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Preclinical studies on the effect of novel NO-releasing molecules on vascular and erectile tone

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List of abbreviations

5’-AMP	5’-adenosine monophosphate
5’-GMP	5’-guanosine monophosphate
7-ER	7-ethoxyresorufin
8-pCPT-cGMP	8-(4-chlorophenylthio)-guanosine3’,5’-cyclic monophosphate
15-ane	trans-[RuCl([15]aneN_4)NO]$^{2+}$
AC	adenylate cyclase
ACh	acetylcholine
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
BH_4	tetrahydrobiopterin
BNP	brain natriuretic peptide
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
Ca-CaM	$Ca^{2+}{}_4$-calmodulin complex
cAMP	cyclic adenosine monophosphate
CAOx	cinnamaldoxime
CC	corpora cavernosa
cGMP	cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
CO	carbon monoxide
CORM	carbon monoxide-releasing molecule
CORM-2	$[Ru(CO)_3Cl_2]_2$
CORM-3	$[Ru(CO)_3Cl(glycinate)]$
cyclam-NO	trans-[RuCl(cyclam)NO]$(PF_6)_2$
DAG	diacylglycerol
DETCA	diethyldithiocarbamic acid
DMSO	dimethylsulfoxide
DPI	diphenyliodonium chloride
ED	erectile dysfunction

EFS	electrical field stimulation
eNOS	endothelial NO synthase
FAD	flavin adenine dinucleotide
FAL	formaldoxime
FAM	formamidoxime
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
FMN	flavin mononucleotide
GAP	GTPase-activating protein
GDI	guanine dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GTN	glyceryl trinitrate
H_2O_2	hydrogen peroxide
HO	heme oxygenase
ICP	intracavernosal pressure
IP_3	inositol-1,4,5-triphosphate
iNOS	inducible NO synthase
KRB	Krebs-Ringer bicarbonate solution
L-NNA	Nω-Nitro-L-arginine
L-NOHA	Nω-hydroxy-L-arginine
MAP	mean arterial pressure
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MYPT-1	myosin phosphatase target subunit isoform 1
NADPH	nicotinamide-adenine-dinucleotide phosphate
NCX	Na^{+}/Ca^{2+}-exchanger
nNOS	neuronal NO synthase

NO	nitric oxide
NOR	norepinephrine
NOS	nitric oxide synthase
$O_2^{\bullet-}$	superoxide
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
$OH^{\bullet}$	hydroxyl
$ONOO^{\bullet-}$	peroxynitrite
PDE	phosphodiesterase
pGC	particulate guanylyl cyclase
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PIP_2	phosphatidyl-inositol-4,5-biphosphate
PMCA	plasmamembrane also contains Ca^{2+}-ATPase
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
ROS	reactive oxygen species
RuBPY	cis-$[Ru(bpy)_2(py)NO_2](PF_6)$
RuNO	trans-$[Ru(NH_3)_4P(OEt)_3NO](PF_6)_3$
RUNOCL	cis-$[RuCl(bpy)_2NO](PF_6)$
SERCA	sarcoplasmic reticulum Ca^{2+}-ATPase
sGC	soluble guanylyl cyclase
SNP	sodium nitroprusside
SOD	superoxide dismutase
TEA	tetraethylammonium
Tempol	4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl
TERPY	$[Ru(terpy)(bdq)NO]^{3+}$
U46619	9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$
VOCC	voltage-operated Ca^{2+} channels

Chapter I

General introduction

Based on the paper:

Ruthenium-based nitric oxide-donating and

carbon monoxide-donating molecules

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I.1 Regulation of vascular smooth muscle tone

In the cardiovascular system, the contractile tone of vascular smooth muscle cells plays a central role in the regulation of blood pressure and distribution of blood flow [1]. The heart continuously pumps blood throughout the body ensuring sufficient blood pressure and blood supply at the entrance of organs and tissues. However, local mechanisms will modulate blood flow within the tissues according to their metabolic and functional needs. This modulation depends on a wide range of extracellular signals including neuronal, humoral, ionic and mechanical forces. Besides the nervous system, also the endothelium, which covers the inner surface of all blood vessels, is very important as it releases various vasoactive factors in response to the external signals. A tightly regulated balance between contractile and relaxant factors eventually determines the level of smooth muscle tone and thus the diameter of the vessels, vascular resistance and blood flow within the organs. Furthermore, the mechanism of penile erection also relies on the contraction/relaxation balance of (vascular) smooth muscle cells [2]. Vasorelaxation is responsible for adequate blood supply in the penis which is necessary to induce erection. Contraction of the penile smooth muscle cells, on the other hand, initiates detumescence of the erected penis returning it to its flaccid state.

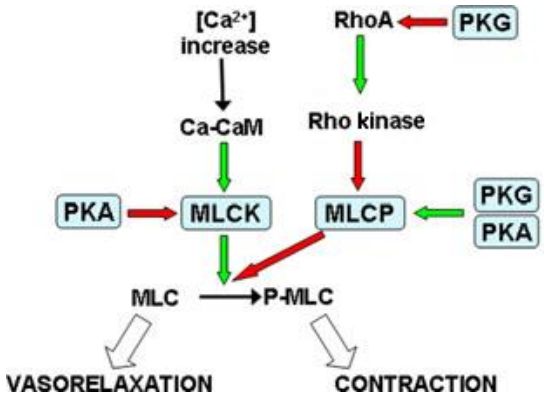

Figure I.1: Influence of $[Ca^{2+}]_i$ and RhoA on vascular smooth muscle tone and its regulation by cyclic nucleotide-dependent protein kinases. *Green arrows* stimulation and *red arrows* inhibition (adapted from [1]).

I.1.1 Vascular contraction

In general, vascular smooth muscle contraction occurs in response to elevated concentrations of intracellular Ca^{2+} ($[Ca^{2+}]_i$) either resulting from an electromechanical or a

pharmacomechanical coupling or a combination of both. A rise of $[Ca^{2+}]_i$ by stimuli which depolarize the cell membrane (KCl, mechanical stretch), subsequently activating voltage-operated Ca^{2+} channels (VOCC) and inducing Ca^{2+} influx, has been referred to as electromechanical coupling. In contrast, pharmacomechanical coupling depends on receptor-mediated stimuli activating G-protein signaling and generation of second messengers and is independent of direct changes in membrane potential [1,3,4]. Both pathways lead to Ca^{2+}-dependent activation of myosin light chain kinase (MLCK) and actin-myosin cross-bridging. Because of the increase in $[Ca^{2+}]_i$, free Ca^{2+} will bind the protein calmodulin with formation of a Ca^{2+}_4-calmodulin complex (Ca-CaM) which removes the pseudo substrate domain from the catalytic core of MLCK. After resolving this auto-inhibition, activated MLCK phosphorylates the 20 kDa myosin light chain (MLC) bound to the head–neck junction of the myosin heavy chains permitting actin access. Ultimately, vascular smooth muscle contraction will occur due to actin-induced activation of the myosin ATPase activity which leads to actin-myosin cross-bridge cycling in the presence of adenosine triphosphate (ATP) (Fig. I.1) [1,3,5-8].

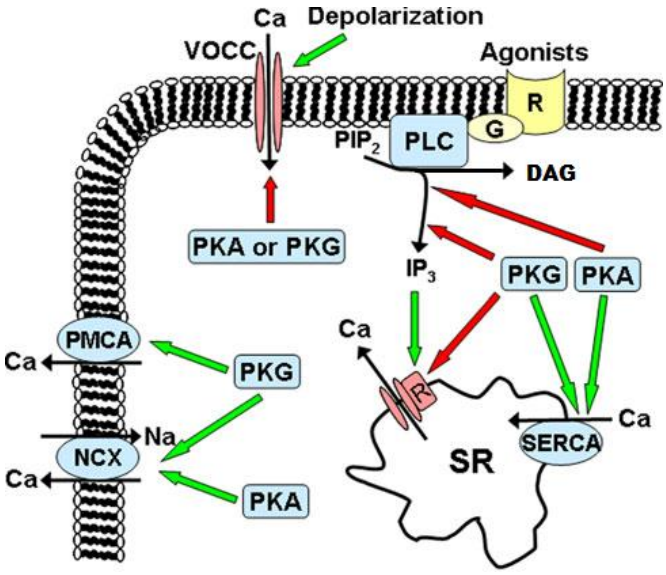

Figure I.2: Mechanisms involved in the increase and decrease of $[Ca^{2+}]_i$ in vascular smooth muscle cells and regulation by cyclic nucleotide-dependent protein kinases. *Green arrows* stimulation and *red arrows* inhibition (adapted from [1]).

Vascular contractile tone is mainly regulated by sympathetic neurons innervating the arterial wall. When an action potential reaches the terminal axon, the elevated open probability of

the plasmamembrane Ca^{2+} channels increases intraneuronal Ca^{2+} concentration triggering the release of norepinephrine (NOR) [9]. NOR will cross the neuromuscular junction to bind predominantly to α_1-adrenergic receptors on the smooth muscle membrane [10]. Stimulation of the α_1-receptor activates phospholipase C (PLC) which in turn catalyzes the conversion of phosphatidyl-inositol-4,5-biphosphate (PIP_2) into two important second messengers: inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Fig. I.2). IP_3 induces the release of Ca^{2+} from intracellular stores by binding its receptors on the sarcoplasmic reticulum. DAG activates protein kinase C (PKC) which controls various cellular processes such as transmembrane Ca^{2+} transport and Ca^{2+} sensitivity of the myofilaments. Besides NOR, several other contractile agonists (tromboxane A2, vasopressine, endothelin, angiotensin II, etc.) elicit vascular contraction by the phosphatidyl-inositol pathway mentioned above after interaction with their respective G-protein coupled receptors [1,3,6,7].

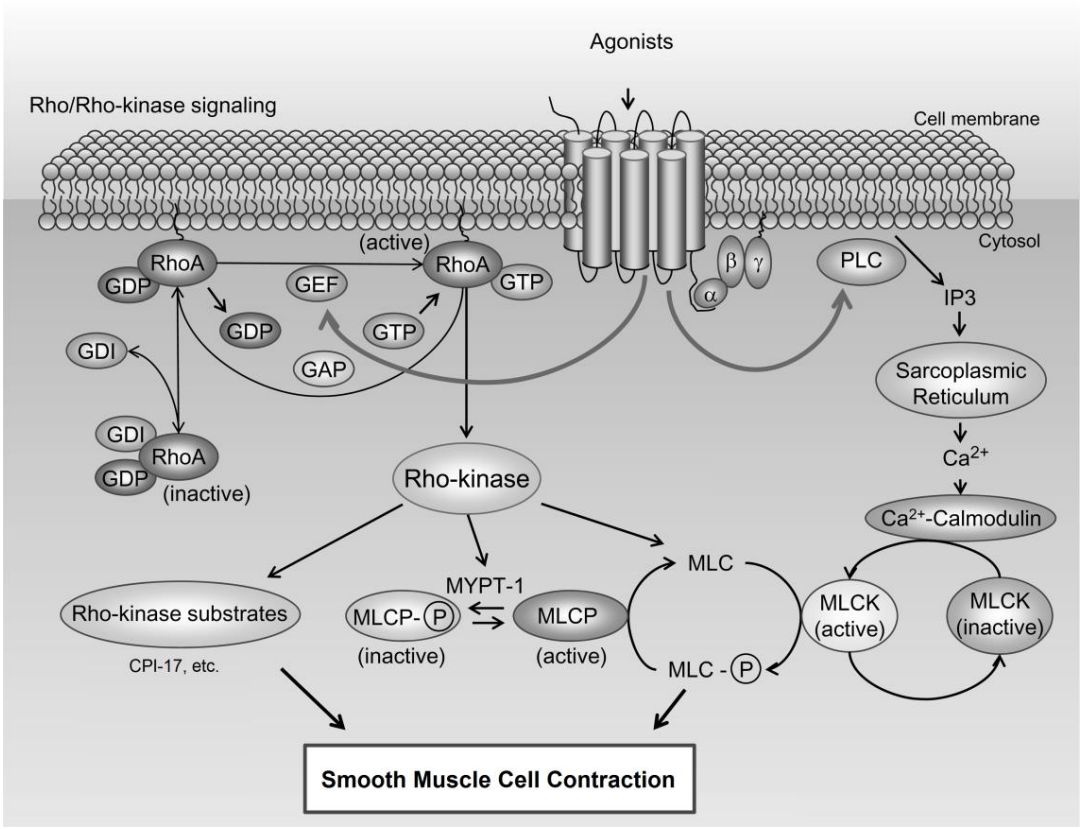

Figure I.3: The RhoA-Rho kinase signaling pathway in which RhoA cycles between an inactive GDP-bound and an active GTP-bound conformation regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (adapted from [11]).

In addition to the initial smooth muscle contraction induced by $[Ca^{2+}]_i$-dependent activation of MLCK, further force generation relies on a Ca^{2+} sensitization mechanism modulating MLC phosphatase (MLCP) activity. MLCP, a heterotrimeric enzyme, regulates the MLC phosphorylation state by removing the high-energy phosphate. The RhoA-Rho kinase pathway inhibits MLCP altering the MLCK/MLCP activity ratio in favor of MLC phosphorylation which leads (indirectly) to smooth muscle contraction [6,7,12,13]. RhoA, a member of the Rho GTPase family, acts as a molecular switch, cycling between an inactive guanosine diphosphate (GDP)-bound and an active guanosine triphosphate (GTP)-bound form. This GDP-GTP cycling is controlled by several regulatory proteins, including guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine dissociation inhibitors (GDIs). Generally, the inactive GDP-bound form of RhoA is locked in the cytosol by GDI, which stabilizes the complex and prevents membrane translocation of RhoA. Agonist-induced activation of G-protein coupled receptors results in an interaction with Rho GEFs, catalyzing the exchange of GDP to GTP and the dissociation of GDI. Activation of RhoA is turned off by GAPs that enhance the intrinsic GTPase activity of the Rho protein, leading to the hydrolysis of GTP to GDP [11,14-17]. Activated RhoA-GTP directly interacts with Rho kinase inducing a conformational change which leads to activation of this serine/threonine kinase towards specific substrates [18]. One of these substrates is the regulatory myosin-binding subunit of MLCP, namely myosin phosphatase target subunit isoform 1 (MYPT-1). Phosphorylation of MYPT-1 at certain threonine residues inhibits MLCP activity [13,19-21]. Rho kinase also inhibits MLCP indirectly by phosphorylating CPI-17, which becomes a potent inhibitor of the catalytic subunit of MLCP [13-15,22]. Moreover, Rho kinase has been shown to directly phosphorylate MLC [23]. Taken together, the RhoA-Rho kinase pathway leads to an increase in MLC phosphorylation to promote smooth muscle contraction independent of changes in $[Ca^{2+}]_i$ and MLCK activity, thereby causing Ca^{2+}sensitization (Fig. I.3).

I.1.2 Vascular relaxation

Reduction of smooth muscle tone is evoked by various exogenous and endogenous vasodilators. These compounds elicit vascular relaxation by inhibiting synthesis of contractile agonists, by blocking receptors which mediate smooth muscle contraction and/or by

decreasing $[Ca^{2+}]_i$ [1,24]. A decrease of $[Ca^{2+}]_i$ will occur at multiple cellular levels as a result of Ca^{2+} uptake and Ca^{2+} efflux as well as inhibition of Ca^{2+} release and Ca^{2+} influx. The sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) can bind Ca^{2+} ions which are then transported over the sarcoplasmic membrane. This Ca^{2+} uptake into the sarcoplasmic reticulum is reversibly inhibited by phospholamban. Phosphorylation of phospholamban relieves its inhibitory effect, therefore increasing SERCA activity and the rate of Ca^{2+} uptake. The plasma membrane also contains Ca^{2+}-ATPases (PMCA) leading to Ca^{2+} efflux into the extracellular space. Moreover, Na^+/Ca^{2+}-exchangers (NCX), located on the plasma membrane, contribute to the Ca^{2+} efflux driven by the transmembrane Na^+ gradient which is maintained by the Na^+/K^+-ATPase pump. Ca^{2+} release from the sarcoplasmic reticulum is decreased by inhibition of IP_3 formation and/or phosphorylation of the IP_3 receptor blocking the Ca^{2+} mobilization in the initial phase of agonist-induced contraction [7,25]. Hyperpolarization of the smooth muscle membrane (indirectly) decreases $[Ca^{2+}]_i$. Activation of K^+ channels causes K^+ efflux and membrane hyperpolarization, resulting in a reduced Ca^{2+} influx through VOCC and subsequent vascular relaxation [3]. Besides influencing $[Ca^{2+}]_i$, vasorelaxant agents can alter Ca^{2+} sensitivity of MLC phosphorylation. Upregulation of MLCP leads to enhanced MLC dephosphorylation whereas phosphorylated MLCK loses its affinity for Ca-CaM which decreases MLC phosphorylation, both shifting the balance towards inactive MLC and thus vasodilation [3,8,12].

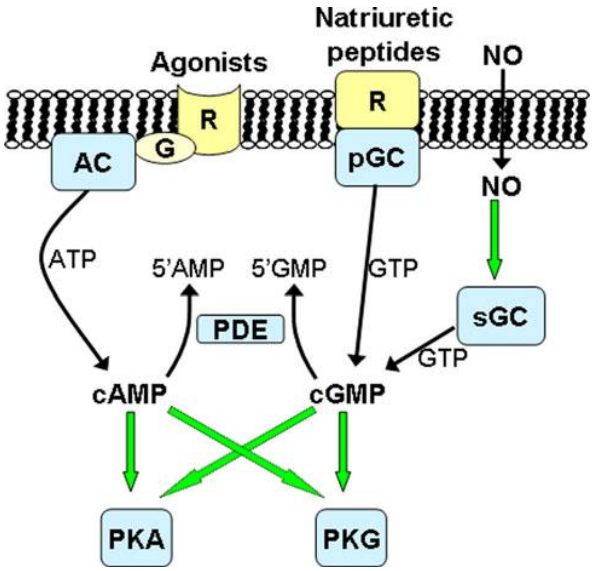

Figure I.4: Regulation of intracellular cyclic nucleotide concentrations and activation of their downstream effector protein kinases. *Green arrows* stimulation [1].

Vasorelaxant agents often generate intracellular second messengers after interaction with specific receptors, which in turn triggers a cascade of molecular reactions. Two important pathways can be distinguished: the cyclic guanosine monophosphate (cGMP) pathway and the cyclic adenosine monophosphate (cAMP) pathway (Fig. I.4). cGMP can be synthesized by particulate guanylyl cyclase (pGC), present in the plasma membrane, which is activated by natriuretic peptides. However, the main mechanism of cGMP production is through nitric oxide (NO)-induced activation of soluble guanylyl cyclase (sGC) in the cytosol. cAMP is formed by adenylate cyclases (AC) in response to G-protein coupled receptor stimulation. The intracellular levels of cGMP and cAMP are also controlled by phosphodiesterases (PDE) converting both into their inactive metabolites 5'-guanosine monophosphate (5'-GMP) and 5'-adenosine monophosphate (5'-AMP) respectively. cGMP exerts its vasorelaxant effect by activating protein kinase G (PKG) whereas cAMP activates protein kinase A (PKA). However, each cyclic nucleotide can act through both serine/threonine kinases. For instance, PKG can also be activated by cAMP, although it requires nearly a 10-fold-higher concentration of cAMP compared to cGMP to activate this kinase. Activated PKG will decrease $[Ca^{2+}]_i$ by phosphorylation of phospholamban (thus SERCA activation and Ca^{2+} uptake), by activation of PMCA and NCX (thus Ca^{2+} efflux), by inhibition of IP_3 synthesis and phosphorylation of the IP_3 receptor (thus inhibiting Ca^{2+} release) and by activation of K^+ channels (thus blocking VOCC and Ca^{2+} influx). Moreover, PKG alters Ca^{2+} sensitization by phosphorylation of RhoA as well as phosphorylation of MYPT-1 at certain serine residues enhancing MLCP activity. Just like PKG, PKA can activate SERCA and NCX, block IP_3 signaling, induce membrane hyperpolarization and increase MLCP activity. Furthermore, PKA also alters MLCK activity by phosphorylation of MLCK preventing interaction with Ca-CaM (Fig. I.1 and I.2) [1,3,24].

Various vasodilators such as prostaglandin E_1, prostaglandin I_2 (prostacyclin), substance P, calcitonin gene related peptide and others have been suggested to influence smooth muscle tone [26-28]. However, their actual contribution to vessel tone regulation remains at question. The principal mediator of vascular relaxation is by far NO released from the endothelial cells acting through activation of the sGC-cGMP-PKG pathway (Fig. I.5).

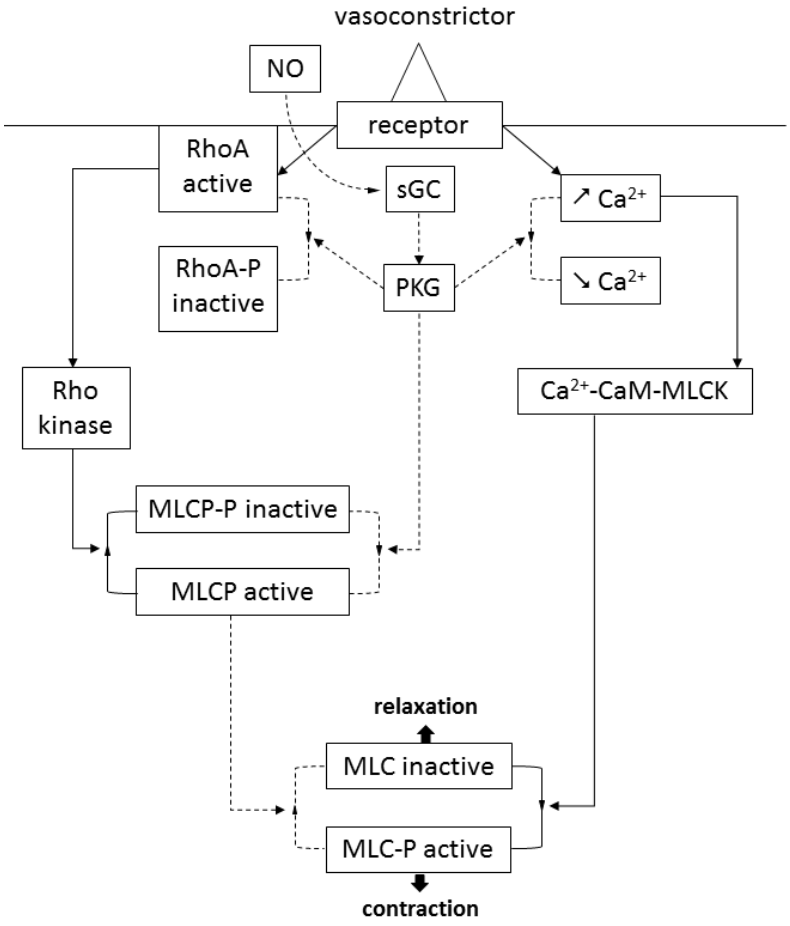

Figure I.5: Opposing actions of NO and vasoconstrictors on vascular smooth muscle tone. *Full arrows* vasoconstriction pathway and *dotted arrows* vasorelaxant pathway (based on [29]).

I.1.3 Vascular (endothelial) dysfunction

In the Western World, cardiovascular diseases such as atherosclerosis, heart failure and coronary heart disease are a major health problem. Despite the significant decline in cardiovascular mortality in Europe over the last three decades, cardiovascular diseases remain the primary cause of death accounting for over 4 million deaths in Europe annually and even 17.3 million deaths worldwide [30,31]. A growing list of risk factors, including hypertension, hypercholesterolemia, smoking and diabetes mellitus are associated with endothelial dysfunction [32,33]. In general, endothelial dysfunction is characterized by an imbalance of vasoconstrictive and vasodilatory compounds, with a shift towards vasoconstriction. In addition, various other endothelium-derived substances and endothelial functions are compromised with concomitant vascular smooth muscle proliferation, leukocyte adhesion and activation, vascular remodeling, platelet aggregation and thrombus

formation [34,35]. The pathophysiological mechanism of endothelial dysfunction is multifactorial, but the major outcome is an impaired NO signaling. NO plays a pivotal role in promoting endothelial homeostasis and maintaining healthy cardiovascular functioning. Therefore, any lowering of NO-bioavailability and/or responsiveness towards NO could result in endothelial dysfunction and thus cardiovascular disease [36].

Impairment of NO functioning can either be an immediate consequence of lowered NO generation or the result of accelerated NO degradation. Altered NO formation may occur due to a lack of substrate or cofactors as well as a reduction of the expression and/or function of NO synthase (NOS), which catalyzes the production of NO. On the other hand, indirect decrease of NO-bioavailability is caused by oxidative stress preventing NO to exert its effects [37,38]. Oxidative stress refers to a condition in which cells are exposed to excessive levels of chemical oxygen derivatives, the so called reactive oxygen species (ROS) [39]. ROS include free radicals such as superoxide ($O_2^{\bullet -}$), peroxynitrite ($ONOO^{\bullet -}$) and hydroxyl ($OH^{\bullet}$), and non-radicals such as hydrogen peroxide (H_2O_2) [40]. ROS formation generally begins with the one-electron reduction of molecular oxygen, giving rise to $O_2^{\bullet -}$. It has a half-life of only a few seconds and is rapidly converted into H_2O_2 either spontaneously or via the catalytic intervention of superoxide dismutase (SOD). Within mammalian cells, several enzyme systems are capable of transferring electrons to molecular oxygen producing $O_2^{\bullet -}$, such as nicotinamide-adenine-dinucleotide phosphate (NADPH) oxidases, the mitochondrial electron transport chain, xanthine oxidase, NOS, cyclooxygenases, lipoxygenases and cytochrome P450 reductases [41]. While all of these systems are important in various disease states, NADPH oxidases seem to play a central role in cardiovascular diseases [40]. $OH^{\bullet}$ is generated from H_2O_2 in the presence of ferrous iron (Fe^{2+}) by the Fenton reaction, but is also formed by the interaction between $O_2^{\bullet -}$ and H_2O_2. Furthermore, $O_2^{\bullet -}$ can also react with NO leading to the highly reactive $ONOO^{\bullet -}$ and, even more importantly, reducing NO availability [40-42]. Hence, the pathological effects of ROS in the cardiovascular system (and $O_2^{\bullet -}$ in particular) result from their ability to scavenge and remove several beneficial vasoprotective compounds such as NO, but also simultaneously result from their direct actions on vascular cell functions [43].

I.1.4 Penile smooth muscle tone

Just like vascular smooth muscle tone, cavernous smooth muscle tone heavily depends on the amount of intracellular free Ca^{2+}. Contraction of the penile vessels and trabecular smooth muscle cells of the corpora cavernosa (CC) is controlled by $[Ca^{2+}]_i$ and the Rho kinase signaling pathway. NOR released from adrenergic neurons interacts with α_1 adrenoceptors on the smooth muscle membrane and elicits smooth muscle contraction as mentioned above in the paragraph 'vascular contraction'. In addition, the Ca^{2+}-sensitizing RhoA-Rho kinase pathway plays a synergistic role in cavernosal (vaso)constriction [44-46].

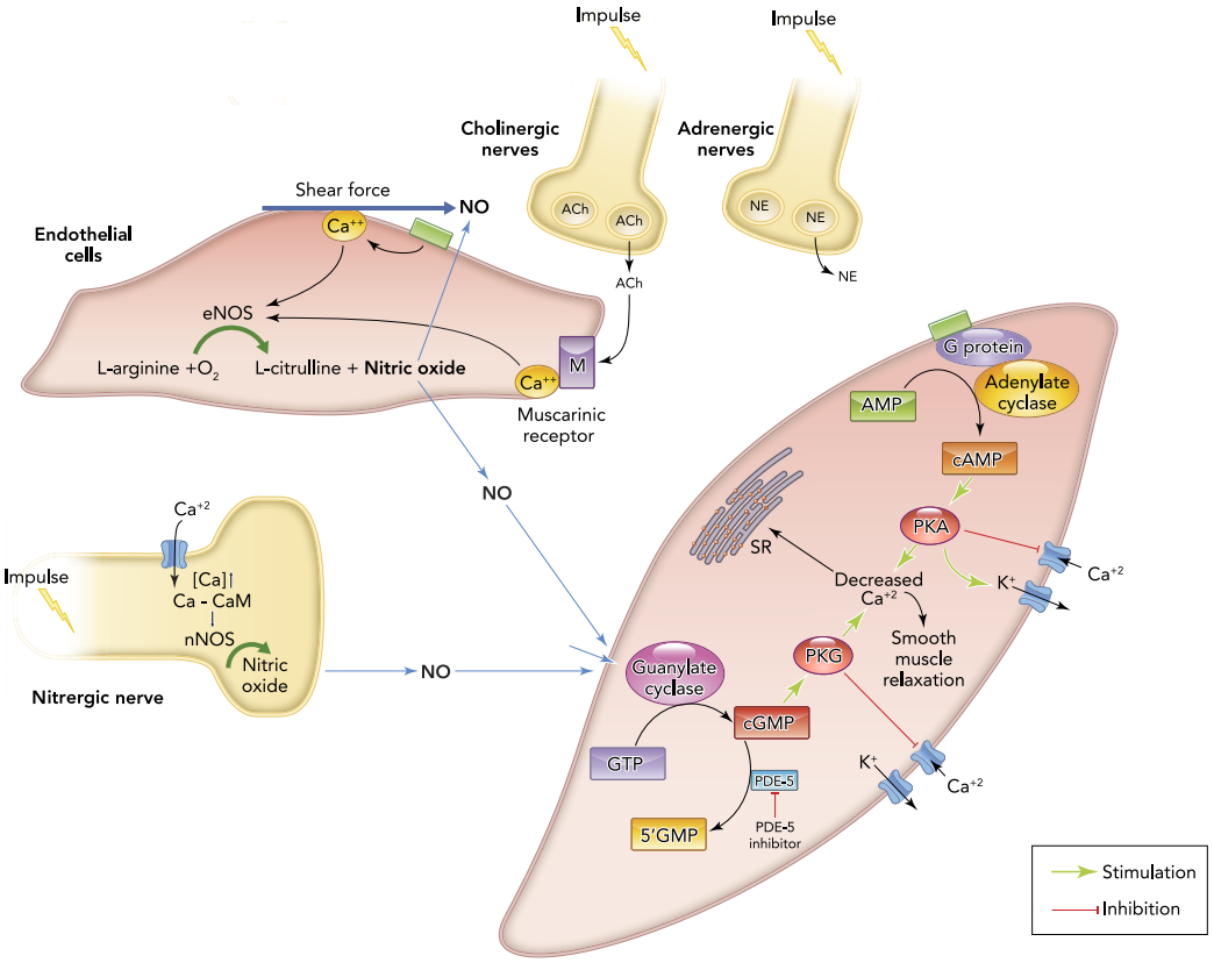

Figure I.6: Regulation of cavernosal smooth muscle relaxation by NO released from the nitrergic nerves and sinusoidal endothelium. Stimulation of the endothelial cells and nitrergic nerves in the penis causes Ca^{2+} influx which promotes production of NO. Binding of NO on sGC in the cavernous smooth muscle catalyzes the conversion of GTP into cGMP. Due to activation of PKA and PKG, both high levels of cGMP and cAMP induce vasodilatation of the arteries and sinusoidal spaces of the corpus cavernosum by decreasing $[Ca^{2+}]_i$, which eventually leads to penile erection (adapted from [51]).

As with vascular relaxation, NO is the most important mediator of penile relaxation [47]. NO is liberated from the endothelial cells, lining the inner surface of the penile arteries and cavernous sinusoids, upon local neurogenic stimulation by acetylcholine and mechanical shear stress by the blood flow. However, in penile tissue an additional source of NO can be found. Electrical impulses in response to sexual stimuli induce the release of NO from noncholinergic parasympathetic neurons innervating the penis [48]. NO, produced by the endothelium as well as by these nitrergic nerves, diffuses into the neighboring smooth muscle cells and activates sGC to increase the intracellular cGMP concentration. cGMP-induced activation of PKG initiates various molecular mechanisms all leading to smooth muscle relaxation. Further, intracellular cGMP concentrations are managed by PDEs of which type 5 is the predominant form present in penile tissue (Fig. I.6) [2,46,49,50].

The smooth muscle cells of the CC and the arteriolar walls play a key role in the erectile process. These cells dynamically regulate the tone of the penile vasculature, and thus the arteriolar blood flow into the sinusoids. Ultimately, the balance between contraction and relaxation will determine the functional state of the penis. Under basal conditions, cavernosal (vaso)constriction allows constant minimal arterial blood flow, covering the nutritional needs and maintaining the penis in flaccid state [52-54]. Upon sexual stimulation, NO, released from nitrergic nerves and/or the endothelium, diffuses into the smooth muscles of the penile arteries and cavernous trabeculae. This activates the relaxant pathways subsequently leading to increased blood flow into the penile sinusoids. Expansion of the CC elicits mechanical pressure on the penile veins, minimizing venous outflow from the sinusoids. Furthermore, the limited elasticity of the tunica albuginea, a tight fibrous layer of connective tissue that surrounds the CC of the penis, causes a rapid increase of intracavernosal pressure and thus penile erection (Fig. I.7) [44,45]. Neurogenic parasympathetic NO release, associated with sexual stimuli, will initiate cavernous tissue relaxation, whereas NO produced by the endothelium will facilitate attainment and maintenance of full erection [48,55]. Physiological, sexually-induced erection is principally mediated through the NO-sGC-cGMP-PKG pathway. Detumescence and returning to basal conditions is carried out primarily through the degradation of cGMP by PDE type 5 [49].

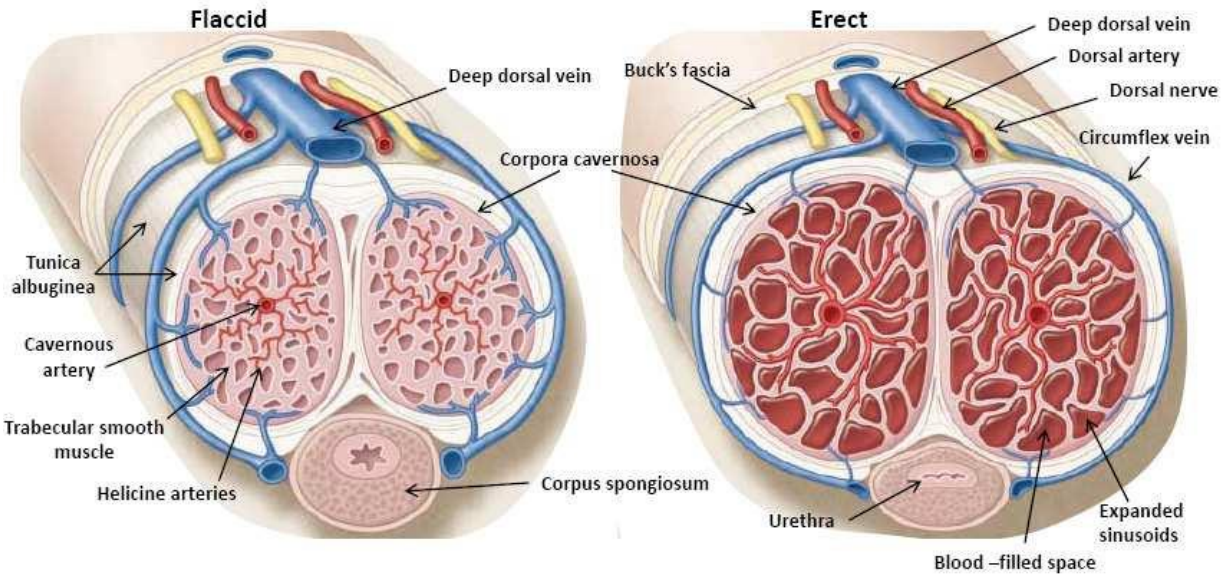

Figure I.7: Anatomy and hemodynamics of penile erection. In the flaccid state, inflow of blood via the constricted helicine arteries is minimal and there is free outflow through the venous plexus. During erection, relaxation of the trabecular smooth muscles and vasodilatation of the arterioles increase blood flow, expanding the cavernous sinusoids. This expansion of the sinusoids compresses the venous plexus reducing outflow of blood which contributes to the rapid increases of intracavernosal pressure [52].

