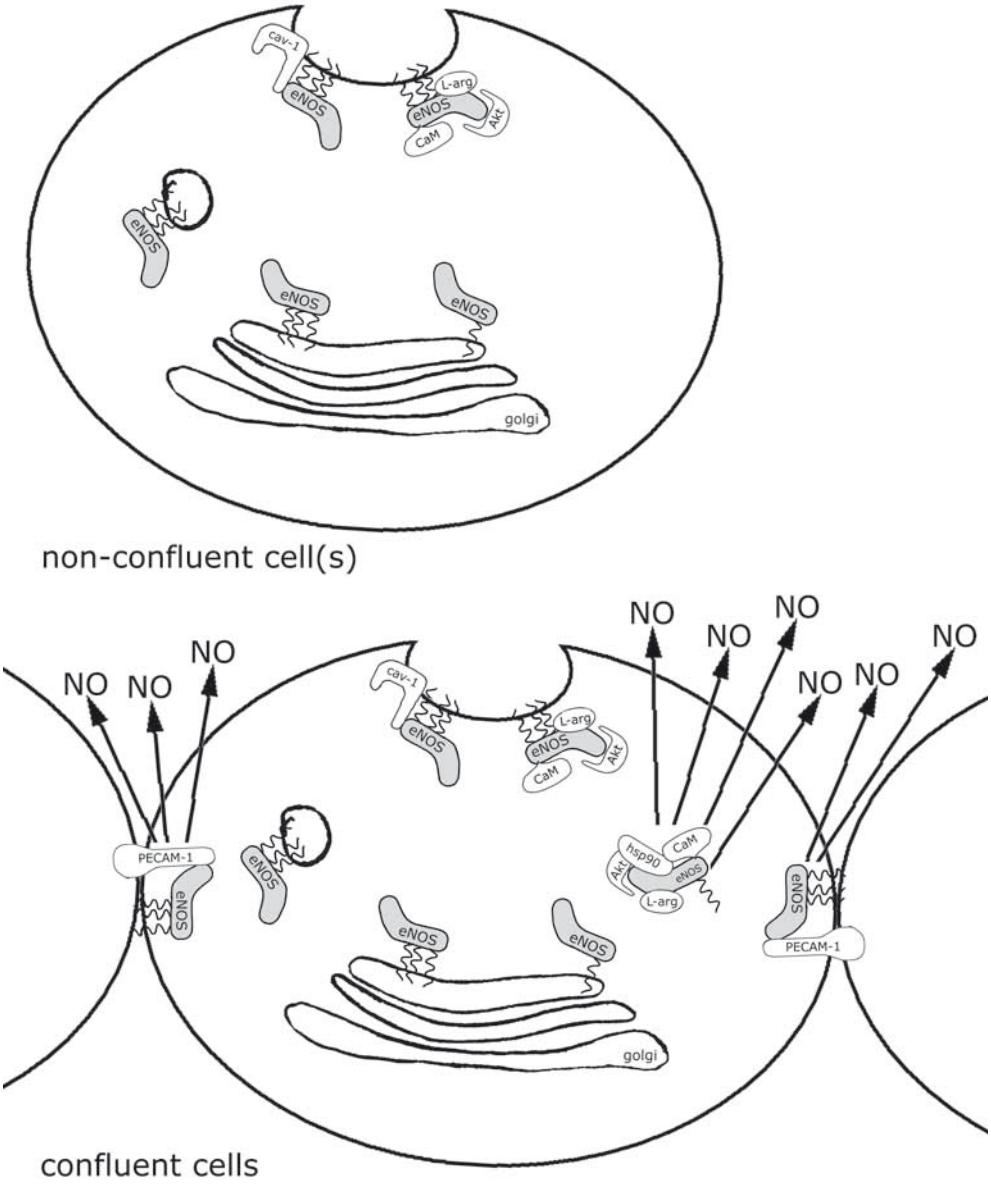


Figure 2. Chapter 4: eNOS localisation.
eNOS localises at cell-cell contact sites, where it is associated with PECAM-1. Presence of eNOS at rafts is essential for eNOS activity and NO production. Akt, protein kinase B; Ca^{2+}, calcium; CaM, calmodulin; cav-1, caveolin-1; eNOS, endothelial nitric oxide synthase; hsp90, heat-shock protein 90; L-arg, L-arginine; NO, nitric oxide; PECAM-1, platelet-endothelial cell adhesion molecule

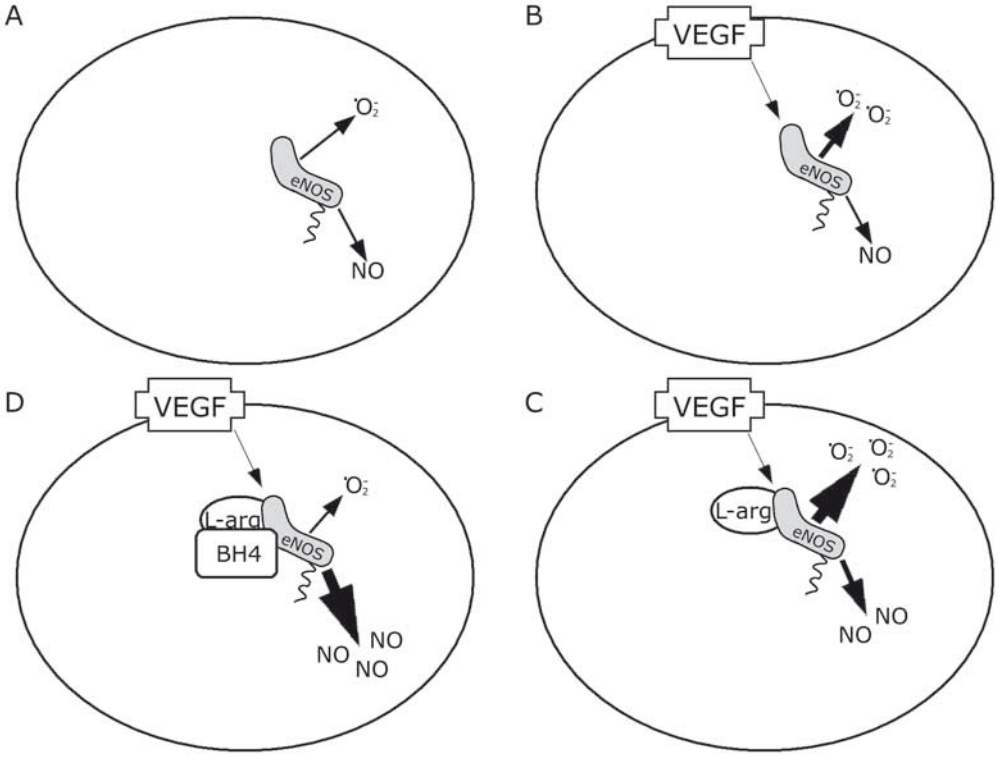


Figure 3. Chapter 5: Substrates and cofactors.
In bEnd.3 cells eNOS is partially uncoupled (A), which is enhanced by stimulation with VEGF (B). High levels of L-arginine further increased eNOS uncoupling and subsequent ROS production (C). Addition of BH4, both in the presence and absence of high levels of L-arginine, prevented eNOS uncoupling to a substantial degree (D). BH4, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; L-arg, L-arginine; NO, nitric oxide; $O_2^{\cdot-}$, superoxide; VEGF, vascular endothelial growth factor

