Evaluating the microcirculation in early phase clinical trials: novel methodologies and interventions

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CHAPTER I

INTRODUCTION

VASCULAR FUNCTION AND ENDOTHELIAL DYSFUNCTION

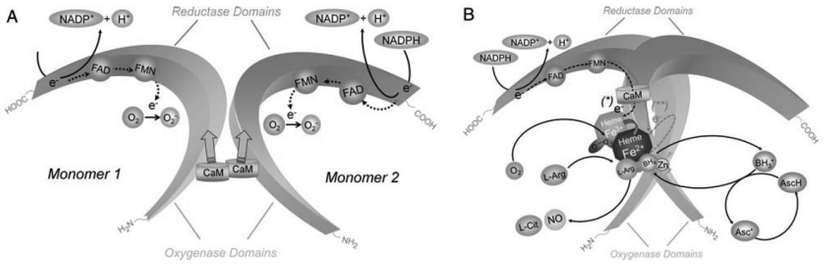
The theme of this thesis is an appraisal of the use of existing and emerging methodologies to gain information on the status of the vascular system in humans. The vascular system is a vital component of the functioning of virtually every organ system of the human body. Central to the physiology of this vascular system is the endothelium, a single layer of cells lining the interior of all blood vessels. Endothelial cells are involved in the regulation of many crucial processes, most importantly the regulation of vascular tone, but also hemostasis and inflammatory processes. Endothelial dysfunction is a precursor in the development of atherosclerosis and is correlated with various cardiovascular risk factors as well as adverse cardiovascular outcomes.[1] Endothelial dysfunction can be summarized in this context as a disbalance between endothelial-derived relaxing factors, such as bradykinin and prostacyclin, and endothelial-derived contracting factors, such as endothelin-1 and angiotensin II, but most crucially nitric oxide (NO).[2–3] Although endothelial-derived may be a misnomer, as cell types other than endothelial cells contribute to blood and perivascular concentrations of these factors,[4–5] endothelial cells are the main source of vascular NO under normal physiological circumstances.[6] NO is a crucial molecule for the functioning of the endothelium, but also fulfills other important roles in a host of physiological processes.

This chapter will first highlight some of the pivotal mechanistic pathways in which NO is involved, then explore therapeutic interventions targeting these pathways, and lastly identify possible imaging strategies to measure the pharmacodynamic effect of interventions on NO bioavailability, some of which will be investigated in this thesis.

NITRIC OXIDE: A PIVOTAL MOLECULE

NO is a highly diffusible, gaseous molecule with an extremely short half-life of 0.05 to 1 second in blood,[7] which exerts its effects through nitration and nitrosation of proteins and molecules such as glutathione and fatty acids, superoxide scavenging, cytochrome c oxidase, but most importantly the NO-soluble guanylyl cyclase (sGC)-cyclic guanosine monophosphate (cGMP) signaling system. Under physiological circumstances, NO is produced by nitric oxide synthases (NOS) from L-arginine, using reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen as co-substrates (Figure 1).[8] Cofactors involved in the function of all NOS isoforms are tetrahydriobiopterin (BH_4), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), while calmodulin acts as a catalysator. Within NOS proteins, electrons are transferred from NADPH through FAD and FMN to L-arginine, oxidizing this substrate and producing L-citrulline and NO. This electron transfer and the binding of BH_4, which are necessary for the function of the enzyme, are only possible when NOS is in its dimeric state, which requires the presence of heme. In their monomeric (uncoupled) state NOS are incapable of binding L-arginine, instead transferring the electron to O_2 to produce superoxide ($O2-\bullet$), a reactive oxygen species (ROS).[8] In endothelial tissue, NO is produced by the endothelial isoform of NOS (eNOS or NOS3), while in various other tissues such as neurons and peripheral mononuclear blood cells, NO is produced by neuronal NOS (nNOS or NOS1) and inducible NOS (iNOS or NOS2), respectively. eNOS and nNOS are dependent on the presence of Ca^{2+} activated calmodulin to form dimers,[9] whereas iNOS is calcium independent.[10] Activity of eNOS in endothelial cells is thus increased significantly by raised intracellular Ca^{2+} levels, in addition to other physiological signals, such as shear stress,[11] bradykinin,[12] and insulin.[13]

FIGURE 1 NOS form and function. A: two uncoupled NOS monomers. B: NOS in dimeric form.