I.1.5 Erectile dysfunction

Penile erection is a complex neurovascular process depending on a tightly regulated balance between psychological, hormonal, neurological, vascular and cavernosal factors. Hence, any alteration in one or more of these factors and mechanisms may lead to erectile dysfunction (ED) [44]. Recent epidemiological studies in the US as well as in Europe reported an ED prevalence of approximately 30 %. With age being an important risk factor for ED, these numbers are expected to rise even further in our aging population [56,57]. ED is generally defined as the inability to achieve and/or maintain an erection for satisfactory sexual intercourse [58]. Even though ED is not life threatening, its influence on social interactions, general wellbeing and patients' quality of life should not be underestimated [59]. In the past ED was mainly considered a psychological problem, but nowadays it is linked to different vascular, psychological, neurological and endocrinological disorders [44,60].

ED is predominantly a disease of vascular origin (approximately 75 % of the patients) and is therefore associated with multiple vascular risk factors such as atherosclerosis, hypertension, hypercholesterolemia, diabetes mellitus, obesity and smoking [58,59,61]. A number of both clinical and preclinical studies on hypertension, hypercholesterolemia, diabetes mellitus and aging have demonstrated endothelial dysfunction to be a critical factor in the development of vasculogenic ED [62]. Since NO is the main mediator of endothelium-dependent penile smooth muscle relaxation, ED associated with endothelial dysfunction is often linked to impaired NO functioning. This can be directly by a reduced NO production and/or indirectly by a lowered NO-bioavailability due to oxidative stress [63,64]. However, there are also several NO-independent mechanisms involved in endothelial and erectile dysfunction. Alterations in prostaglandin synthesis and signaling may contribute to an imbalance of penile eicosanoid homeostasis resulting in (vaso)constriction of penile blood vessels and corporal smooth muscles. In addition, an increase of angiotensin II and endothelin-1 concentrations may further promote a vasoconstrictor effect and thus ED [64].

I.2 Nitric oxide

Since its discovery in the 1980s as being the endothelium-derived relaxing factor, NO rapidly gained interest and is nowadays regarded as one of the most important signaling molecules of various biological processes [65]. NO is the principle mediator in both the cardiovascular system and penile erection, promoting (vascular) smooth muscle relaxation.

I.2.1 Endogenous NO production

Biosynthesis of NO involves a two-step oxidation of L-arginine to L-citrulline, with concomitant production of NO. In a first step, L-arginine is hydroxylated by a standard mono-oxygenation reaction forming the stable intermediate Nω-hydroxy-L-arginine (L-NOHA). In a second step, L-NOHA is further oxidized to L-citrulline and NO. Molecular oxygen and reduced NADPH are utilized as co-substrates, whereas flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin (BH_4) serve as cofactors (Fig. I.8) [66,67].

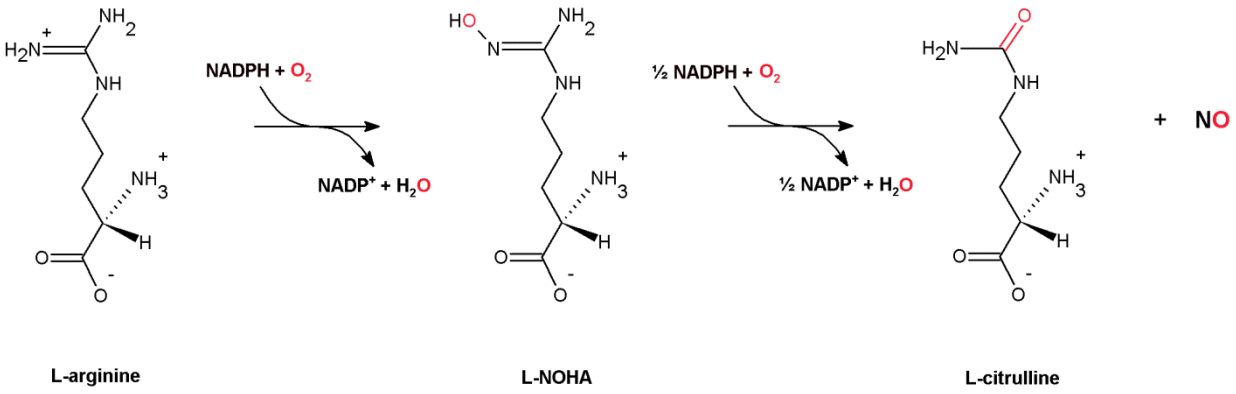

Figure I.8: Endogenous synthesis of NO from L-arginine.

Endogenous NO production is catalyzed by a family of NOS enzymes. These dimeric enzymes consist of two identical monomers, which can be functionally divided into two major regions: a carboxy-terminal reductase domain and an amino-terminal oxygenase domain. Active NOS transfers electrons from NADPH, via the flavins FAD and FMN in the reductase domain, to the heme in the oxygenase domain. The oxygenase domain also binds the essential cofactor BH_4, molecular oxygen and the substrate L-arginine. Between the two regions lies a calmodulin recognition site, which is important for both the structure and function of the

enzyme (Fig. I.9) [68,69]. Three quite distinct isoforms of NOS (eNOS, nNOS and iNOS) have been identified as products of different genes, with different localization, regulation, catalytic properties and inhibitor sensitivity [69]. Endothelial NOS (eNOS) is constitutively expressed mainly in (vascular) endothelial cells. Its enzymatic activity depends on a sufficient increase of $[Ca^{2+}]_i$ which enhances calmodulin affinity to eNOS, facilitating the flow of electrons from NADPH in the reductase domain to the heme in the oxygenase domain. Neuronal NOS (nNOS) is also Ca^{2+}- and calmodulin-dependent and is constitutively present throughout the brain as well as in the spinal cord and peripheral nervous system. Unlike eNOS and nNOS, inducible NOS (iNOS) already binds calmoduline at very low $[Ca^{2+}]_i$ and can therefore be activated without the need for a Ca^{2+} increase. However, iNOS expression is highly regulated by cytokines, some of which promote and others inhibit induction of the enzyme. Although primarily identified in macrophages, expression of iNOS can be stimulated in virtually any cell or tissue, given that the appropriate inducing agents are provided [66,67,70].

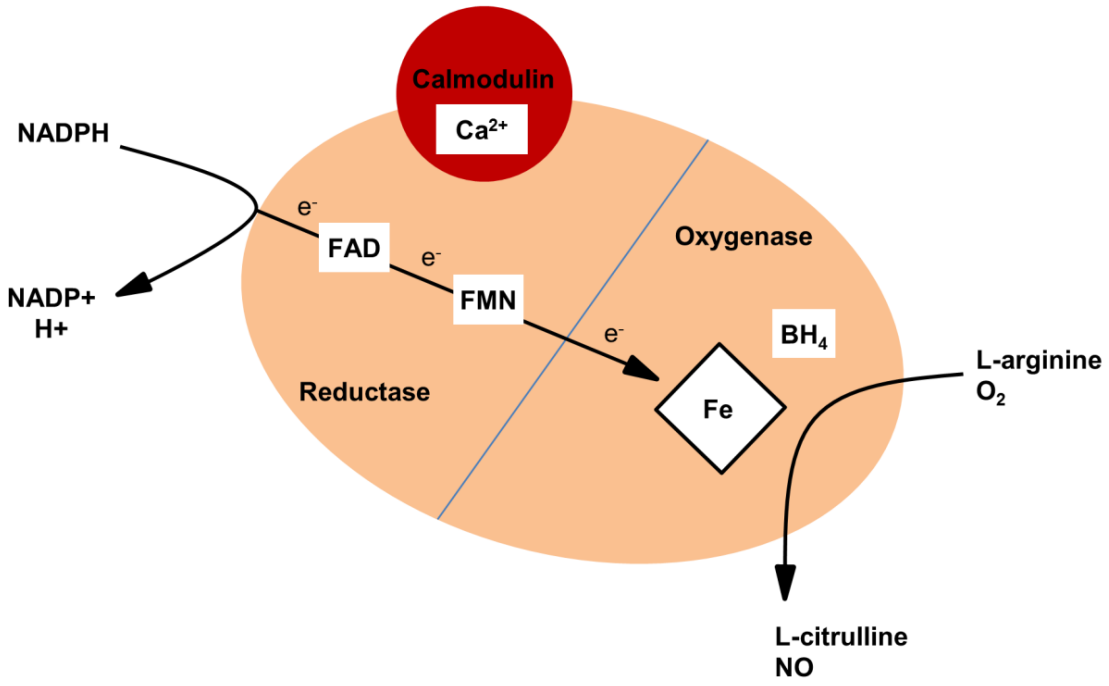

Figure I.9: Schematic representation of the overall reaction catalyzed by NOS (based on [69]).

I.2.2 NO-related therapy and drawbacks

NO exerts various vasculoprotective roles in the cardiovascular system including inhibition of smooth muscle proliferation and apoptosis, stimulation of endothelial proliferation, suppression of platelet aggregation and prevention of vascular inflammation [71]. A quantitative and/or functional NO deficiency limiting NO-dependent signaling pathways has been implicated in a number of cardiovascular diseases. In these clinical conditions it would be an attractive therapeutic option to mimic endogenous NO functioning by administration of (low concentrations of) exogenous NO [70,72]. Because of the limited feasibility and short half-life of gaseous NO, NO donor molecules are being used for over a century to treat coronary artery disease (angina pectoris) and, to a lesser extent, as a therapy for congestive heart failure, myocardial infarction and hypertension [73]. To date, organic nitrates (e.g. glyceryl trinitrate/nitroglycerin: GTN) are still the NO donors most frequently used in clinical practice for the treatment of ischemic syndromes. These drugs mimic the actions of endogenous NO by bioconversion to NO or NO-related compounds [74]. Well-known side effects of nitrates are headache and hypotension as a result of (abrupt) systemic vasodilation. However, the major drawback of this NO replacement therapy is the occurrence of resistance and tolerance upon prolonged use [75]. Progressive attenuation of nitrate-induced vasodilation can result from an increased production of substances with contractile effects, such as catecholamines, angiotensin II and endothelin-1, and has been referred to as “pseudo”-tolerance. This phenomenon contributes to the rebound effects after discontinuation of nitrate therapy. Tolerance is also associated with a progressive impairment of the biochemical properties of nitrates. Although the underlying mechanisms remain complex, it has been reported that oxidative stress and the production of ROS seem to be involved. ROS may impair the bioconversion of the nitrates but also interferes with the NO signaling by scavenging NO, which reduces NO-bioavailability, and by oxidation of sGC towards a ferric- and eventually heme-free state, which makes it unresponsive to NO and thus NO donors [76-78]. Besides tolerance, (cGMP-independent) adverse effects occur, secondary to the non-specific interactions with other biological molecules. Indeed, NO does not only activate sGC but also other proteins by nitrosation or interaction with metals, which could induce beneficial as well as harmful effects [79].

Because of its central role in the normal physiological erectile functioning, the NO pathway is an important target in the therapeutic strategies of vasculogenic ED associated with endothelial dysfunction. In the past, various NO-donating compounds have been tested in the context of ED and penile erection. Because of its vasodilatory capacity in cardiovascular diseases, GTN was evaluated as a treatment for ED. To avoid systemic cardiovascular effects, GTN was applied topically either by an ointment or transcutaneous patches. Studies showed that this transdermal GTN application could alter penile vascular diameter and enhance the quality of erection [80-84]. However, later on a placebo controlled clinical trial could not prove a significant effect of transcutaneous GTN. Nevertheless, the authors still recommended to try this penile application of GTN before starting with large-scale investigation or invasive treatment [85]. Another extensively studied NO donor is sodium nitroprusside (SNP). Intracavernosal as well as transurethral administration of SNP elicits penile erection associated with an increase of intracavernosal pressure and penile length [86-88]. However it was reported that the potency of SNP is lower compared to intracavernosal injection of papaverine and prostaglandin E_1 [89,90]. Moreover, systemic hypotension often occurs as an undesired side effect [89-91]. The NO donor linsidomine chlorhydrate is also able to increase arterial blood flow and to relax penile smooth muscles thus leading to penile erection. Even so, this treatment strategy did not seem to be superior to intracavernosal injection of papaverine/phentolamine and prostaglandin E_1 [92-95]. It has been suggested that sodium nitrite can act as a storage form of vasoactive NO. Although less potent than SNP, sodium nitrite increases intracavernosal pressure as a result of its conversion to NO by NOS and xanthine oxidoreductase [96]. However, sodium nitrite is not able to restore erectile functioning in aged rats [97]. Notwithstanding the sometimes promising results, all of the NO-donating molecules mentioned above are nowadays not available on the pharmaceutic market for the treatment of ED. This could probably be explained by to poor efficacy as compared to other available treatment strategies as well as the occurrence of adverse effects and/or the development of tolerance upon prolonged use [85,89-94,97,98].

Currently, PDE type 5 inhibitors are regarded as the first-line treatment of ED. The overall efficacy rate of these drugs is around 60 to 70% but is substantially lower in patients with severe neurological damage, ED after radical prostatectomy, diabetes or severe vascular

disease [99,100]. After all, for these drugs to work, a 'critical' amount of NO is necessary. Severe nerve damage and conditions in which expression or activity of NOS and/or release of NO is impaired, will negatively influence NO availability. Lack of adequate amounts of NO excludes sufficient sGC activation and subsequent cGMP formation undermining the mechanism of action of PDE type 5 inhibitors [101]. So although efficacy and safety of PDE type 5 inhibitors have been proven in large clinical trials, at least 30 to 35 % of the patients fail to respond [102]. Moreover, PDE type 5 inhibitors can potentiate the vasodilatory effect of nitrate therapy resulting in a potentially life-threatening hypotension. Hence, concomitant use of PDE type 5 inhibitors with nitrates, including occasional and short-acting administration, is contraindicated [103].

Development of tolerance, low bioavailability and adverse effects limit the therapeutic value of current NO replacement strategies. Moreover, although PDE type 5 inhibitors seem to be the most successful, they still remain ineffective in roughly 30% of ED patients, present various undesirable side effects and are only a short-term cure. Therefore the search for new alternative treatments is still at hand. Recently, oxime derivatives, bearing an oxime (C=NOH) functional group, were presented as new group of compounds with vasodilatory effects due to the release of NO. Furthermore, as a result of their interesting biochemical properties, a wide range of metallocomplexes have been developed which are capable of releasing NO (or CO) under certain conditions.

I.2.3 Oximes

As mentioned above, the first step of endogenous NO production is the formation of the stable intermediate L-NOHA which is specifically and exclusively catalyzed by NOS. Further oxidative cleavage of the C=NOH bond of L-NOHA will eventually lead to NO. Studies have demonstrated that oxidative cleavage of the C=N bond in the C=NOH functional group is not only mediated by NOS but also by other hemoproteins such as microsomal cytochromes P450 and peroxidases [104-106]. Because of their structural resemblance with L-NOHA, oxime derivatives, which also contain a C=NOH bond, gained interest as alternative NO-delivery strategy. Based on their structure and chemical composition, oxime derivatives can be subdivided into different groups including non-aromatic substituted oximes, aromatic amidoximes and aromatic ketoximes. It was established that oxidative cleavage of these

oximes, by hemoproteins other than NOS such as microsomal cytochromes P450, could lead to NO production [107-109]. This NOS-independent NO release could be of potential value, restoring NO deficiency in pathological conditions typified by endothelial (and in many cases also NOS-) dysfunction such as cardiovascular diseases and erectile dysfunction.

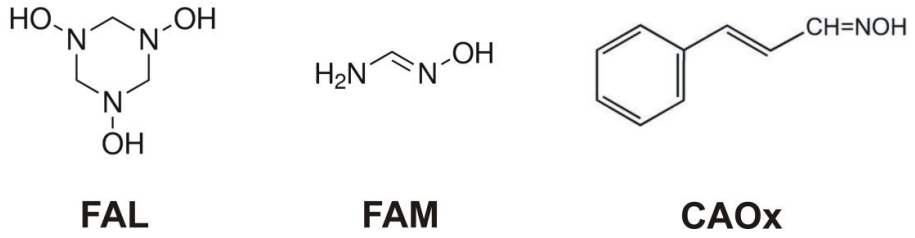

Figure I.10: The chemical structure of formaldoxime (FAL), formamidoxime (FAM) and cinnamaldoxime (CAOx).

However, as yet research on the effect of oximes on vessel tone and its underlying mechanism(s) is very limited [110-113]. In these few studies, formaldoxime (FAL) seemed to be the most potent compound to establish in vitro vasorelaxation of rat aortic ring segments (Fig. I.10). Chalupsky at al. demonstrated that the oxime-induced vasorelaxation does not occur from spontaneous NO release in the organ bath solution but rather from the production of NO in the smooth muscle layer. Since the cell-permeable NO scavenger PTIO could alter the oxime-induced relaxation, the production of NO as a result of oxime metabolization is held responsible for the oxime effect. This metabolization of oximes into NO seems to be mediated by 7-ER-sensitive NADPH-reductases whereas NOS and monoamine oxidases are not involved. The produced NO subsequently activates sGC and PKG as proven by the inhibitory effect of ODQ and Rp-8-Br-cGMPS respectively [110]. Furthermore in vivo experiments were performed with the oxime compounds in which formamidoxime (FAM) induced the most pronounced blood pressure lowering effect when endogenous NO formation was blocked (Fig. I.10). Jaros et al. reported that inhibition of sGC by methylene blue could almost completely prevent the blood pressure decrease elicited by the oximes which was believed to indicate a relative contribution of NO to the vasodilatory action of the oxime compounds [111]. In addition to the non-aromatic oximes FAL and FAM, the underlying mechanisms of the aromatic oxime cinnamaldoxime (CAOx) were elucidated in vitro on rat mesenteric artery (Fig. I.10). Again NO release seems to occur through

NADPH-dependent reductases, leading to the activation of the sGC-cGMP-PKG pathway. The vasorelaxant effect of CAOx is also associated with the activation of large-conductance Ca^{2+}-activated K^+ channels [113]. Similar molecular pathways were also proven to underlay the vasorelaxing effect of other C=NOH bearing molecules such as L-NOHA and 4-chloro-benzamidoxime [112].

I.2.4 Ruthenium-based NO-donating molecules

I.2.4.1 Ruthenium metallocomplexes

Many metal ions play a crucial role in various biological processes. Hence, a potential role for metal-containing compounds as medicinal agents can be considered [114]. Preparations of iron, zinc, copper, gold, mercury and bismuth were used in medical practice throughout history. The use of metal compounds in medicine already dates back to 3000 BC when the Egyptians used copper to sterilize water. 3500 years ago gold was included in a variety of medicines in Arabia and China. Around 1500 BC different iron-based therapies were introduced in Egypt and zinc was found to promote wound healing. The Greek physician Hippocrates already used mercury in 400 BC and in the Renaissance period mercurous chloride was proposed as a diuretic [115-118].

The last five decades, metal complexes have attracted attention of many drug designers in the perpetual search for new therapeutic approaches. One of the oldest and best-known metallodrugs is the platinum-based anticancer drug cisplatin, which was discovered by accident in the 1960s [117,119]. Platinum belongs to the group of precious metals like gold and silver. Although in ancient times these metals were used because of their rare nature rather than its known medicinal activities, precious metals have been shown useful in a clinical setting. A wide range of platinum compounds are developed for cancer treatment, gold compounds are implemented in the treatment of rheumatoid arthritis and silver compounds present antimicrobial properties [115,116].

More recently a less-known member of the precious metal class, namely ruthenium, entered drug research. Nowadays, ruthenium is already found in drugs with immunosuppressant [120], antimicrobial [121], antibiotic [122] and anticancer activity [123,124]. Over the years,

ruthenium became popular due to its interesting physico-chemical characteristics. First of all, the ability of ruthenium to coordinate ligands in a three dimensional configuration allows functionalization of these groups aiming at defined molecular targets. Moreover, rational ligand design provides control over the kinetic properties. In this way ruthenium complexes tend to slowly exchange its ligands when in contact with biological tissues in order to exert a biological effect. Secondly, ruthenium can access a range of oxidation states (II, III, IV) under physiological conditions. These oxidation states permit participation in biological redox chemistry influencing optimal dose and bioavailability. As a result of the reducing environment of diseased tissues, the more inert ruthenium(III) complexes are converted into active ruthenium(II) complexes which creates a certain selectivity causing minimal damage to healthy tissues. Thirdly, ruthenium shows a relatively low toxicity probably due its ability to mimic iron in binding different biomolecules, such as serum albumin and the iron transport protein transferrin [116,118,125].

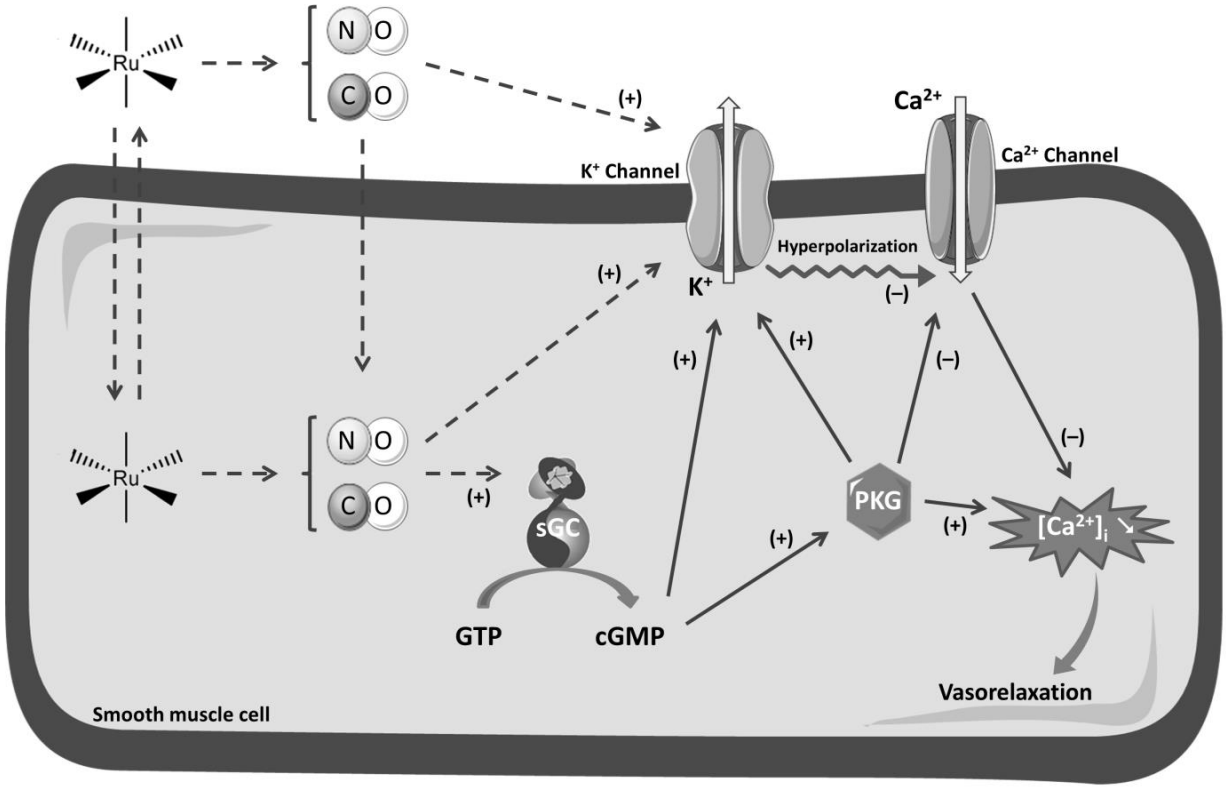

Figure I.11: Overview of the main pathways involved in the vasorelaxation induced by ruthenium-based NO- or CO-donating molecules. (sGC = soluble guanylyl cyclase; PKG = cGMP-dependent protein kinase; $[Ca^{2+}]_i$ = intracellular calcium concentration)

Besides their own possible therapeutic effect, metals can have a passive accompanying role in the delivery of drugs. The formation of a metal complex can modify toxicity, solubility and lipophilicity of the drugs, altering pharmacokinetics and pharmacodynamics [118,126]. Because of its favourable properties, ruthenium seems to be a perfect candidate for the design of such delivery agents. Recently, several NO-containing ruthenium compounds have been developed as an alternative delivery strategy of NO in disease states associated with an impaired NO functioning including cardiovascular diseases and erectile dysfunction. These ruthenium-based NO-complexes were tested on their ability to release NO but more importantly also their ability to relax (vascular) smooth muscle cells and the underlying mechanism(s) of effect were investigated (Fig. I.11 and Table I.1).

Class	NO species	NO release	Effect	Mechanism	Ref.
$[Ru(terpy)(bdq)NO]^{3+}$ *(TERPY)*					
polypyridine	$NO^{\bullet}$ / NO^{-}	light irradiation	vasorelaxation *(rat aorta/ mesenteric artery)* blood pressure decrease *(rat)*	sGC K^+ channels Ca^{2+}-ATPase	[127-136]
trans-$[RuCl([15]aneN_4)NO]^{2+}$ *(15-ane)*					
macrocyclic	$NO^{\bullet}$ / NO^{-}	chemical reduction light irradiation	vasorelaxation *(rat aorta)* blood pressure decrease *(rat)*	sGC K_{ATP} K_{Ca} K_v	[137-143]
cis-[RuCl(bpy)2NO](PF6) *(RUNOCL)*					
polypyridine	not specified	light irradiation	vasorelaxation *(rat aorta)* cytosolic $[Ca^{2+}]$ decrease *(rat aorta smooth muscle cells)*	sGC K_{Ca}	[144,145]
cis-$[Ru(bpy)_2(py)NO_2](PF_6)$ *(RuBPY)*					
polypyridine	$NO^{\bullet}$	metabolization	vasorelaxation *(rat aorta)*	sGC K^+ channels	[146-148]
trans-$[Ru(NH_3)_4P(OEt)_3NO](PF_6)_3$ *(RuNO)*					
tetraamine	$NO^{\bullet}$	chemical reduction	blood pressure decrease *(rat)*	sGC	[149-152]
trans-$[RuCl(cyclam)NO](PF_6)_2$ *(cyclam-NO)*					
macrocyclic	$NO^{\bullet}$	chemical reduction light irradiation	blood pressure decrease *(rat)*	sGC	[153-155]

Table I.1: Ruthenium-based NO complexes. ($NO^{\bullet}$ = free radical NO; NO^{-} = nitroxyl anion; sGC = soluble guanylyl cyclase; K_{ATP} = ATP-sensitive K^+ channel; K_{Ca} = Ca^{2+}-activated K^+ channel; K_v = voltage-dependent K^+ channel)

I.2.4.2 $[Ru(terpy)(bdq)NO]^{3+}$

To date $[Ru(terpy)(bdq)NO]^{3+}$, abbreviated as TERPY, is the most extensively described ruthenium-based NO-releasing molecule in literature (Fig. I.12). This nitrosyl polypyridine ruthenium complex has been shown to induce smooth muscle cell relaxation of rat aortic rings due to the release of NO by light irradiation. The time to reach maximum relaxation in the dark was around 14 times longer as compared to the time it took under visible light irradiation [127]. Comparing the vasorelaxant effect of TERPY and the commonly used NO donor SNP led to some interesting insights in the underlying mechanisms of their effect. Although both compounds induce a pronounced relaxation of endothelium-denuded rat aorta, SNP seems to be more potent. The response to SNP only involves free radical NO, whereas both free radical NO and nitroxyl anions are involved in TERPY-induced relaxation. Moreover in contrast to SNP, activation of sarcoplasmic reticulum Ca^{2+}-ATPase does not contribute to the vasorelaxation by TERPY. Taken together, these differences could explain the lower potency of TERPY when compared to SNP. However, the relaxing effect of both NO donors shows some similarities too. The effect of both compounds is mediated by stimulation of sGC and K^+ channels as proven by the inhibitory effect of ODQ and tetraethylammonium (TEA) respectively [128,129]. In rat mesenteric resistance arteries, these pathways are also involved and even sarcoplasmic reticulum Ca^{2+}-ATPase seems to contribute to the vascular relaxation induced by TERPY since thapsigargin blocked its effect [130].

Surprisingly, TERPY failed to relax rat basilar artery as a result of the absence of NO release. However this observation could be viewed as positive (lack of) effect since cerebral vasodilation is thought to be the major cause of NO donor-induced headache, which is the most common side effect of nitrovasodilator therapy [131]. Another interesting finding is the fact that TERPY exhibits a less potent vascular relaxation in the presence of intact endothelium by uncoupling endothelial NOS enhancing tissue $O_2^{\bullet-}$ levels. Subsequent conversion of $O_2^{\bullet-}$ to H_2O_2 or $ONOO^{\bullet-}$ activates the cyclooxygenase enzyme followed by the production of thromboxane A_2 which negatively modulates the TERPY response. Keeping in mind that various disease states are characterized by impairment of the endothelium, the higher potency of TERPY in endothelium-denuded arteries could imply a higher effectiveness in patients suffering from endothelial dysfunction as compared to healthy individuals [132].

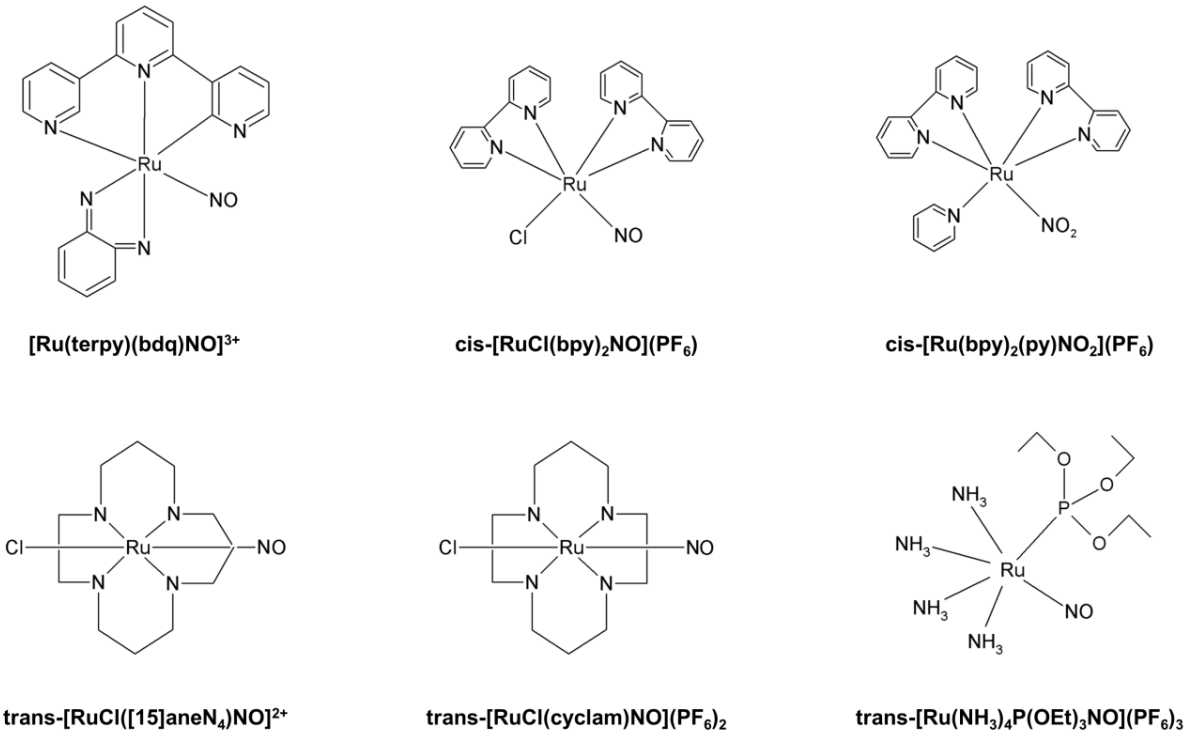

Figure I.12: The chemical structures of the NO-donating ruthenium complexes.

Nevertheless, the absence of functional endothelium associated with cardiovascular disease is not the only alteration that could modify TERPY responses. In a rat model of renal hypertension, the vasorelaxant effect of TERPY seemed to be lower in endothelium-denuded aorta ring segments of hypertensive rats as compared to normotensive control rats. This decreased vasodilatation in response to TERPY was attributed to impaired activation of K^+ channels [133]. Moreover it was previously observed that the antioxidant vitamin C can normalize the altered TERPY effect on aorta's of renal hypertensive rats, providing evidence for the involvement of $O_2^{\bullet-}$ [136]. In contrast, TERPY-induced vasorelaxation was found similar in endothelium-denuded mesenteric resistance arteries of renal hypertensive and normotensive rats [130]. Furthermore the vasorelaxation by TERPY does not differ between normotensive and spontaneously hypertensive rats [129]. Recently, it was reported that the underlying mechanism of the latter observation involves uncoupling of endothelial NOS. In normotensive rats, TERPY leads to uncoupling of endothelial NOS reducing NOS expression and resulting in increased production of $O_2^{\bullet-}$. Subsequent interaction between NO and $O_2^{\bullet-}$, forming $ONOO^{\bullet-}$, lowers NO availability and therefore alters TERPY effect. In spontaneously hypertensive rats, NOS expression is low and $O_2^{\bullet-}$ concentrations are already high altering

the TERPY-induced vasorelaxation. So both in normotensive and spontaneously hypertensive rats, the presence of $O_2^{\bullet-}$influences the vasorelaxation induced by TERPY resulting in an equally potent effect in both rat models [135].

Although these seemingly contradictory in vitro results regarding the added value of TERPY in case of disease and in particular hypertension, TERPY showed promising blood pressure lowering effects in vivo. TERPY has a long-lasting dose-dependent hypotensive effect which is more pronounced in renal hypertensive rats than in normotensive rats [136]. In spontaneously hypertensive rats the hypotension induced by TERPY was also more potent than in normotensive controls. In addition this hypotensive effect does not lead to reflex tachycardia [129]. TERPY also presents a sexual dimorphism as its hypotensive effect is higher in male than in female spontaneously hypertensive rats. This phenomenon seems to be associated with oxidative stress and deficient superoxide dismutase activity in female spontaneously hypertensive rats, as the superoxide dismutase mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (tempol) abolished the sexual dimorphism [134]. Overall, TERPY could represent a new therapeutic approach in the treatment of hypertension associated with vascular dysfunction.