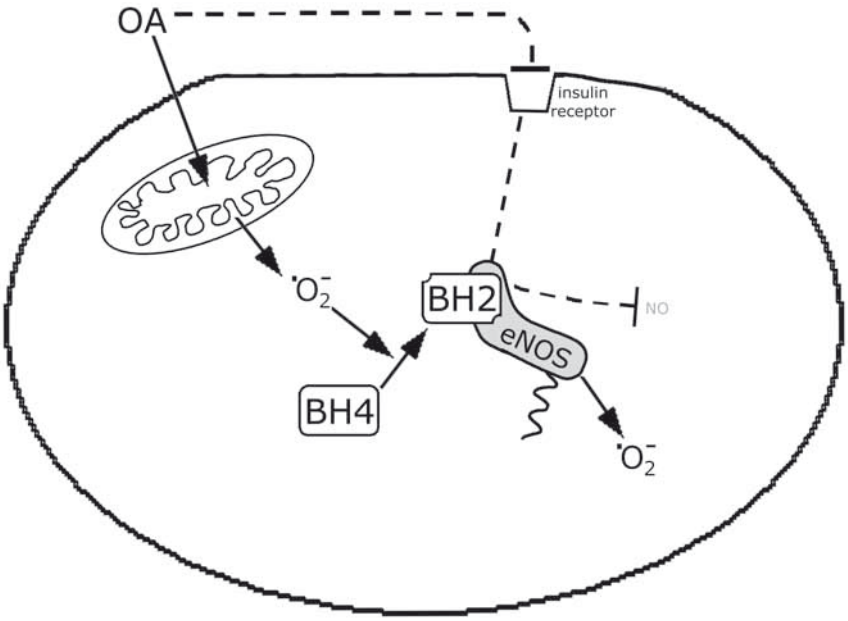
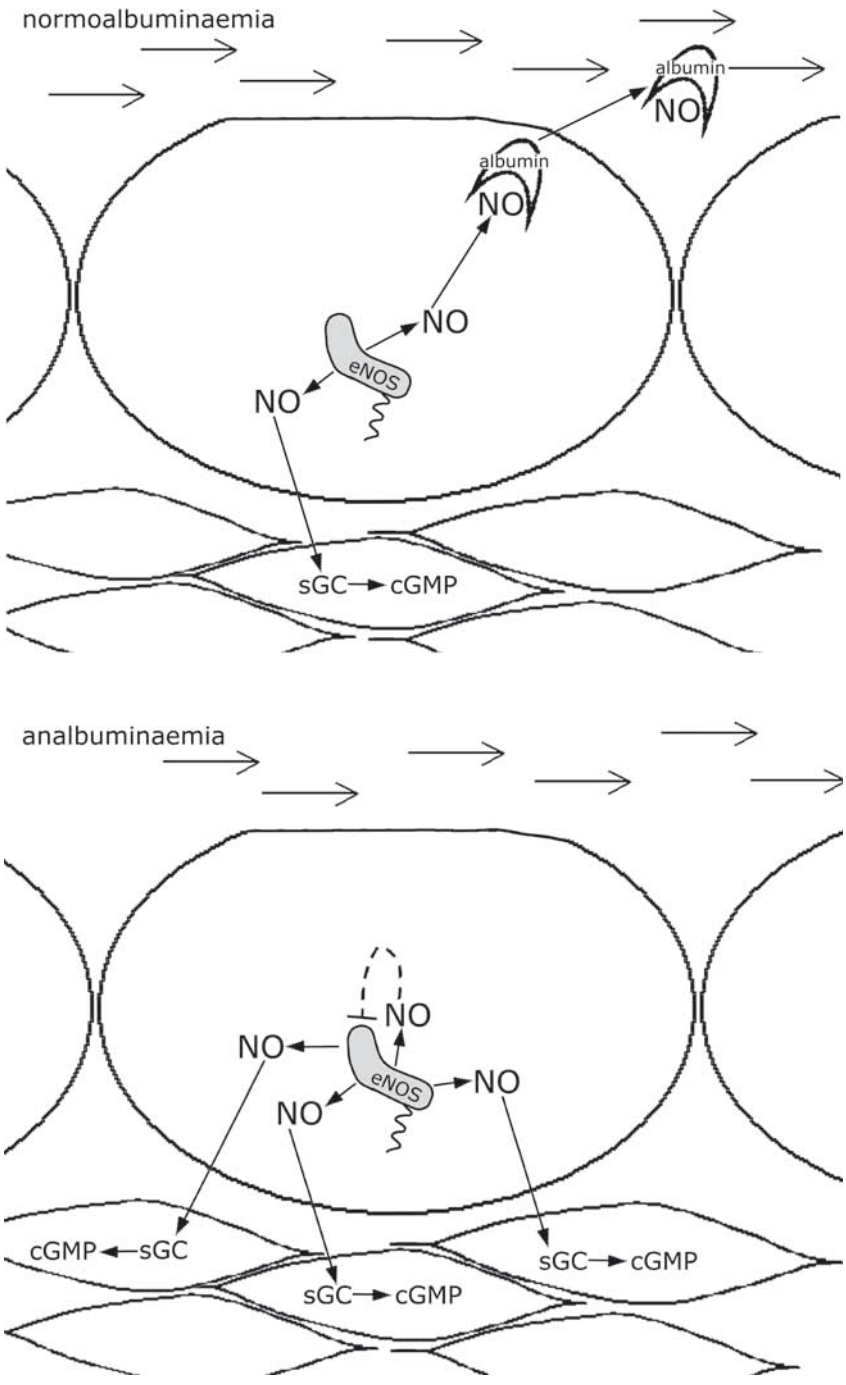


Figure 4. Chapter 6: Hyperlipidaemia.
OA increases ROS production in bEnd.3 cells, which is predominantly generated by mitochondria. In the presence of OA, both basal and insulin-induced eNOS activity is decreased. OA presumably inhibits the insulin receptor at the plasma membrane of bEnd.3 cells. In addition, mitochondrial-derived ROS probably reduces BH4 by oxidation to BH2, resulting in eNOS uncoupling. Indeed, addition of BH4 partially prevented the generation of OA-induced ROS. Dotted lines indicate inhibitory pathways. BH4, tetrahydrobiopterin; BH2, dihydrobiopterin; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; $O_2^{\cdot-}$, superoxide; OA, oleic acid

Figure 5. Chapter 7: Hypoalbuminaemia - model: analbuminaemia. →
eNOS activity and NO production are increased in the absence of albumin *in vitro*. In aortic rings of analbuminaemic rats ACh-induced relaxation is increased as compared to control. cGMP, cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; sGC, soluble guanylate cyclase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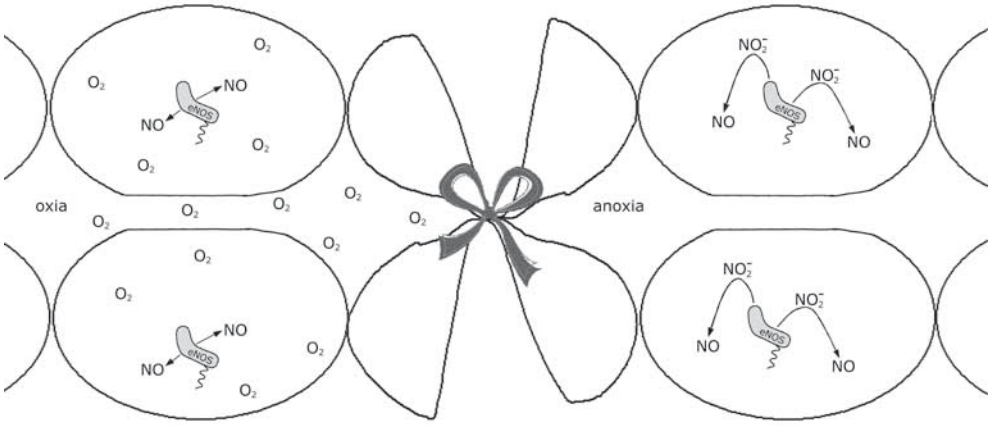

Figure 6. Chapter 8: Hypoxia - model: anoxia.
Under anoxic conditions, eNOS does not produce NO via common arginine-citrulline conversion. In contrast, eNOS is involved in the reduction of NO2- to NO, through the anoxic nitrite reductase pathway. Thus eNOS provides an alternative NO source in conditions deprived of oxygen. eNOS, endothelial nitric oxide synthase; NO, nitric oxide; NO_2^-, nitrite; O_2, oxygen

Localisation of eNOS

In chapter 4, we showed that localisation of eNOS at cell-cell contact sites may be important for regulation of vascular permeability. We aimed to determine how cellular localisation of eNOS and activity of the enzyme were correlated. Our data indicated that eNOS needs to be present at cell-cell contacts to become activated (figure 2). At these contact sites, eNOS is localised at membrane domains which also contain platelet-endothelial cell adhesion molecule-1 (PECAM-1).
The endothelial lining of the vessel wall is a permeable barrier, which is located at the interface between the vascular and the perivascular compartments. Vascular permeability of endothelial cells is controlled by regulation of the tightness of interendothelial junctions, regulation of transendothelial transport and the formation of focal adhesion complexes that promote binding of endothelial cells to the basement membrane[333]. The role of NO in regulating the relative permeability of the endothelial lining of the vessel wall is still a matter of debate. NO has been shown to increase microvascular permeability[334], but there is equally compelling evidence that NO decreases microvascular permeability[335]. These observations raise the possibility that NO production may tonically regulate endothelial barrier function.
PECAM-1 has been reported to physically associate with the eNOS complex at intercellular contact sites[35]. However, it is not known whether this association is a direct interaction of eNOS and PECAM-1 or whether other proteins are involved in the