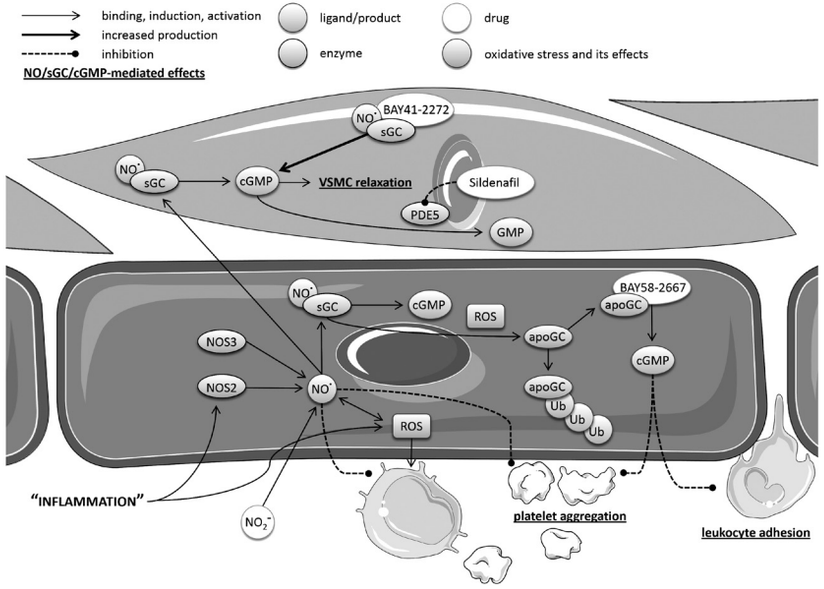


ASCH = ascorbic acid; BH_4 = tetrahydrobiopterin; CAM = calmodulin; FAD = flavin adenine dinucleotide; FM = flavin mononucleotide; L-ARG = L-arginine; L-CIT = L-citrulline; NADP = nicotinamide adenine dinucleotide phosphate; NO = nitric oxide. (Adapted from Förstermann et al.[8]) (see inside front cover for image in fullcolor)

Nitric oxide performs several functions depending on its location. In the endothelium, NO activates sGC in the smooth muscle cells surrounding the endothelial wall to convert guanosine triphosphate to cGMP. sGC is an enzyme consisting of two subunits, one of which contains a heme-NO-oxygen binding domain (HNOX). Physiological activation of sGC by NO is dependent on heme being present in its reduced state, allowing binding of NO and production of cGMP.[14] Some pharmacological compounds targeting NO signaling circumvent this requirement by activating sGC even when the HNOX site is oxidized.[15] When produced by sGC, cGMP

targets cGMP-gated cation channels, phosphodiesterases (PDES) and protein kinase G I and II in vascular smooth muscle, causing vasodilation, but also reduction of platelet aggregation, leukocyte adhesion, and vascular smooth muscle cell proliferation, illustrating the role of NO in the pathophysiology of inflammation and atherosclerosis (Figure 2).[16] Through this pathway, NO exerts protective effects on cardiovascular disease and associated disorders.[17]

FIGURE 2 The effects of NO, sGC and CGMP in endothelium and vascular smooth muscle.



APOGC = heme-free guanylyl cyclase; CGMP = cyclic guanosine monophosphate; NO = nitric oxide; NO2 = nitrogen dioxide; NOS = nitric oxide synthase; PDE = phosphodiesterase; ROS = reactive oxygen species; SGC = soluble guanylyl cyclase; UB = ubiquitin; VSMC = vascular smooth muscle cell. (Adapted from Vandendriessche et al.[16]) (see inside front cover for image in fullcolor)

iNOS, deriving its name both from being inducible by pathogenic triggers and being independent of Ca^{2+} levels,[18] is expressed in response to inflammatory stimuli in a wide array of cell types, including microglia, astrocytes, hepatocytes, macrophages, Kupffer's cells, and chondrocytes.[19–21] iNOS exerts immunomodulatory effects and is involved in defence against pathogens.[22] When activated, iNOS generates NO in large quantities, contributing to protection against micro-organisms and exhibiting anti-tumoral effects.[23] However, in case of excessive NO production, ROS are formed, which may result in deleterious effects such as DNA and mitochondrial damage.[24] Medical conditions characterized by such a heightened inflammatory response include endotoxemia, psoriasis, colitis, arthritis, and end-stage renal disease. These conditions are associated with increased whole body NO production due to increased expression of iNOS.[24–26]