I.2.4.3 Trans-$[RuCl([15]aneN_4)NO]^{2+}$

Trans-$[RuCl([15]aneN_4)NO]^{2+}$ is a nitrosyl macrocyclic ruthenium complex which is occasionally abbreviated as 15-ane (Fig. I.12). This ruthenium complex is able to induce a pronounced vasorelaxation of NOR-contracted rat aortic rings. The time-course for maximal relaxation of 15-ane was up to 3 time longer than for SNP. Relaxation by 15-ane occurred through release of NO as proven by the complete abolishing effect of the NO scavenger oxyhemoglobin. When tissues are contracted with a high concentration of K^+, instead of NOR, the relaxant effect of 15-ane disappears. This was partly explained by the participation of K^+ channels in the 15-ane-induced relaxation. But more importantly this observation indicates that 15-ane needs a reducing agent, such as the catecholamines NOR and phenylephrine, to exert its effect [137,141]. Besides chemical reduction, photoinduction of 15-ane by UV light irradiation is able to release NO from this ruthenium complex, subsequently leading to complete aorta relaxation [137-139].

Just like with TERPY, the NO species contributing to the 15-ane relaxant responses are both nitroxyl anion and free radical NO. Elucidating the molecular pathways underling the relaxant effect of 15-ane, ODQ partially inhibited the vascular relaxation indicating the involvement of sGC activation followed by cGMP formation. cGMP-independent activation of K^+ channels also seems to take place since co-incubation of ODQ with the non-selective K^+ channel inhibitor TEA resulted in an additional inhibition. More specific, 15-ane is able to activate voltage-dependent, ATP-sensitive and Ca^{2+}-activated K^+-channels as its relaxation is impaired in the presence of respectively 4-aminopyridine, glibenclamide as well as apamin and iberiotoxin. Nevertheless, iberiotoxin-sensitive Ca^{2+}-activated K^+ channel showed to be the most prominent subtype involved in the vasorelaxation by 15-ane [143]. The deficient 15-ane-induced relaxation in renal hypertensive rat aortas originates from an impaired functional activity of these K^+ channels, further emphasizing their importance in the 15-ane effect [142]. Although the vasodilatory effect of 15-ane is lower in renal hypertensive rat aortas as compared to normotensive controls, 15-ane only establishes a significant and distinct blood pressure lowering effect in severe renal hypertensive rats. In moderate hypertensive rats the hypotensive effect of 15-ane was limited and dose-dependent, whereas in normotensive rats 15-ane did not present any effect at all [140].

I.2.4.4 Cis-[RuCl(bpy)$_2$NO](PF$_6$)

Cis-[RuCl(bpy)$_2$NO](PF$_6$), also known as RUNOCL, is another polypyridine ruthenium complex which is only activated by irradiation, controlling its release of NO (Fig. I.12). Photoinduction of RUNOCL using a visible light system induces relaxation of phenylephrine-contracted rat aorta, independent of the presence of intact endothelium. Pretreatment of the aortic tissue with ODQ or with apamin and iberiotoxin, both inhibitors of the Ca^{2+}-sensitive K^+ channels, significantly altered the RUNOCL-induced relaxations, implying a role for sGC activation and stimulation of small- and large conductance Ca^{2+}-activated K^+ channels [145]. These observations are in agreement with the decrease of cytosolic Ca^{2+} concentration in vascular smooth muscle cells in response to RUNOCL. Also ODQ inhibits this reduction of cytosolic Ca^{2+}. Moreover TEA is able to block the Ca^{2+} decrease by RUNOCL, hence an activation of K^+ channels also seems to be involved [144].

I.2.4.5 Cis-[$Ru(bpy)_2(py)NO_2$](PF_6)

A third polypyridine complex is the cis-[$Ru(bpy)_2(py)NO_2$](PF_6) compound (= RuBPY) consisting of ruthenium, bipyridine ligands and a nitrite group (Fig. I.12). It was reported that the release of NO by RuBPY was attributed to a reduction process [146]. However later it was proven that RuBPY does not need to be chemically reduced nor does NO release occur upon photoinduction. On the contrary RuBPY-induced relaxations only depend on the presence of tissue [147]. Furthermore it was demonstrated that the complex RuBPY requires metabolization in order to elicit vasorelaxation. It was suggested that the nitrite group of RuBPY is converted into radicalar NO which appears to be catalyzed by the sGC enzyme [147,148]. RuBPY does not only activates sGC, but also induces hyperpolarization of the cell membrane and subsequent vasodilation which is blocked by TEA as well as by high K^+ concentration [148].

I.2.4.6 Trans-[$Ru(NH_3)_4P(OEt)_3NO$](PF_6)$_3$

In light of its NO-delivering capacity, the potential hypotensive effect of the ruthenium tetraamine complex trans-[$Ru(NH_3)_4P(OEt)_3NO$](PF_6)$_3$ (= RuNO) was tested (Fig.I.12) [149]. RuNO can establish a distinct decrease in mean arterial pressure in conscious rats due to NO release after activation by reducing agents. This blood pressure lowering effect of RuNO is completely blocked by the sGC inhibitor methylene blue and the NO scavenger carboxy-PTIO, indicating that its mechanism of action is primarily through the NO-sGC-cGMP pathway. However, when in solution, RuNO rapidly loses its hypotensive properties. Addition of $NaNO_2$ seemed to help maintain the stability of RuNO in physiological conditions. After all, co-administration of $NaNO_2$ and RuNO helped to preserve the hypotensive effect of this ruthenium complex [150]. It was suggested that the nitrite ions could react with the reduced metabolite of RuNO yielding trans-[$Ru(NH_3)_4P(OEt)_3NO_2$]$^+$ and thus renewing the source of NO [151]. Furthermore, RuNO shows some promising results in different rat models of hypertension. The hypotensive response to RuNO was more pronounced in acute hypertension, induced by phenylephrine or angiotensin II, as well as in chronic spontaneously hypertensive rats compared to the normotensive controls. However, when hypertension was obtained by Nω-nitro-L-arginine methyl ester, no difference could be observed between the RuNO effect in hypertensive and normotensive animals [152].

I.2.4.7 Trans-[RuCl(cyclam)NO]$(PF_6)_2$

Trans-[RuCl(cyclam)NO]$(PF_6)_2$, which can be abbreviated as cyclam-NO, is a macrocyclic ruthenium complex (Fig. I.12). Like the macrocyclic 15-ane, NO release of cyclam-NO is controlled by light irradiation as well as chemical reduction [138]. Dissociation of NO occurs relatively slow in contrast to for instance RuNO which rapidly loses NO under physiological conditions. However, in this way cyclam-NO can deliver NO in a controlled manner, acting as a long-lasting, but less potent vasodilator [153,155]. Due to the release of NO following in situ reduction, cyclam-NO exhibits hypotensive properties. In phenylephrine-induced hypertensive rats cyclam-NO produces a reduction in blood pressure which is 3-fold larger than in normotensive rats. Moreover, this decrease of mean arterial pressure proceeds up to 20-times longer than the SNP-mediated blood pressure drop. As suspected the hypotensive effect by cyclam-NO is mainly dependent on cGMP production as a result of sGC activation [154].

I.2.4.8 Other ruthenium-based NO donors

The ruthenium complex cis-[Ru(H-dcbpy$^-$)$_2$(Cl)NO$^+$], although more potent in terms of inducing vascular relaxation, is rapidly converted into the more stable cis-[Ru(H-dcbpy$^-$)$_2$(Cl)NO_2^-] nitrite analog. However, once converted, cis-[Ru(H-dcbpy$^-$)$_2$(Cl)NO_2^-] does not act as a nitrite donor but rather as a NO generator due to fast conversion of the released nitrite to free radical NO. cis-[Ru(H-dcbpy$^-$)$_2$(Cl)NO_2^-]-induced rat aorta relaxation, following a decrease in cytosolic Ca^{2+} concentration, primarily depends on sGC activation. Yet, in part, also activation of K^+ channels takes place [156]. Similar to cis-[Ru(H-dcbpy$^-$)$_2$(Cl)NO$^+$], cis-[Ru(NO_2)(bpy)$_2$NO]$(PF_6)_2$ is converted to its more stable nitrite analog. Likewise, activation of sGC and K^+ channels are the underlying mechanisms of Ca^{2+}decrease and vasorelaxation. Moreover this stable configuration has shown to dose-dependently reduce blood pressure [157].

I.3 Carbon monoxide

It has been shown that CO signaling may become relevant as some kind of rescue mechanism when NO-bioavailability is reduced [158,159]. Since the therapeutic value of NO replacement therapy is limited by the development of tolerance, CO administration has been suggested as an alternative treatment strategy.

I.3.1 Endogenous CO production

CO is generated physiologically, primarily (>85 %) as a side product from the oxidative degradation of heme by the heme oxygenase (HO) enzyme (Fig. I.13). HO catalyzes the cleavage of heme at the α-methene bridge carbon to generate biliverdin-IXα, which is accompanied by the release of ferrous iron and CO. This reaction requires three molecules of molecular oxygen as well as reducing agents from NADPH:cytochrome P450 reductase. Subsequently, biliverdin-IXα is rapidly converted to bilirubin by biliverdin reductase and iron is stored into ferritin [160,161]. The degradation of heme, and thus the endogenous formation of CO, is visible during the development of bruises [162].

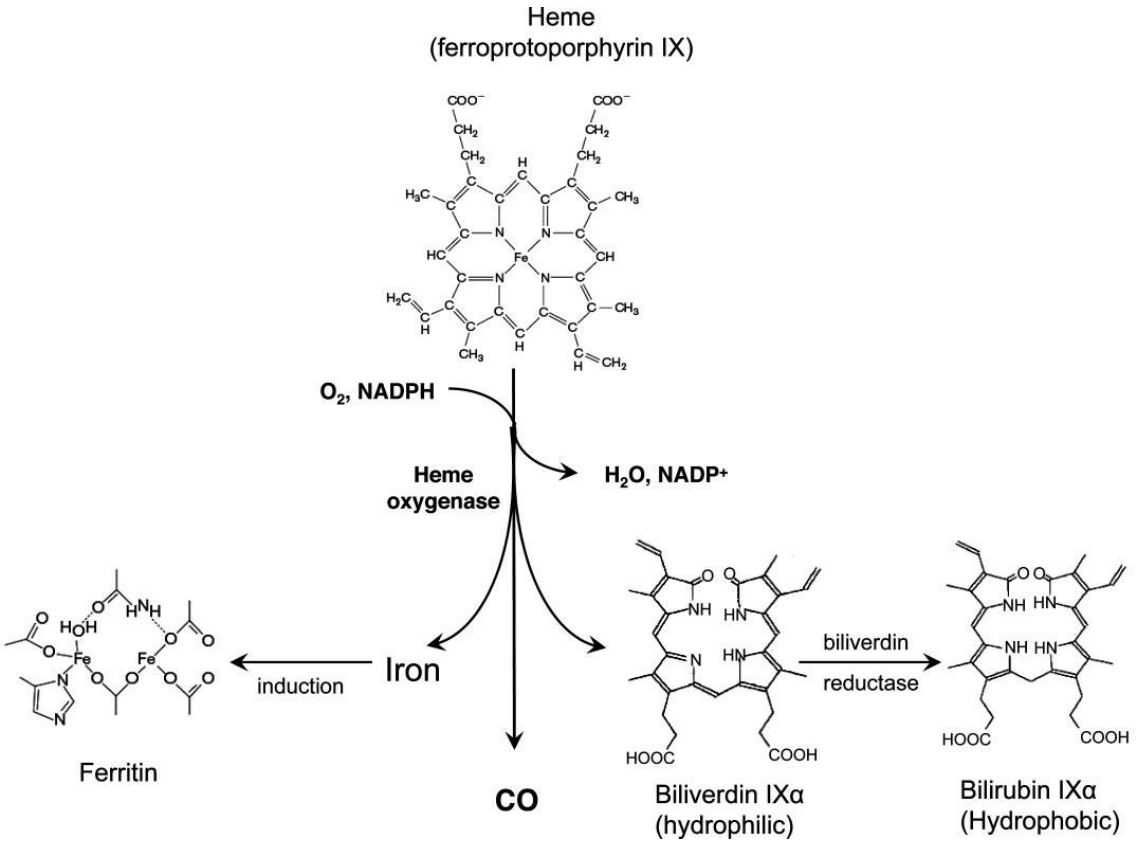

Figure I.13: Oxidative degradation of heme by heme oxygenase [163].

Two major isoforms of the HO enzyme have been characterized, indicated as HO-1 and HO-2 [160,164]. Under basal conditions, HO-2 is constitutively expressed in most human tissues. In contrast, HO-1 is an inducible isoform that may be induced through various conditions and agents including its substrate heme but also hypoxia and hyperoxia, smoking, metalloporphyrins, cytokines, growth factors, hormones and heavy metals. Moreover, HO-1 gene transcription is induced by ROS and oxidative stress, suggesting that HO-1 could serve as an inducible defense mechanism against oxidative cellular stress [165-167]. Some studies also describe a third isoform, HO-3, which closely resembles HO-2 but with much lower catalytic activity. However, this isoform does not seem to be expressed in humans [164].

Just like NO, CO has been shown to act through the activation of sGC increasing cGMP concentrations [168,169]. CO is also able to regulate different classes of ion channels directly. [170] The most widely studied ion channel target of CO is the large-conductance, Ca^{2+}-activated K^{+} channel which is activated by CO in smooth muscle cells [171]. Moreover, various other (heme-dependent) pathways have been described as CO targets such as the cytochrome P450 system [172]. Although the signaling pathway of CO shows some analogy with the NO pathway, CO also substantially differs from NO. The potency of CO to activate sGC is distinctively lower as compared to NO. Binding of NO stimulates sGC activity by 100- to 200-fold, whereas CO only induces a 4-fold increase [173]. Furthermore, CO is essentially a stable molecule as it does not react with intracellular metabolites, unlike NO which is a highly reactive free radical susceptible to inactivation by $O_2^{\bullet -}$ and other oxidative pathways [161].

I.3.2 Ruthenium-based CO-releasing molecules

The feasibility of inhaled CO gas as a therapy remains questionable due to its lack of specificity and its toxicity at high concentrations. After all, binding of CO to hemoglobin, forming carboxy-hemoglobin, concentration-dependently interferes with oxygen transport and -delivery. Additional inhibition of cytochrome C oxidase by CO contributes to the observed cell hypoxia. At high concentrations, CO is associated with various clinical symptoms including dizziness and lowered consciousness. Prolonged and excessive exposure could be even lethal [174]. Hence, CO-releasing molecules (CORMs) were developed to

release CO locally in a safe and controlled way [175]. As with NO, ruthenium seemed a perfect fit for the creation of such metallocomplexes. Several of these ruthenium-based CO-donating compounds were tested on their capacity to relax smooth muscles as well as on their usefulness for the treatment of cardiovascular diseases and erectile dysfunction (Fig. I.14 and Table I.2).

Solubility	CO release	Effect	Mechanism	Ref.
$[Ru(CO)_3Cl_2]_2$ *(CORM-2)*				
organic solvents	ligand substitution	vasorelaxation *(rat aorta/ renal arteriole mice aorta/ femoral artery)* blood pressure decrease *(rat)* corporal relaxation *(mice)* protection against ischemia/reperfusion injury *(rat)*	sGC K_v p38 mitogen-activated protein kinase β protein kinase C phosphatidylinositol 3-kinase	[24,42,73-82]
$[Ru(CO)_3Cl(glycinate)]$ *(CORM-3)*				
water	ligand substitution water-gas shift	vasorelaxation *(rat aorta)* blood pressure decrease *(rat)* corporal relaxation *(rat)* positive inotropic *(rat)* anti-ischemic *(rat)* protection against ischemia/reperfusion injury *(rat/mice)*	sGC K_{ATP} K_{Ca} sGC Na^+/H^+ exchange regulation of Na^+, K^+ and Ca^{2+} levels K_{ATP}	[73,83-92]

Table I.2: Ruthenium-based CO complexes. (sGC = soluble guanylyl cyclase; K_v = voltage-dependent K^+ channel; K_{ATP} = ATP-sensitive K^+ channel; K_{Ca} = Ca^{2+}-activated K^+ channel)

I.3.2.1 $[Ru(CO)_3Cl_2]_2$

$[Ru(CO)_3Cl_2]_2$ (= CORM-2) is the first ruthenium-based CO-releasing molecule that has been developed (Fig. I.14). Since the solubility of CORM-2 is restricted to organic solvents, dimethyl sulfoxide has been used as vehicle to test this compound in vitro and in vivo. CORM-2 has shown to deliver CO in biological systems making it a useful tool to mimic the actions of the endogenous heme oxygenase pathway and to elucidate the effectiveness of CO under pathological conditions as well as its underlying pharmacological mechanism(s) [176]. It was proven that CORM-2 significantly relaxes isolated aortic ring segments and increases arteriolar diameter of afferent renal arterioles in a concentration-dependent manner [158,175,177,178]. In the presence of reduced myoglobin this vasorelaxing effect

was abolished, confirming that the pharmacological effect is mediated by liberation of CO [175]. The guanylyl cyclase inhibitor ODQ significantly attenuates the CORM-2 effect demonstrating, at least in part, the involvement of the sGC-cGMP pathway [176-178]. K^+ channels also contributed to the CORM-2 induced vasodilation. Although it was published that CO is able to act directly on Ca^{2+}-activated K^+ channels, CORM-2 rather activates the voltage dependent K^+ channels in mice thoracic aorta [171,178]. Co-inhibition of sGC and K^+ channels did not produce a more pronounced inhibition of the CORM-2 relaxation providing indirect evidence for the involvement of an additional molecular mechanism [178].

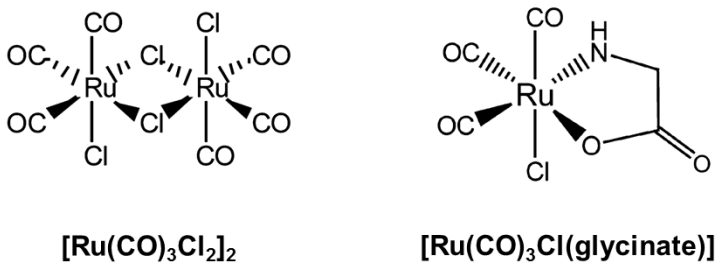

Figure I.14: The chemical structure of $[Ru(CO)_3Cl_2]_2$ (= CORM-2) and $[Ru(CO)_3Cl_2(glycinate)]$ (= CORM-3).

The vasorelaxant capacity of CORM-2 potentially implies a blood pressure lowering effect in vivo. Intravenous administration of increasing CORM-2 concentrations elicits a transient decrease of blood pressure. Hence, it was hypothesized that CORM-2 could be beneficial in case of hypertension. In a rat model of acute hypertension, CORM-2 significantly counteracted L-NAME-induced pressor responses [175]. Although CORM-2 responses seem to be impaired during hypertension as compared to normotensive rats, CORM-2 is still able to induce a significant relaxation of thoracic aorta. Exercise training in hypertensive rats fully restores the impaired relaxant effects of CORM-2 due to an increased K^+ channel activity [179].

In addition to its vasorelaxant effect, CORM-2 exhibits profound cardioprotection. Soni et al. demonstrated a significant concentration-dependent and endothelium-independent reduction of ischemia/reperfusion injury in perfused rat hearts [180]. Further research delineated the underlying mechanisms of this cardioprotective effect, being activation of the p38 mitogen-activated protein kinase β and protein kinase C pathways before ischemia and

the phosphatidylinositol 3-kinase pathway during reperfusion [181]. Moreover, CORM-2 protects cardiomyocytes from ischemia/reperfusion-induced apoptosis most likely via inhibition of a mitochondrial apoptotic pathway and an improvement of the energy metabolism [182]. During the postresuscitation period CORM-2 also improves cardiac function by reducing the production of cardiac mitochondrial ROS, attenuating oxidative stress of the heart [183].

CORM-2 does not only relax vascular smooth muscles, but also relaxes mice CC. CO gas and CORM-2 are both able to concentration-dependently induce corporal smooth muscle relaxation offering a valuable treatment option for erectile dysfunction. The CO gas response seems to be partially mediated by the sGC-cGMP pathway. However, the underlying mechanism(s) of the CORM-2 effect on CC remain(s) as yet unknown [184].

I.3.2.2 $[Ru(CO)_3Cl(glycinate)]$

Due to its poor water solubility, the compatibility of CORM-2 with biological systems is relatively low limiting its pharmacological value. Therefore a new series of ruthenium carbonyls were developed by attaching different amino acid groups to the metal core. After screening these compounds for their potency to release CO and to mediate vasorelaxation, $[Ru(CO)_3Cl_2(glycinate)]$ (= CORM-3) was identified as most promising agent (Fig. I.14) [176]. Just like CORM-2, CORM-3 readily releases CO in a biological milieu. Its vasoactive properties in isolated vessel ring segments were tested, showing a profound aortic relaxation within a few minutes after addition to the organ bath. The lack of response to iCORM-3 (inactivated CORM-3 which is depleted of CO) demonstrates that CO is directly responsible for the vascular effects of CORM-3. Blocking the ATP-dependent K^+ channels with glibenclamide or the sGC activity with ODQ considerably reduced CORM-3-dependent relaxation, confirming that K^+ channel activation and cGMP partly mediate the CORM-3 effect. CORM-3-induced vasorelaxation was significantly lower in endothelium-denuded vessels as well as after inhibition of NOS activity. Three possible underlying reasons were proposed: (1) a synergistic/permissive role of an endothelial factor augmenting the vasodilatory activity of CO, (2) the production of a substance in the endothelium such as NO which interacts with the ruthenium core of CORM-3 facilitating CO release or (3) displacement of NO from an

intracellular storage pool by CO [185]. However, Alshehri et al. proved that the aforementioned hypotheses are unlikely to explain the endothelium- and cGMP-dependent relaxant effect of CORM-3. They suggested that this effect results from a stimulation of NOS. Furthermore, they found that CORM-3 also produces relaxation independent of the endothelium and sGC by direct activation of smooth muscle K^+ channels [186].

Besides its vasorelaxant activities in vitro, CORM-3 also elicits vasorelaxation in vivo leading to a rapid decrease in mean arterial pressure. This blood pressure lowering effect was further enhanced in the presence of YC-1, which is known to sensitize sGC to activation by CO, confirming the involvement of sGC [185,187]. To look further into the actions of CORM-3 in the cardiovascular system during hypertension, the effect of CORM-3 was investigated on aortas of normotensive and spontaneously hypertensive rats. Pretreatment with ODQ or charybdotoxin reduced the CORM-3 relaxation in both strains, indicating a contribution of sGC and Ca^{2+}-activated K^+ channels respectively. In contrast to the NO donor S-Nitroso-N-Acetyl-D,L-Penicillamin, the CORM-3 mediated vascular relaxation was conserved in spontaneously hypertensive rat aortas. Hence, CORM-3 could serve as a potential alternative for the treatment of hypertension, when response to NO donor therapy turns out to be sub-optimal or absent [188].

The vasodilatory properties of CORM-3 are associated with positive inotropic and anti-ischemic effects observed in cardiac tissue. CO release from CORM-3 exerts a direct positive inotropic effect on isolated perfused rat heart in which both cGMP and stimulation of Na^+/H^+ exchange appear to be involved [189]. Many research groups studied the cardioprotective effect of CORM-3 on myocardial ischemia/reperfusion injury. CORM-3 improves haemodynamic, biochemical and histological parameters of isolated hearts subjected to ischemia/reperfusion by the activation of mitochondrial ATP-dependent K^+ channels. CORM-3 has a beneficial effect on myocardial perfusion and contractility, reduces the release of myocardial creatine kinase (a specific marker of cardiac tissue injury) and attenuates infarct size [190]. CORM-3 reduces the incidence of reperfusion-induced ventricular fibrillation and tachycardia by regulating Na^+, K^+ and Ca^{2+} levels. Normally, myocardial ischemia/reperfusion injury is associated with cellular loss of K^+ as well as increased intracellular concentrations of Na^+ and Ca^{2+}. When hearts are treated with CORM-3, significant decreases in cellular Na^+/Ca^{2+} gains and K^+ loss occur resulting in antiarrhythmic

protection and associated reduction in infarct size. However, the direct mechanism(s) underlying this normalization of ion balance remain(s) to be fully identified. CORM-3 also diminishes myocardial infarct size in vivo when given just before reperfusion [191,192]. Even more important is the fact that CORM-3 has been shown to induce cardioprotection not only when administered at the time of reperfusion but also when given 24 or 72 h prior to coronary occlusion. This sustained cardioprotective influence of CORM-3 may be useful as prevention in patients at risk for myocardial infarction [193].

Since erectile dysfunction is often attributed to a decrease in cGMP content, CORM-3 has been investigated to see if it could alter cavernous cGMP content due to its release of CO and subsequent activation of sGC. Intracavernosal injection of CORM-3 increased cGMP by twofold compared with the inactive negative control. Moreover it increased endogenous heme oxygenase-1 protein. Hence, CORM-3 seems feasible to compensate for the lowered cGMP content in erectile dysfunction [194].

I.3.2.3 Other ruthenium-based CO donors

Besides the two prototypic ruthenium-based CORMs described above, a few other CO-delivery agents were developed, with ruthenium being the metal core, such as ruthenium imidazole oxime carbonyls [195]. Although previously most studies with CORMs were performed with the commercially available CORM-2 and CORM-3, nowadays the potential usefulness of both compounds is a matter of debate. Researchers have tried to elucidate the chemistry and mechanisms of both CORMs, however the exact mode of action remains obscure [196]. CORM-2 and CORM-3 formerly accepted as fast CO-releasers recently appear to require a reducing agent in order to release CO at an appropriate rate. More importantly there seem to be some discrepancies between CORM effects, CO gas effect and heme oxygenase induction, although all three strategies aim to increase CO concentrations [178,197]. Furthermore these CORMs have shown to elicit effects independently of CO-release [170,198]. Even though in most cases these CO-independent effects seem to be beneficial, possible undesired side effects should be kept in mind. Romao et al. presented a model for the rational design of CORMs with the appropriate pharmaceutical properties [199]. Based on the principles of this model, new complexes with

improved properties were developed. [$RuCl_2$-thiogalactopyranoside$(CO)_3$], abbreviated as ALF492, showed some improved druglike properties as well as modified CO-release profiles [200]. Moreover alternative CORM-delivery strategies are being tested in which for instance CORMs are combined with a micellar system [201]. However, to date none of these newer "next-generation" ruthenium-based CORMs were tested in the context of smooth muscle relaxation or as potential therapeutics for cardiovascular diseases and/or erectile dysfunction.

I.4 Soluble guanylyl cyclase

sGC is the principal physiological target of NO (and CO), playing a pivotal role in vascular as well as penile smooth muscle relaxation. Therefore, exploring the involvement of sGC in the relaxant effect of new alternative (NO-related) treatment strategies is inevitable.

I.4.1 Guanylyl cyclases

cGMP was first identified and purified in rat urine in 1963 [202]. A few years later, the enzymes responsible for the conversion of GTP into cGMP were discovered [203-205]. Over time it became clear that these so called guanylyl cyclases (GC) are ubiquitous enzymes regulating critical functions in vivo. In mammals, 7 different membrane-bound/particulate GCs (pGC) and 2 physiologically relevant cytosolic/soluble GCs (sGC) exist. Although pGCs and sGCs show certain sequence homology and structural resemblance, their function and regulation is substantially different. sGC is a heterodimeric protein regulated by the gaseous molecules NO and CO [206]. In contrast, pGC is generally expressed as homodimer serving as a receptor for peptide ligands including the atrial, brain and C-type natriuretic peptides (resp. ANP, BNP and CNP) [207,208]. The natriuretic peptide family is primarily involved in the regulation of cardiovascular homeostasis. ANP and BNP are both produced predominantly in the heart and seem to bind to the same signaling receptor (NPR-A). CNP is expressed in (vascular) endothelial cells and has its own receptor (NPR-B). Being a potent inhibitor of smooth muscle proliferation, CNP acts as an antagonist for a variety of growth factors [209].

I.4.2 Structure and isoforms

Isolation of sGC from the cytosol has proven that the soluble isoforms of GC exist as heterodimers composed of two homologous subunits, α and β, with a molecular mass of ~73-82 kDa and ~70 kDa respectively [212]. Expression of both subunits is required for catalytic activity [213]. In mammals, for each subunit two isoforms are identified (α_1/α_2 and β_1/β_2). The isoforms of α-subunits are highly homologous with ~48% sequence identity whereas β-subunits have an overall sequence identity of ~41% [214]. Both the α_1 and β_1 subunit are found in most tissues opposed to the α_2 subunit which is only highly expressed in the brain, lung, colon, heart, spleen, uterus, and placenta. Unlike β_1, the β_2 isoform is not

ubiquitously expressed and is primarily found in the kidney [215]. The best-characterized and physiologically relevant heterodimers are the α_1/β_1 and the α_2/β_1 forms. Although the primary structure of both α-isoforms substantially differs, no difference could be observed when comparing the kinetic and pharmacological properties of the α_1/β_1 and α_2/β_1 heterodimers [216]. For the β_2 subunit it was found that it does not exhibit cyclase activity when expressed with α_1 or α_2. In vitro, β_2 seems to be active in the absence of an α-subunit, suggesting that it can function as a homodimer. However, to date the physiological role of β_2 in cGMP signaling remains unclear [217].

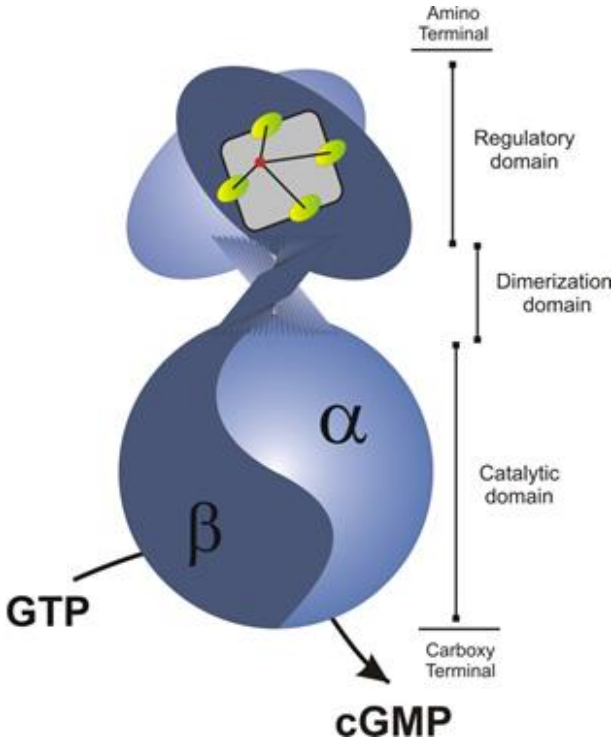

Figure I.15: Schematic structure of the heterodimeric sGC enzyme (based on [210] and [211])

Each subunit consists of three functional domains: an amino-terminal heme-binding regulatory domain, a central dimerization domain and a carboxy-terminal catalytic domain (Fig. I.15) [211]. Compared to the central and catalytic domains, the regulatory region of sGC subunits exhibits lower homology between isoforms and across species. However, this regulatory amino-terminal domain plays a critical role in the sGC function as it binds heme which is required for the activation of sGC by NO [215]. sGC binds 1 molecule of heme per heterodimer which is sandwiched between the two subunits [218]. The central dimerization region is involved in the formation of the heterodimeric structure, which is also necessary for the sGC enzyme to exhibit catalytic activity [213]. The carboxy-terminal domain of the

sGC subunits is the most conserved region of these proteins sharing a substantial sequence homology with the corresponding domains in pGCs and adenylyl cyclases. This domain is responsible for substrate recognition and the catalytic activity of sGC [211,215].

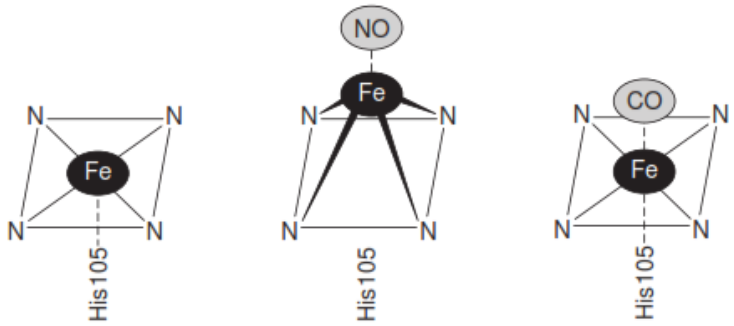

Figure I.16: Schematic representation of NO and CO binding to the prosthetic heme moiety of sGC [219].

The prosthetic heme group of sGC is a five-membered nitrogen-containing porphyrinic ring in which four nitrogen atoms are coordinated with a central ferrous (Fe^{2+}) iron (Fig. I.16). The axial ligand of the penta-coordinated iron center is histidine at position 105 (His_{105}) in the amino terminus of the β subunit [220]. Binding of NO results in the formation of a transient hexa-coordinated histidine-heme-NO intermediate. By breaking the bond between the axial histidine and iron, this intermediate is rapidly converted into a penta-coordinated ring with NO in the fifth position, displacing the iron from the plane of the porphyrinic ring. Activation of sGC by binding of NO and subsequent conformational change will increase basal catalytic activity up to 200 fold [221]. His_{105} is the essential amino acid required for binding the prosthetic heme moiety in the β subunit. Mutation of His_{105} results in the inability of sGC to bind heme and produces an enzyme that is unresponsive to NO [222,223]. Moreover, oxidation of the heme group to the ferric iron (Fe^{3+}) state also prevents both binding and activation by NO and is even often associated with a complete loss of the heme moiety from the protein [224]. Besides NO, sGC can bind CO at its heme group, yielding a hexa-coordinated complex. Leaving the iron-histidine bond intact, interaction between CO and sGC leads only to a 4-fold increase of catalytic activity [225]. When compared to other hemoproteins, such as hemoglobin and myoglobin, sGC is unique because it preferentially binds NO (or CO) rather than oxygen forming a ferrous-nitrosyl species even under aerobic conditions. This peculiar characteristic of sGC is important for its function as NO sensor since in vivo oxygen is present at much higher levels than NO [211,214].

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Chapter II

Aims

It is generally accepted that NO act as an essential gaseous neurotransmitter in the human body. In the cardiovascular system NO regulates normal blood pressure, blood flow and vascular resistance [1]. Moreover, NO plays a crucial role in the mechanism of penile erection [2]. Therefore it is reasonable that impairment of NO-bioavailability results in various disease states, including hypertension and erectile dysfunction (ED) [3,4]. Because of the limitations and adverse effects of current NO-related therapies, the search for new and better alternatives still remains [5,6]. In this continuous urge for improvement, oxime derivatives have been put forward due to their NO-releasing capacities [7]. Furthermore, (ruthenium-based) metallocomplexes have been suggested as delivery agents of (gaseous) molecules such as NO and CO [8]. By compensating the loss of normal NO functioning, both oximes and ruthenium-based NO/CO-releasing molecules potentially offer some (new) therapeutic perspectives.