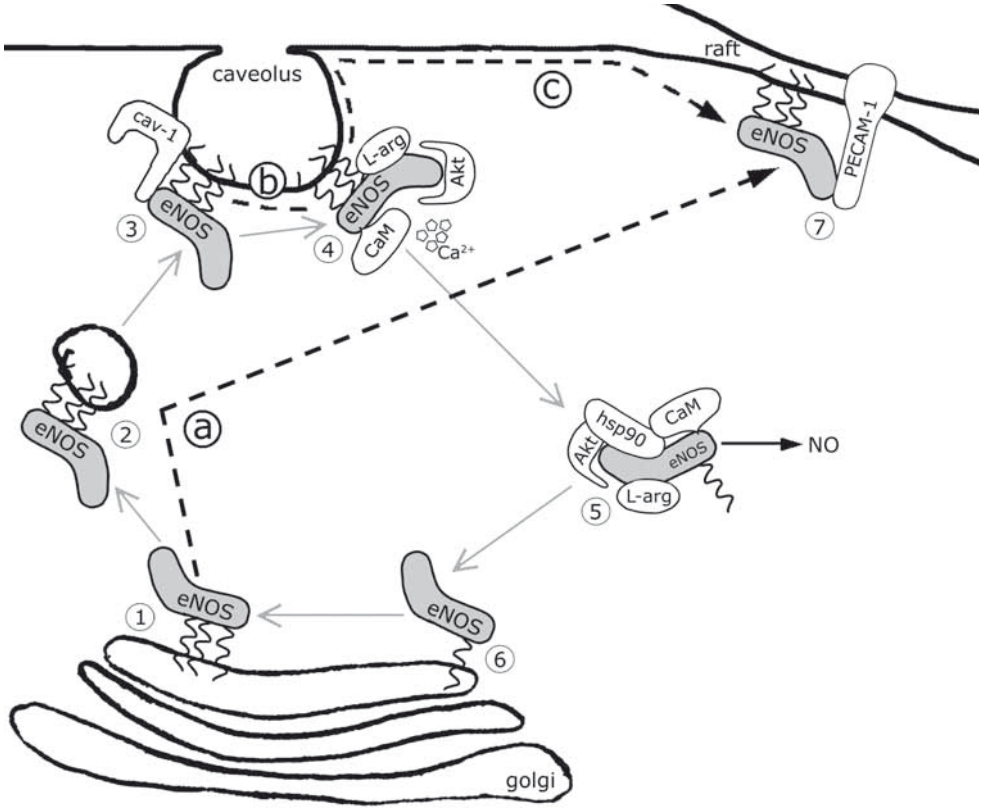

Figure 7. The eNOS activation/deactivation cycle (modified from [4]).
eNOS is translocated from the Golgi complex (1) to caveolae (3) via vesicular transport (2). When CaM binds to eNOS (4), the enzyme is translocated to the cytosol (5), where it produces NO. After deactivation, eNOS is relocated to the Golgi complex (6). At cell-cell contact sites, eNOS co-localises with PECAM-1 (7). The mechanisms that lead to the localisation of eNOS at rafts are unknown. It is possible that eNOS is directly transported from the Golgi complex to rafts, probably via vesicles (a). It is also conceivable that eNOS travels from caveolae to rafts via lateral diffusion through the plasma membrane, either while associated to cav-1 (b) or in the form of the semi-active complex with CaM and Akt (c). Akt, protein kinase B; Ca^{2+}, calcium; CaM, calmodulin; cav-1, caveolin-1; eNOS, endothelial nitric oxide synthase; hsp90, heat-shock protein 90; L-arg, L-arginine; PECAM-1, platelet-endothelial cell adhesion molecule

formation of the complex. We[36] and others[37, 38] have shown that receptor-stimulated activation of eNOS is enhanced in the presence of PECAM-1. However, the role of eNOS-PECAM-1 association is unclear. Upon stimulation of endothelial cells, both

decreased[35] and increased[38] protein interaction between eNOS and PECAM-1 has been reported.
The molecular and cellular mechanisms that lead to the localisation of eNOS at intercellular junctions remain to be determined. As outlined in chapter 1, eNOS localisation within the endothelial cell is determined by the activation/deactivation cycle (figure 7[4]). How eNOS is translocated to cell-cell contact sites where it co-localises with PECAM-1 is unknown. It can be speculated that the enzyme is directly transported from the Golgi complex to rafts at the plasma membrane, most likely via vesicles (figure 7, route a). Another possibility is that eNOS is translocated from caveolae to rafts via lateral diffusion through the plasma membrane as has previously been described for other proteins[336, 337], either while associated to caveolin-1 (cav-1; figure 7, route b) or in the form of the semi-active complex with calmodulin (CaM) and protein kinase B (Akt; figure 7, route c). Direct translocation of the fully activated, cytosolic eNOS to cell-cell contact sites is not likely to occur, since the eNOS-PECAM-1 complex dissociates during the first 60 seconds of eNOS activation[35]. Energetically it would be rather inconvenient for a cell to transport an active enzyme to a site where it is deactivated by interaction with another protein, only to activate the enzyme again in order for it to exert its actions. In order to solve this translocation problem, additional studies are needed.

In conclusion, NO plays an important role in the dynamic regulation of the intercellular junctions of the endothelium. eNOS is enriched at these junctions, which is a prerequisite for its activation by agonists. At the junctions, eNOS co-localizes with PECAM-1. The nature of the molecular and cellular mechanisms that lead to the localisation of eNOS at intercellular junctions remain to be determined.

Substrates and cofactors

In chapter 5, we hypothesised that under conditions of sustained high expression of eNOS, acute stimulation of eNOS induces uncoupling of the eNOS enzyme due to a relative shortage of substrate and/or cofactors resulting in superoxide production. We determined whether eNOS uncoupling occurs in bEnd.3 cells and if stimulation of the cells with VEGF would enhance eNOS uncoupling (figure 3). In addition, we verified whether uncoupling of eNOS was due to a shortage of L-arginine, tetrahydrobiopterin (BH4) or both. We demonstrated that in bEnd.3 cells, which chronically express high eNOS levels, eNOS is partially in the uncoupled state. Addition of vascular endothelial growth factor (VEGF) enhanced ROS production particularly at high L-arginine concentrations, which was corrected to a substantial degree by addition of exogenous BH4. These data suggest that under conditions of chronically increased eNOS expression and particularly if eNOS activity is stimulated, relative shortage of BH4 may lead to

eNOS uncoupling, resulting in superoxide production.
Although L-arginine is thought to prevent eNOS uncoupling, we[129] and others[64, 65] have shown that supplementation of L-arginine to a system with sustained high expression of eNOS may contribute to rather than reduce eNOS uncoupling. Since bEnd.3 cells do not express any neuronal NOS (nNOS) or inducible NOS (iNOS) and ROS producing systems, such as NADPH oxidase and xanthine oxidase (XO), are not active under these conditions, it can be concluded that the increased ROS production in the presence of excess L-arginine is generated by eNOS, due to uncoupling.
The results of the study described in chapter 4 show that simple administration of L-arginine to improve endothelial function is not the answer to inhibit atherogenesis. In patients with cardiovascular disease, the data for the benefits of L-arginine supplementation are inconsistent; in fact, L-arginine may worsen existing endothelial dysfunction. More experimental work is required to determine in which patients L-arginine therapy will be consistently beneficial, and in which it should be used with caution.

Metabolic influences

Elevated levels of free fatty acids
In chapter 6, the effect of oleic acid (OA), a monounsaturated fatty acid, on eNOS activity and NO production *in vitro* is described. We found that OA increased ROS and decreased NO production in bEnd.3 cells (figure 4).
A possible mechanism by which treatment of bEnd.3 cells with OA lead to a decrease in eNOS activity is by disturbance of intracellular calcium (Ca^{2+}) concentrations, which has been described previously for endothelial cells[338-340]. Under physiological conditions, mitochondria play a central role in the gating of store-operated channels[341, 342], which is the major Ca^{2+} entry pathway in endothelial cells[79]. Mitochondrial overload, i.e. by elevated FFA levels, leads to inhibition of this capacitative or store-operated Ca^{2+} entry (CCE)[338-340]. Since eNOS is Ca^{2+}-dependent, disturbance of CCE may contribute to diminished eNOS activity[338]. We showed that OA attenuated eNOS activity under basal conditions. Stimulation with calcium ionophore significantly enhanced eNOS activity. In fact, eNOS activity increased much more in the presence of OA as compared to basal circumstances. This indicates that diminished eNOS activity in the presence of OA is not due to a defect in calcium signalling.
NO plays an important role in the regulation of insulin sensitivity and arterial pressure. We showed that OA decreased insulin-induced NO production in bEnd.3 cells, most likely due to a potential defect at the level of the insulin receptor. In line with these results, it has been reported that an oral fat load containing primarily oleic acid significantly impaired endothelial function in healthy volunteers, as measured by

flow-dependent vasodilatation in the fore-arm[276]. It has been shown that endothelial function and levels of oxidative stress are affected by fatty acids[264, 343]. In addition to the total amount of fat, the composition of dietary fat is an important contributor to the postprandial response[344, 345]. A diet predominantly containing unsaturated fatty acids has been shown to decrease mortality due to coronary heart disease as compared to diets consisting of mainly saturated fatty acids[99, 100]. In patients suffering from insulin resistance, stimulation of eNOS with insulin is impaired[101], most likely due to increased levels of free fatty acids (FFA)[102].
Interestingly, eNOS knockout mice are insulin-resistant and hypertensive[346, 347]. eNOS deficiency in humans has not been reported so far, therefore extrapolation of the findings in eNOS knockout mice to humans is problematic. However, there is evidence that cardiovascular disease states such as hypertension, coronary artery disease and myocardial infarction are associated with eNOS gene polymorphisms[348-350] and impaired NO synthesis[348], which could predispose to insulin resistance[351]. Partial deletion of the eNOS gene does not alter insulin sensitivity or blood pressure in mice under normal conditions[352]. However, during metabolic stress, i.e. elevated levels of FFA, eNOS deficiency leads to increased insulin resistance and arterial hypertension in mice. In addition, it has been reported that free fatty acid elevation is a highly significant risk factor for hypertension[353]. In conclusion, a Western type diet may predispose to the development of insulin resistance and arterial hypertension.