Last, nNOS or NOS1 is mainly localized in dendritic spines of specific neurons,[27] but also present in cardiac, skeletal, and smooth muscle.[28–30] NO produced in and around neurons by nNOS, eNOS and iNOS3[1] modulates neurovascular coupling,[32] long-term synaptic potentiation,[33] neurogenesis,[34] vascular permeability[35] and by extension neuronal function and higher-level cognitive functions such as learning and memory.[34–36] Dysfunction of neuronal NO signaling is implicated in central nervous system disorders as varied as neurodegenerative disease,[37] chronic depression,[38] multiple sclerosis,[39] and stroke.[40] For example, NOS activity and expression is lower in patients with Alzheimer's disease compared to healthy controls[41] and associated with cognitive decline,[42] while decreased cGMP levels in the brain are associated with memory impairment,[43] supporting the pivotal role for NO in cognitive function. Inhibitors of NOS reduce learning and cause amnesia in preclinical animal models,[44–45] but their exact role in memory is controversial.[46–48] Modulation of NO signaling by targeting nNOS in the brain has been extensively researched in the clinic, with until now ambiguous results.[49]

POTENTIAL INTERVENTIONS TARGETING NITRIC OXIDE

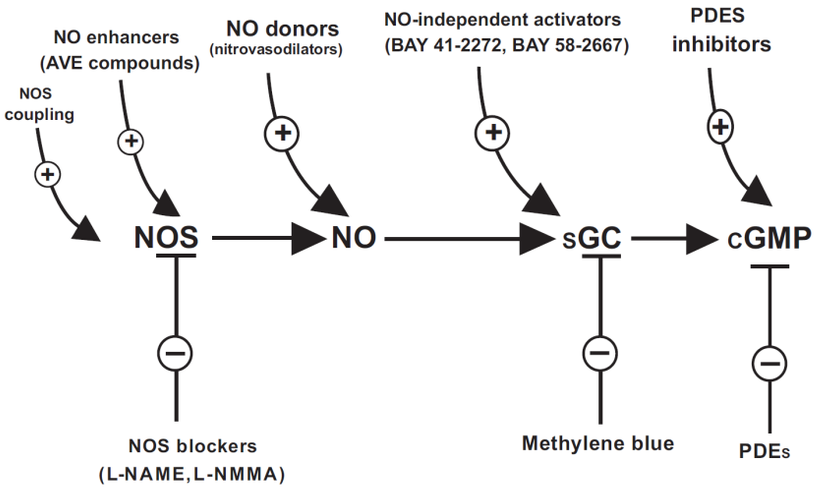
Endothelial NO has been the subject of pharmacological research aiming to reduce blood pressure, improve outcomes of cardiovascular disease and, most famously, alleviate erectile dysfunction. NO is also considered an attractive pharmacological target in the central nervous system to improve cognitive function or alleviate cognitive dysfunction, while NO produced by iNOS has been the subject of investigation for treatment of infectious or inflammatory disorders.[50–51] There are several suitable places for intervention in the NO-sGC-cGMP cascade (Figure 3).[52] First, increased NO production can be achieved by stimulating NOS activity via NOS isoform coupling. An example of this approach is administration of BH_4, which is approved by the FDA for treatment of phenylketonuria,[53] but was also shown to exert beneficial cardiovascular effects through eNOS coupling in animal models.[54–55] Second, NO enhancers such as AVE9488 can increase NO production by elevating expression of NOS, possibly resulting