Oxime derivatives had already shown to induce vascular relaxation of rat aorta ring segments as a result of their NO-release [7]. This vasorelaxant effect could also be of value in the context of penile erection and ED. Hence, in our first study, described in **Chapter IV**, we examined the ability of the oximes FAL and FAM to relax isolated mice corpora cavernosa (CC) by performing in vitro tension measurements. We also aimed to elucidate the underlying mechanisms of the oxime effects in vitro, without the influence of counteracting neuronal, humoral and hemodynamic factors seen in in vivo conditions.

However, in order to find out the physiological relevance of our in vitro results, we also carried out some in vivo experiments. In this second study, we aimed to monitor the changes in mean arterial pressure (MAP) and intracavernosal pressure (ICP) after intravenous and intracavernosal injection of the oxime compounds respectively. Furthermore, we tried to confirm the importance of sGC as the main target of FAL-/FAM-induced effects, both in vitro and in vivo, by using genetically modified sGCα_1 knock-out mice (sGC$\alpha_1^{-/-}$). The results of this study are presented in **Chapter V**.

An important factor responsible for the lowered NO functioning in ED is the presence of reactive oxygen species [9]. Oxidative stress and the associated production of reactive oxygen species, such as superoxide, diminish the available effective NO concentrations by forming peroxynitrite, thus impairing NO-mediated relaxations. So in additional in vitro experiments, we tested the influence of oxidative stress on (NO-mediated) corporal relaxations with special attention to the effect on FAL-/FAM-induced responses. The results are also presented in **Chapter V**.

Over the years, various ruthenium-containing metallocomplexes were developed for the safe and controlled release of NO and CO. Since NO and CO are able to induce vasorelaxation, these ruthenium-based compounds were also expected to present a vasorelaxing effect which was already reported various times in literature [8]. However, some ambiguous results regarding the correlation between the CO-release from CO-releasing molecule-2 (CORM-2) and its vascular effects, made us wonder if ruthenium itself could influence vascular tone [10]. Therefore, we designed a study to explore the vasorelaxing effect of different ruthenium-containing compounds on mice aorta ring segments. In addition, former studies had demonstrated that CORM-2 and its water soluble alternative CORM-3 attenuate NO donor-induced vasorelaxation [11,12]. To evaluate the influence of ruthenium as such on exogenous and endogenous NO-mediated signaling, we performed in vitro experiments with some ruthenium-containing compounds. The results of the ruthenium experiments are described in **Chapter VI**.

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Chapter III

Materials and Methods

III.1 In vitro tension measurements

In vitro tension measurements are a sensitive technique to evaluate the mechanical responses of isolated vascular ring segments and corpora cavernosa (CC). It allows complete control over the experimental conditions without the interference of competing or compensating regulatory in vivo mechanisms. This technique, using freshly isolated tissues, is the golden standard to investigate the influence of molecules on vascular tone and makes it possible to determine the underlying molecular pathways.

III.1.1 The experimental setup

The changes in tissue tension were measured by a myograph constructed by the technical staff of our laboratory (Fig. III.1 and Fig. III.2) [1]. This myograph consists of a 10 ml organ bath containing two holders to fix the isolated tissue preparations. One of the holders is connected to a micrometer which allows adjustment of the distance between the two holders. While maintaining this distance and therefore the length of the mounted tissues constant, isometric tension changes are recorded by a force transducer which is connected to the other holder. Subsequently, the measured force is transformed into an electrical signal which is amplified and registered by a reading system. In order to create in vivo-like conditions the myograph was placed on a heating plate at 37°C and carbogen gas (95 % O_2 en 5 % CO_2) was supplied continuously. The organ bath solutions were also preheated in a warm water bath at 37°C and bubbled with carbogen gas.

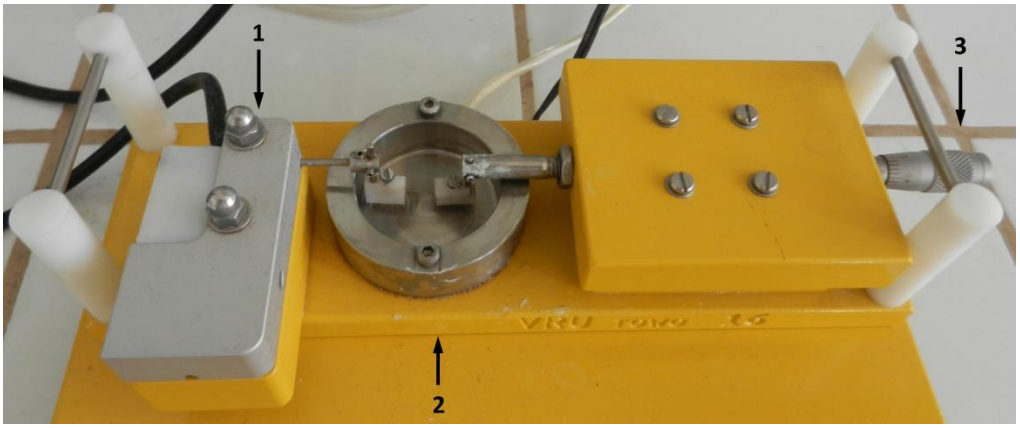

Figure III.1: Myograph with (1) force transducer, (2) organ bath and (3) micrometer.

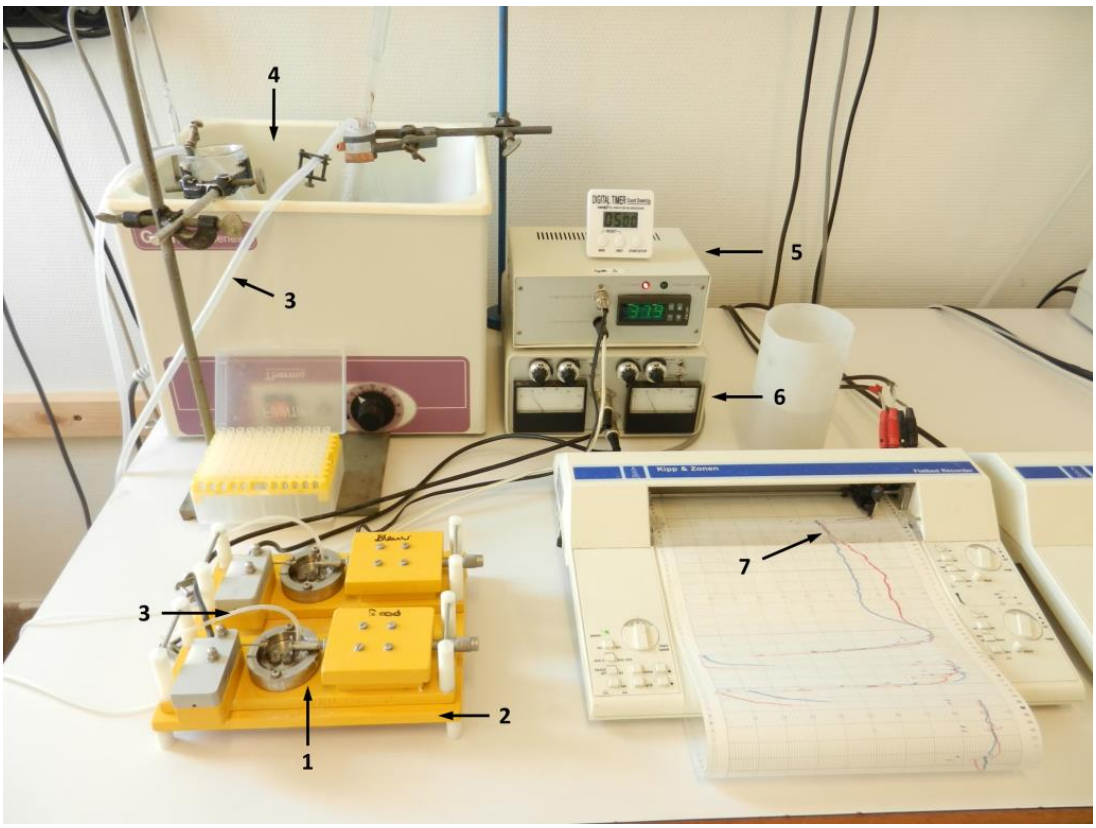

Figure III.2: Overview of the experimental in vitro setup. (1) myograph (2) heating plate (3) carbogen gas supply (4) warm water bath (5) thermoregulator (6) amplifier (7) reading system.

III.1.2 Mice models

Most experiments were performed with mature (age 8-12 weeks) male Swiss mice which were obtained from Janvier (Saint-Berthevin, France). For determining the role of sGC in the molecular pathway of the studied vasorelaxant compounds, transgenic $sGC\alpha_1$ knock-out mice ($sGC\alpha_1^{-/-}$) and $sGC\beta_1$ knock-in mice ($sGC\beta_1^{ki/ki}$) as well as their wild type controls (129SvJ) were used. These transgenic mice were generated, genotyped and bred in the Department of Biomedical Molecular Biology (Ghent University) at the Laboratory of Molecular Pathology and Experimental Therapy (Inflammation Research Center, VIB, Ghent, Belgium). The genotyping of the mice was carried out prior to the experiments by PCR and Southern blot analysis. All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Food and water were provided ad libitum and the animals were kept in a 12h/12h day/night cycle. All experiments were also approved by the local ethical committee for animal experiments (ECD 10/42, Ghent University, 14/02/2011 and ECD 14/48, Ghent University, 14/07/2014).

III.1.2.1 $sGC\alpha_1^{-/-}$

The $sGC\alpha_1$ knock-out mice ($sGC\alpha_1^{-/-}$) were developed by a targeted deletion of exon 6 of the $sGC\alpha_1$ gene. Deletion of exon 6, which codes for a conserved region of the catalytic domain, does not cause a frame shift but results in a mutant protein. When co-expressed with $sGC\beta_1$, this mutant protein is able to form a heterodimer, which is however found to be inactive and could not be stimulated by NO. Hence, the $sGC\alpha_1^{-/-}$ mice lack a functional $sGC\alpha_1\beta_1$ isoform which is the predominant form in vascular smooth muscle cells. However, the $sGC\alpha_2\beta_1$ activity still remains. Quantitative RT–PCR experiments and immunoblot techniques have also indicated that $sGC\alpha_2\beta_1$ does not compensate for the loss of cGMP production by $sGC\alpha_1\beta_1$ [2].

III.1.2.2 $sGC\beta_1^{ki/ki}$

Transgenic $sGC\beta_1$ knock-in mice ($sGC\beta_1^{ki/ki}$) were generated by replacing exon 5 of the endogenous Gucy1b3 gene with a mutant exon 5 carrying a point mutation of the histidine 105 residue. Normally, this histidine 105 is responsible for ligation of the heme-group to sGC. However, replacement of the histidine residue with phenylalanine results in the expression of heme-free sGC. Although basal activity is preserved, the heme-free sGC can no longer be activated by NO [3]. This modification of the β_1 subunit is generally considered as a global alteration of sGC activity because both $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$ are affected and the α_1 and α_2 subunits do not form functional heterodimers with the β_2 subunit (as mentioned in Chapter I.4).

III.1.3 Tissue preparation

At the day of the experiment, the tissues were dissected from the mice after cervical dislocation (Fig. III.3). The thoracic part of the aorta was isolated from the thoracic cavity alongside the spine and displaced in fresh, cold Krebs-Ringer bicarbonate (KRB) solution. Remaining adherent connective tissue as well as surrounding adipose tissue were carefully removed. In the upper leg, the femoral artery was separated from the femoral vein and nerve. Furthermore, the CC were cleansed from surrounding connective and adipose tissue and the dorsal arteries, dorsal veins, glans penis, corpus spongiosum as well as the urethra

were removed. The CC were separated and excised at the base. After excision of the femoral artery and CC, the isolated tissues were also transferred in cold KRB solution. Isolation of the tissues has to be done with precision and great care in order to preserve intact smooth muscle contraction and functionality of the endothelium.

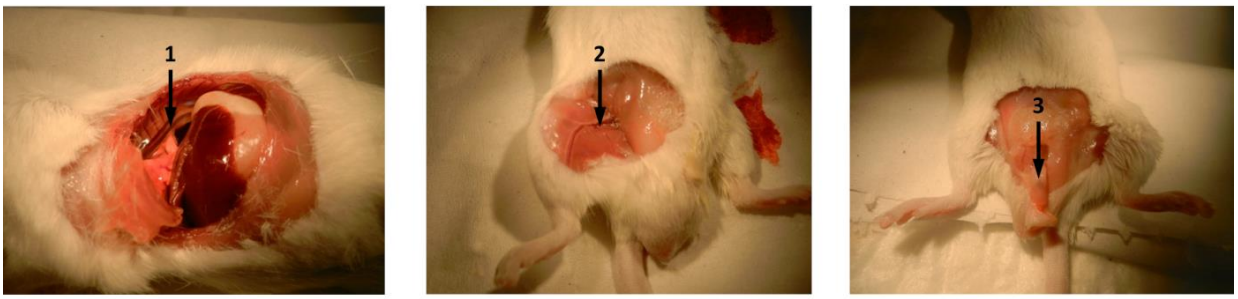

Figure III.3: Isolation of mice (1) aorta, (2) femoral artery and (3) corpora cavernosa.

III.1.4 Tissue mounting

After isolation, the tissues were mounted into the organ bath of the myograph by fixing them between two holders (Fig. III.4). The arterial ring segments were mounted by passing two wires through the lumen of the vessels whereas the CC strips were clamped between the holders.

Figure III.4: Organ bath with half-moon shaped holders for mounting arterial ring segments (left) and holders for clamping CC strips (right).

III.1.4.1 Arterial ring segments

The isolated thoracic aorta and femoral artery were cut into ring segments of around 2.5 mm in length. Under a microscope, these arterial ring segments were then mounted in an organ bath between a pair of holders by guiding two thin stainless steel wires (40 µm) through the lumen.

First, one wire was clamped between the holders by turning the micrometer (Fig. III.5.2). The wire was fixed on the top corner of the left holder with a screw keeping the free end pointed towards the operator (Fig. III.5.3). Next, the arterial ring segment was gently pulled over the wire using a forceps (Fig. III.5.4). Slowly, the ring segment was slided towards the center of the holders and the wire was tightened with a screw (Fig. III.5.5). In the following step, a second wire was guided through the arterial lumen and again clamped between the holders by turning the micrometer (Fig. III.5.6). The second wire was screwed to the top and bottom corner of the right holder (Fig. III.5.7). Finally, the remaining loose ends of the wires were cut off to avoid interference the experiments (Fig. III.5.8). This mounting procedure has to be done with due diligence to remain the endothelium intact.

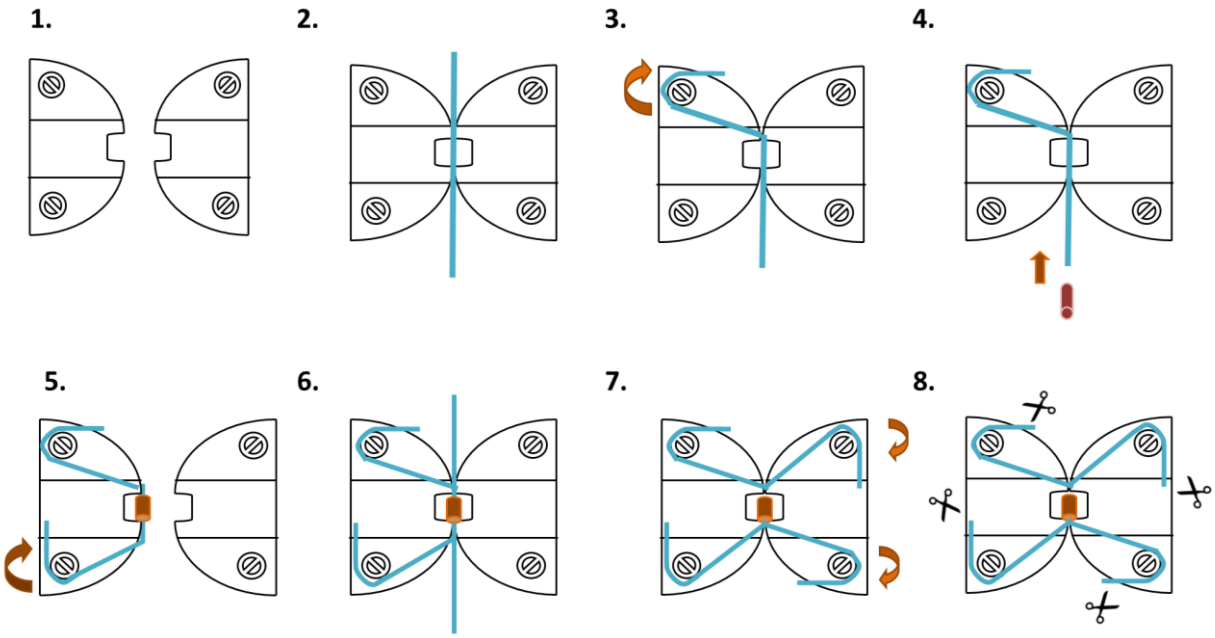

Figure III.5: Schematic overview of the mounting procedure of arterial ring segments.

III.1.4.2. CC strips

In contrast to the small arterial ring segments, mounting the CC strips is much easier. Each CC was mounted longitudinal as a whole. Instead of the half-moon shaped holders for the arterial tissues, the holders for the corporal tissue consist of two blocks in between which the CC can be pressed. Both ends of the CC were clamped between the two holders and tightened with two screws. Because of the relatively larger size, this procedure can be done without the need for a microscope.

III.1.5 Preliminary Protocol

After mounting, the tissue preparations were left to equilibrate for 30 minutes in KRB solution (37 °C; pH 7.4) and continuously oxygenated with carbogen gas. In order to obtain maximal, stable contractions and relaxations, each type of tissue was subjected to a slightly different preliminary protocol before starting with the actual experiments.

III.1.5.1 Aortic ring segments

The mounted aortic tissues were gradually stretched by turning the micrometer until a fixed stable preload of 4.905 mN was obtained, as it has been shown that this results in a maximal response. It takes around 30 minutes to reach a stable equilibrium during which the aortic ring segments spontaneously lose part of their tension. Therefore regular adjustment is necessary until the tension remains stable. Every 10 minutes the KRB solution in the organ bath was refreshed with new, preheated and oxygenated KRB solution. After submitting the preload, the aortic rings were maximally contracted for 20 minutes by replacing the KRB solution with an adjusted high K^+ (120 mmol/L) KRB solution and concomitant addition of 5 µmol/L norepinephrine (NOR). Afterwards, the aortic tissues were rinsed with KRB solution until they returned to basal tone. Subsequently, functionality of the endothelium was tested by first contracting the aortic ring segments with 5 µmol/L NOR. When a stable plateau was reached, 10 µmol/L acetylcholine (ACh) was added. The endothelium was considered intact in tissues that relaxed more than 50 % of the obtained contraction level after addition of ACh. The preparations were intensively washed and ready to start with the actual experiments when basal tone was reached again.

III.1.5.2 Femoral artery ring segments

In small arteries, such as the femoral artery, the optimal internal diameter for maximal active force production was determined by using a normalization procedure [1,4]. Gradually stretching the femoral ring segments influences the length of the circumferentially oriented smooth muscle cells. At a certain internal circumference or diameter, which correlates with a specific length of the smooth muscle cells, the force development reaches a maximum. Therefore, optimal experimental conditions can be obtained by calculating the position of the holders corresponding to the vessels' ideal internal circumference which has been found

to be 0.9 times the internal diameter a vessel would have in situ when relaxed and under a transmural pressure of 100 mmHg. After normalizing the femoral artery ring segments to their optimal diameter, precontraction of the tissues was induced with high K^+ KRB solution and 10 µmol/L NOR. To have a maximal contraction response during the experiments, this precontraction step was repeated twice. After each precontraction the femoral artery rings were washed to obtain basal tissue tension. The presence of intact endothelium was checked by addition of 10 µmol/L ACh when the rings segments reached a stable plateau with 10 µmol/L NOR. When the contraction level dropped more than 50 % after ACh addition, the endothelium was considered fully functional. Afterwards the tissues were rinsed again.

III.1.5.3. CC strips

Just like with the aortic tissues, the CC were gradually stretched to offer nearly maximal reactivity during the experiments. After 60 minutes of regular tension adjustment, a stable preload of 4.415 mN was reached. Then, the CC were precontracted two times by adding only 5 µmol/L NOR since the use of a high K^+ KRB solution does not result in stable contractions. When a plateau was reached after approximately 10 minutes, the CC strips were extensively rinsed until they returned to their basal tension level. Control of the endothelial functioning was performed by contraction with 5 µmol/L NOR and subsequent relaxation with 1 µmol/L ACh. To consider the endothelium intact, a relaxation of at least 50 % was required again. Afterwards the tissues were washed to basal tone.

III.1.6 Mechanistic studies

In the different in vitro studies presented in this thesis, various pharmacological agents were used because of their specific effects when incubating the isolated tissues with these substances. An overview of the compounds used can be found in Table III.1. Various pharmacological inhibitors were added to elucidate the underlying molecular pathways involved in the FAL- and FAM-induced relaxations. Hydroquinone and DETCA were used to create an in vitro model for oxidative stress. L-ascorbic acid and N-acetyl L-cysteine were applied in the in vitro experiments with $RuCl_3$ because of their antioxidant capacities.

For the arterial tissues, experiments were performed in parallel (unpaired observations). A concentration-response curve was performed in a ring segment, while at the same time in another, parallel ring segment the concentration-response curve was performed in the presence of an antagonist. In contrast, the corporal strips were each used as their own control (paired observations). A concentration-response curve was established on a corporal strip and after a washout period, the concentration-response curve was repeated on the same corporal strip in the presence of an antagonist.

Abbr.	(Chemical) Name	Effect	Conc.	Incubation Time
ODQ	1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one	sGC inhibition	10 µmol/L	10 min
DPI	diphenyliodonium chloride	Flavoprotein inhibition	10 µmol/L	30 min
7-ER	7-ethoxyresorufin	NADPH-dependent reductases inhibition CYP450 1A1 inhibition	10 µmol/L	20 min
apocynin	4'-hydroxy-3'-methoxyacetophenone	NADPH oxidases inhibition	300 µmol/L	20 min
miconazole	1-[2-(2,4-Dichlorobenzyloxy)-2-(2,4-dichlorophenyl)ethyl]-1H-imidazole	CYP450 inhibition	10 µmol/L	20 min
L-NNA	Nω-Nitro-L-arginine	NOS inhibition	100 µmol/L	20 min
TEA	tetraethylammoniumchloride	K^+ channels inhibition	3 mmol/L	20 min
apamin	apamin	Small conductance calcium activated K^+ channel inhibition	1 µmol/L	20 min
SQ 22,536	9-(Tetrahydro-2-furanyl)-9H-purin-6-amine	Adenylate cyclase inhibition	100 µmol/L	20 min
compound C	6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo [1,5-a]pyrimidine	AMP-activated protein kinase inhibition	10 µmol/L	20 min
hydroquinone	1,4-Dihydroxybenzene	Superoxide generation Free radical scavenging	50 µmol/L	20 min
DETCA	diethyldithiocarbamic acid	Superoxide dismutase inhibition	8 mmol/L	20 min
L-ascorbic acid	L-ascorbic acid	Antioxidant	100 µmol/L	20 min
N-acetyl L-cysteine	N-acetyl L-cysteine	Antioxidant	1 mmol/L	20 min

Table III.1: Overview the pharmacological molecules used in the in vitro experiments, their main effect(s), the concentration used and the duration of tissue incubation before inducing contraction with norepinephrine or phenylephrine.

III.1.7 Electrical Field Stimulation

Neurogenic-mediated relaxations of the CC were examined by applying electrical field stimulation (EFS; parameters: train duration 40 s; frequency 1, 2, 4 and 8 Hz; pulse duration 5 ms; 80 V). When a stable contraction was obtained with phenylephrine (5 µmol/L), two parallel platinum electrodes were lowered into the organ baths using a lever (Fig. III.6). The L-shaped electrodes are placed at the level of the mounted CC in a way that the CC strip is located in-between both ends of the electrodes (Fig. III.6). The electrodes are connected to a Grass stimulator (80 V). The CC strips were stimulated with consecutive frequencies of 1, 2, 4 and 8 Hz. Each stimulus was applied for 40 seconds with a pulse duration of 5 ms.

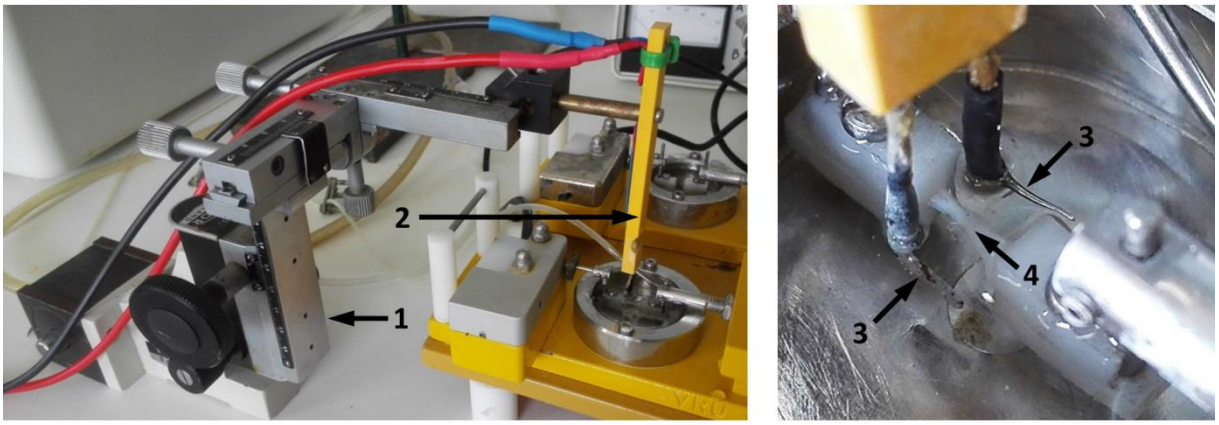

Figure III.6: Overview of the experimental setup for the in vitro electrical field stimulation (left) showing the lever (1) and electrode holder (2). Close up of the organ bath (right) showing the position of the L-shaped platinum electrodes (3) relative to the mounted CC strip (4).

III.2 In vivo pressure measurements

Although in vitro tension measurements are well suited for elucidating the molecular mechanisms of contractile and relaxant compounds, it is not necessarily correlated to relevant in vivo effects. Therefore, additional in vivo experiments were performed evaluating simultaneously blood pressure and intracavernosal pressure (Fig. III.7).

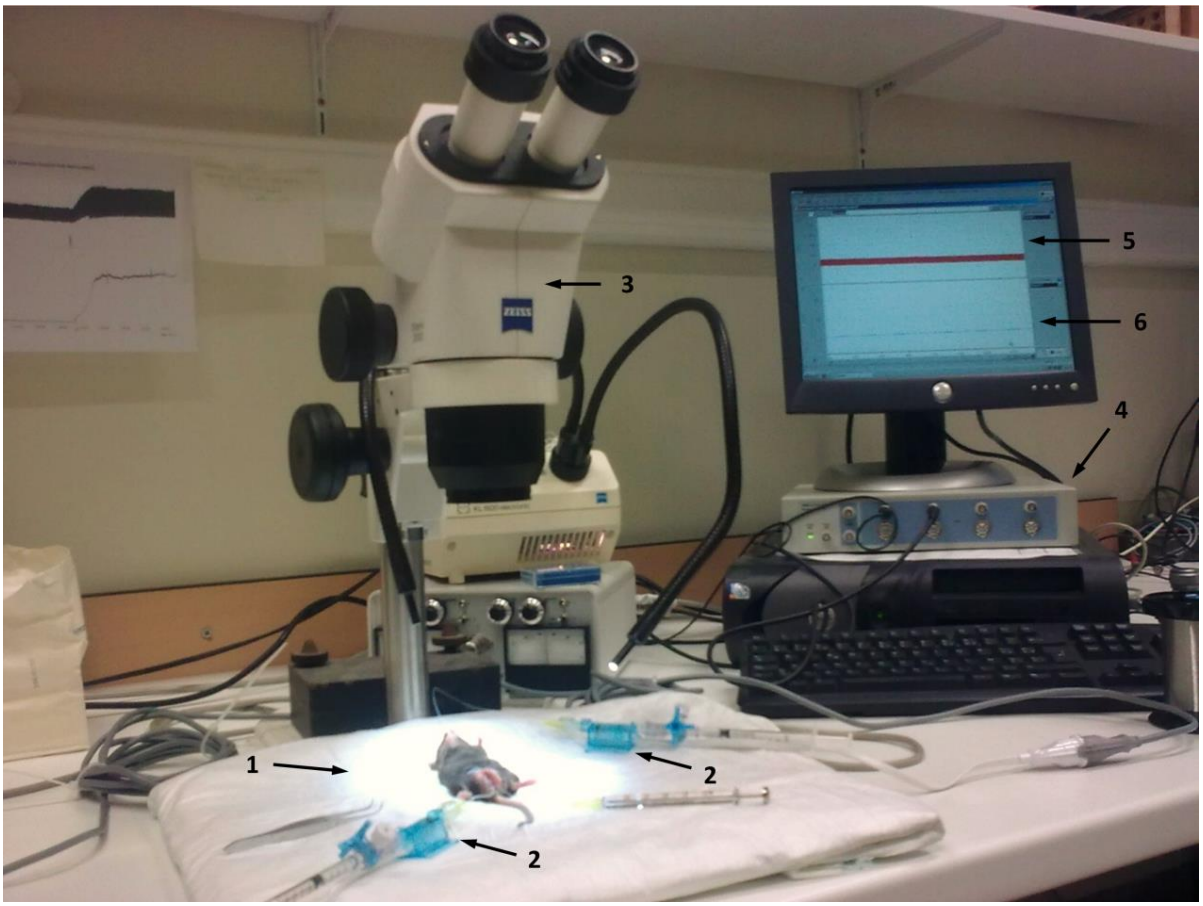

Figure III.7: Overview of the experimental in vivo setup. (1) heated blanket (2) pressure sensor (3) microscope (4) recording system (5) mean arterial pressure (MAP) monitoring (6) intracavernosal pressure (ICP) monitoring.

III.2.1 Mouse preparation

The mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) prior to the experiments. To maintain their body temperature at 37 °C, the mice were placed on a heated blanket in supine position. During anesthesia, the mice breathed spontaneously without the need for additional oxygen supply.

III.2.2 Blood pressure and intracavernosal pressure measurements

To evaluate systemic blood pressure, a polyethylene (PE-10) tube was inserted into the left carotid artery and connected to a pressure transducer (Fig. III.7). To avoid blood clotting, the PE-10 catheter was filled with heparinized saline (25 U/mL). Using the Powerlab recording system (AD instruments), the mean arterial pressure (MAP) was recorded on a computer with the LabChart software (AD instruments). To monitor penile hemodynamics, intracavernosal pressure (ICP) was assessed simultaneously with the MAP [5]. For that, a 30-G needle attached to another PE-10 tube, filled with heparinized saline, was introduced into the right CC (Fig. III.8). Again this catheter was connected to a pressure transducer and ICP was registered on a computer through the Powerlab system and LabChart software.

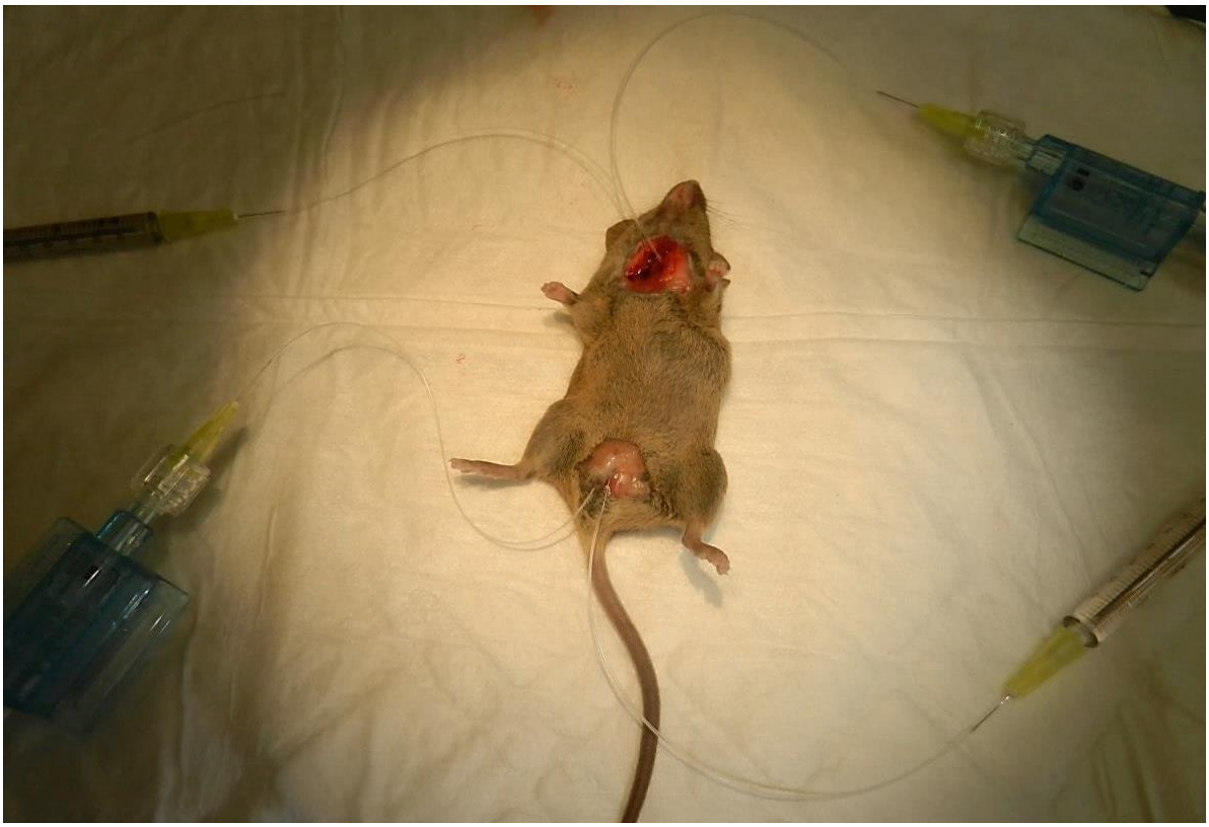

Figure III.8: Photograph of an anesthetized mouse in which 4 catheters are inserted: a catheter in the left carotid artery connected to a pressure sensor to monitor MAP (right upper corner), a catheter in the right jugular vein for intravenous injection (left upper corner), a catheter in the right CC connected to a pressure sensor to monitor ICP (left lower corner) and a catheter in the left CC for intracorporeal injection (right lower corner).