We reported that increased levels of OA lead to mitochondrial-derived ROS production in bEnd.3 cells, which may, in turn, contribute to diminished eNOS activity. Oxidation of BH4 by ROS leads to a decrease in BH4 levels which may subsequently lead to uncoupling of eNOS. Inhibition of the mitochondrial respiratory chain with thenoyltrifluoroacetone (TTFA) and addition of BH4 only partly restored eNOS activity and prevented OA-induced ROS production. Co-treatment of TTFA and BH4 did not further abolish OA-induced ROS production. NADPH oxidase and XO were excluded as potential sources of ROS production, as specific inhibitors of these enzyme systems did not have any effect on OA-induced ROS production. This implies that either an additional ROS source is present or that the antioxidant capacity is decreased in bEnd.3 cells.
Glutathione peroxidases are a family of antioxidant enzymes that utilize glutathione in the reduction of hydrogen peroxide and alkyl hydroperoxides. Among the various glutathione peroxidases, phospholipid hydroperoxide glutathione peroxidase (PHGPx) is an antioxidant enzyme involved in detoxification of lipid hydroperoxides in cellular membranes and lipoproteins[354]. This enzyme may play a critical role in antioxidant protection against oxidative stress induced by FFA. It has been shown that OA increased PHGPx mRNA levels in HUVECs[355], but whether this occurs in bEnd3 cells remains to be shown.

Oxidation of the zinc-thiolate cluster in eNOS by peroxynitrite ($ONOO^-$) has been proposed as a mechanism of eNOS uncoupling[356]. However, the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) was reported to prevent zinc loss from eNOS and thus prevent uncoupling of the enzyme. Since L-NAME did not prevent OA-induced ROS production in our study, oxidation of the zinc-thiolate cluster can be excluded as a potential ROS source.
Heat shock protein 90 (hsp90) may also play a role in eNOS-uncoupling. Inhibition of hsp90 prevents its association with eNOS, resulting in eNOS uncoupling[357]. The expression of heat shock proteins is increased when cells are exposed to elevated temperatures. In addition, increased expression of heat shock proteins can also be triggered by oxidative stress. Incubation of bEnd.3 cells with OA leads to the formation of $O_2^{\cdot-}$ by mitochondria. Presumably, the production of ROS leads to upregulation rather than inhibition of hsp90. Therefore it is questionable whether eNOS uncoupling is caused by inhibition of hsp90 in the presence of OA. However, to confirm these speculations, expression levels and activity of hsp90 should be determined.
Increased levels of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) may also cause eNOS uncoupling[63]. ADMA levels are affected by oxidative stress and are associated with abnormalities in lipid regulation, although to date, no effect of FFA on ADMA has been reported[358, 359]. In addition, increased ADMA levels decrease L-arginine availability, resulting in eNOS uncoupling. However, we[129] and others[64, 65] have shown that supplementation of L-arginine may contribute to the generation of ROS via eNOS uncoupling and subsequent atherosclerotic lesion formation. Therefore, it is unlikely that increased ADMA levels contribute to OA-induced ROS production in bEnd.3 cells.

Hypoalbuminaemia
In chapter 7, we determined eNOS activity and NO production *in vitro* and endothelium-independent and endothelium-dependent aortic vascular function *ex vivo* under albumin-free conditions. We used cultured endothelial cells under analbuminaemic (<1 g/L) conditions and analbuminaemic rats to study the effect of acute and chronic exposure to low albumin levels as such, in the absence of other systemic factors. We found that NO production *in vitro* was not decreased, but in fact increased in the absence of albumin (figure 5). These results are in line with observations by others that the causes of hypoalbuminaemia (<35 g/L) rather than reduced albumin levels itself explain the relation with endothelial dysfunction in chronic kidney disease (CKD) and proteinuric conditions[112, 113, 115, 300].
It should be acknowledged that analbuminaemic rats are born without albumin. Therefore it is conceivable that these animals have developed a complex and chronic adaptation system to account for the absence in albumin. We used an *in vitro* analbuminaemic model to examine the direct effect of albumin on eNOS activity and NO production,

thereby purposely excluding the influences of other factors that are certainly present in the *in vivo* situation. Similar considerations resulted in the use of vascular rings from male analbuminaemic rats. Although analbuminaemic rats are not the ideal model to study hypoalbuminaemia, it is a model that is not 'contaminated' by inflammation or renal disease. In addition, dyslipidaemia is mild in the male animals, excluding a change in lipid levels as a possible cause for the observed effects[310].

Albumin has many important effects in the physiology of normal health. It binds and transports a number of substances, such as NO, free fatty acids, calcium and drugs including aspirin and penicillin[360]. The NO-binding capacities of albumin explain the anticoagulant, antithrombotic and vasodilatory characteristics of the protein[302]. It plays a role in the scavenging of ROS and other toxins[361] and albumin accounts for 75-80% of the colloid osmotic pressure of human plasma[362]. Despite these important functions, hereditary analbuminaemia is compatible with life, as is proven by the fact that analbuminaemic rats have normal renal function and blood pressure and are able to reproduce[363, 364].
Hypoalbuminaemia is associated with CKD and cardiovascular events. However, administration of albumin to patients suffering from hypoalbuminaemia does not improve survival or reduce morbidity. In fact, it has been shown that albumin infusion in critically ill hypoalbuminaemic patients resulted in a higher mortality as compared with control groups[365]. In addition, analbuminemia in humans is generally only found as an incidental laboratory finding, rather than presenting a disease[362]. In line with our results, these observations suggest that the cause of hypoalbuminaemia and not low albumin levels per se, is associated with morbidity and mortality.

Hypoxia

In chapter 8, we studied the effect of hypoxia on eNOS function and activity. We used a model of anoxia and determined whether eNOS provides an alternative source of NO for tissues under these conditions, via the anoxic nitrite reductase pathway. We reported a marked eNOS-dependent increase in NO production in bEnd.3 cells under anoxic conditions, indicating that eNOS indeed serves as an alternative source of NO (figure 6). Under hypoxic conditions where vasodilatation is most required, enzymatic activity of eNOS is blocked, since the conversion of L-arginine into L-citrulline and NO requires oxygen[122]. Several factors have been shown to reduce circulating nitrite (NO_2^-) to NO during hypoxia[123, 124], in addition to eNOS[123].

Hypoxia has been associated with both up- and downregulation of eNOS expression. In rats exposed to hypoxia aortic levels of eNOS mRNA were decreased[366], whereas

increased eNOS mRNA and protein levels were found in the lungs[367, 368]. In addition, in cultured bovine aortic endothelial cells[369] and porcine aortic endothelial cells[370] exposed to hypoxia eNOS mRNA and protein levels were increased, whereas hypoxia decreased eNOS mRNA and protein expression in HUVECs[371] and bovine pulmonary endothelial cells[372]. The reason for the variability of eNOS expression is unknown, but it may reflect differences in species, the vascular bed from which the endothelial cells were derived, and the duration and severity of hypoxic exposures.
Since eNOS is involved in the reduction of NO_2^- to NO, it is conceivable that increasing the (intracellular) levels of the substrate NO_2^-, increasing eNOS activity or upregulating eNOS protein expression may protect against ischaemic injury. Indeed, administration of sodium nitrite during hepatic and cardiac ischaemia in mice resulted in profound, dose-dependent protective effects on cellular necrosis, apoptosis and infarct size[311]. In addition, it has been reported that administration of an NO donor (SNP and 3-morpholine syndonimine)[373, 374] within 30 minutes after the onset of ischaemia, reduces the infarct size in rats by improving blood flow. Furthermore, eNOS knockout mice show a defect in arteriogenesis and functional blood flow after an ischaemic challenge. The local delivery of an adenovirus encoding constitutively active eNOS reduced the clinical manifestations of ischaemia[375]. However, attempts to prevent ischaemic injury by increasing eNOS protein expression should be performed with great care. Non-diabetic mice, which systemically overexpressed eNOS, were protected against ischaemic injury in the liver. However, in diabetic mice with systemically increased eNOS expression, the ischaemic injury was significantly increased. Treatment with BH4 prevented the hepatic ischaemic injury, indicating that eNOS is uncoupled in diabetic mice[376]. These results suggest that reduction of NO_2^- to NO could potentially help to counter the negative effects of ischaemia by increasing NO levels near the endothelium during hypoxia. However, under certain conditions, such as diabetes, eNOS cofactor co-therapy may be necessary in order to prevent eNOS uncoupling.
In addition to its importance in ischaemia-reperfusion, the ability of eNOS to mediate NO production from NO_2^- may also play a role during microangiopathy, stenosis or other pathophysiological conditions resulting in decreased levels of oxygen.

The results presented in chapter 8 suggest that increasing NO_2^- levels *in vivo* may provide an interesting therapeutic method to protect the body against the harmful effects of hypoxia. However, we showed that addition of extracellular NO_2^- did not result in increased eNOS-mediated NO production during anoxia. This is probably due to the fact that NO_2^- anions do not spontaneously enter the cell. Therefore, administration of NO_2^- can not be used therapeutically to increase NO production at sites of hypoxia.