in reduced ischemia-reperfusion injury[56] and improved cardiac remodeling after infarction.[57] Third, NO donors can directly increase NO or NO-related molecules and enhance sGC-cGMP signaling independently of endogenous NO production. Nitroglycerin, which induces vasodilation of coronary arteries and relieves anginal chest pain,[58] is probably the best-known example of this approach. NO donors have also produced beneficial effects in animal models of stroke[40] and show promise in human clinical trials for treatment of acute stroke,[59] stroke-like episodes in the context of mitochondrial disorders,[60] and ischemia-reperfusion injury.[61] Fourth, some drugs aim to either activate or stimulate sGC to produce cGMP.[62] Activation of sGC can increase production of cGMP regardless of the redox status of the enzyme or presence of heme. This strategy has however been proven risky due to the high potential for hypotensive adverse events and unfavorable pharmacokinetic and pharmacodynamic characteristics of compounds activating sGC, as illustrated by the effects of the experimental drugs cinaciguat and ataciguat.[63–65] Alternatively, drugs can stimulate sGC in its reduced state, acting independently of, or synergistically with, NO. Examples of this mechanism include riociguat, an NO-independent sGC-stimulator approved for treatment of pulmonary hypertension,[66] and zagociguat, a novel drug that stimulates sGC both NO-dependently and NO-independently in order to potentially treat cognitive dysfunction. Finally, the NO-sGC-cGMP axis can be affected by inhibiting PDE-mediated breakdown of cGMP. The most widely known PDE inhibitor is sildenafil, which targets the PDE5 family of the enzyme to alleviate erectile dysfunction.[67] Drugs that target other families of PDE are used in treatment of, or are in development for, a wide array of indications, including heart failure, asthma,[68] chronic obstructive pulmonary disease,[69] mitochondrial disorders,[70] and cognitive dysfunction caused by Alzheimer's disease[71] or schizophrenia.[72–73]

DEVELOPMENT OF TREATMENTS TARGETING NITRIC OXIDE

Early phase development of pharmacological treatments targeting the NO-sGC-cGMP chain is hampered by the fact that NO itself is difficult to measure due to its short half-life and low (sub-nanomolar) concentrations.[74] Most *in vivo* assessments have poor sensitivity and high variability, interference of ROS with assays, or other drawbacks.[75–76] Measurements of products derived from NO such as nitrogen dioxide, peroxynitrite, nitrite or nitrate are similarly limited by a short half-life and high reactivity of their targets,[77] while measurements of NO precursors such as arginine do not necessarily reflect *in vivo* NO production.[78] Moreover, due to the complex interactions present in the NO system, no single concentration measurement of related molecules directly translates to NO bioavailability or effects of NO on a cellular or tissue level.[76–77,79] As a result there is a gap in knowledge in how to measure the efficacy of NO-modulating drugs. This is particularly true for studies in healthy volunteers, a population most commonly participating in early phase clinical trials.

FIGURE 3 Therapeutic targets in the NO-sGC-cGMP pathway.



CGMP=cyclic guanosine monophosphate; NO=nitric oxide; NOS=nitric oxide synthase; PDE=phosphodiesterase; L-NAME=L-Nitro-Arginine Methyl Ester; L-NMMA=NG-Monomethyl-L-Arginine; sGC=soluble guanylyl cyclase. (Adapted from Evora et al.)[52]

IMAGING METHODS TO MEASURE NITRIC OXIDE IN VIVO

One way to circumvent the issues associated with blood-based biomarkers to assess NO bioavailability and the resulting endothelial function *in vivo* is to use imaging endpoints. The advantages of this approach are non-invasiveness and ability to measure the effects of NO in the most important target organ: the vascular bed. Various imaging modalities have been used, or are in development for, assessment of NO bioavailability and endothelial (dys)function, including doppler flowmetry, often combined with flow mediated dilation (FMD),[80–81] laser speckle contrast imaging (LSCI),[82] passive leg movement (PLM) ultrasonography,[83] peripheral arterial tonometry (PAT),[84] and magnetic resonance imaging.[85] Some methods, such as FMD and PLM, assess macrovascular reactivity by