III.2.3 Intravenous and intracavernosal injection

To study the effect of pharmacological agents on blood pressure and intracavernosal pressure, the compounds where administered through intravenous and intracavernosal injection respectively. For intravenous bolus injection, the right jugular vein was catheterized with a PE-10 tube, again filled with heparinized saline (Fig. III.7). Only small amounts of fluid could be injected to avoid volume-induced alteration of blood pressure. Moreover, the injection volume was standardized to 10 µL in order to interpret the dose-response relation correctly, independent from potential volume-related effects. Intracavernosal administration was obtained via a separate catheter inserted into the left CC (Fig. III.7). As a catheter, a 30-G needle attached to a PE-10 tube was used which was also filled with heparinized saline. Because of the limited volume expansion of the CC, the intracavernosal injection volume was standardized to 5 µL. For each compound tested, either after intravenous or after intracavernosal injection, a dose-response curve was established. Between each injected dose, the injection catheter was flushed several times with heparinized saline to remove all residual agent.

III.3 Data analysis and statistical procedures

Data are presented as mean values ± standard error of the mean; N represents the number of preparations. For the in vitro experiments, the relaxations are expressed as the percentage decrease in contraction level. The level of NOR-induced contraction is presented in mN. For the in vivo experiments, blood pressure changes are shown as absolute changes in MAP (mmHg) whereas the ICP effects are calculated as the ICP adjusted for the MAP, expressed in percentage (ICP/MAP x 100). Statistical significance was evaluated using the nonparametric test for paired (Wilcoxon: CC strips), unpaired (Mann-Withney U: arterial ring segments) and multiple unpaired (Kruskal-Wallis with Bonferroni-corrected pairwise comparison: analysis of the different ruthenium-containing compounds on aorta ring segments) observations when appropriate (SPSS, version 18; IBM Corporation, Armonk, NY, USA). Data were considered statistically significant when $P<0.05$.

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Chapter IV

NO-donating oximes relax corpora cavernosa through mechanisms other than those involved in arterial relaxation

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IV.1 Abstract

INTRODUCTION: Erectile dysfunction (ED) as well as many cardiovascular diseases are associated with impaired NO-bioavailability. Recently, oxime derivatives have emerged as vasodilators due to their NO-donating capacities. However, whether these oximes offer therapeutic perspectives as alternative NO-delivery strategy for the treatment of ED is unexplored.

AIMS: This study aims to analyze the influence of formaldoxime (FAL), formamidoxime (FAM) and cinnamaldoxime (CAOx) on corporal tension and to elucidate the underlying molecular mechanisms.

METHODS: Organ bath studies were carried out measuring isometric tension on isolated mice corpora cavernosa (CC), thoracic aorta and femoral artery. After contraction with norepinephrine (NOR), cumulative concentration-response curves of FAL, FAM and CAOx (100 nmol/L – 1 mmol/L) were performed.

MAIN OUTCOME MEASURES: FAL-/FAM-induced relaxations were evaluated in the absence/presence of various inhibitors of different molecular pathways.

RESULTS: FAL, FAM and CAOx relax isolated CC as well as aorta and femoral artery from mice. ODQ (sGC inhibitor), diphenyliodonium chloride (nonselective flavoprotein inhibitor) and 7-ethoxyresorufin (inhibitor of CYP450 1A1 and NADPH-dependent reductases) substantially blocked the FAL-/FAM-induced relaxation in the arteries, but not in CC. Only a small inhibition of the FAM response was observed with ODQ.

CONCLUSIONS: This study shows for the first time that NO-donating oximes relax mice CC. Therefore oximes are a new group of molecules with potential for the treatment of ED. However, the underlying mechanism(s) of the FAL-/FAM-induced corporal relaxation clearly differ(s) from the one(s) involved in arterial vasorelaxation.

Keywords: Corpora cavernosa; oxime; formaldoxime; formamidoxime; cinnamaldoxime; erectile function

IV.2 Introduction

Over the past decades nitric oxide (NO) has evolved as an important (patho)physiological signaling molecule with therapeutic potential. The endogenous production occurs via NO synthase (NOS) that hydroxylates L-arginine to the stable intermediate Nω-hydroxy-L-arginine (L-NOHA). L-NOHA is further oxidized to L-citrulline and NO [1]. NO acts as the principle mediator of penile erection and is also the best known vasodilator in the cardiovascular system. Several disease states such as erectile dysfunction (ED) and hypertension are associated with impaired NO-mediated relaxations due to loss of endothelial and/or neuronal production of and/or response to NO [2,3]. To compensate for this loss, some oxime derivatives have been presented as new NO donor molecules, mimicking the action of endogenous NO by their bioconversion to NO or NO-related compounds [4,5]. In the past, the NO-donating capacities of oximes were demonstrated both by direct (electron paramagnetic resonance spectroscopy) and indirect (NO-scavenging with PTIO) strategies [4-6]. In rat aorta both formaldoxime (FAL) and formamidoxime (FAM) have been shown to induce a substantial vasorelaxant effect [4]. Moreover, participation of the NO pathway in the cinnamaldoxime (CAOx)-induced relaxation has been demonstrated in rat mesenteric artery [5]. As proven vasodilators of arteries, oximes could also be of value in the treatment of ED. However, no studies support this hypothesis yet.

IV.3 Aims

In our study we examined the ability of FAL, FAM and CAOx to relax isolated mice corpora cavernosa (CC). Furthermore, the mechanisms underlying this effect were examined and compared with the mechanisms involved in the relaxation of mice aorta and femoral artery.

IV.4 Materials and methods

IV.4.1 Animals

Mature (age 8-12 weeks) male Swiss mice were obtained from Janvier (Saint-Berthevin, France). Food and water were provided ad libitum and all animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. This study was approved by the local Ethical Committee for Animal Experiments.

IV.4.2 Tissue preparations and mounting

After cervical dislocation thoracic aorta, femoral artery and corpora cavernosa (CC) were carefully isolated, displaced in cold Krebs-Ringer bicarbonate (KRB) solution, mounted into 10 ml organ baths from a myograph for isometric tension measurements as previously described [7,8] and left to equilibrate for 30 minutes in KRB solution (37°C; pH 7.4, bubbled with 95% O_2 – 5% CO_2).

IV.4.3 Preliminary protocol

Before starting with the actual experiments, each tissue was subjected to a slightly different preliminary protocol in order to obtain maximal, stable contractions and relaxations as previously described [7-9]. To test the functionality of the endothelium, 5 µmol/L (aorta and CC) or 10 µmol/L (femoral artery) norepinephrine (NOR) was used to induce contraction and when a stable plateau was reached, 10 µmol/L (arteries) or 1 µmol/L (CC) acetylcholine (ACh) was added. Thereafter, preparations were washed until basal tone was obtained.

IV.4.4 Experimental protocol

All preparations were contracted with NOR, and when a stable plateau was obtained, cumulative concentration-response curves for FAL, FAM and CAOx (100 nmol/L – 1 mmol/L) were established. The molecular pathways involved in these effects were tested using different inhibitors. In some experiments, the endothelium of aortic or femoral artery segments was removed by gently rubbing the intimal surface with a rough polyethylene tube or small hair respectively, while the CC were carefully squeezed between 2 fingers for 30 seconds. The absence of functional endothelium was evaluated by the loss of response to ACh.

IV.4.5 Drugs, chemicals and reagents

The experiments were performed in a KRB solution with the following composition (mmol/L): NaCl, 135; KCl, 5; $NaHCO_3$, 20; glucose, 10; $CaCl_2$, 2.5; $MgSO_4$, 1.3; KH_2PO_4, 1.2 and EDTA, 0.026 in H_2O. Norepinephrine bitartrate (NOR), dimethylsulfoxide (DMSO), acetylcholine chloride (ACh), formaldoxime trimer hydrochloride (FAL), formamidoxime (FAM), cinnamaldoxime (CAOx), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 4'-hydroxy-3'-methoxyacetophenone (apocynin), diphenyliodonium chloride (DPI), 7-ethoxyresorufin (7-ER), miconazole nitrate, Nω-Nitro-L-arginine (L-NNA), tetraethylammoniumchloride (TEA), apamin, SQ 22,536, and compound C were obtained from Sigma-Aldrich (St.Louis, MO, USA). DPI, apocynin, 7-ER, miconazole and compound C were dissolved in DMSO and ODQ in ethanol. All other drugs were dissolved in distilled water. Incubation with ODQ, 7-ER and L-NNA elicited a significant increase in the NOR-induced contraction of the arterial ring segments, whereas DPI and miconazole evoked a substantial decrease. However, these experiments were also performed on ring segments of mice with a 129SvJ background in which the contraction level was not/barely influenced. These results led to the same conclusions as compared to our results with the Swiss mice. The final concentrations of vehicle solution in the organ bath never exceeded 0.1 %.

IV.4.6 Data analysis and statistical procedures

Data are presented as mean values ± SEM; N represents the number of preparations. The relaxations are expressed as the percentage decrease in contraction level. Statistical significance was evaluated by using the Mann-Withney *U*-test (aorta and femoral artery) and Wilcoxon test (CC) (SPSS, version 18; IBM Corporation, Armonk, NY, USA). $P<0.05$ was considered as significant.

IV.5 Main outcome measures

FAL- and FAM-induced concentration-response curves were evaluated in the absence and presence of different inhibitors.

IV.6 Results

IV.6.1 Effect of FAL, FAM and CAOx

In precontracted corporal strips FAL, FAM and CAOx (100 nmol/L – 1 mmol/L) elicited a concentration-dependent relaxant effect (Fig. IV.1). However, FAM induced a substantially smaller relaxation than FAL and CAOx (maximal effect FAL: 73.26% ± 5.62 – FAM: 40.52% ± 4.59 – CAOx: 92.6% ± 6.42 – control: 4.17% ± 6.42). The onset of FAL relaxation was relatively fast, while the FAM and CAOx relaxations were rather gradual (Fig. IV.1). After intensive washout, a similar NOR-induced contraction level is obtained when compared to the first contraction with NOR. This illustrates the reversibility of the relaxations induced by oximes. Moreover, the concentration-response curves of FAL, FAM and CAOx could be reproduced on the same preparation (data not shown). FAL, FAM and CAOx also elicited a concentration-dependent relaxation in precontracted aorta and femoral artery rings. The FAL-/FAM-induced relaxations were quite similar, while CAOx induced a more pronounced relaxation at the highest concentrations in both aorta (maximal effect FAL: 71.13% ± 4.34 – FAM: 80.4% ± 3.2 – CAOx: 104.7% ± 1.76 – control: -4.65% ± 1.09) and femoral artery (maximal effect FAL: 61.89% ± 5.18 – FAM: 88.16% ± 6.7 – CAOx: 103.84% ± 1.29 – control: 9.06% ± 4.46).

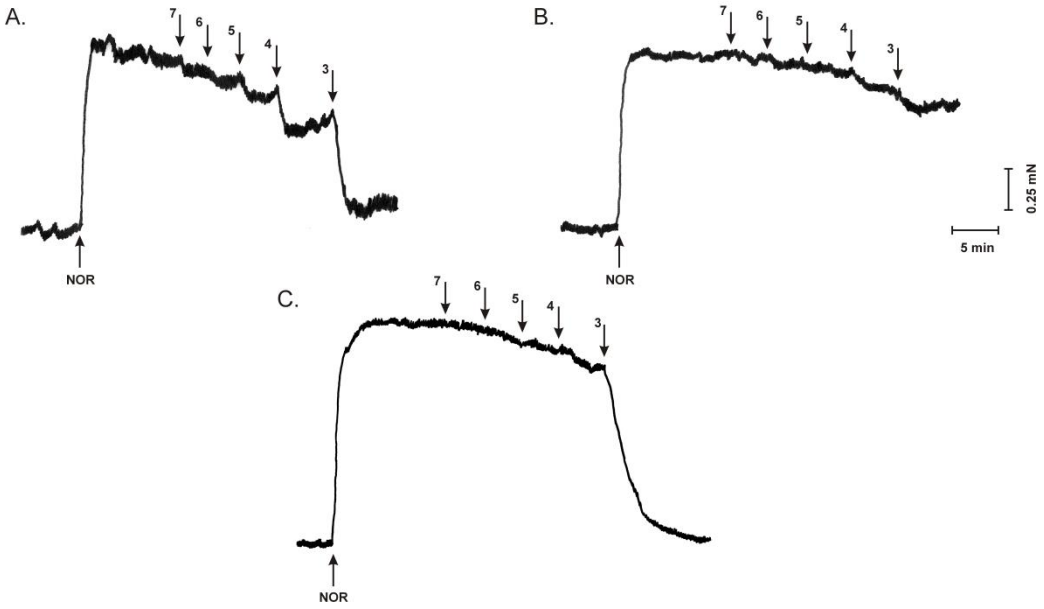

Figure IV.1: Original tracings showing a concentration-response curve to formaldoxime (FAL) (-log mol/L) (A); formamidoxime (FAM) (-log mol/L) (B) and cinnamaldoxime (CAOx) (-log mol/L) (C) in mice CC precontracted with NOR.

IV.6.2 Involvement of sGC

CC incubated with the soluble guanylyl cyclase (sGC) inhibitor ODQ (10 µmol/L, 10 min) showed a somewhat smaller relaxation with FAM (Fig. IV.2F) but not with FAL (Fig. IV.2E). However, ODQ significantly decreased the FAL effects in aorta as well as femoral artery (Fig. IV.2A and C) and almost abolished FAM-induced relaxations (Fig. IV.2B and D).

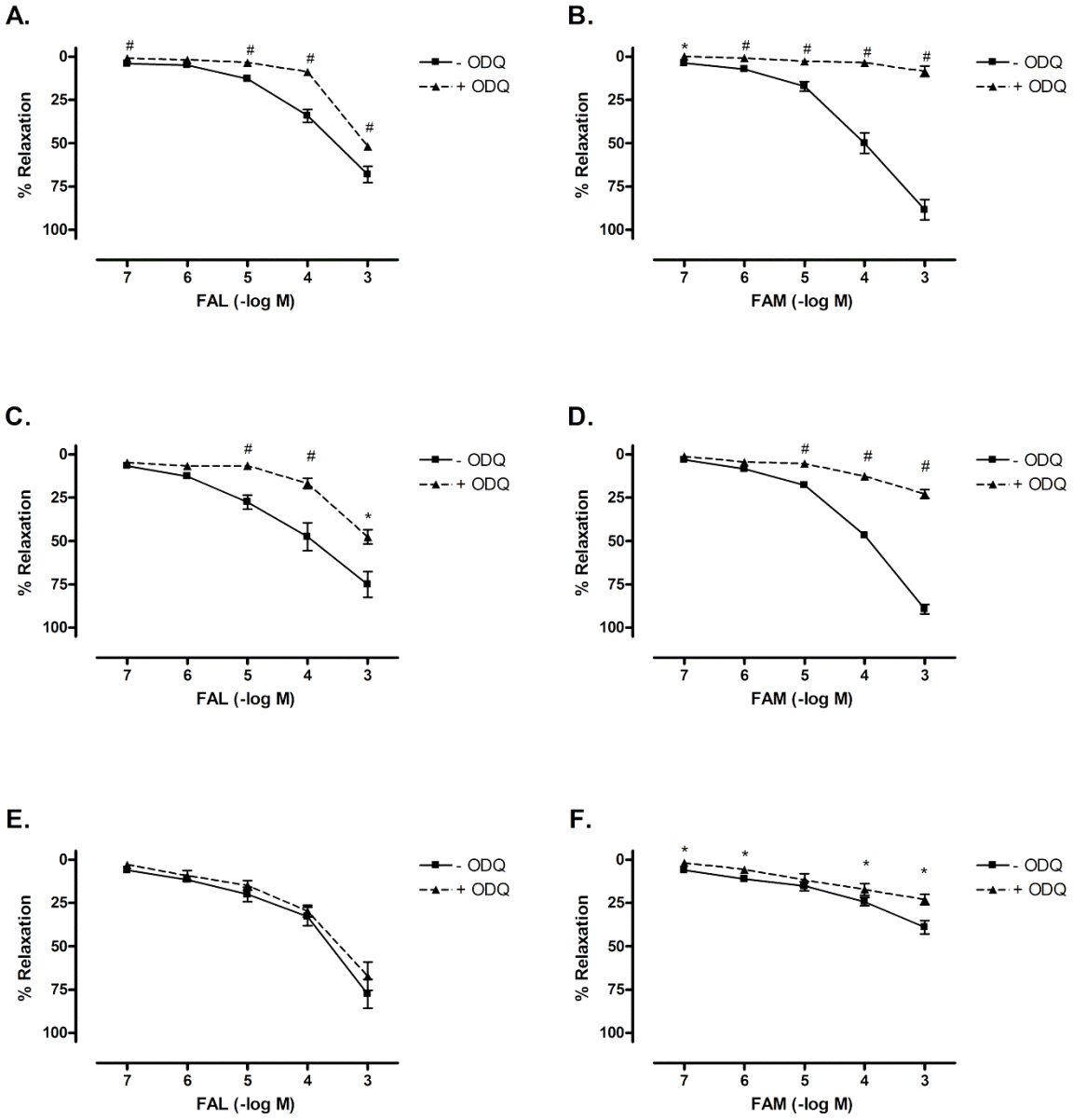

Figure IV.2: The effect of formaldoxime (FAL) and formamidoxime (FAM) in the absence (■) or presence (▲) of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 µmol/L incubated during 10 minutes) in thoracic aorta (A and B); in femoral artery (C and D) and in CC (E and F). Data are expressed as % relaxation of the NOR-induced tone (N = 5-6); **P*<0.05 and #*P*<0.01

IV.6.3 Involvement of NADPH oxidase/reductase and CYP450

The nonselective flavoprotein inhibitor DPI (10 µmol/L, 30 min) caused a very small inhibition only at the highest concentration of FAL and FAM in CC (Fig. IV.3E and F). In contrast, a strong inhibition of the FAL-/FAM-induced relaxations occurred in aortic and femoral artery rings after incubation with DPI (Fig. IV.3A–D).

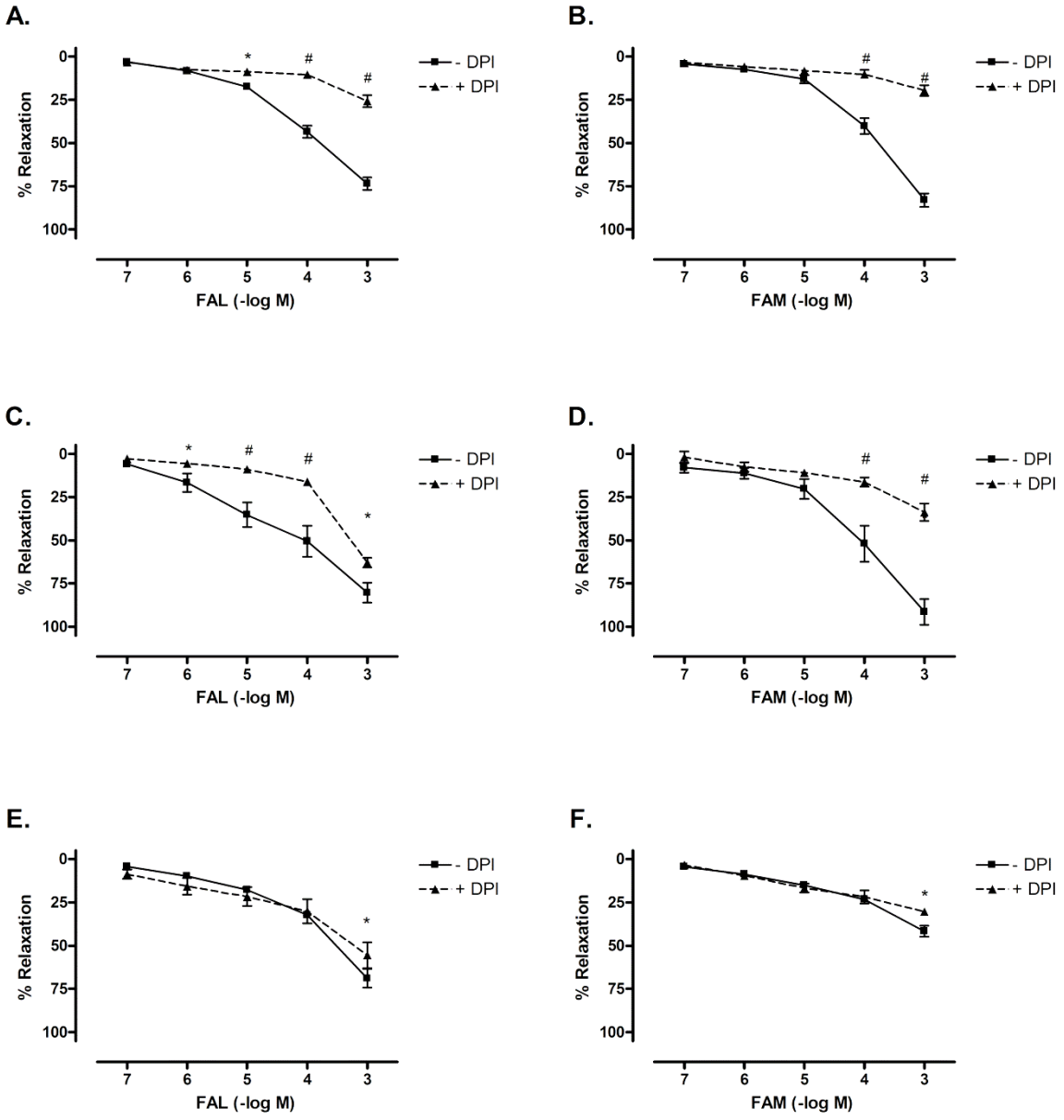

Figure IV.3: The effect of formaldoxime (FAL) and formamidoxime (FAM) in the absence (■) or presence (▲) of diphenyliodonium chloride (DPI) (10 µmol/L incubated during 30 minutes) in thoracic aorta (A and B); in femoral artery (C and D) and in CC (E and F). Data are expressed as % relaxation of the NOR-induced tone (N = 5-9); **P*<0.05 and #*P*<0.01

In the CC FAL-/FAM-induced relaxations were not influenced in the presence of apocynin (300 µmol/L, 20 min), a specific blocker of NADPH oxidases (Fig. IV.4E and F). Moreover, apocynin did not affect the FAL response in the arteries (Fig. IV.4A and C) but significantly diminished the vasorelaxant effect of FAM (Fig. IV.4B and D).

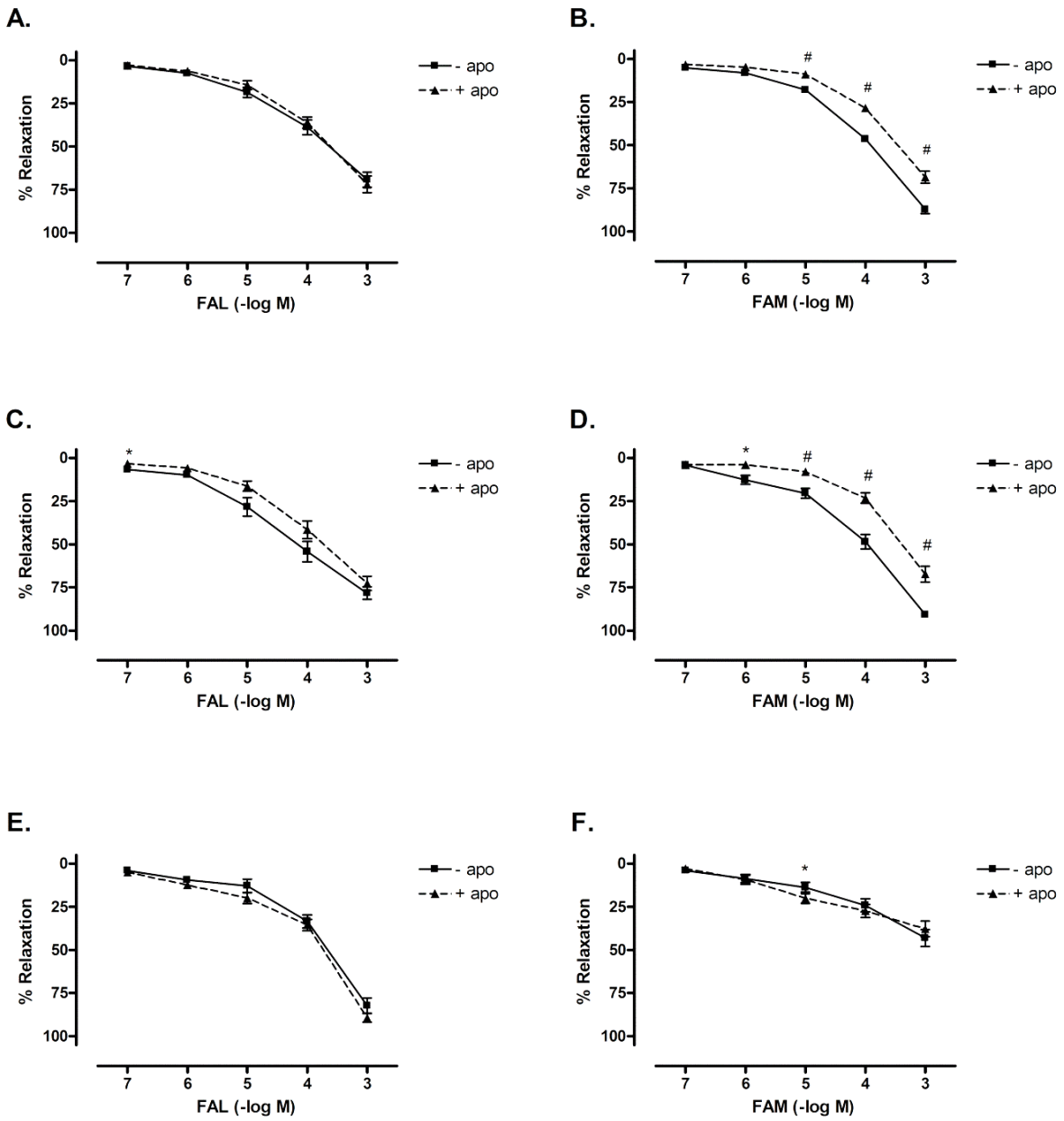

Figure IV.4: The effect of formaldoxime (FAL) and formamidoxime (FAM) in the absence (■) or presence (▲) of apocynin (300 µmol/L incubated during 20 minutes) in thoracic aorta (A and B); in femoral artery (C and D) and in CC (E and F). Data are expressed as % relaxation of the NOR-induced tone (N = 6-9); **P*<0.05 and #*P*< 0.01

7-ER (10 µmol/L, 20 min), an inhibitor of NADPH-dependent reductases and CYP450 1A1, failed to inhibit the FAL-/FAM-responses in CC (Fig. IV.5E and F), but significantly diminished the FAL effect and even abolished the FAM relaxation in arteries (Fig. IV.5A–D). The CYP450-inhibitor miconazole (10 µmol/L, 20 min) did not modify the FAL-/FAM-induced relaxation neither in the CC nor in the arteries (data not shown).

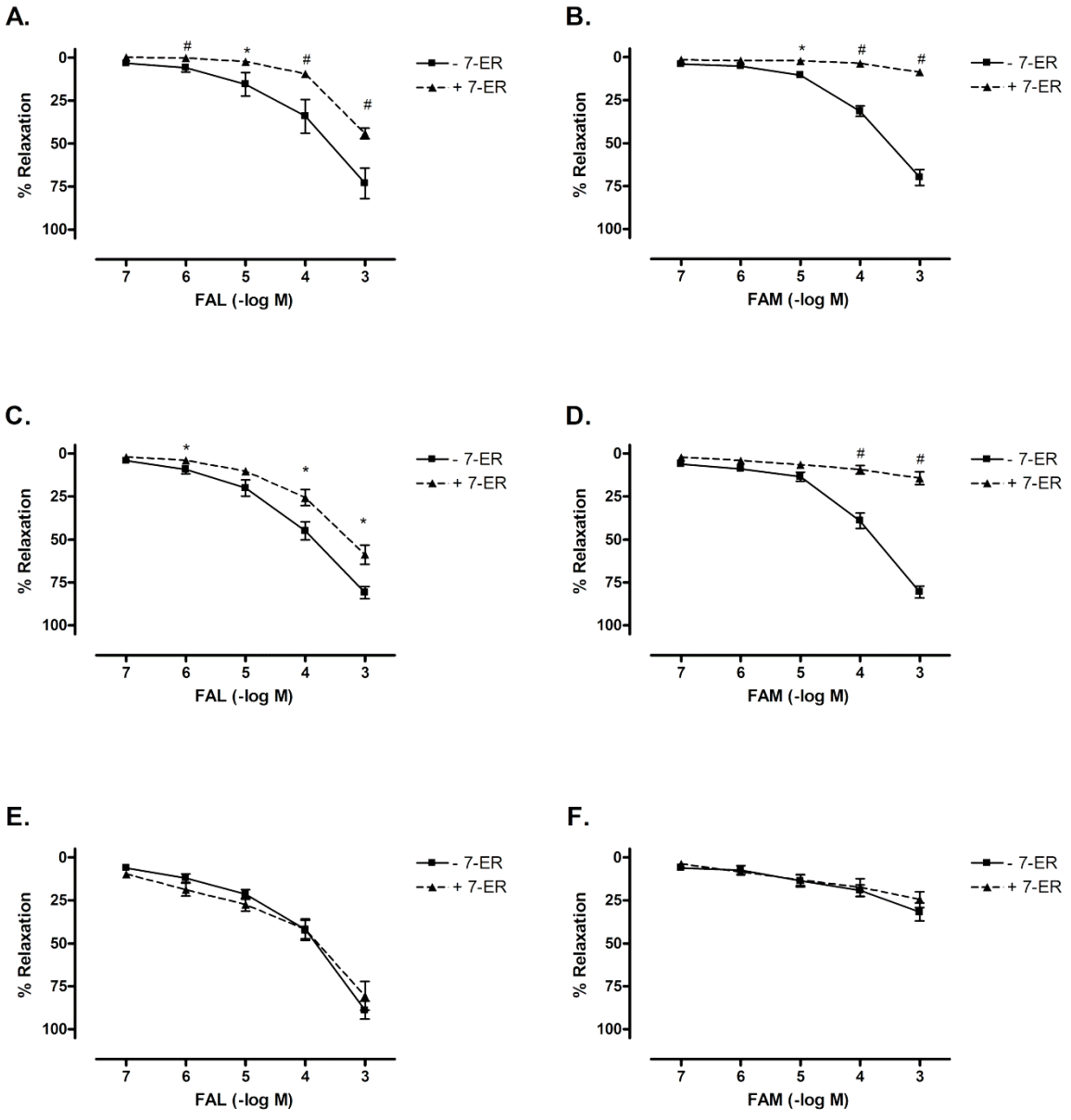

Figure IV.5: The effect of formaldoxime (FAL) and formamidoxime (FAM) in the absence (■) or presence (▲) of 7-ethoxyresorufin (7-ER) (10 µmol/L incubated during 20 minutes) in thoracic aorta (A and B); in femoral artery (C and D) and in CC (E and F). Data are expressed as % relaxation of the NOR-induced tone (N = 5-7); *P<0.05 and #P<0.01

IV.6.4 Involvement of NOS and the endothelium

The NOS inhibitor L-NNA (100 µmol/L, 20 min) did not alter the FAL-/FAM-induced responses in the CC (Fig. IV.6E and F). Surprisingly, in the arteries the relaxant effect of FAL was substantially enhanced (Fig. IV.6A and C), whereas the FAM-induced relaxations showed only a very small increasing trend at higher concentrations (Fig. IV.6B and D).

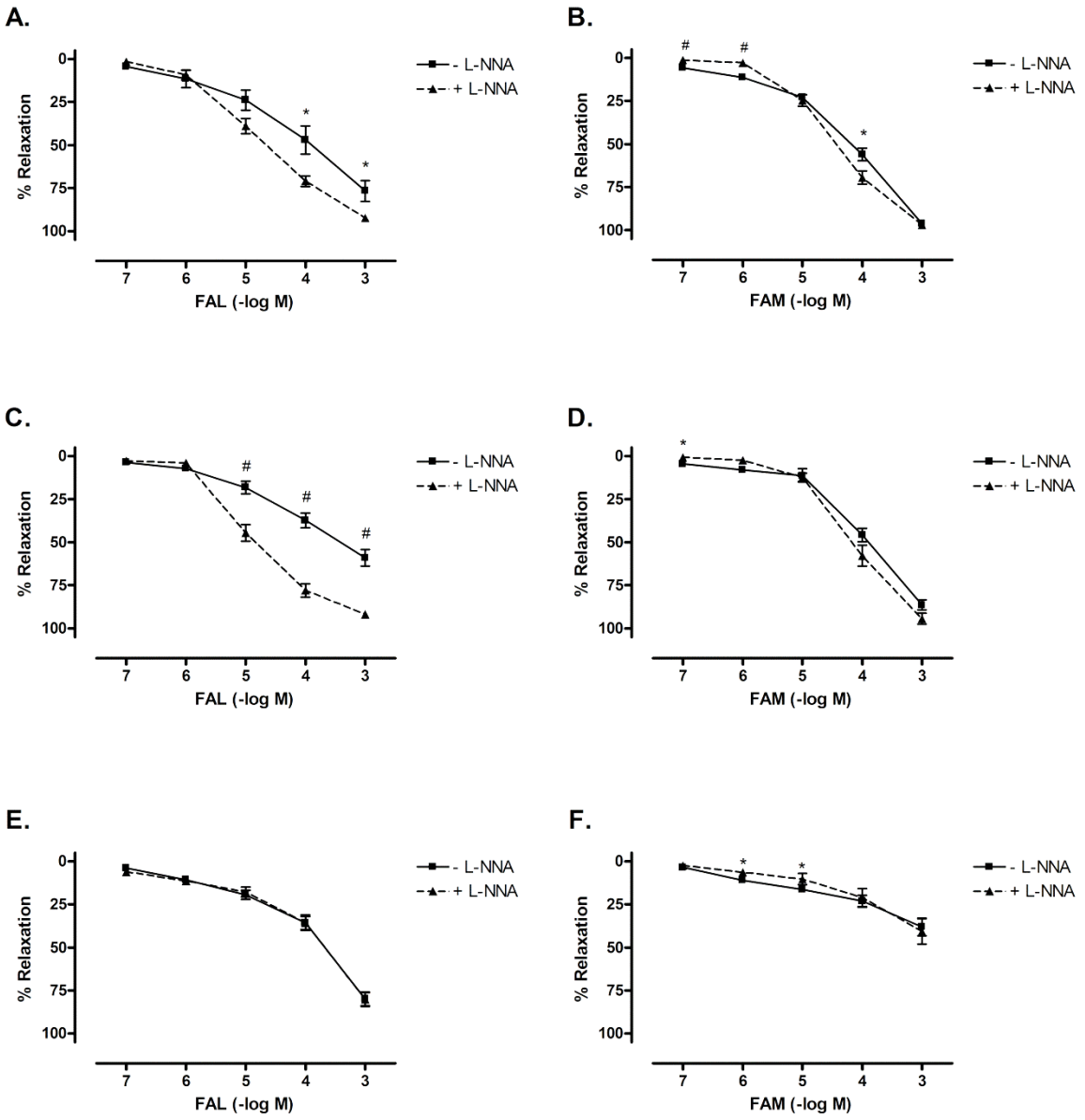

Figure IV.6: The effect of formaldoxime (FAL) and formamidoxime (FAM) in the absence (■) or presence (▲) of Nω-Nitro-L-arginine (L-NNA) (100 µmol/L incubated during 20 minutes) in thoracic aorta (A and B); in femoral artery (C and D) and in CC (E and F). Data are expressed as % relaxation of the NOR-induced tone (N = 6-8); **P*<0.05 and #*P*<0.01

Removal of the endothelium did not modify the FAL-/FAM-effects in the CC (Fig. IV.7E and F). In endothelium-denuded aorta a significantly enhanced relaxation of FAL was seen, whereas in femoral artery this enhancement was limited to one concentration. (Fig. IV.7A and C). Removal of the endothelium elicited only a limited, but statistically significant, increase of the FAM response in aortic rings but had no effect in femoral artery (Fig. IV.7B and D).