Perspectives

The results of the studies described in this thesis support the general perspective that eNOS plays an important role in cardiovascular diseases. Improving NO availability has emerged as a major therapeutic goal to reduce cardiovascular risk. In an ideal situation, eNOS is sufficiently expressed, produces adequate amounts of NO and is not uncoupled, i.e. does not produce $O_2^{\cdot-}$. The produced NO then reaches its signalling target, mainly soluble guanylate cyclase (sGC), and elicits a cellular response.

Several strategies can be applied to target eNOS with the ultimate goal to increase NO bioavailability. Statins have been shown to substantially modify eNOS. Statins, or 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGR) inhibitors, form a class of hypolipidemic agents used to lower cholesterol levels in people at risk for cardiovascular disease. Statins inhibit endogenous cholesterol synthesis and increase the clearance of low-density lipoprotein from the bloodstream. However, recent data indicate that statins are cardioprotective by a number of mechanisms independent of lipid lowering. Statins have been shown to increase eNOS mRNA and protein expression[377] and enhance eNOS mRNA stability[378, 379]. In addition, statins decrease the expression of caveolin-1 mRNA and increase the association between eNOS and hsp90, thereby increasing eNOS activity and NO production[380]. Statins have been reported to activate Akt via the PI3K-Akt pathway, leading to the phoshporylation of S1177 in eNOS and a subsequent increase in NO production[381-383]. On the level of eNOS uncoupling, statins may also play a role by elevating GTP cyclohydrolase 1 (GTPCH1) gene expression, the rate-limiting enzyme in the synthesis of BH4[73], and BH4 synthesis, thereby increasing NO production and preventing relative shortages of BH4[384, 385]. Statins can prevent scavenging of NO by ROS either directly by exhibiting anti-oxidant properties[386] or by decreasing the expression of essential NADPH oxidase subunits and upregulating catalase expression[387-389].

In addition to statins, there are several other approaches to regulate NO bioavailability, for example by increasing eNOS activity. High density lipoprotein (HDL) has been shown to induce eNOS activity through the PI3K/Akt pathway[390]. Exercise, diets rich in monounsaturated fatty acids and fibres, alcohol (1 to 2 drinks a day) and the female hormone oestrogen all lead to an increase in HDL levels in the blood. In addition, decreasing ADMA concentrations may increase endogenous NO production by increasing eNOS substrate availability or preventing eNOS uncoupling[63]. Overexpression of dimethylarginine dimethylaminohydrolase (DDAH), the enzyme involved in ADMA degradation, reduced ADMA levels and increased NOS activity[391].

Gene therapy is based on the delivery of exogenous genetic material in order to treat gene defects. But in addition, gene therapy can also be used to influence the endogenous genetic components involved in disease development. Gene transfer of VEGF and eNOS has been reported to lead to the production of measurable amounts of both VEGF

protein and NO, respectively[392]. In addition, VEGF gene delivery improves blood flow in ischemic myocardium[393,394]. Another strategy is to introduce cells genetically manipulated *ex vivo*, such as skeletal myoblasts, that on injection secrete proteins that can increase local tissue concentrations of a biologically active substance[395-397]. Direct gene transfer methods are often transient, whereas the introduction of genetically manipulated cells is a more long-term process, resulting in more efficient delivery and concentration of the gene of interest. However, gene transfer of VEGF has been associated with malignant side-effects. For example constitutive overexpression of VEGF in non-ischaemic murine hearts can lead to the formation of endothelial cell-derived intramural vascular tumours near the implantation site[398]. In addition, adenovirus-mediated delivery of recombinant VEGF to the carotid artery in rabbits stimulates production of superoxide anion most likely caused by upregulation of NADPH oxidase expression and enzymatic activity[399].
Reducing ROS levels, either directly by anti-oxidants or indirectly by inhibition of ROS production, is another approach to increase NO bioavailability. Inhibitors of the angiotensin II pathway, e.g. the angiotensin-converting enzyme (ACE) inhibitors or angiotensin II type 1 (AT1) receptor antagonists, can act as direct anti-oxidants, preventing scavenging of NO by ROS[400, 401]. The principal lipid-soluble and water-soluble natural antioxidants α-tocopherol[402] (the principal component of vitamin E) and ascorbic acid[403, 404] (vitamin C), respectively, are also involved in the scavenging of ROS and increasing NO bioavailability. In addition, enzymes removing ROS, such as SOD, glutathione peroxidase and glutathione reductase, proteins and low molecular weight compounds, such as transferrin and haptoglobin, that limit the availability of pro-oxidant free metal ions like copper or non-haem iron, and low molecular weight molecules that are capable of scavenging radicals, such as glutathione and uric acid, are also involved in the regulation of NO bioavailability by decreasing ROS levels.
To prevent eNOS uncoupling either in a 'normal' disease situation or after gene transfer of VEGF or eNOS, supplementation of BH4 is essential. GTPCH1 expression and BH4 synthesis are stimulated by a wide array of factors, such as statins[384, 385], as described above, phenylalanine[405], insulin[406], inflammatory cytokines, including interleukin-1, interferon-γ, and tumour necrosis factor-α[407], cyclosporin A[408] and H_2O_2[409]. Vitamin C[410] and folate[411, 412] enhance endothelial BH4 bioavailability through chemical stabilisation or scavenging of reactive oxygen species, thereby contributing to the maintenance of physiological homeostasis in the endothelium. However, there is conflicting evidence on the positive effects of folate. It has been argued that folate exerts its actions through stabilisation of BH4[413]. In addition, it was recently reported that supplementation of folic acid did not reduce the risk of major cardiovascular events in patients with vascular disease[414].
In conclusion, elevation of eNOS expression and activity may be beneficial in treatment of endothelial dysfunction and cardiovascular disease. However, a potential caveat of inducing eNOS expression and/or activity in these pathological conditions is a

concomitant induction of eNOS uncoupling with the generation of potentially harmful reactive oxygen species as a consequence. Therefore, therapy aimed at elevation of eNOS production and/or activity should be designed in such a way that this unwanted side-effect is circumvented. An overview of the therapeutic strategies is shown in table 1. Note that some approaches are disputed. Considering its beneficial effects, treatment with statins alone should be sufficient to prevent eNOS-mediated endothelial dysfunction and cardiovascular disease. However, statin therapy may not be enough to cure existing cardiovascular complications. Therefore, in cardiovascular patients a combination of eNOS and GTPCH1 gene therapy, statins and vitamin C could be the best strategy. This combination would lead to increased eNOS and BH4 expression levels, increased eNOS activity, increased generation of BH4 and decreased ROS levels, all together providing a possible cure for endothelial dysfunction and cardiovascular disease.

Table 1. Potential interventional targets to optimize eNOS availability and function

Mechanism / Treatment	transcription	eNOS activity	eNOS uncoupling	NO bioavailability
Statins	Increase eNOS expression[377]	Decrease cav-1 mRNA expression[380]	Increase GTPCH1 expression[384, 385]	Inhibit NADPH oxidase[387-389]
	Enhance eNOS mRNA stability[378, 379]	Increase eNOS-hsp90 association[380]		Increase catalase expression[389]
		Stimulate Akt[381-383]		
Gene therapy (eNOS, VEGF)	Increase eNOS expression[392, 398, 399] - *disputed*			
HDL		Stimulate Akt[390]		
L-arginine			Increase L-arginine levels[64, 65, 129, 254-258] - *disputed*	
Gene therapy (DDAH)		Decrease ADMA[391]		
Vitamin C			Increase BH4 stability[410]	Scavenge ROS[403, 404]
Folic acid			Increase BH4 stability[411-414] - *disputed*	
ACE inhibition				Scavenge ROS[401]
AT1 receptor antagonists				Scavenge ROS[400, 401]
Vitamin E				Scavenge ROS[402]

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SAMENVATTING

Samenvatting

Inleiding

Het menselijk bloedvatenstelsel is een gesloten systeem bestaande uit ongeveer 96000 km vaten. Het bloed dat via de vaten naar de organen stroomt, zorgt voor aan- en afvoer van zuurstof, koolstofdioxide en afvalstoffen en bevat hormonen en afweerstoffen. De binnenkant van de bloedvaten is bekleed met een enkele laag endotheelcellen. Deze cellen vormen een barrière tussen het bloed en de rest van de vaatwand en reguleren de uitwisseling van allerlei stoffen en cellen van en naar het bloed.
Endotheelcellen scheiden verschillende stoffen uit die ervoor zorgen dat bloedvaten uitzetten (vasodilatatie) of juist krimpen (vasoconstrictie). Wanneer de werking van de endotheelcellen, en daardoor dus het functioneren van de bloedvaten, verstoord is, kan dat leiden tot het ontstaan van verschillende ziektes, waaronder hartinfarcten, beroertes en nierziektes. Roken, hoge bloeddruk, overgewicht, een te hoog cholesterolgehalte van het bloed en suikerziekte zijn allemaal factoren die de werking van het endotheel kunnen verstoren (endotheeldisfunctie).
Twee factoren die een grote rol spelen in het functioneren of disfunctioneren van endotheel zijn stikstofoxide (NO) en reactieve zuurstofradicalen (ROS). NO wordt gemaakt in endotheelcellen door het enzym 'endotheliaal stikstofoxide synthase' (eNOS) en is onder andere belangrijk voor vasodilatatie. NO is essentieel voor het goed functioneren van het endotheel. Een goede werking van eNOS is dus van belang om de vaatwand in optimale conditie te houden. ROS kunnen eveneens geproduceerd worden in endotheelcellen. Een opmerkelijk gegeven is dat eNOS, wanneer het niet goed functioneert, zelf in staat is om ROS te maken (zie verderop in deze samenvatting). ROS zijn ondermeer belangrijk voor het doorgeven van signalen binnen een cel, maar ROS zijn ook potentieel gevaarlijk omdat ze schade aan alle belangrijke biomoleculen kunnen aanrichten. De hoeveelheid ROS in een cel wordt gereguleerd door antioxidant systemen die een teveel aan ROS onschadelijk maken. Antioxidant systemen zorgen er ook voor dat de hoeveelheid NO en ROS met elkaar in balans zijn. Echter, wanneer het endotheel niet goed functioneert, kan er zoveel ROS geproduceerd worden dat de antioxidant systemen het niet meer aankunnen. Grote hoeveelheden ROS kunnen leiden tot schade aan de vaatwand en, uiteindelijk, hart- en vaatziektes. In **hoofdstuk 1** van dit proefschrift worden een aantal algemene zaken omtrent eNOS besproken.