measuring vasodilation and/or increases in flow velocity in response to a stimulus. In the case of FMD, this stimulus is most often shear stress induced by occlusion and reperfusion of the upper arm, during which changes in the diameter of the brachial artery are measured. Alternatively, PLM aims to measure increases in blood flow velocity in the femoral artery induced by passively moving the lower leg. LSCI and PAT mainly assess perfusion in the microcirculation, LSCI by deriving the flow of red blood cells in the skin from changes in laser reflection, and PAT by assessing changes in finger blood volume through plethysmography. Given this variety in assessments, a combination of imaging techniques can provide information from a range of vessel types. Further emphasizing the need for integration of different techniques is that each imaging modality has different drawbacks, including variability between individuals and operators, cost of equipment and training, subject burden, and a necessity to combine the imaging modality with physiological challenges to induce tissue responses. Hence, at the Centre for Human Drug Research, where all studies described in this thesis were conducted, a battery of tests containing a variety of imaging modalities was developed to evaluate vascular function, with an emphasis on microcirculation as a proxy for NO bioavailability. This test battery could theoretically fill the gap between proximal endpoints measured in the blood and tissue-level effects of NO. The implemented imaging methods included LSCI with occlusion-reperfusion and local thermal heating challenges, PLM, near infrared spectroscopy (NIRS) combined with venous and arterial vessel occlusion challenges, sidestream dark field imaging (SDFM) of sublingual and skin vasculature, and reduced nicotinamide adenine dinucleotide (NADH) skin fluorescence imaging.

LSCI is an imaging procedure that uses changes in laser speckle pattern reflection of the skin to assess local blood flow changes. When combined with occlusion-reperfusion and heating challenges, LSCI can measure the response of vasculature to physiological stimuli and thus assess endothelial function.[86–87] Similarly, PLM is an imaging method in which changes in blood flow in the femoral artery are measured in response to movement of the lower leg to evaluate vasodilatory capacity of vessels in the leg, again providing a measure of NO bioavailability.[88] NIRS can be used to quantify the oxygenation of tissues by measuring relative and total concentrations of oxygenated and deoxygenated hemoglobin. When combined with occlusion challenges, NIRS provides information on oxygen consumption and blood flow.[89–90] SDFM measures the density and flow of small vessels through a portable microscope that emits light in a wavelength absorbed by hemoglobin. This allows visualization of vessels and red blood cells in real time. Vascular SDFM is typically performed sublingually since the oral mucosa allows penetration of the light necessary to image the underlying blood vessels, which are superficially located.[91–92] Last, flow mediated skin fluorescence measurements (FMSF) of NADH can measure vascular function by quantifying the level of NADH fluorescence in the skin before and after an occlusion-reperfusion cycle.[93] FMSF also provides partial measurement of NAD+/NADH ratio and thereby information on cellular redox status,[94] which is of particular interest given the close interaction of NO with ROS and the dependence of NO production and NO function on reduced sites in the NOS and sGC enzymes.

AIMS AND OUTLINE OF THIS THESIS

The aim of this thesis was to explore the value of non-invasive imaging to assess the functional status of the vasculature. The goals of the studies described in this thesis were to 1) investigate if imaging can be used to reliably evaluate NO-dependent processes, 2) assess whether effects of physiological challenges or interventions on those processes were detectable and finally, 3) explore if early-phase clinical trials evaluating NO-mediated effects can become more informative when these techniques are utilized. First, in **Chapter II**, imaging modalities are employed in healthy volunteers undergoing a mixed-meal metabolic challenge to assess inter- and intraindividual variability of the imaging methods and their potential to detect effects of a challenge. **Chapter III** describes a study exploring if vascular imaging can differentiate between disordered NO function in patients with mitochondrial disease, a population with known elevated reactive oxygen species and lower NO bioavailability, and normal NO function in matched healthy volunteers. In **Chapter IV** the performance of a medical device intended to improve endothelial function and microcirculation in the skin was tested in healthy volunteers through imaging of the skin. The second half of this thesis describes two different approaches to target the NO-sGC-cGMP axis in the brain. In **Chapter V and VI** sGC stimulation was explored in a first-in-human and subsequent proof-of-concept trial with an sGC stimulator that reaches the cerebrospinal fluid. **Chapter VII** describes the effects of a central nervous system penetrant PDE2 inhibitor on cGMP levels in the cerebrospinal fluid. Finally, in **Chapter VIII**, the reliability of imaging modalities in assessment of endothelial function and NO bioavailability, and their value in measuring effects of challenges or potential treatments are discussed. This chapter includes recommendations for the inclusion of imaging-based endpoints to assess pharmacodynamic effects of drugs or therapies targeting the NO system in future clinical trials.

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