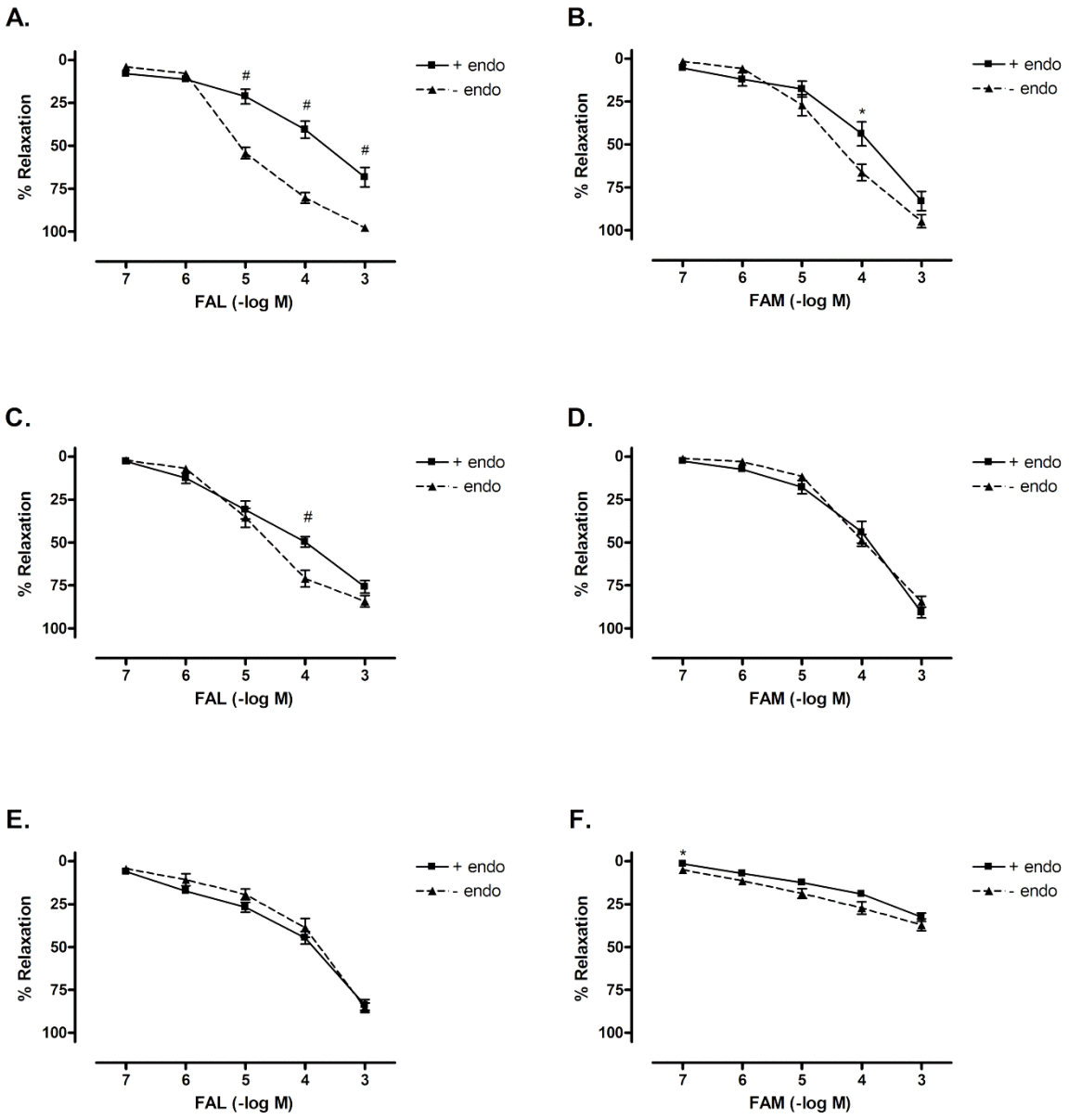

Figure IV.7: The effect of formaldoxime (FAL) and formamidoxime (FAM) in the presence (■) or absence (▲) of the endothelium in thoracic aorta (A and B); in femoral artery (C and D) and in CC (E and F). Data are expressed as % relaxation of the NOR-induced tone (N = 6-9); *P<0.05 and #P<0.01

IV.6.5 Involvement of other pathways

To explore the role of alternative pathways in the FAL-/FAM-induced corporal relaxation, the K^+ channel blocker TEA (3 mmol/L, 20 min), the small-conductance Ca^{2+}-activated K^+ channel blocker apamin (1 µmol/L, 20 min), the adenylyl cyclase inhibitor SQ 22,536 (100 µmol/L, 20 min) and the AMP kinase inhibitor compound C (10 µmol/L, 20 min) were used. None of these inhibitors influenced the FAL-/FAM-related responses in the CC (data not shown).

IV.7 Discussion

In this study, we show for the first time that oximes such as FAL, FAM and CAOx relax mice corporal tissue and thus may be of interest in the treatment of ED. Surprisingly, the mechanism(s) underlying this relaxant effect differ(s) considerably from the one(s) involved in the oxime-induced relaxation of arteries.

In the search for NO donor drugs, oxime derivatives have been presented as a new group. Oximes have attracted attention because of their close resemblance with L-NOHA, the stable intermediate in the endogenous NO production [10]. Since L-NOHA elicits a concentration-dependent vasodilation, the presence of a C=NOH bond in oxime derivatives raised the question whether they also could serve as an alternative NO-delivery strategy [11]. The relaxant effect of different substituted non-aromatic oximes, including FAL and FAM, as well as the aromatic CAOx has already been demonstrated in isolated rat aorta and mesenteric artery respectively [4,5]. In our study, we tested these compounds in the context of penile erection and demonstrated that FAL, FAM and CAOx indeed elicit a pronounced relaxation of mice CC. Parallel experiments on mice aorta and femoral artery were performed in which their profound vasorelaxing capacity was confirmed. However, the CAOx-related effect was not entirely concentration-dependent and required higher concentrations in comparison with the gradual FAL-/FAM-induced relaxations. Moreover, the very poor solubility in water and the strong arterial relaxant (side)effects would limit the therapeutic value of CAOx for the treatment of ED. Therefore, we focused on the FAL-/FAM-induced relaxations.

The main endogenous target of NO is sGC which stimulates various (vaso)relaxing pathways through the formation of cGMP. Obviously, the sGC/cGMP-pathway should, at least in part, be involved in the effect of any possible NO donor. Hence, an activation of sGC was implicated in the vasorelaxation by L-NOHA and other compounds bearing a C=NOH function [5,6,12,13]. Even more specifically for FAL and FAM, it was proven on rat aorta that their effect was strongly diminished when sGC was inhibited by ODQ [4]. Also we found that ODQ substantially decreased the FAL vasorelaxation in the mice arteries and the FAM response was even completely abolished, definitely supporting the importance of the sGC/cGMP pathway. Surprisingly, our results in the CC only revealed a small inhibition of the FAM relaxation by ODQ, whereas ODQ had even no influence at all on the FAL effect.

As oxime-induced relaxation does not result from spontaneous NO release, but rather occurs via biotransformation within the tissue, we were interested in the underlying metabolization pathway(s) [4]. It was reported that diphenyleneiodonium, an analog of the non-specific flavoprotein inhibitor DPI, inhibited the metabolic activation of glyceryl trinitrate (GTN) which is considered as the prototypic NO donor through enzymatic biotransformation [14,15]. Besides for GTN, flavoproteins are involved in the rat aorta relaxation of L-NOHA and 4-chloro-benzamidoxime, which also possess a C=NOH function, as DPI could block their effect [6]. Also in our experiments, DPI almost abolished FAL-/FAM-related responses in aorta and femoral artery, clearly indicating the participation of (a) flavoprotein-based mechanism(s). However, this was not the case for the FAL-/FAM-induced relaxations in the CC.

Diphenyleneiodonium and DPI are frequently used for the nonselective inhibition of NADPH oxidase [16]. Though apocynin, the best known NADPH oxidase inhibitor, did not modify the FAL-induced relaxations and caused only a limited inhibition of the FAM response in the arteries. These results suggest that not NADPH oxidase inhibition, but other mechanisms are responsible for the strong inhibition of the FAL-/FAM-related effects in the presence of DPI.

Besides NADPH oxidase, diphenyleneiodonium and DPI block multiple other enzyme systems including NOS, glucose phosphate dehydrogenase, xanthine oxidase, mitochondrial electron transport, aldehyde dehydrogenase-2, aldehyde oxidase, and NADPH-CYP450 reductase [15-17]. In terms of oxime-induced relaxations NADPH-CYP450 reductase seems to be of particular interest. Several studies demonstrated the importance of the CYP450/NADPH-CYP450 reductase system in the NO-mediated responses of ACh, GTN and sodium nitroprusside [18,19]. Furthermore 7-ER, an inhibitor NADPH-dependent reductases and CYP450 1A1, diminished the relaxations induced by L-NOHA, 4-chloro-benzamidoxime, FAL, FAM and other oxime derivatives [4-6,12,20]. Unfortunately in our mouse model no decrease of the FAL-/FAM-induced relaxations by 7-ER could be observed in the CC. At the same time, a pronounced inhibition of the FAL response occurred in the aorta and femoral artery whereas the FAM effect was even completely blocked. So in the arteries 7-ER-sensitive mechanisms, e.g. CYP 450 and/or NADPH-dependent reductases, do seem to be involved. However, miconazole had no influence on either FAL-/FAM-induced relaxations, ruling out the significance of CYP450. This is in accordance with the failure of the CYP450-

inhibitors proadifen, 17-ODYA and/or miconazole to inhibit the vasorelaxing effect of L-NOHA and some oximes [4,6,11-13]. In contrast, for some other oxime derivatives it was reported that NO formation was mediated by CYP450 [21,22].

The combined results of 7-ER and miconazole are pointing towards a contribution of NADPH-dependent reductases in the FAL-/FAM-induced relaxation. Because of their structural and functional resemblance it is not surprising that besides NADPH-CYP450 reductase also the NADPH-dependent reductase domain of NOS can be blocked by 7-ER [19,23,24]. The oxidative cleavage of the C=NOH function can occur through NOS-dependent pathways [8], but the relaxant effect of various oximes is NOS-independent [4-6]. In correspondence L-NNA had no effect on the FAL-/FAM-induced responses in the CC as well as the FAM response in the arteries. Surprisingly, L-NNA strongly enhanced the FAL-induced vasorelaxation. Previously, a similar but non-significant trend for the FAL relaxation in rat aorta was noticed [4]. An explanation for the enhanced response after NOS-inhibition remains to be found, but this phenomenon can be associated with some in vivo experiments. In one study oxime derivatives, including FAL and FAM, elicited only an acute systemic vasodilation in vivo when the endogenous NO production was blocked [25]. L-NNA only blocks the endogenous NO formation, but other (endothelial) pathways, such as the endothelium-derived hyperpolarizing factor and vasodilatory prostanoids, are preserved and should be considered. Nevertheless, in our study, endothelium-denuded preparations exhibited analogous results. The FAM effect remained unchanged after removal of the endothelium. In contrast, the FAL-induced vasorelaxation was even more pronounced in the absence of the endothelium. This partially correlates with experiments on rat aorta in which no difference was observed in FAL-/FAM-related responses between tissues with or without endothelium [4]. So although addition of L-NNA and removal of the endothelium do not affect the exact same molecular mechanisms, their effect on our FAL and FAM results reflects that there exists some kind of overlap. The results with L-NNA as well as with endothelium removal indicate that an absence of endogenous NO and/or endothelium promotes FAL-induced relaxation. A possible explanation for these enigmatic observations is that the absence of functional NOS shifts breakdown of the oximes to other mechanisms leading to vasorelaxing metabolites. Nevertheless, elimination of these endogenous systems would entail a stronger efficacy of FAL, which could be beneficial under pathological

conditions. After all, different types of ED as well as multiple cardiovascular diseases are linked to endothelial dysfunction which is mainly related to a decreased NO formation and/or lowered NO responsiveness [2,26]. The more pronounced relaxant effect of FAL and FAM in the absence of NOS and the endothelium could therefore compensate for this diminished NO and reduce the deleterious consequences of impaired NOS activity.

Our study reveals a clear difference between arteries and CC. Although both FAL and FAM elicit corporal smooth muscle relaxation as well as arterial vasorelaxation, the pathways activated by FAL and FAM in the arteries are more evident than those in the CC. In the arteries, a pronounced inhibition with ODQ proves the importance of the sGC/cGMP-pathway. Since both miconazole and L-NNA fail to influence the FAL-/FAM-induced responses, inhibition by 7-ER implicates the involvement of NADPH-CYP450 reductase. This observation is further supported by the fact that DPI blocked the FAL-/FAM-related effects to the same extent as 7-ER. The NO donor capacities of FAL and FAM were previously demonstrated by electron paramagnetic resonance spectroscopy and by the blunting of their vasorelaxing effect by the NO-scavenger PTIO [4]. Taking this into account we assume that the underlying mechanism of FAL-/FAM-induced vasorelaxation involves the metabolic conversion of the C=NOH function to NO or NO-related compound by (a) NOS- and endothelium-independent, but NADPH-CYP450 reductase-dependent mechanism(s) and subsequent activation of the sGC/cGMP pathway. One should keep in mind that this pathway does not cover the complete FAL response because ODQ, 7-ER and DPI only have a partial inhibitory effect. So (an) other (NO-independent) mechanism(s) take(s) part in the FAL-induced relaxation. Recently, an analogous pathway was suggested for CAOx [5]. Concerning the FAM-induced vasorelaxation, a small role can be addressed to NADPH oxidase. The surprising enhancement of FAL responses in the absence of endogenous NO and/or endothelium offers therapeutic perspectives in several disease states.

On the contrary, in the CC the exact mechanism of action remains elusive. NOS, NADPH oxidase, NADPH-dependent reductases, CYP450, AMP kinase, adenylate cyclase and K^+ channels do not appear to be involved. Only a limited involvement of the sGC/cGMP pathway in the FAM response is found. Because of the sGC/cGMP participation, FAM could be beneficial in combination with PDE5-inhibitors, since in many ED variants lack of sufficient

amounts of cGMP undermines the efficacy of PDE5-inhibitors [27-29]. Besides that, the NOS- and endothelium-independency of the FAL-/FAM-related responses makes them still valuable in ED associated with endothelial dysfunction.

IV.8 Conclusion

In conclusion, this study shows for the first time that FAL, FAM and CAOx elicit relaxation in mice CC. The mechanism(s) involved in the FAL-/FAM-induced relaxations are clearly different from the mechanism(s) underlying their arterial vasorelaxing effect. In the CC NOS- and endothelium-independency could be demonstrated. In the arteries NADPH-CYP450 reductase and the sGC/cGMP pathway seem to mediate the vasorelaxation elicited by FAM and partially by FAL. NADPH oxidase also plays a limited role in the FAM vasorelaxation. In addition, when functional NOS and/or endothelium are absent FAL-induced vasorelaxation is stronger. Thus, oximes can further be considered as alternative NO-donating drugs for the treatment of ED.

IV.9 Acknowledgements

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Chapter V

Oximes induce erection and are resistant to oxidative stress

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V.1 Abstract

INTRODUCTION: Because of their NO-donating capacities, oxime derivatives have shown to offer some therapeutic perspective for the treatment of erectile dysfunction (ED) as well as cardiovascular diseases. However, to date the in vivo effect of these oximes on erectile function remains unknown. In many disease states oxidative stress occurs, impairing NO-mediated relaxations. Hence the influence of oxidative stress on oxime-induced effects is also of interest.

AIMS: This study aimed to evaluate the in vivo effect of formaldoxime (FAL) and formamidoxime (FAM) on blood pressure and intracavernosal pressure (ICP); and to examine the role of sGC and the influence of oxidative stress on the FAL and FAM responses.

METHODS: Blood pressure and intracavernosal pressure were monitored in vivo after resp. intravenous or intracavernosal injection of FAL and FAM. Moreover isometric tension was measured in vitro on isolated mice corpora cavernosa (CC), thoracic aorta and femoral artery in organ baths. The role of sGC was investigated by using transgenic mice lacking the alpha 1 subunit of sGC.

MAIN OUTCOME MEASURES: Mean arterial pressure (MAP) and intracavernosal pressure (ICP) were measured after FAL/FAM injection. In vitro relaxation of CC strips was evaluated in response to addition of FAL/FAM.

RESULTS: In vivo both FAL and FAM elicit a dose-dependent lowering of blood pressure (maximal ΔMAP: 33.66 ± 4.07mmHg (FAL) and 20.43 ± 2.06mmHg (FAM)) as well as an increase of intracavernosal pressure (maximal increase of ICP/MAP: 70.29 ± 2.88% (FAL) and 52.91 ± 8.61% (FAM)). The FAL/FAM effect is significantly lower in knockout versus wild-type mice. Oxidative stress has an inhibitory effect on corporal NO-mediated relaxations induced by electrical field stimulation (EFS), acetylcholine (ACh) and sodium nitroprusside (SNP) whereas the responses to 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate sodium salt, FAL and FAM were not influenced.

CONCLUSIONS**:** Oximes induce erection which is mediated by sGC. The oxime-induced relaxations are resistant to oxidative stress, which increases their therapeutic potential for the treatment of ED.

V.2 Introduction

The nitric oxide(NO)/soluble guanylyl cyclase(sGC)/cyclic guanosine monophosphate(cGMP)-pathway plays a pivotal role in the normal regulation of penile erection as well as in the complex balance of vascular tone. Consequently, any profound lowering in NO production and/or decreased response to endogenously formed NO can result in erectile dysfunction (ED) and/or various cardiovascular diseases [1]. NO-donors have been used for several years to compensate for lost NO-bioavailability. Recently, oxime compounds such as formaldoxime (FAL) and formamidoxime (FAM) (Fig. V.1) have been presented as a new class of NO-donating molecules because of their structural resemblance with Nω-hydroxy-L-arginine (L-NOHA), a stable intermediate in the endogenous NO production. These oximes relax vascular tissues as well as mice corpora cavernosa (CC) through metabolization within the tissue [2-4]. Furthermore, oxime derivatives show a distinct blood pressure-lowering effect in rats after inhibition of the endogenous NO synthesis [5]. However, so far the in vivo effect of oximes on intracavernosal pressure (ICP) and their ability to induce erection is not studied yet.

The lowered NO functioning in ED often results from an increased production of reactive oxygen species, diminishing the effective NO concentration for cavernosal smooth muscle relaxation [6]. Therefore oxidative stress associated with hypertension [7], diabetes [8,9], aging [10,11], hypercholesterolemia [12] and radiation damage [13] can lead to ED. It was reported that oxidative stress impairs NO-mediated relaxations of mice CC [14]. Hence, we were interested if oxidative stress also has an influence on the FAL-/FAM-induced corporal relaxations, as an impairment would limit their therapeutic relevance.

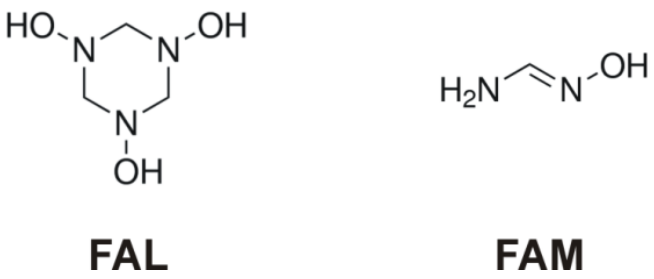

Figure V.1: The chemical structure of formaldoxime (FAL) and formamidoxime (FAM).

V.3 Aims

In our study, we examined the in vivo effect of FAL and FAM on blood pressure and ICP. The importance of sGC in the FAL-/FAM-induced effects was characterized both in vitro and in vivo. Moreover the influence of oxidative stress on (NO-mediated) corporal relaxation was evaluated in vitro.

V.4 Materials and methods

V.4.1 Animals

For our in vitro and in vivo experiments mature (8-14 weeks) male soluble guanylyl cyclase alpha 1 knockout ($sGC\alpha_1^{-/-}$) mice and $sGC\alpha_1^{+/+}$ mice (129SvJ) were used [15]. All animals were bred in the SPF facility of the Department of Molecular Biomedical Research (VIB, Ghent, Belgium) and were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. This study was also approved by the local Ethical Committee for Animal Experiments (Faculty of Medicine and Health Sciences, Ghent University, Belgium).

V.4.2 In vitro study

V.4.2.1 Tissue preparations and mounting

Thoracic aorta, femoral artery as well as CC were isolated from the animals after cervical dislocation. The tissues were then mounted into 10 ml organ baths from a myograph for isometric tension measurements as previously described [16,17]. The mounted tissues were left to equilibrate for30 minutes in Krebs-Ringer bicarbonate (KRB) solution at 37°C (pH 7.4) and continuously bubbled with carbogen gas (95% O_2 – 5% CO_2).

V.4.2.2 Preliminary protocol

In order to obtain maximal, stable contractions and relaxations during the actual experiments, each tissue was subjected to a slightly different preliminary protocol as previously described [16-18]. Functionality of the endothelium was tested by addition of 10 µmol/L (arteries) or 1 µmol/L (CC) acetylcholine (ACh) to the organ baths after

contraction with 5 µmol/L (aorta and CC) or 10 µmol/L (femoral artery) norepinephrine (NOR). Afterwards the preparations were rinsed extensively until they returned to their basal tension level.

V.4.2.3 Experimental protocol

In a first series of experiments both the effect of FAL and FAM was tested in $sGC\alpha_1^{-/-}$ and compared to their effect in the wild-type control mice ($sGC\alpha_1^{+/+}$). All preparations were contracted with NOR and when a stable plateau was obtained cumulative concentration-response curves for FAL and FAM (100 nmol/L – 1 mmol/L) were established. In a second series of experiments the influence of oxidative stress on corporal relaxations was evaluated. CC were incubated with the superoxide generator/free radical scavenger hydroquinone (50 µmol/L) and the superoxide dismutase inhibitor diethyldithiocarbamic acid (DETCA) (8 mmol/L) for 20 minutes. After reaching a stable contraction with phenylephrine (5 µmol/L), electrical field stimulation (EFS; train duration 40s; frequency 1, 2, 4 and 8 Hz; pulse duration 5ms; 80V) as well as cumulative concentration response curves for ACh, sodium nitroprusside (SNP), 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate sodium salt (8-pCPT-cGMP), FAL and FAM were performed.

V.4.3 In vivo study

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed on a heated blanket maintaining their body temperature at 37°C. A PE-10 tube filled with heparinized saline (25 U/mL) was inserted into the carotid artery and connected to a pressure transducer to monitor the mean arterial pressure (MAP). A 30-gauge needle attached to another PE-10 tube was introduced into the right CC and also connected to a pressure transducer to measure the intracavernosal pressure (ICP). The effect of FAL and FAM (1–8 mg/kg) on blood pressure was evaluated after intravenous injection via a PE-10 tube inserted into the jugular vein (standardized injection volume of 10 µL). In a second set of experiments, the ICP response to FAL and FAM (1–8 mg/kg) was investigated through intracavernosal administration via a separate cannula (30-gauge needle attached to a PE-10 tube) inserted into the left CC (standardized injection volume of 5 µL). Between each injected dose the cannulas were flushed several times with heparinized saline to remove residual agent.

V.4.4 Drugs, chemicals and reagents

The experiments were performed in a KRB solution with the following composition (mmol/L): NaCl, 135; KCl, 5; $NaHCO_3$, 20; glucose, 10; $CaCl_2$, 2.5; $MgSO_4$, 1.3; KH_2PO_4, 1.2 and EDTA, 0.026 in H_2O. NOR bitartrate, ACh, FAL hydrochloride, FAM, SNP, 8-pCPT-cGMP, hydroquinone, DETCA sodium salt and phenylephrine hydrochloride were obtained from Sigma-Aldrich (St.Louis, MO, USA). All drugs were dissolved in water (in vitro) or heparinized saline (25 U/mL) (in vivo). Concentrations are expressed as final molar concentrations in the organ bath experiments, while for the in vivo study the amount of agent injected is given as mg/kg.

V.4.5 Data analysis and statistical procedures

Data are presented as mean values ± SEM; N represents the number of preparations. For the in vitro experiments, relaxations are expressed as the percentage decrease in contraction level. For the in vivo study, blood pressure effects are shown as absolute changes in MAP whereas the intracavernosal pressure results are calculated as the ICP adjusted for the MAP, expressed in percentage (ICP/MAP x 100). Statistical significance was evaluated by using the non-parametric tests for paired (Wilcoxon) and unpaired (Mann-Withney U) observations when appropriate (SPSS, version 18; IBM Corporation, Armonk, NY, USA). $P<0.05$ was considered as significant.

V.5 Main outcome measures

MAP and ICP were measured in vivo after respectively intravenous and intracavernosal FAL/FAM injection. In vitro relaxation of CC strips was evaluated in response to addition of FAL/FAM. The results of the wild-type control mice were compared to the effects in the knockout mice to assess the role of sGC. The influence of oxidative stress was determined after generation of superoxide and inhibition of superoxide dismutase.

V.6 Results

V.6.1 Role of sGC in vitro

Both FAL and FAM (100 nmol/L – 1 mmol/l) relaxed the different tissues of $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ mice in a concentration-dependent manner. The responses in $sGC\alpha_1^{-/-}$ preparations of aorta and femoral artery were significantly reduced but not completely abolished (Fig. V.2A-D). Moreover, FAM induced a lower effect in $sGC\alpha_1^{-/-}$ CC compared to the wild-type control strips (Fig. V.2F). In contrast, FAL elicited identical concentration-dependent relaxations of both $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ CC (Fig. V.2E).

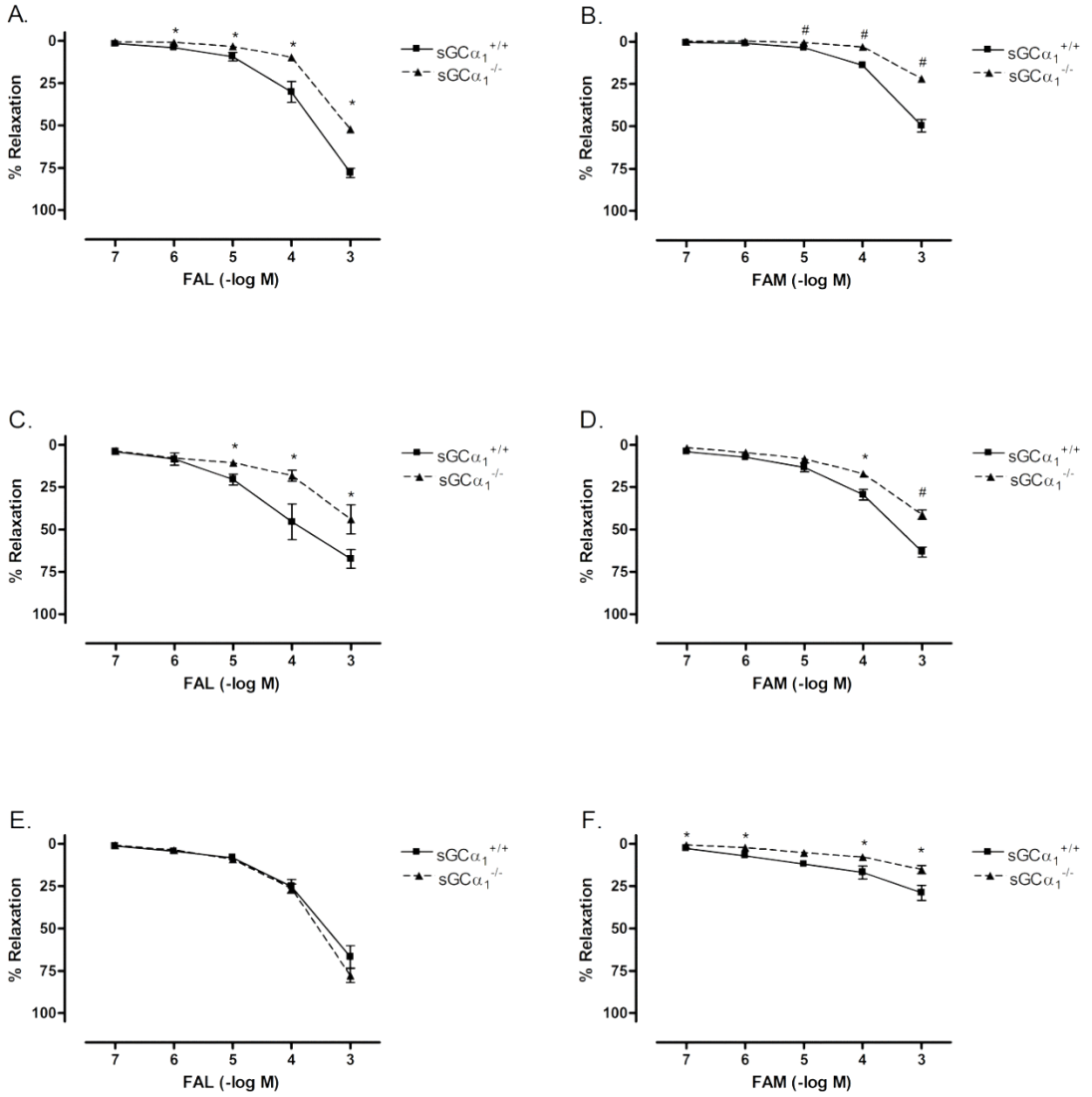

Figure V.2: The effect of formaldoxime (FAL) and formamidoxime (FAM) in thoracic aorta (A and B); in femoral artery (C and D) and in corpora cavernosa (E and F) from $sGC\alpha_1^{+/+}$ (■) and $sGC\alpha_1^{-/-}$ (▲) mice. Data are expressed as % relaxation of the norepinephrine (NOR)-induced tone (N = 4-6); *P<0.05 and #P<0.01

V.6.2 Role of sGC in vivo

V.6.2.1 Blood pressure

Basal MAP of $sGC\alpha_1^{+/+}$ mice (134 mmHg ± 2.28; N=31) was significantly ($P < 0.001$) lower compared to the basal MAP of $sGC\alpha_1^{-/-}$ mice (157 mmHg ± 1.75; N=36) as previously described [15]. Intravenous injection of both FAL and FAM decreased MAP of $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ mice dose-dependently (Fig. V.3A). However this MAP decrease was significantly less pronounced in the $sGC\alpha_1^{-/-}$ mice. In contrast, the effects of vehicle control solutions did not differ between the $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ mice. The characteristics of this MAP decrease seem to differ between FAL and FAM. Intravenous administration of FAL induces a biphasic response: a very fast blood pressure drop followed by a fast rise in blood pressure. Injection of FAM on the other hand elicits a more slow and maintained lowering of MAP, followed by a gradual recovery to the basal blood pressure level (Fig. V.3B).

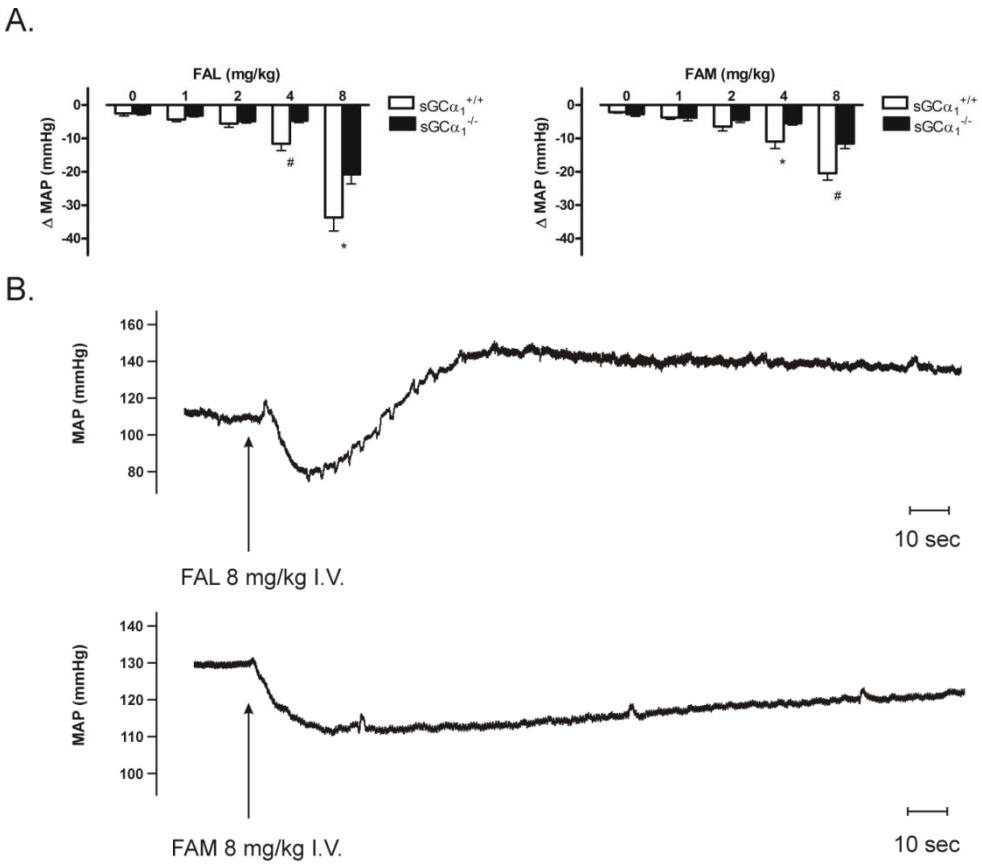

Figure V.3: (A) The changes in mean arterial pressure (MAP) after intravenous administration of formaldoxime (FAL) and formamidoxime (FAM) in $sGC\alpha_1^{+/+}$ versus $sGC\alpha_1^{-/-}$ mice. ($\Delta MAP = MAP_{after} - MAP_{before}$); (N = 6-10); *$P$<0.05 and #$P$<0.01. (B) Original tracings showing the change in mean arterial pressure (MAP) after intravenous (I.V.) administration of FAL and FAM (8 mg/kg) in $sGC\alpha_1^{+/+}$ mice.