Om hart- en vaatziektes te genezen of zelfs te voorkomen, is het essentieel dat we weten welke mechanismen endotheeldisfunctie veroorzaken. Daarvoor zouden we het liefst ín endotheelcellen willen kijken. Aangezien endotheelcellen zich aan de binnenkant van de bloedvaten bevinden en we er dus niet eenvoudig bij kunnen om te zien wat zich binnenin afspeelt, wordt vaak een modelsysteem gebruikt: endotheelcellen die in een bakje gekweekt kunnen worden. In de studie beschreven in dit proefschrift hebben we gebruik gemaakt van endotheelcellen die we vrijwel eindeloos door kunnen kweken. Deze

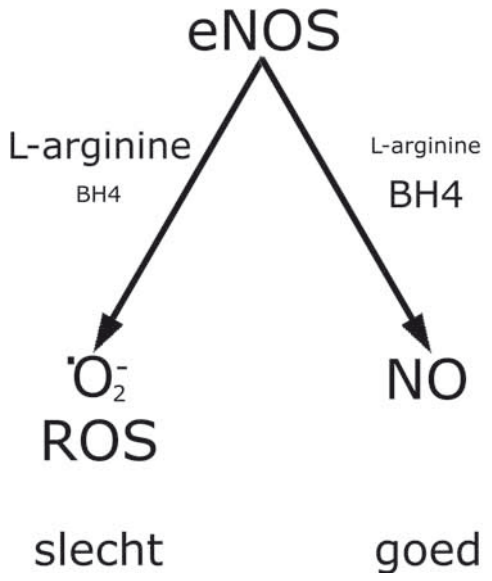
bEnd.3 cellen, afkomstig uit muizen, bevatten grote hoeveelheden eNOS en produceren veel NO. Ze lijken erg op de endotheelcellen zoals die in de mens voorkomen. Daarom zijn bEnd.3 cellen ideaal voor het bestuderen van condities die leiden tot (kleine) verschillen in NO en/of ROS productie, met endotheeldisfunctie als gevolg.

Voor iets dat je groots wil aanpakken, moet je klein beginnen. De eerste stap in het bepalen van de oorzaken van endotheeldisfunctie is het kunnen detecteren van de belangrijkste spelers, NO en ROS. Er zijn verschillende manieren om de hoeveelheid NO en ROS in een cel te bepalen, maar de meeste technieken zijn niet erg gevoelig en sommige zijn zelfs niet specifiek. Daarom is het belangrijk altijd meerdere technieken te gebruiken. Een aantal verschillende technieken voor het meten van NO en ROS staan beschreven in **hoofdstuk 2**.
In **hoofdstuk 3** van dit proefschrift wordt een techniek beschreven waarmee men, in tegenstelling tot andere methoden, in staat is exact te meten hoeveel NO geproduceerd wordt in een endotheelcel. We kunnen op deze manier goed het verschil meten tussen de hoeveelheid NO die geproduceerd wordt door een cel in rust en een cel waarin eNOS "aan staat" (geactiveerd is) en dus meer NO maakt. Deze techniek zou ook goed gebruikt kunnen worden om te bepalen welke factoren de werking van eNOS verminderen en daarmee bijdragen aan het ontstaan van hart- en vaatziektes.

Werking van eNOS

Om de effecten van verschillende factoren op de NO productie te kunnen bepalen, moeten we eerst de werking van eNOS in kaart brengen. Een endotheelcel bestaat uit allerlei verschillende compartimenten, celorganellen genoemd. Ieder organel bevat verschillende enzymen die belangrijk zijn voor een goede werking van de cel. Endotheelcellen in een vaatwand zullen over het algemeen met elkaar in aanraking zijn, ze vormen cel-cel contacten. In **hoofdstuk 4** hebben we gekeken naar de plaats (lokalisatie) van eNOS in een endotheelcel. eNOS maakt een "rondje" door de endotheelcel langs verschillende organellen. Op de meeste van deze plaatsen is eNOS niet actief, dat wil zeggen, er wordt geen NO geproduceerd. Wanneer eNOS zich bevindt in het cytosol, de vloeistof waarin alle celorganellen drijven, is het enzym wel actief en produceert het NO. Wij hebben ontdekt dat eNOS, naast andere plaatsen in de cel, ook aanwezig is op cel-cel contacten en dat deze lokalisatie eveneens essentieel is voor de activatie van het enzym. Waarschijnlijk is de lokalisatie van eNOS op cel-cel contacten belangrijk voor de barrièrefunctie van het endotheel. Stoffen en cellen uit het bloed moeten getransporteerd kunnen worden naar de rest van de vaatwand en andersom. Daarvoor moeten ze eerst langs het endotheel. NO speelt waarschijnlijk een rol bij het "openen" van de endotheellaag, waardoor cellen en stoffen kunnen passeren. Lokalisatie van eNOS op cel-cel contacten is nuttig, omdat de NO die geproduceerd wordt ter plekke zijn werk kan doen.

Samenvatting



Figuur 1. De balans tussen functioneel en disfunctioneel eNOS. In aanwezigheid van L-arginine en afwezigheid van BH4, zal eNOS ontkoppeld raken en superoxide produceren. Wanneer er zowel voldoende L-arginine als BH4 aanwezig is, zal eNOS goed functioneren en NO produceren.

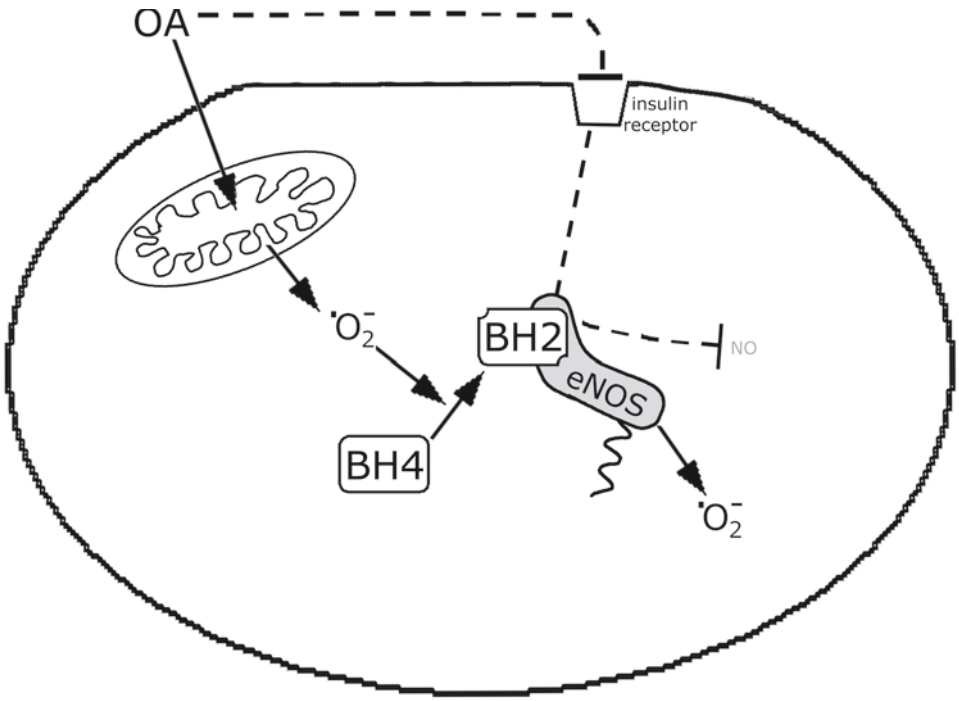
Naast de lokalisatie van eNOS in de cel, zijn er nog meer factoren van invloed op een goede werking van het enzym. De twee belangrijkste factoren zijn het substraat L-arginine en de co-factor tetrahydrobiopterine (BH4). L-arginine wordt door eNOS omgezet in L-citrulline. Bij deze reactie wordt NO gevormd. Zonder L-arginine zal eNOS dus geen NO kunnen produceren. BH4 is een co-factor die ervoor zorgt dat de elektronen die nodig zijn voor de omzetting van L-arginine op een goede manier door het eNOS enzym heen stromen en op de juiste plaats terechtkomen. Wanneer er een tekort is aan BH4, zal eNOS de elektronen afgeven aan zuurstof. Dit proces wordt 'eNOS ontkoppeling' genoemd. Een zuurstofmolecuul met extra elektronen wordt superoxide genoemd en is een zeer schadelijk zuurstofradicaal (een vorm van ROS). Men denkt dat zowel een tekort aan L-arginine als een tekort aan BH4 zal leiden tot eNOS ontkoppeling. In **hoofdstuk 5** laten wij echter zien dat deze opvatting niet klopt.
Omdat ons modelsysteem van bEnd.3 cellen grote hoeveelheden eNOS bevat, is het aannemelijk dat er een tekort is aan de essentiële factoren L-arginine en BH4. Inderdaad hebben we gezien dat bEnd.3 cellen in rust naast NO ook superoxide produceren. Wanneer we eNOS activeren, zien we dat er niet alleen meer NO geproduceerd wordt, maar ook meer superoxide. Hieruit kunnen we concluderen dat in bEnd.3 cellen eNOS dus voor een deel ontkoppeld is. Om te bepalen welke factor van invloed is op deze

ontkoppeling, hebben we in eerste instantie extra L-arginine toegevoegd. Wanneer de ontkoppeling veroorzaakt wordt door een tekort aan L-arginine, zou een toevoeging moeten leiden tot minder ontkoppeling en dus minder superoxide productie. Tot onze verbazing resulteerde de toevoeging van L-arginine echter niet in een vermindering, maar juist in een toename van superoxide productie. Dus, in tegenstelling tot de algemene opvatting wordt ontkoppeling van eNOS verergerd door de aanwezigheid van L-arginine. Toevoeging van BH4 leidde echter wel tot een afname in de hoeveelheid superoxide. Samenvattend kunnen we dus stellen dat ontkoppeling van eNOS voorkomen kan worden door de aanwezigheid van voldoende BH4 en dat een overmaat van L-arginine juist kan leiden tot een verergering van de ontkoppeling (figuur 1). L-arginine blijft echter wel essentieel voor de productie van NO. In endotheelcellen is het dus van belang dat de hoeveelheid L-arginine en BH4 met elkaar in balans zijn, zodat eNOS goed kan functioneren en ontkoppeling voorkomen kan worden.

eNOS in ziekte

Endotheeldisfunctie kan veroorzaakt worden door verschillende factoren. Zo kunnen verhoogde hoeveelheden vrije vetzuren in het bloed, een aandoening die vaak voorkomt bij patiënten met suikerziekte (diabetes mellitus) of patiënten met het nefrotisch syndroom, bijdragen aan het ontstaan van endotheeldisfunctie. Ook de samenstelling van de verschillende soorten vetzuren zoals die voorkomen in de voeding kan van invloed zijn op de functie van het endotheel. Zo kan voeding met voornamelijk onverzadigde vetten (ω3, ω6), het risico op hart- en vaatziektes verminderen, terwijl maaltijden rijk aan verzadigde vetten het risico op die ziektes juist vergroten. In een studie in gezonde vrijwilligers is aangetoond dat de endotheelfunctie afneemt na toediening van het enkelvoudig onverzadigd vetzuur oliezuur (OA). Het mechanisme achter de door vetzuren veroorzaakte endotheeldisfunctie is echter nog niet bekend. In **hoofdstuk 6** hebben we een poging gedaan dit mechanisme te ontrafelen.
Kweken van bEnd.3 cellen in een medium met oliezuur resulteerde in een toename in ROS productie. Het overgrote deel van de gevormde ROS bleek afkomstig uit mitochondriën, de energiecentrales van de cel, en slechts een klein deel werd veroorzaakt door ontkoppeling van eNOS. Naast een verhoogde productie van ROS, vonden we ook een afname in de NO productie. Deze afname bleek veroorzaakt te worden door het feit dat de eNOS activiteit geremd werd in aanwezigheid van oliezuur. Insuline, een hormoon dat betrokken is bij de stofwisseling van vetzuren en een bekende activator is van eNOS, was niet in staat de eNOS activiteit in aanwezigheid van oliezuur te verhogen. Dit wordt waarschijnlijk veroorzaakt door het feit dat de insulinereceptor, het aangrijpingspunt voor insuline op de cel, geblokkeerd wordt door oliezuur. Toevoeging van BH4 resulteerde in een toename in eNOS activiteit en NO productie en een geringe afname in de ROS productie.

Samenvatting



Figuur 2. Een verhoogde concentratie oliezuur (OA) leidt tot superoxideproductie door mitochondriën. BH4 wordt door superoxide omgezet tot BH2, wat resulteert in verlaagde BH4 concentraties, waardoor eNOS ontkoppeld raakt en superoxide produceert. Oliezuur blokkeert ook de insulinereceptor, waardoor eNOS niet geactiveerd kan worden door insuline en dus geen NO produceert. BH2, dihydrobiopterine; BH4, tetrahydrobiopterine; eNOS, endotheliaal stikstofoxide synthase; $O_2^{\cdot-}$, superoxide; OA, oliezuur

Deze resultaten leiden tot de volgende hypothese (figuur 2): Verhoogde concentraties vrije vetzuren leiden tot ROS productie door mitochondriën. BH4 kan door ROS omgezet worden tot BH2, waardoor de co-factor zijn werk niet meer kan doen. Daardoor raakt eNOS ontkoppeld en produceert het superoxide. Aan de andere kant blokkeert oliezuur de insulinereceptor, waardoor activatie van eNOS door middel van insuline niet goed werkt en eNOS dus geen NO produceert. Deze resultaten kunnen (voor een deel) verklaren waarom verhoogde concentraties vrije vetzuren in het bloed een risico-factor zijn voor het ontstaan van endotheeldisfunctie.

Albumine is het meest voorkomende eiwit in het bloed. Het speelt een belangrijke rol bij het handhaven van de bloeddruk. Daarnaast is het betrokken bij het transport van stoffen

in het bloed, waaronder NO, vrije vetzuren en medicijnen. Wanneer de concentratie albumine in het lichaam te laag is, spreekt men van hypoalbuminemie. Patiënten die lijden aan het nefrotisch syndroom of andere vormen van nierziektes, hebben vaak ook last van hypoalbuminemie. Hypoalbuminemie is geassocieerd met endotheeldisfunctie. Of de endotheeldisfunctie een direct gevolg is van de hypoalbuminemie of veroorzaakt wordt door condities die aanleiding geven tot het ontstaan van verlaagde albumine concentraties, zoals chronische ontsteking, is niet duidelijk.
In **hoofdstuk 7** hebben we gekeken naar het effect van albumine op eNOS activiteit en NO productie. In aanwezigheid van albumine bleek eNOS in bEnd.3 cellen minder actief te zijn en minder NO te produceren dan wanneer het systeem geen albumine bevatte. Om te bepalen wat het effect van de toegenomen NO productie is in een bloedvat, hebben we een bloedvat uit een rat geïsoleerd. Hiervoor hebben we twee verschillende typen ratten gebruikt. De ene rat (Sprague Dawley Rat; SDR) is vergelijkbaar met gezonde mensen, de andere rat (Nagase Analbuminaemic RAT; NAR) heeft geen albumine en is daarom vergelijkbaar met mensen die lijden aan hypoalbuminemie. Door ringetjes te snijden van het geïsoleerde bloedvat en ze vervolgens in een meetopstelling te brengen, kan vasodilatatie, het uitzetten van de bloedvaten, gemeten worden. Het bloedvat van de NAR liet een betere vasodilatatie zien dan het bloedvat van de SDR. Dit is in overeenkomst met de resultaten in de bEnd.3 cellen waarin we zagen dat de eNOS activiteit hoger is in afwezigheid van albumine. Deze resultaten wijzen erop dat hypoalbuminemie niet de oorzaak is van endotheeldisfunctie in patiënten. Waarschijnlijk zijn de condities die aanleiding geven tot het ontstaan van verlaagde albumine concentraties ook verantwoordelijk voor het ontstaan van endotheeldisfunctie in patiënten die lijden aan nierziektes.

Wanneer een bloedvat wordt afgesloten, bijvoorbeeld door een bloedstolsel, kan er geen zuurstof meer getransporteerd worden naar het achterliggende weefsel. Als gevolg daarvan zal het weefsel afsterven. Daarom is het van levensbelang dat het bloedvat zo snel mogelijk weer geopend wordt, zodat er weer zuurstof getransporteerd kan worden.
In **hoofdstuk 8** hebben we bekeken in hoeverre eNOS betrokken is bij het opnieuw openen van een afgesloten bloedvat. Wanneer er geen zuurstof aanwezig is, kan eNOS L-arginine niet omzetten tot L-citrulline en wordt er dus geen NO geproduceerd. We ontdekten dat nitriet, dat veel voorkomt in het menselijk lichaam onder meer door opname uit voedsel, in een zuurstofarme situatie omgezet kan worden tot NO. Hiervoor bleek eNOS verantwoordelijk te zijn. eNOS produceerde in dit geval NO niet via de gebruikelijke route door de omzetting van L-arginine tot L-citrulline, maar via een alternatieve reactie die nitriet omzet tot NO. Dus in het geval van een afgesloten bloedvat, kan eNOS “eerste hulp” verlenen door NO te produceren uit het aanwezige nitriet en zo zorgen voor vasodilatatie en het herstel van de bloedstroom.