V.6.2.2 ICP

Intracavernosal injection of increasing amounts FAL and FAM resulted in a dose-dependent increase in ICP of $sGC\alpha_1^{-/-}$ as well as $sGC\alpha_1^{+/+}$ mice. Basal ICP, when adjusted for the MAP, as well as vehicle controls did not differ between both groups. On the contrary, the FAL-induced rise in ICP/MAP was significantly lower in $sGC\alpha_1^{-/-}$ mice compared to $sGC\alpha_1^{+/+}$ mice. Moreover, in $sGC\alpha_1^{-/-}$ mice the effect of FAM on ICP was almost completely abolished (Fig. V.4A). Intracavernosal injection of both FAL and FAM also induces a small and transient decrease in MAP (Fig. V.4B).

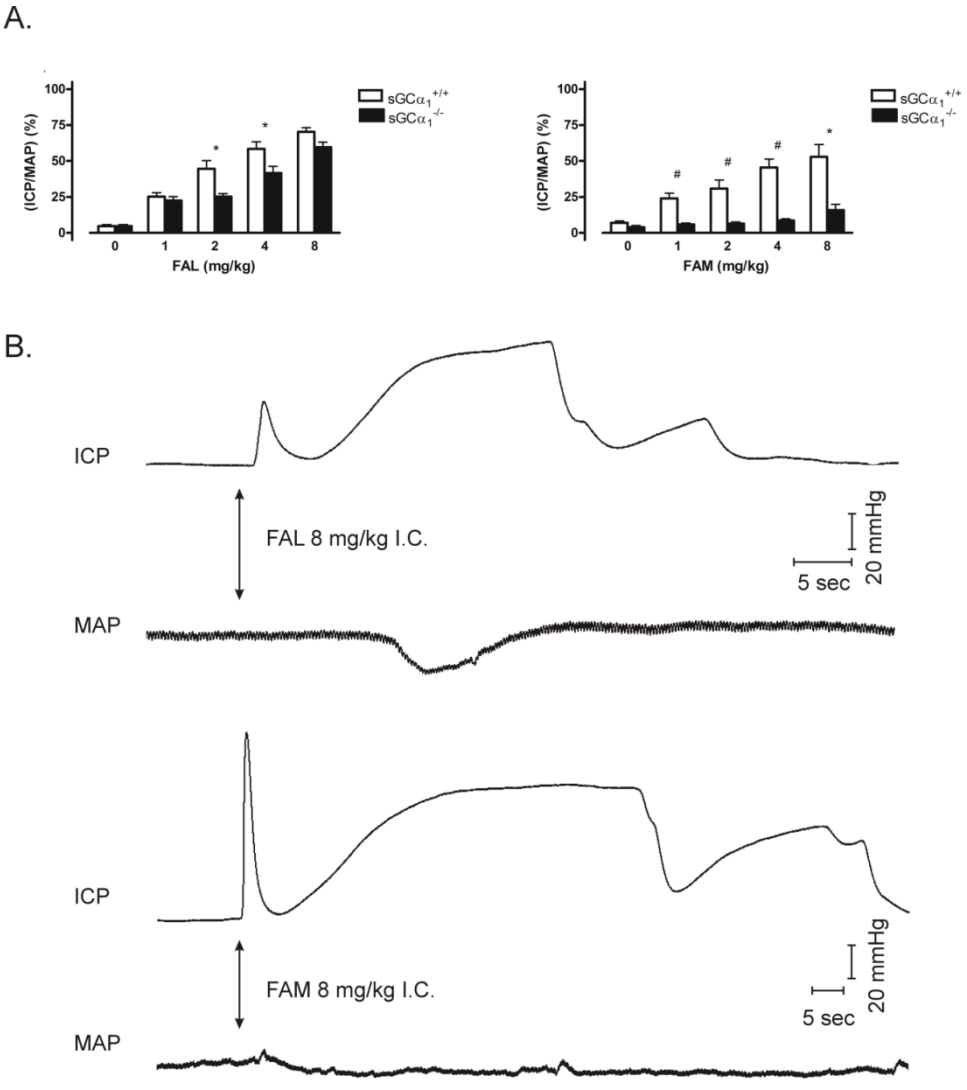

Figure V.4: (A) The intracavernosal pressure/mean arterial pressure (ICP/MAP) ratio (in %) after intracavernosal administration of formaldoxime (FAL) and formamidoxime (FAM) in $sGC\alpha_1^{+/+}$ versus $sGC\alpha_1^{-/-}$ mice. (N = 6-9); **P*<0.05 and #*P*<0.01. (B) Original tracings showing the change in intracavernosal pressure (ICP) as well as mean arterial pressure (MAP) after intracavernosal (I.C.) administration of FAL and FAM (8 mg/kg) in $sGC\alpha_1^{+/+}$ mice.

V.6.3 Influence of oxidative stress on corporal relaxations

Incubation with the superoxide generator hydroquinone (50 µmol/L) and the superoxide dismutase inhibitor DETCA (8 mmol/L) strongly inhibited NO-mediated relaxations induced by EFS, ACh and SNP (Fig. V.5A-C) in isolated CC. In contrast, the corporal relaxation by 8-pCPT-cGMP, FAL and FAM remained unaffected (Fig. V.5D-F).

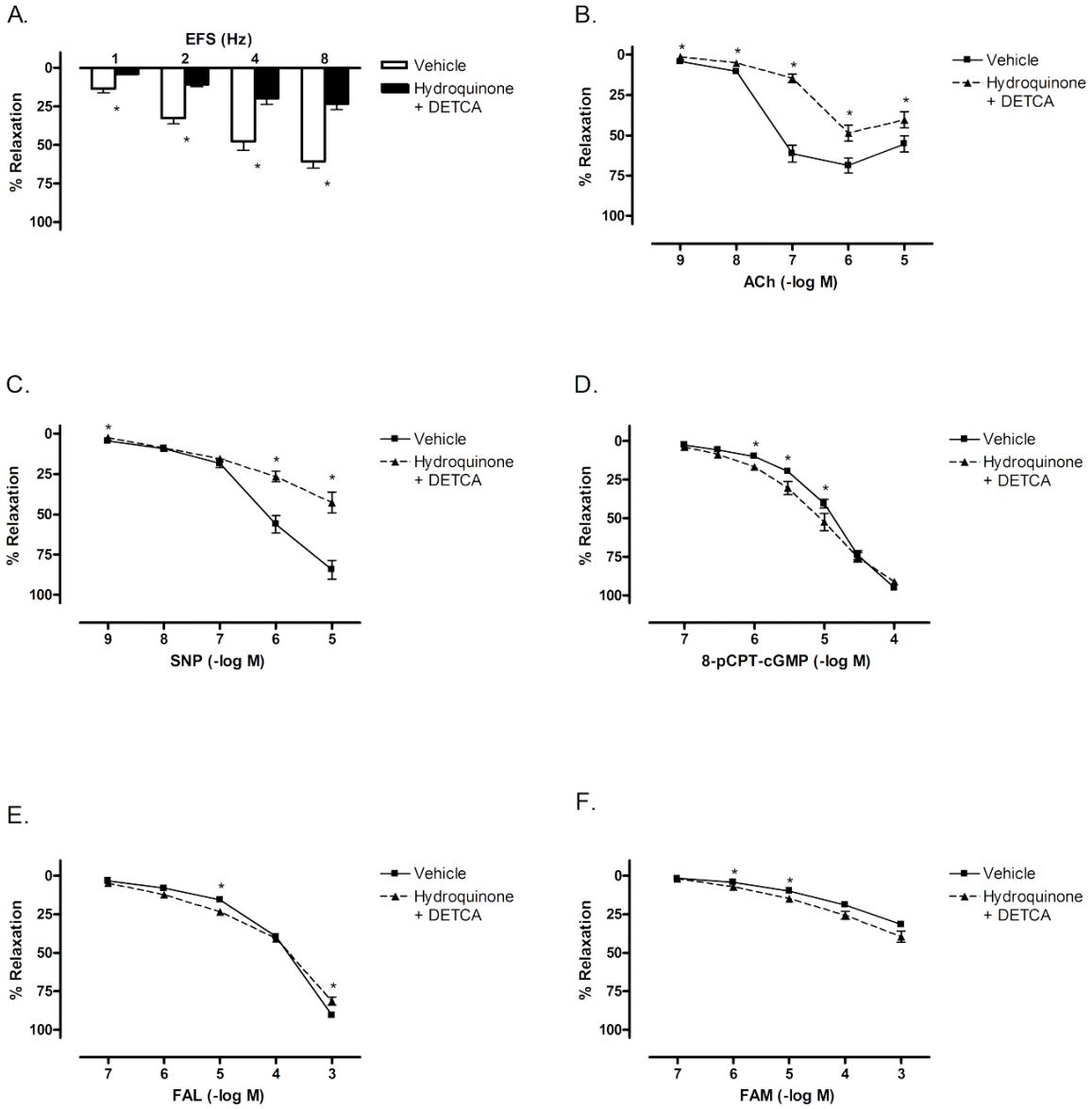

Figure V.5: The influence of hydroquinone (50 µmol/L) and diethyldithiocarbamic acid (DETCA) (8 mmol/L) on relaxations of isolated corpora cavernosa induced by electrical field stimulation (EFS) (A); acetylcholine (ACh) (B); sodium nitroprusside (SNP) (C); 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate (8-pCPT-cGMP) (D); formaldoxime (FAL) (E) and formamidoxime (FAM) (F) in corpora cavernosa (CC) of $sGC\alpha_1^{+/+}$ mice. Data are expressed as % relaxation of the phenylephrine-induced tone (N = 5-8); **P*<0.05

V.7 Discussion

Our results show for the first time that the oxime compounds FAL and FAM are able to induce erection in vivo. The blood pressure lowering effect of FAL and FAM as well as the importance of sGC in their responses were confirmed. Furthermore we demonstrated that both FAL and FAM seem to be resistant to oxidative stress offering some perspective as a potential new treatment strategy for ED.

Oxime compounds were proposed as a new class of NO-donating molecules which can compensate the impaired NO functioning in various disease states. It was reported that metabolization of oxime compounds occurs through (a) 7-ethoxyresorufin sensitive pathway(s), resulting in the release of NO. Subsequently NO interacts with its main target sGC, eventually leading to smooth muscle relaxation [2-4]. A previous study in our lab already established the importance of sGC in both FAL- and FAM-mediated relaxations on isolated preparations. The sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ) blocked FAL-induced vasorelaxation in mice aorta and femoral artery ring segments whereas the effect of FAM was even completely abolished. In contrast, ODQ had no influence on the FAL response in the isolated mice CC and only a limited involvement of sGC was demonstrated for the FAM effect [4]. Besides a pharmacological approach, the role of sGC in the

FAL-/FAM-induced relaxations can further be characterized by a genetic approach using transgenic mice. After all ODQ lacks specificity as there are reports indicating that ODQ also influences the redox state of other heme-containing proteins than sGC such as cytochrome P450 [19]. Moreover, the use of genetically modified mice enables to differentiate between the different isoforms of sGC elucidating their individual role in the FAL/FAM responses. sGC is a αβ-heterodimer of which two isoforms exist for each subunit (α_1/α_2 and β_1/β_2), but only the $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$ heterodimers are catalytically active [20]. In the $sGC\alpha_1^{-/-}$ mice a deletion in the catalytic domain of the α_1 subunit results in a selective loss of activity of the $sGC\alpha_1\beta_1$ isoform [15]. Both FAL- and FAM-induced relaxations are significantly lower in the aorta and femoral artery ring segments of $sGC\alpha_1^{-/-}$ mice compared to the wild-type control mice, implicating a role for the $sGC\alpha_1\beta_1$ isoform in these responses. Also the FAM-effect in the CC seems to be mediated by the activation of the $sGC\alpha_1\beta_1$ isoform, while this was not the case for the FAL-response. Remarkably, in our genetically modified $sGC\alpha_1^{-/-}$ mice only a

limited lowering of the FAL-/FAM-induced relaxations of aorta and femoral artery occurred whereas ODQ almost abolished their responses, especially the FAM-response [4]. This can be explained by the fact that ODQ is able to inhibit both sGC isoforms. In the $sGC\alpha_1^{-/-}$ mice on the other hand only the $sGC\alpha_1\beta_1$ isoform is catalytically inactive while the $sGC\alpha_2\beta_1$ isoform maintains its functionality. Thus the remaining $sGC\alpha_2\beta_1$ activity could be responsible for the FAL-/FAM-induced vasorelaxation which is still seen in the $sGC\alpha_1^{-/-}$ mice. In general we can say that sGC plays an important role in the FAL-/FAM-mediated relaxations. However, sGC does not appear to take part in the FAL-induced corporal relaxation which is in correlation with our previous in vitro study [4].

In contrast to our findings, FAL and FAM do not elicit a blood pressure lowering effect in control rats. However, a blood pressure drop could be demonstrated after inhibition of endogenous NO synthesis and this blood pressure drop was further enhanced when also the arterial baroreflex was interrupted [5]. Our in vivo experiments demonstrate that both FAL and FAM induce a dose-dependent blood pressure reduction in mice even without blockade of endogenous NO synthesis. This blood pressure lowering is at least in part mediated by the activation of sGC since the in vivo FAL-/FAM-response is significantly lower in $sGC\alpha_1^{-/-}$ mice compared to their wild-type controls, which corresponds with our in vitro results. Jaros et al. [5] also reported an involvement of sGC in the blood pressure lowering effect of FAL and FAM in rats by using methylene blue, which is however a non-specific sGC inhibitor and also has redox effects.

As yet, nothing is known about the effect of FAL and FAM on erectile function in vivo. In our study both compounds were administered through intracavernosal injection. This resulted in a dose-dependent increase of ICP. So, FAL and FAM not only relax corporal smooth muscles in vitro, but also in vivo, leading to a rise of ICP and ultimately erection. However, this ICP rise is less pronounced when compared to standard NO-donors such as SNP and Spermine-NO. Lower doses of these NO-donors (µg/kg instead of mg/kg range) give even higher ICP values [21]. In accordance with the in vitro results in the CC, sGC is involved in the effect of FAM on ICP as its response was almost completely abolished in the $sGC\alpha_1^{-/-}$ mice. Surprisingly sGC also plays a limited role in the FAL-induced ICP rise whereas in vitro no sGC-involvement could be demonstrated in the corporal FAL-relaxation.

Various disease states are characterized by oxidative stress due to an increased production of reactive oxygen species, including superoxide. Excessive levels of superoxide lower NO-bioavailability by forming peroxynitrite [22]. The interaction between NO and superoxide is one of the important pathophysiological mechanisms implicated in ED [6]. In steptozotocin-induced diabetes in rats it was proven that antioxidant therapy improves NO-mediated neurogenic (EFS) and endothelium-dependent (ACh) corporal relaxation [23]. Lowered ICP values after cavernosal nerve stimulation in diabetic rats could be restored by treatment with the superoxide dismutase mimetic tempol [24] and adenoviral gene transfer of extracellular superoxide dismutase [25]. Furthermore, experiments on human CC and penile resistance arteries showed that antioxidant therapy ameliorates impaired NO-mediated relaxations (both ACh and EFS) in diabetic patients with ED [26]. Oxidative stress alters NO-mediated relaxations in mice CC, as the combination of the superoxide generator LY 83583 and the superoxide dismutase inhibitor DETCA inhibited corporal relaxations induced by EFS, ACh and acidified sodium nitrite [14]. In our model of oxidative stress we increased superoxide concentrations by using the superoxide generator (free radical scavenger) hydroquinone [27,28] in combination with DETCA, an irreversible inhibitor of the Cu^{2+}/Zn^{2+}-containing superoxide dismutase [29]. The combination of hydroquinone and DETCA significantly lowered EFS-, ACh- and SNP-induced corporal relaxations. This confirms that oxidative stress influences respectively neuronal, endothelial as well as exogenous NO-mediated responses in CC. As expected oxidative stress had no effect on the 8-pCPT-cGMP, an analog of cGMP, which causes NO-independent corporal relaxation. Surprisingly both FAL and FAM responses were unaffected by oxidative stress as the combination of hydroquinone and DETCA had no influence on the FAL-/FAM-induced relaxations. However, in our previous study we could not elucidate the underlying mechanism of corporal FAL-/FAM-effect [4]. So it is possible that in the CC FAL and FAM elicit relaxation mainly through (a) NO-independent mechanism(s), which could explain the lack of sensitivity towards oxidative stress. Moreover, it was already reported that superoxide leads to the production of nitrites from glyceryl trinitrate whereas FAL was resistant to its action [30].

The pathophysiology of ED is multifactorial, but is often linked to impaired NO functioning. This can be caused either directly by a lowered NO production because of altered NO synthase expression, NO synthase function and endothelial dysfunction [31] or indirectly by a decreased NO-bioavailability due to oxidative stress [6]. Previously we already showed that the FAL-/FAM-induced corporal relaxations are independent of NO synthase and the endothelium [4]. In this study we demonstrated that FAL and FAM are not susceptible to oxidative stress. Moreover we could also prove that FAL and FAM induce erection in vivo. Taken together, all these results point out that oximes such as FAL and FAM should be considered as potential new and alternative drugs for the treatment of ED.

V.8 Conclusion

In this study we showed for the first time that both FAL and FAM induce a concentration-dependent erectile effect in vivo. However, the in vivo ICP increase seems to be mediated by (a) different mechanism(s) than the FAL-/FAM-induced in vitro corporal relaxations. Moreover, both compounds elicit a distinct blood pressure lowering effect. Activation of sGC plays an important role in the FAL-/FAM-effect on blood pressure as well as on ICP, but FAL and FAM could partially exert their effects by different mechanisms. In addition, FAL-/FAM-induced corporal relaxations seem to be unaffected by oxidative stress. Overall, oximes offer favourable perspectives for the treatment of ED.

V.9 Acknowledgements

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Chapter VI

The influence of ruthenium on vascular tone

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VI.1 Abstract

OBJECTIVES: Over the past few years ruthenium became under the attention for development of organometallic drugs with various therapeutic applications. Because of its favorable characteristics ruthenium is perfectly suitable for drug design. Ruthenium-containing complexes exert a wide range of biological effects. However, so far the influence of ruthenium itself on vascular tone has never been studied.

METHODS: The effect of ruthenium was analyzed through organ bath studies measuring isometric tension on mice thoracic aorta. After obtaining a stable contraction plateau, cumulative concentration-response curves of the ruthenium-compounds ($RuCl_3$, Ruthenium Red, $[RuCl_2(CO)_3]_2$ and $RuCl_2(DMSO)_4$) (30 – 600 µmol/L) were performed. The effect of $RuCl_3$ after contraction with different contractile agents was evaluated. Furthermore the influence of ruthenium-containing molecules on endogenous (ACh) and exogenous (SNP) NO-mediated relaxations was determined.

KEY FINDINGS: All studied ruthenium-compounds elicit to some extent a decrease of the contraction level. Looking further into the underlying mechanism we found that $RuCl_3$ relaxes aortic rings only when contracted with norepinephrine (NOR). This $RuCl_3$-induced relaxation can be prevented by the antioxidants ascorbic acid and N-acetyl L-cysteine. In addition ruthenium-compounds may diminish acetylcholine (ACh)- and/or sodium nitroprusside (SNP)-induced relaxations.

CONCLUSIONS: Ruthenium-containing molecules can influence vascular tone induced by NOR due to oxidative inactivation. Moreover they can undermine NO-mediated responses. This should be considered when developing ruthenium-containing drugs.

Keywords: Aorta; ascorbic acid; carbon monoxide-releasing molecule; nitric oxide; ruthenium

VI.2 Introduction

Ruthenium is a precious metal with some intriguing properties making it useful for various pharmacological applications. In the past, precious metals have already been successful for drug development, such as platinum compounds for cancer treatment, silver compounds as antimicrobial agents and gold compounds in the treatment of rheumatoid arthritis [1]. Nowadays ruthenium can be found in drugs with immunosuppressant, antimicrobial, antibiotic and anticancer activity [2-5]. Over the years ruthenium became popular for drug designers as it has interesting characteristics. First of all, ruthenium complexes exchange ligands when in contact with biological tissues, which is often necessary to exert biological effects. Another important property is that ruthenium shows different oxidation states under physiological conditions namely Ru(II), Ru(III) and Ru(IV) with Ru(III)-complexes being biological more inert. Hypoxic conditions, which occur in multiple disease states, lead to a reductive environment reducing the relatively inert Ru(III)-complexes to active Ru(II)-complexes. This activation-by-reduction mechanism creates certain selectivity towards the hypoxic, diseased tissues [1,6]. Another advantage of ruthenium is its low toxicity due to the ability of ruthenium to mimic iron in binding to biomolecules, including serum transferrin and albumin, resulting in effective biodistribution and minimum systemic toxicity [1,7].

Based on its beneficial characteristics several ruthenium-containing drugs were developed for safe and controlled delivery of carbon monoxide (CO) and nitric oxide (NO) in tissues. Since CO and NO induce vasorelaxation, these CO- and NO-releasing ruthenium-complexes were tested for their vasorelaxing effect. Many of them indeed elicit vasorelaxation which is then attributed to the release of CO or NO [8-11]. However, in a recent study using the ruthenium-containing CO-releasing molecule $[RuCl_2(CO)_3]_2$ (= CORM-2) we found that its vasorelaxing effect could not completely be explained by CO-release as CORM-2 showed a stronger relaxation than CO as such. Moreover, tissues that do no relax in response to CO do so in response to CORM-2 [12]. Therefore we wondered if the ruthenium-core of CORM-2 could be responsible for this enigmatic observation. As far, the influence of ruthenium itself on vascular tone has never been studied.

Some ruthenium-containing compounds can rapidly and strongly bind NO and this could be useful for the treatment of diseases involving NO overproduction such as septic shock [13-15]. However, this mechanism can be considered as an undesired side-effect of ruthenium-compounds in disease states with a compromised NO-bioavailability. CO-releasing ruthenium-drugs are often proposed as compensating therapy to overcome loss in endogenous NO functioning. Hence, scavenging of the already low NO-levels by ruthenium-based drugs would seriously counteract their beneficial effect. It was already demonstrated that CORM-2 attenuates NO donor-induced relaxations and that the water-soluble ruthenium-based CO-releasing molecule $RuCl(CO)_3$-glycinate (= CORM-3) inhibits endothelium- and NO-dependent relaxations [16,17]. However, the influence of ruthenium itself on NO-mediated signalling is not completely explored.

VI.3 Aims

The present study was designed to elucidate the vasorelaxing effect of different ruthenium-containing compounds on isolated mice aortic ring segments. Furthermore, the influence of these compounds on endogenous as well as exogenous NO-mediated relaxations was evaluated.

VI.4 Materials and methods

VI.4.1 Animals

For this study mature (age 8-12 weeks) male Swiss mice were obtained from Janvier (Saint-Berthevin, France). All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Food and water were provided ad libitum and animals were kept in a 12h/12h day/night cycle. All experiments were approved by the local Ethical Committee for Animal Experiments (Ghent University, 14/02/2011, ECD 10/42).

VI.4.2 Tissue preparations and mounting

After cervical dislocation thoracic aorta was carefully isolated from surrounding tissues and displaced in cold Krebs-Ringer bicarbonate (KRB) solution. Aortic ring segments were mounted into 10 ml organ baths from a wire myograph for isometric tension recordings as previously described [12,18]. The mounted aortic rings were then left to equilibrate for 30 minutes in KRB solution (37°C; pH 7.4, bubbled with 95% O_2 – 5% CO_2).

VI.4.3 Preliminary protocol

To obtain maximal, stable contractions and relaxations of the aortic ring segments, a preliminary protocol was performed as previously described [12]. Subsequently, the tissues were contracted with 5 µmol/L NOR. When a stable plateau was reached 10 µmol/L ACh was added to test the functionality of the endothelium. The endothelium was considered intact when ACh induced more than 50 % tissue relaxation. Preparations were washed until they returned to basal tone before starting with the actual experiments.

VI.4.4 Experimental protocol

In a first set of experiments the effect of various ruthenium-containing molecules was tested. Aortic rings were contracted with 5 µmol/L NOR and when a stable plateau was obtained cumulative concentration-response curves for $RuCl_3$, Ruthenium Red, $[RuCl_2(CO)_3]_2$ and $RuCl_2(DMSO)_4$ (30 – 100 – 300 – 600 µmol/L) were established. Furthermore the

responses to $RuCl_3$ were evaluated after contraction with different contractile agents. The $RuCl_3$ responses after NOR-induced contraction were compared with those after contraction with K^+_{30}, serotonin (1 µmol/L), 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$ (U46619; 5 nmol/L), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 1.5 µmol/L) and phenylephrine (1 µmol/L). In another series of experiments the influence of ascorbic acid and N-acetyl L-cysteine on the $RuCl_3$-induced effects were analysed. First, tissues were incubated with vehicle, $RuCl_3$ (200 µmol/L) or $RuCl_3$ (200 µmol/L) + ascorbic acid (100 µmol/L) before inducing contraction with 5 µmol/L NOR. In a second experiment, the effect of ascorbic acid (100 µmol/L) and N-acetyl L-cysteine (1 mmol/L) on the cumulative concentration-response curve for $RuCl_3$ was determined. In a third experiment, the organ bath solution was replaced by either 10 mL KRB + NOR (5 µmol/L) or 10 mL KRB + NOR (5 µmol/L) + $RuCl_3$ (200 µmol/L) or 10 mL KRB + NOR (5 µmol/L) + $RuCl_3$ (200 µmol/L) + ascorbic acid (100 µmol/L), which were first left at 37°C and continuously bubbled with 95% O_2 – 5% CO_2 for 1 hour. In a last series of experiments aortic ring segments were incubated with 200 µmol/L of either $RuCl_3$, $RuCl_2(DMSO)_4$ or $[RuCl_2(CO)_3]_2$ and contracted with 1.5 µmol/L $PGF_{2\alpha}$. After obtaining a stable plateau cumulative concentration-response curves for ACh and SNP (1 nmol/L – 10 µmol/L) were performed.

VI.4.5 Drugs, chemicals and reagents

The experiments were performed in a KRB solution with the following composition (mmol/L): NaCl, 135; KCl, 5; $NaHCO_3$, 20; glucose, 10; $CaCl_2$, 2.5; $MgSO_4$, 1.3; KH_2PO_4, 1.2 and EDTA, 0.026 in H_2O. K^+_{30} was made as modified KRB solution containing 30 mmol/L K^+ (changes in the KCl concentration of the KRB solution were compensated by equimolar adjustment of the NaCl concentration). Norepinephrine bitartrate (NOR), dimethylsulfoxide (DMSO), acetylcholine chloride (ACh), sodium nitroprusside (SNP), $RuCl_3$, Ruthenium Red, $[RuCl_2(CO)_3]_2$ (CORM-2), $RuCl_2(DMSO)_4$, U46619, serotonin, L-phenylephrine hydrochloride, L-ascorbic acid and N-acetyl L-cysteine were obtained from Sigma-Aldrich (St.Louis, MO). $PGF_{2\alpha}$ (Dinolytic®) was acquired from Pfizer (Puurs, Belgium). All stock solutions were prepared in distilled water, except for CORM-2 (dissolved in DMSO; maximal DMSO concentration: 0.6%) and U46619 (dissolved in methylacetate). All concentrations are expressed as final molar concentrations in the organ baths.

VI.4.6 Data analysis and statistical procedures

Data are presented as mean values ± SEM; N represents the number of preparations. The relaxations are expressed as the percentage decrease in contraction level. The level of NOR-induced contraction is presented in mN. Statistical significance was evaluated using the Mann-Withney U test and Kruskal-Wallis test with Bonferroni corrected pairwise comparison when appropriate (SPSS, version 18; IBM Corporation, Armonk, NY, USA). Data were considered significant when $P<0.05$.

VI.5 Results

VI.5.1 Effect of ruthenium-containing compounds

All tested ruthenium-compounds lowered, to some extent, NOR-induced tone of mice aortic rings (Fig. VI.1). Both $RuCl_3$ and Ruthenium Red were able to elicit a strong relaxant effect (maximal effect $RuCl_3$: 87.77% ± 3.63 – Ruthenium Red: 73.37% ± 6.49). The ruthenium-based CO-releasing molecule $[RuCl_2(CO)_3]_2$ (= CORM-2) also showed a concentration-dependent vasorelaxation. However, its relaxant capacity was significantly lower as compared to $RuCl_3$ (maximal effect $[RuCl_2(CO)_3]_2$: 48.73% ± 5.83). In contrast, the CO-free ruthenium-complex $RuCl_2(DMSO)_4$ did only present a limited influence on the NOR-induced contraction level (maximal effect $RuCl_2(DMSO)_4$: 15.73% ± 8.96). Remarkably the lowest concentration of Ruthenium Red (30 µmol/L) induced a significant contractile effect (14.06% ± 3.58), whereas the other ruthenium-compounds did not show any contractile effect at all.

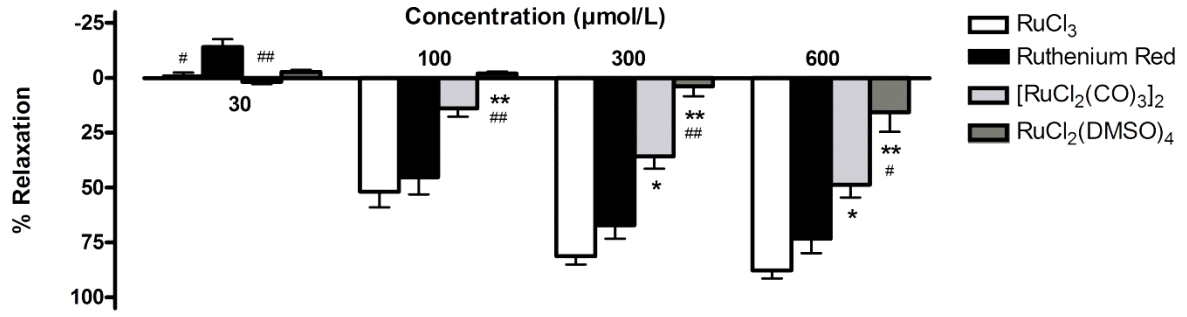

Figure VI.1: Cumulative concentration-responses to $RuCl_3$, Ruthenium Red, $[RuCl_2(CO)_3]_2$ (=CORM-2) and $RuCl_2(DMSO)_4$ in mice aortic ring segments precontracted with 5 µmol/L norepinephrine (NOR). Data are expressed as % relaxation of the NOR-induced tone (N = 5-7); **P*<0.05 (as compared to $RuCl_3$); ***P*<0.01 (as compared to $RuCl_3$); #*P*<0.05(as compared to Ruthenium Red) and ##*P*<0.01 (as compared to Ruthenium Red).

VI.5.2 Effect of $RuCl_3$ on different contractile agents

$RuCl_3$ almost completely relaxes NOR-contracted aortic rings. However when contraction was elicited using a K^+-rich KRB-solution (K^+_{30}), $RuCl_3$ did not seem to have any relaxant effect (Fig. VI.2 A). Also, when the aortic segments were contracted with serotonin, $PGF_{2\alpha}$ or the thromboxane A_2 mimetic U46619, $RuCl_3$ did not show any vasorelaxation (Fig. VI.2 B-D). Even after contraction with phenylephrine $RuCl_3$ did not induce relaxation (Fig. VI.2 E).

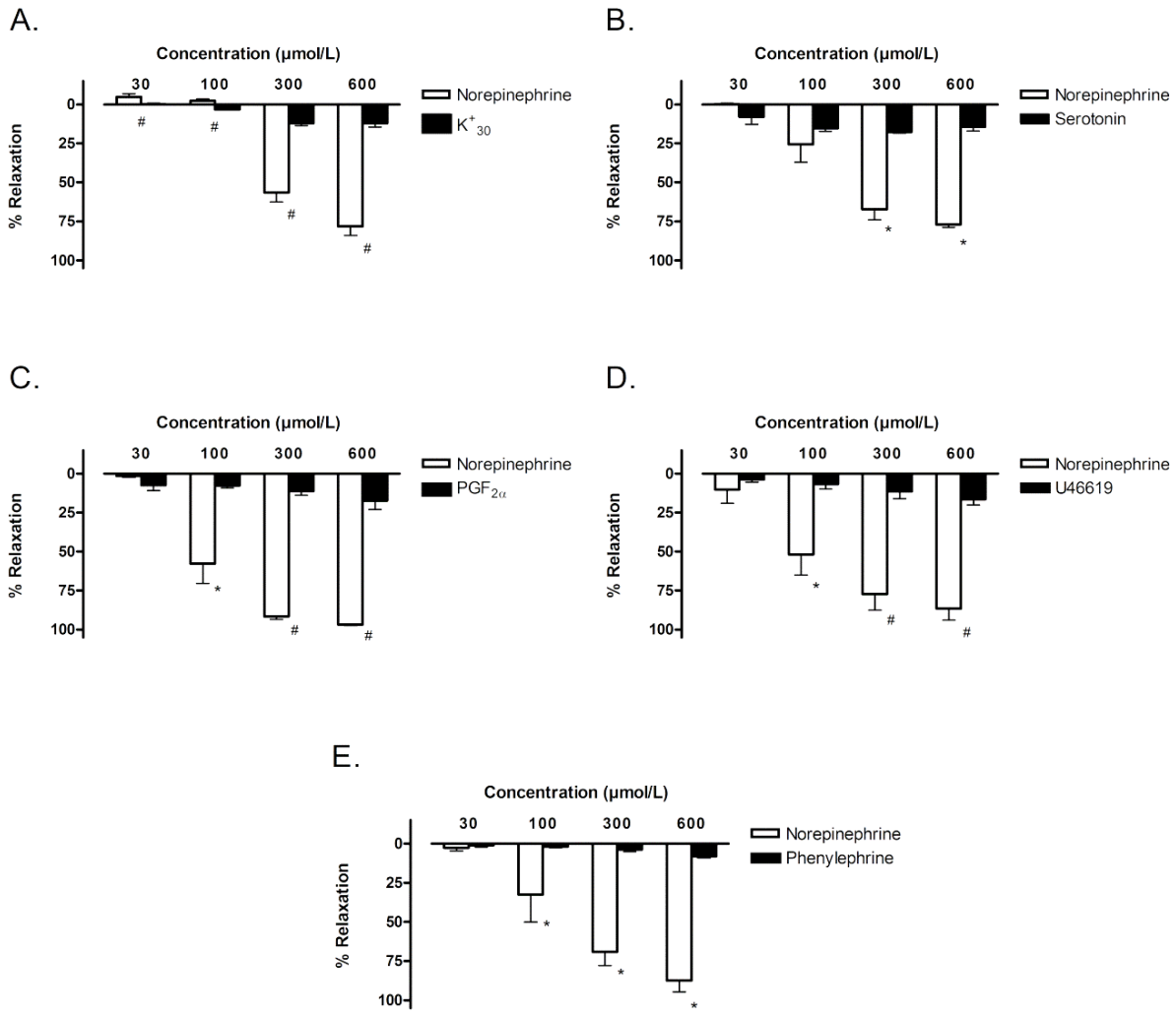

Figure VI.2: The effect of $RuCl_3$ on norepinephrine(NOR)-precontracted mice aortic ring segments compared to the $RuCl_3$ response after contraction with K^+_{30} (A); 1 µmol/L serotonin (B); 1.5 µmol/L $PGF_{2\alpha}$ (C); 5 nmol/L U46619 (D) and 1 µmol/L phenylephrine (E). Data are expressed as % decrease in contractile tone (N = 4-7); **P*<0.05 and #*P*<0.01

VI.5.3 Influence of ascorbic acid and N-acetyl L-cysteine

NOR is susceptible to oxidative degradation by traces of heavy metals. This oxidation diminishes vascular reactivity which can be prevented by the addition of ascorbic acid [19]. We wondered if ascorbic acid would be able to influence the effect of $RuCl_3$ on NOR-induced contraction. Incubation of aortic ring segments with 200 µmol/L $RuCl_3$ for 20 minutes counteracted NOR-contraction. Although the aortic rings started to contract after addition of NOR, at some point the contraction level rapidly declined and the vessels returned to basal tone (Fig. VI.3 B). However, co-incubation with 100 µmol/L ascorbic acid prevented the $RuCl_3$-induced loss of vascular tone (Fig. VI.3 C).