Conclusie

De resultaten beschreven in dit proefschrift dragen bij aan de basale kennis over de biologie van eNOS, waarvan al bekend is dat dit een belangrijke speler is in het gezond houden van het bloedvatenstelsel. Tevens werden specifieke ziektebeelden gemodelleerd, op grond waarvan nieuwe strategieën voor de behandeling van endotheeldisfunctie voorgesteld worden. Op basis van de resultaten beschreven in dit proefschrift lijkt toediening van eNOS co-factor BH4 in specifieke gevallen te leiden tot een substantiële verbetering van de vaatfunctie. Daarnaast zou verhoging van NO productie door eNOS gentherapie een klinische toepassing kunnen hebben. Echter tegelijkertijd zullen ook de eNOS co-factoren moeten worden toegediend omdat anders een averechts effect wordt bereikt. Als laatste is de bevinding dat eNOS ook in zuurstofarme omgeving NO kan maken mits aan bepaalde voorwaarden wordt voldaan van belang voor nieuwe therapeutische strategieën voor behandeling van aandoeningen zoals hartinfarcten en beroertes.

List of Publications

List of Publications

1. Govers R, Bevers L, de Bree P, Rabelink TJ. Endothelial nitric oxide synthase activity is linked to its presence at cell-cell contacts. Biochem J. 2002 Jan 15; 361(Pt 2): 193-201.

2. Bouwman JJM, Visseren FLJ, Bevers LM, van der Vlist WE, Bouter KP, Diepersloot RJA. Azithromycin reduces Chlamydia pneumoniae induced attenuation of eNOS and cGMP production by endothelial cells. Eur J Clin Invest. 2005 Sep; 35(9): 573-82.

3. Bevers LM, Braam B, Post JA, van Zonneveld AJ, Rabelink TJ, Koomans HA, Verhaar MC, Joles JA. Tetrahydrobiopterin, but not L-arginine, decreases NO synthase uncoupling in cells with high levels of endothelial NOS. Hypertension 2006 Jan; 47(1): 87-94.

4. Bevers LM, van Faassen EE, Vuong TD, Ni Z, Boer P, Koomans HA, Braam B, Vaziri ND, Joles JA. Low albumin levels increase endothelial NO production and decrease vascular NO sensitivity. NDT 2006, in press.

5. Vanin AF, Bevers LM, Mikoyan VD, Kubrina LN, van Faassen EE. Reduction improves spin trapping of nitric oxide in biological systems. Nitric Oxide 2006, in press.

6. Bevers LM, Verhaar MC, Boer P, Rabelink TJ, Dallinga-Thie GM, de Bree PM, van Zonneveld AJ, Koomans HA, Braam B, Joles JA. Oleic acid increase mitochondrial ROS production and decrease eNOS activity in cultured endothelial cells. Submitted.

7. Vanin AF, Bevers LM, Schwok A, van Faassen EE. Nitric oxide acts as nitrite reductase under anoxia. CMLS 2006, in press.

Dankwoord

Dankwoord

’k Zat tegen beter weten in, te wachten op een nieuwe zin. Maar alles wat er kwam was eerder al gezegd, misschien niet door mij maar net zo slecht. (Acda&deMunnik – *Tien seconden*)

Jaap, in anderhalf jaar tijd hebben wij samen voor elkaar gekregen waar anderen vier jaar de tijd voor hebben. Ik bewonder het enthousiasme waarmee je mij onder je vleugels hebt genomen en hebt begeleid naar een prachtig eindresultaat. Ik ga me nu een ander hoekje van de wetenschap verdiepen, maar ik zal je wijze lessen in gedachten houden!

Branko, jouw inbreng gaven de hoofdstukken altijd nét dat beetje extra. Ik vond het geweldig dat je, terwijl je druk bezig was met de voorbereidingen voor je vertrek naar Canada, toch tijd voor mij vrijmaakte om een middag te brainstormen over de discussie. En ik moet je eerlijk bekennen, de egoboost die jij me iedere keer wist te geven… Wauw!

Hein, het begon met een fles wijn die ik kreeg als kerstgeschenk toen VGK was “overgenomen” door de nefro en het eindigde in dit proefschrift. Ook jou wil ik heel hartelijk bedanken voor wat je allemaal gedaan hebt. Na een half jaar van de decaan, zorgde jij ervoor dat ik nog een heel jaar extra kreeg. Het heeft tot dit fantastische resultaat geleid.

Anton Jan, drie jaar lang hebben we iedere week heerlijk zitten brainstormen. Helaas hebben niet alle (wilde) plannen tot een goed resultaat geleid, maar wat er nu ligt, mag er wezen, nietwaar?!

Ton, ons kennismakingsgesprek is me altijd bijgebleven. Je was erg gecharmeerd van het feit dat ik van klassieke muziek houd. Ik kreeg als opdracht van je mee de mensen op het lab wat kennis van de klassiekers bij te brengen. Helaas is dat niet gelukt, maar de rest wel!

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Ernst, jij kan EPR doen lijken als ware het iets simpels als het runnen van een gelletje. Toch gingen er heel wat uurtjes zitten in het berekenen van de hoeveelheid NO die één endotheelcel produceert. Together with Anatoli, you have contributed to a major part of this thesis.

Geesje, jouw kennis van vetzuren en alles wat daarmee samenhangt, is in mijn ogen onbegrensd. Op iedere vette vraag van mijn kant, had jij een antwoord. Zonder jou was ik dan ook niet zo ver gekomen!

David, here we are now, defending our thesis on which we worked so long and hard. Congrats, we did it! Good luck in your future job and we'll meet again, somewhere. Doei!

Petra, jazeker sta je erin! Zonder jou was dit boekje namelijk niet geworden wat het was! Vijf jaar lang hebben we samen geploeterd. Je presteerde het om tussen je drukke werkzaamheden als hoofdanaliste door, toch steeds weer tijd te hebben voor een DAF assay of de zeer gewaardeerde kopjes thee-met-een-goed-gesprek.

Des, onze samenwerking was als een achtbaan en ik ben gek op achtbanen! We begonnen naast elkaar, toen verhuisde je naar het buurlab en inmiddels zit je aan de andere kant van het ziekenhuis. Maar je wist ons altijd te vinden voor de laatste roddels en een goede lachbui. De meeltjes die je stuurde in de tijd dat ik thuis zat te schrijven, waren steeds een welkome afleiding. Ik hoop er nog veel te mogen ontvangen!

Jelger, mijn eerste, enige en vooral beste student! Samen hebben we de grenzen van eNOS opgezocht. Helaas heeft jouw geknutsel geen hoofdstuk in het proefschrift opgeleverd, maar we hebben wel lol gehad. Ik "lees" nog vaak je verhaal van een bijna afgestudeerde. Wat zullen wij later worden?

Dankwoord

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Rob, dankjewel voor je geweldige hulp bij de lay-out en drukken van dit boekje. We drinken er onder het genot van Good Company een biertje op!

Paul, zou het gelukt zijn, het allereerste boek zonder fouten? Bedankt voor alles!

Dankwoord

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Lief Tonneke, vanaf nu zal ik geen saaio meer zijn. Fegtu?!

Lonneke

Curriculum Vitae

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 3 juni 1975 te Utrecht. In 1994 werd het VWO diploma behaald aan het Sint Maartenscollege in Maastricht. In datzelfde jaar werd begonnen met de studie scheikunde aan de Universiteit Utrecht. In mei 2000 werd het doctoraal diploma behaald met als bijvakken Biochemie van Lipiden (dr. A.J. Aarsman en prof. H. van den Bosch, Faculteit Scheikunde, Universiteit Utrecht) en Neurologie (dr. E.A.J. Joosten, dr. L.H. Schrama en prof. P.R. Bär, Faculteit Geneeskunde, Universiteit Utrecht) en als hoofdvak Biochemie van Lipiden en Veterinaire Biochemie (dr. T.B. Dansen, dr. J.J. Batenburg en prof. K.W.A. Wirtz, Faculteit Scheikunde en Faculteit Diergeneeskunde, Universiteit Utrecht). De aanstelling als assistent in opleiding volgde op 1 juni 2000 bij het Laboratorium voor Vasculaire Geneeskunde van het Universitair Medisch Centrum Utrecht. Het eerste onderzoeksjaar vond plaats onder begeleiding van dr. R.M.T. Govers en prof. T.J. Rabelink. Vervolgens werd er drie jaar lang onderzoek verricht onder leiding van prof. A.J. van Zonneveld en prof. T.J. Rabelink. Tenslotte werd de onderzoeksperiode afgerond onder begeleiding van dr. J.A. Joles, dr. B. Braam en prof. H.A. Koomans.
Met ingang van 1 augustus 2006 is zij als postdoc werkzaam bij het Tumor Immunologisch Laboratorium in het Nijmegen Centre for Molecular Life Sciences te Nijmegen.