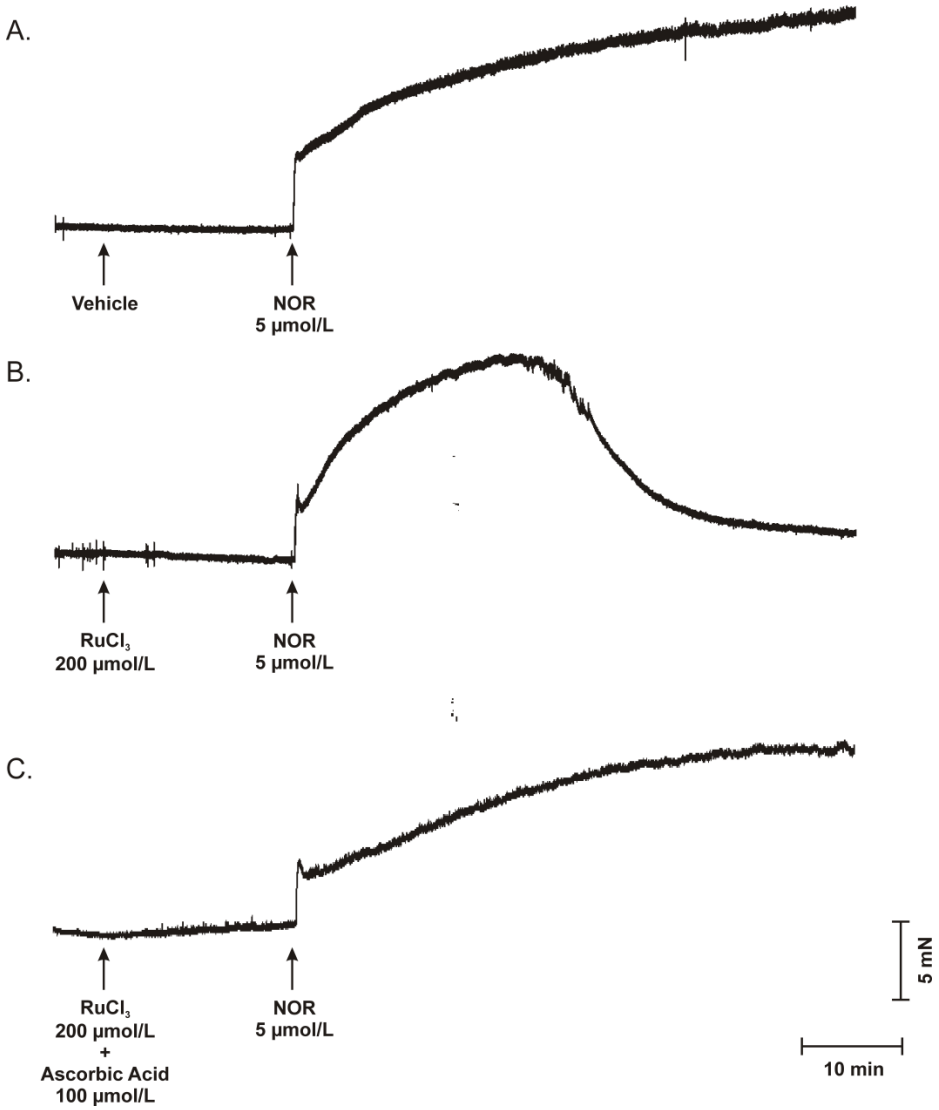

Figure VI.3: Original tracings showing the contraction of mice aortic ring segments with 5 µmol/L norepinephrine (NOR) in the presence of vehicle (A), $RuCl_3$ (200 µmol/L) (B), and $RuCl_3$ (200 µmol/L) + ascorbic acid (100 µmol/L) (C).

Ascorbic acid also significantly diminished $RuCl_3$-induced vasorelaxation of NOR-contracted aortic rings (Fig. VI.4 A). However, ascorbic acid as such has no influence on NOR-induced contraction (maximal contraction: without ascorbic acid: 10.48 mN ± 0.81 - with ascorbic acid: 9.01 mN ± 0.96; N = 8; p = 0.336). Moreover, the antioxidant N-acetyl L-cysteine (1 mmol/L) strongly blocked the vasorelaxing effect of $RuCl_3$ after aortic contraction with NOR (Fig. VI.4 B). In another experiment, 200 µmol/L $RuCl_3$ was left to react with 5 µmol/L NOR in KRB solution at 37 °C for 1 hour. In contrast to the control solution (KRB + NOR), changing the organ bath solution of the tissues by this $RuCl_3$- and NOR-containing KRB solution did not result in contraction (maximal contraction without $RuCl_3$: 12.50 mN ± 1.32 – with $RuCl_3$: 0.39 mN ± 0.20; N = 8). However, when $RuCl_3$ is left to react with NOR in the presence of 100 µmol/L ascorbic acid, contraction does occur and is not significantly different from the contraction induced by the control solution (maximal contraction in the presence of $RuCl_3$ and ascorbic acid: 8.95 mN ± 1.86; N = 8).

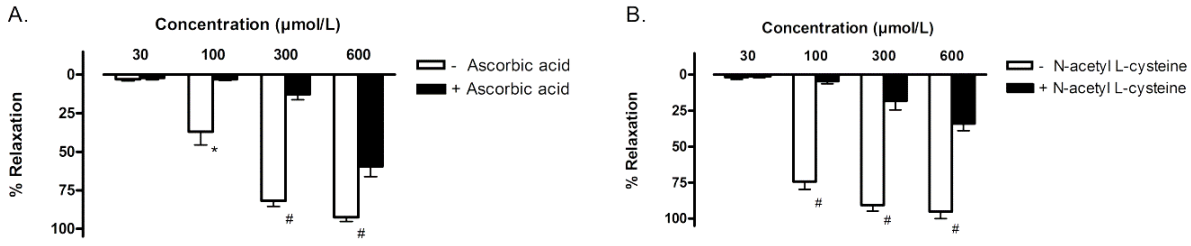

Figure VI.4: Cumulative concentration-responses to $RuCl_3$ in the absence or presence of ascorbic acid (100 µmol/L incubated during 20 minutes) (A) or N-acetyl L-cysteine (1 mmol/L incubated during 20 minutes) (B) in mice thoracic aorta contracted with 5 µmol/L norepinephrine (NOR). Data are expressed as % relaxation of the NOR-induced tone (N = 7); **P*<0.05 and #*P*<0.01

VI.5.4 Influence of ruthenium-containing compounds on NO-mediated relaxations

For these incubation experiments we used a contractile agent which was not influenced by $RuCl_3$, namely $PGF_{2\alpha}$. The presence of $RuCl_3$ had an inhibitory effect on SNP- but not on ACh-induced relaxations (Fig. VI.5 A and B), whereas the ruthenium-complex $RuCl_2(DMSO)_4$ had no influence on both ACh- and SNP-mediated relaxations (Fig. VI.5 C and D). In contrast, $[RuCl_2(CO)_3]_2$ (= CORM-2) showed a strong inhibitory effect on ACh- as well as SNP-induced relaxations (Fig. VI.5 E and F).

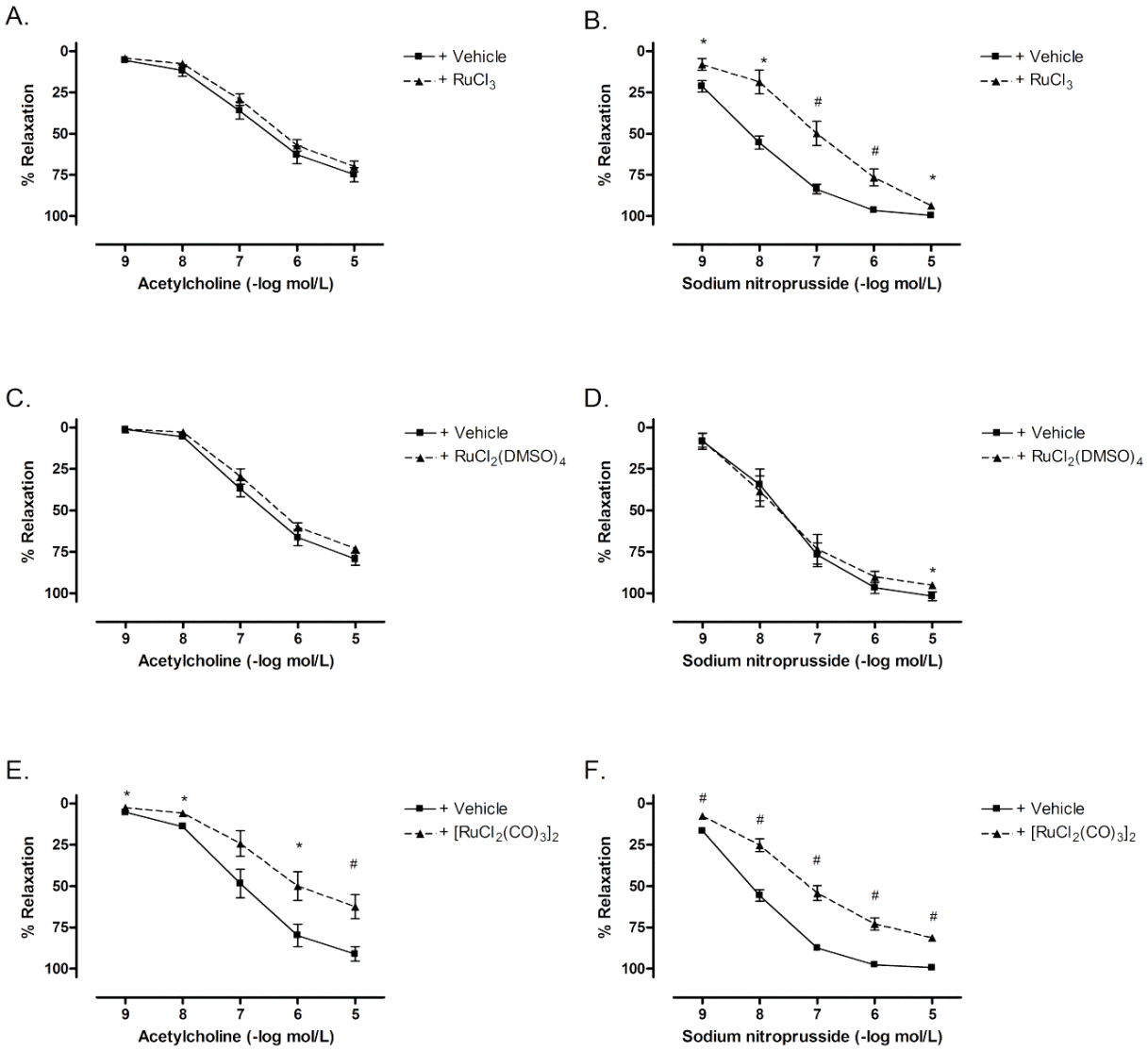

Figure VI.5: The effect of incubation with 200 µmol/L $RuCl_3$ (A), $RuCl_2(DMSO)_4$ (C) or $[RuCl_2(CO)_3]_2$ (= CORM-2) (E) compared to vehicle control on acetylcholine-mediated relaxations in mice thoracic aorta. The effect of incubation with 200 µmol/L $RuCl_3$ (B), $RuCl_2(DMSO)_4$ (D) or $[RuCl_2(CO)_3]_2$ (= CORM-2) (F) compared to vehicle control on sodium nitroprusside-mediated relaxations in mice thoracic aorta. Data are expressed as % relaxation of the $PGF_{2\alpha}$-induced tone (N = 5-9); **P*<0.05 and #*P*< 0.01

VI.6 Discussion

This study showed that ruthenium as such and ruthenium-containing complexes can influence vascular tone. These compounds do not only counteract NOR-induced contractions, but can also impair NO-mediated relaxations.

Organometallic complexes possess unique physico-chemical properties for drug development. They provide a great structural variety and rational ligand design offers control over its kinetic properties [20]. Hence, ruthenium-based molecules were developed delivering vasorelaxing CO and NO to the tissues in a controlled and safe way. However, the vasorelaxant effect of these ruthenium-complexes could not always be linked to their CO-/NO-release. Decaluwé et al. observed that part of the effects of the CO-releasing ruthenium-complex $[RuCl_2(CO)_3]_2$ (= CORM-2) is unrelated to CO-release [12]. Therefore, we hypothesized that the ruthenium-core of CORM-2 could be (partially) responsible for the CORM-2 effects. Hence, the influence of various ruthenium-containing compounds on vascular tone was tested.

We found that $RuCl_3$ as well as Ruthenium Red strongly relaxed mice aorta. CORM-2 showed a less pronounced relaxation. $RuCl_3$ is often used as "inactive" CO-free control for the CO-donor CORM-2 [8,21,22]. However, $RuCl_3$ is perhaps not the best candidate to study the effects of the ruthenium-core of CORM-2. First of all the oxidation state of ruthenium differs in both molecules. Ruthenium is trivalent in $RuCl_3$ whereas it is bivalent in CORM-2. Moreover, $RuCl_3$ is an ionic compound in contrast to the ruthenium-based metal complex CORM-2. Another "inactive" CO-free control molecule for CORM-2 is the ruthenium-based metal complex $RuCl_2(DMSO)_4$ [23]. In contrast to $RuCl_3$, $RuCl_2(DMSO)_4$ had only a limited influence on contractile tone. Therefore, it remains unclear to what extent the release of CO and the ruthenium-core is responsible for the CORM-2 relaxation. Whatsoever, all ruthenium-compounds studied somehow influence vascular tone. Hence the possible vascular effects of ruthenium should be taken into account when investigating ruthenium-based compounds.

Remarkable is the contractile effect induced by the lowest concentration of Ruthenium Red, what was not observed with the other ruthenium-compounds. Generally Ruthenium Red is used as an inhibitor of the ryanodine receptors localized on the smooth endoplasmic

reticulum releasing Ca^{2+} ions into the cytoplasm upon activation [24]. Hence, inhibition of this Ca^{2+}-release would imply a lower cytoplasmic Ca^{2+}-concentration and subsequent smooth muscle cell relaxation which is in contrast with the contractile effect in our results. However, activation of the ryanodine receptors can establish local increases of the Ca^{2+}-concentration at the plasma membrane, leading to opening of Ca^{2+}-activated K^{+}-channels and hyperpolarization of the smooth muscle cell [25]. Thus it is possible that the low concentration of Ruthenium Red opposes the hyperpolarization due to ryanodine receptor activation and K^{+}-channel opening, resulting in a contractile response. Moreover Ruthenium Red has shown to possess various nonspecific effects such as inhibition of voltage-gated Ca^{2+}-channel activity, plasma membrane Ca^{2+}-ATPase, Ca^{2+}-activated K^{+}-channels, IP_3-mediated Ca^{2+} release, mitochondrial Ca^{2+} uniporter and calmodulin activity. There are also important direct effects on myosin light chain kinase and myosin light chain phosphatase [24]. This wide range of possible effects of Ruthenium Red could all explain in their own way the relaxant as well as contractile responses seen on our aortic tissues.

This study is the first to demonstrate a strong vasorelaxant effect of $RuCl_3$. Only one study reported a vasoactive effect of $RuCl_3$ namely a limited contractile effect on rat aortic rings [17]. This divergence could be due to species differences as already shown for CO and CORM-2 [12], but this is rather unlikely. Another possible explanation could be the difference in agents used to contract the aortic segments: NOR in our mouse vessels whereas phenylephrine in their rat experiments. This led us to test the effect of $RuCl_3$ also after contraction with phenylephrine. Despite the fact that both vasoconstrictors are α_1-adrenergic agonists, the large relaxant effect of $RuCl_3$ after contraction with NOR was completely abolished when the tissues were contracted with phenylephrine. This surprising observation led us to investigate the response of $RuCl_3$ after contraction with other vasoconstrictors. The relaxant effect of $RuCl_3$ was not seen when contraction was induced by K^{+}_{30}, serotonin, $PGF_{2\alpha}$ or the thromboxane A2 mimetic U46619. Thus the strong vasorelaxant effect of $RuCl_3$ only occurs after contraction with NOR.

This specificity of the $RuCl_3$-effect after NOR-contraction cannot be explained by an (in)direct interaction with α_1-adrenergic mechanisms since $RuCl_3$-responses are absent after contraction with phenylephrine. Besides the specificity for NOR, another remarkable observation was that the $RuCl_3$ relaxation showed a large variability. The potency of $RuCl_3$

varied strongly from experiment to experiment and did not seem to be concentration-dependent. It looked more like a mechanism that has been set in motion steadily and then leads to a complete relaxation. This cannot be explained by cell death as then it should occur after contraction with all contractile agents. Moreover, addition of another bolus dose of NOR after the $RuCl_3$-induced relaxations was again able to elicit a strong contraction of the vessels (data not shown). Furthermore, preliminary TUNEL-staining experiments, tracing DNA fragmentation due to cell death, could not prove any sign of cell death under our experimental conditions (data not shown). Another hypothesis could be that $RuCl_3$ gradually destroys NOR. In contrast to the more stable phenylephrine, NOR is quite sensitive to oxidative degradation by traces of heavy metals resulting in a loss of contraction [19]. To scavenge these metals, EDTA is routinely added to the KRB solution leading to stable contractions with NOR. However it is possible that $RuCl_3$ somehow counteracts the chelation of heavy metals by EDTA or on itself stimulates oxidation of NOR. A higher concentration of EDTA (1 mmol/L) was added to the organ baths to test our hypothesis but unfortunately had no significant influence (data not shown). It has been shown that the antioxidant ascorbic acid is able to prevent the loss of vasoconstriction due to the oxidation ofNOR [19]. Therefore we analyzed the effect of ascorbic acid on $RuCl_3$-induced responses. Ascorbic acid strongly diminished the relaxant effect of $RuCl_3$ on NOR-contracted aortic rings. Moreover, ascorbic acid prevented the loss of contractile tone after incubation with $RuCl_3$. Also the antioxidant N-acetyl L-cysteine was able to counteract the $RuCl_3$ effect on NOR-contracted aortic rings. So, our data suggest that $RuCl_3$ undermines NOR activity by oxidative degradation. Since NOR is one of the most important regulators of vascular tone in vivo, the counteracting effect of ruthenium/$RuCl_3$ should be considered in future research of ruthenium-containing compounds. Furthermore, our data showed that this oxidative degradation of NOR by $RuCl_3$ is independent of the presence of vascular tissue. After all, when $RuCl_3$ is left to react with NOR for 1 hour before adding it to the aortic ring segments, no more contraction could be obtained.

A ruthenium-core is not only present in CO- and NO-donors but also in compounds with NO scavenging capacities which are useful in various disease states that are characterized by NO-overproduction such as septic shock [13-15]. However, other diseases such as hypertension and erectile dysfunction are associated with impaired NO-production and

CO-(and NO-)releasing ruthenium-based molecules have been proposed as drugs to compensate for this lost NO-bioavailability. However, for these drugs, an additional NO scavenging effect of the ruthenium core can be regarded as an unwanted side effect limiting their therapeutic value. Marazioti et al. showed that CORM-2 diminishes NO donor-induced relaxations in rat aorta [16]. Furthermore, experiments on murine gastric fundus demonstrated that responses to exogenous NO were inhibited by CORM-2, whereas endogenous NO relaxations by electrical field stimulation were not affected. On the other hand $RuCl_3$ only inhibited exogenous NO responses [26]. Also, CORM-3 attenuates ACh- but not SNP-induced relaxations in rat aorta while $RuCl_3$ reduced both ACh- and SNP-effects [17]. We examined the effect of pre-incubation with $RuCl_3$, $RuCl_2(DMSO)_4$ and $[RuCl_2(CO)_3]_2$ (= CORM-2) on endogenous (ACh) as well as exogenous (SNP) NO-mediated relaxations. We used $PGF_{2\alpha}$ as contractile agent since the contraction level of $PGF_{2\alpha}$ is not altered by pre-incubation with the ruthenium-containing compounds. We found that incubation of CORM-2 had an inhibitory effect on exogenous NO-mediated relaxations, which is in accordance with former studies [16,26]. Moreover CORM-2 diminished also ACh-induced relaxations. A possible explanation for the latter effect could be an interaction between CO released from CORM-2 and the NO-signaling pathways. CO is able to stimulate as well as to inhibit NO synthase activity depending for instance on concentrations. For its inhibitory effect a CO concentration of 1 mmol/L seems to be needed [27-29]. Therefore NO synthase inhibition is unlikely to be responsible for the CORM-2 effect in our study. Moreover, if CO, released form CORM-2, causes NO synthase inhibition it could justify the decrease in ACh-induced relaxation, but inhibition of SNP responses would not be expected. Another hypothesis could be quenching of NO by the transition metal ruthenium-core. Indeed also $RuCl_3$ altered the SNP-induced relaxation which is related to previous results [17,26]. In contrast, $RuCl_3$ had no effect on ACh responses in mice aorta, whereas in rat aorta an inhibitory effect was proven [17]. Furthermore, the CO-free ruthenium-complex $RuCl_2(DMSO)_4$ did not modify ACh nor SNP responses which correlates with the lack of effect by incubation of CO-free inactive CORM-3 [17]. Although the results are not clear-cut, the possible unwanted effect of ruthenium-containing compounds on NO-mediated responses should not be underestimated.

VI.7 Conclusion

In conclusion, this study shows for the first time that $RuCl_3$ specifically relaxes aortic rings due to oxidative inactivation of NOR. Therefore the influence of ruthenium on vascular tone induced by NOR, being the most important physiological regulator of the cardiovascular system as neurotransmitter of the orthosympathetic nervous system, should be considered. Moreover, ruthenium-based molecules may alter NO-mediated relaxations. In future studies on ruthenium-containing compounds, their influence on vascular tone and their possible NO scavenging (side) effects should be kept in mind as this could limit their net therapeutic potential.

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Chapter VII

Discussion and future perspectives

Nowadays, the (patho)physiological relevance of NO as a key signaling molecule has been well established. In the cardiovascular system, NO is the best known and most documented vasodilator. NO is continuously generated in vascular endothelial cells by basally expressed eNOS. These low levels of NO participate in the maintenance of basal vascular tone and regulation of blood pressure [1]. In the mechanism of penile erection, NO also acts as the principal mediator causing vasodilation of cavernous and helicine arteries and relaxation of trabecular smooth muscles [2]. Initiation of cavernosal relaxation is caused by NO liberated from parasympathetic nerve endings, whereas activation of eNOS may facilitate attainment and sustainment of penile erection [3]. In several disease states such as hypertension and erectile dysfunction (ED), (vascular) NO-mediated relaxations are impaired due to loss of (endothelial) NO production and/or unresponsiveness to NO [4]. Current cardiovascular therapy includes the use of organic nitrates, which release NO through metabolization, to compensate for this lost NO-bioavailability [5]. In patients suffering from ED, PDE type 5 inhibitors, which block the degradation of (NO-induced) cGMP, are still the first choice drugs [6]. However, the therapeutic value of both NO-related treatment strategies is limited by the development of tolerance, low bioavailability, unwanted side effects and the occurrence of a substantial number of non-responders [7-10]. As a result, the search for new better alternatives is still ongoing.

VII.1 Oxime derivatives

About a decade ago, a few studies proved the potential use of oxime derivatives as a new group of vasodilatory and blood pressure lowering compounds due to their NO-releasing capacities [11,12]. The presence of the oxime (C=NOH) functional group had aroused interest of researchers to explore the vasorelaxant effect of these compounds. After all, L-NOHA, which also bears a C=NOH group, had shown to induce endothelium-dependent relaxation of porcine coronary arteries by acting as a substrate for eNOS leading to enhanced NO concentrations [13]. Hence, various (non-)aromatic oximes were tested on their ability to relax vascular tissue and to elicit a decrease of blood pressure. Amongst them, formaldoxime (FAL) seemed to be the most potent compound in vitro whereas formamidoxime showed the most promising decline of blood pressure in vivo [11,12]. However, none of these compounds was studied in the context of penile erection and ED.

As a first goal of this thesis we tried to establish the ability of both FAL and FAM to relax mice corpora cavernosa (CC) as well as to elucidate the underlying mechanism(s) of their effect. To correlate our results with previous findings in literature and to explore the possible differences between vascular and corporal tissue, parallel experiments on mice aorta and femoral artery were performed. As expected from a former study on rat aorta, both FAL and FAM were able to concentration-dependently relax mice aorta and femoral artery [11]. However, it was for the first time that we could prove that both compounds also relax mice corporal tissue and thus could be of interest in the treatment of ED.

Since sGC is the main target of NO, it is obvious that estimating the contribution of the sGC-cGMP pathway is the first step in determining the underlying molecular pathway(s) of any (new) NO donor and thus also of the oxime derivatives. For over twenty years now, ODQ has been widely used as potent inhibitor of sGC [14]. Irreversible oxidation of the prosthetic heme group of the sGC enzyme by ODQ results in a poor NO-sensitive ferric state [15]. Incubation of our mice aorta and femoral artery ring segments with ODQ leads to a substantial inhibition of the FAL-induced vasorelaxation and even completely abolished the FAM effect. On the contrary, ODQ was not able to alter corporal relaxation by FAL and had only a limited effect on the FAM-induced responses in the mice CC. However, the pharmacological application of ODQ as sGC inhibitor has a few drawbacks. It has been reported that ODQ also shows redox activity against other heme-containing proteins including haemoglobin and cytochrome P450 [16,17]. Moreover, high concentrations of NO seem to overcome the inhibitory effect of ODQ which implies that NO donor-induced effects in the presence of ODQ do not necessarily prove sGC independence [18]. Therefore, we defined the role of sGC in the FAL-/FAM-induced relaxations more accurately by using genetically modified mice. Furthermore, this genetic approach enabled us to distinguish the individual role of the different isoforms of sGC in the FAL-/FAM-responses. Despite the existence of two isoforms for each subunit of sGC, only the $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$ heterodimers are catalytically active and found in vivo [19]. A deletion in the catalytic domain of the α_1 subunit selectively inactivates the $sGC\alpha_1\beta_1$ isoform [20]. In these so called $sGC\alpha_1^{-/-}$ mice, both FAL-/FAM-induced relaxations of aorta and femoral artery ring segments were significantly lower as compared to the wild-type control mice. In mice CC, the $sGC\alpha_1\beta_1$ isoform also seemed to mediate the FAM-effect but not the FAL-effect. Although $sGC\alpha_1\beta_1$ is

the predominant form in most tissues, including the vascular and penile smooth muscle cells, residual activity of the $sGC\alpha_2\beta_1$ isoform should not be underestimated [21]. Hence, genetically modified knock-in mice ($sGC\beta_1^{ki/ki}$) were developed in which mutation of the histidine 105 residue of $sGC\beta_1$ to phenylalanine results in the expression of heme-free NO-insensitive sGC which affects both heterodimeric isoforms [22]. The results of our FAL/FAM experiments in these $sGC\beta_1^{ki/ki}$ mice are presented in supplementary figure 1. The even lower response of FAL and FAM in aorta and femoral artery of the $sGC\beta_1^{ki/ki}$ mice in comparison with their effect in the $sGC\alpha_1^{-/-}$ mice indicates that besides the $sGC\alpha_1\beta_1$ isoform also the $sGC\alpha_2\beta_1$ isoform is involved. However, in the CC no difference was observed in the FAM-induced relaxations of the $sGC\alpha_1^{-/-}$ and $sGC\beta_1^{ki/ki}$ mice suggesting that only the $sGC\alpha_1\beta_1$ isoform plays a role.In general we can say that both isoforms of sGC play an important role in the FAL-/FAM-mediated arterial relaxations whereas in the CC activation of the $sGC\alpha_1\beta_1$ isoform is primarily responsible for the FAM effect. In contrast, sGC does not appear to take part in the FAL-induced corporal relaxation.

The lack of NO release in the absence of vascular tissue suggests that oxime-induced vasorelaxation unlikely results from spontaneous NO formation, but rather from metabolization within the tissue [11]. Our research has led to a proposed metabolization pathway underlying the FAL-/FAM-mediated vascular relaxation. We started with a broad, general approach by blocking non-specifically all flavoproteins with diphenyliodonium (DPI). After all, it has been reported that flavoproteins are involved in the vasorelaxant effect of the prototypic metabolic NO donor glyceryl trinitrate as well as some C=NOH bearing compounds [23-25]. Because DPI greatly blocked the FAL/FAM responses in our mice aorta and femoral artery ring segments to the same extent as ODQ, we assumed (a) flavoprotein(s) contribute(s) to their effects. Further stepwise inhibition of the different flavoproteins that could be involved in the FAL/FAM effect ruled out all irrelevant pathways. Taken together our results, we propose that (a) NADPH-CYP450 reductase-dependent mechanism(s) participates in the FAL-/FAM-induced relaxation of mice aorta and femoral artery which lead(s) to the biotransformation of the C=NOH function to NO or a NO-related compound subsequently activating the sGC-cGMP pathway. Recently, a similar pathway was suggested for the aromatic oxime derivative cinnamaldoxime [26]. However, the hypothesized pathway above does not fully explain the arterial oxime effect, especially not for FAL. It is possible

that these unexplained effects are related to (a) NO-independent mechanism(s). Unfortunately, we were also unable to elucidate the exact molecular mechanism(s) of FAL and FAM in the CC. Based on our results, we can already eliminate a possible contribution of NADPH oxidase, NADPH-dependent reductases, CYP450, AMP kinase, adenylate cyclase and K^+ channels. In the CC, only a limited involvement of sGC in the FAM effect was demonstrated.

Even though in literature SNP is the most frequently used comparator for research on exogenous NO sources, this NO donor does not relate well to our experiments. First of all, SNP does not only decompose into NO but also other substances such as cyanide, which can also influence smooth muscle tone [27]. More importantly, SNP generates NO rather spontaneously whereas oximes seem to undergo a metabolization step to release NO [28]. In a way this whole metabolization process of the oxime compounds is more comparable to the NO generation from GTN, a nitrovasodilator commonly used in cardiovascular diseases [5]. GTN has also proven to elicit relaxation of human CC strips [29,30]. Though there is no metabolization mechanism that can fully explain the vascular effect of GTN, several pathways appear to play a role including CYP450 [31], CYP450 reductase [32], glutathione S-transferase [33], xanthine oxidase [34] and aldehyde dehydrogenase [35].In accordance with our oxime results the flavoprotein inhibitor diphenyleneiodionum, an analog of DPI, blocked the enzymatic bioactivation of GTN [23,24]. Moreover, GTN-induced vasorelaxation is also sensitive to the CYP450 reductase inhibitor 7-ER, which also significantly alters the oxime responses [36,37]. However, while CYP450 seems to be important for GTN biotransformation, it does not take part in the oxime effect. A common side effect of GTN administration is the occurrence of tolerance upon prolonged use [7]. Chalupsky et al. demonstrated that pre-exposition of aortic ring segments to GTN decreased the relaxant effect of a subsequent addition of GTN confirming the existence of tolerance. Unlike GTN, FAL does not seem to be subjected to tolerance. In addition, no cross tolerance was observed between GTN and formaldoxime [11].

It is one thing to elicit vasorelaxation in normal physiological conditions, it is however another thing to be effective in pathological conditions. The C=NOH containing stable intermediate of endogenous NO production, L-NOHA, has proven to be a substrate of eNOS implicating the need for an intact endothelium to exert its effect [13]. However, many

(cardiovascular and erectile) disease states are characterized by endothelial dysfunction which often includes impairment of NOS activity [38]. Therefore it seemed interesting to explore the endothelium- and/or NOS-dependence of FAL and FAM. The NOS inhibitor L-NNA did not influence the FAL/FAM effect in the CC as well as the FAM-induced vasorelaxation. Surprisingly, the FAL response in the arteries was even more pronounced in the presence of L-NNA but an explanation for this enigmatic observation remains to be found. Although inhibition of NOS entails elimination of the most important endothelial signaling pathway, other pathways are preserved and should be taken into account [38]. Nevertheless, in correspondence with our L-NNA results, removal of the endothelium did not alter the FAM effect and even significantly enhanced FAL-induced vasorelaxation. These experiments in the presence of L-NNA as well as in endothelium-denuded tissue suggest that both FAL and FAM could be at least equally active in case of endothelial dysfunction-related pathologies as compared to normal physiological conditions. Various disease states, such as ED, are also associated with increased oxidative stress which can contribute to the endothelial dysfunction [39]. The excessive levels of reactive oxygen species, including superoxide anion, can quench NO by forming peroxynitrite which will (indirectly) lead to a lowered NO-bioavailability [40]. We used the superoxide generator hydroquinone in combination with the superoxide dismutase inhibitor DETCA to increase superoxide levels in the CC thus creating an in vitro model of oxidative stress. As expected, the combination of hydroquinone and DETCA impaired neuronal, endothelial and exogenous NO-induced corporal relaxations as it significantly blocked electrical field stimulation-, acetylcholine- and sodium nitroprusside responses respectively, which confirms our oxidative stress model. However, both FAL and FAM did not seem to be susceptible to the induced oxidative stress. Taken together, the NOS- and endothelium-independence as well as the resistance towards oxidative stress of both FAL and FAM offer some promising perspectives as alternative treatment strategies.

In vitro tension measurements of vessel ring segments and corporal strips are an ideal, fast and cost-effective technique to unravel the molecular pathways underlying the contractile or relaxant effect of a wide range of compounds. Ion concentrations of the organ bath solutions, temperature and oxygen supply are adjusted to mimic the ‘real life’ conditions as much as possible. However, in these organ bath experiments not all interfering in vivo

factors can be taken into account. A vasorelaxant or corporal relaxant effect in vitro does not necessarily imply an in vivo, clinically relevant effect such as lowering of blood pressure or induction of erection respectively. Therefore, we performed some in vivo mice experiments in which blood pressure as well as intracavernosal pressure were monitored after intravenous or intracavernosal injection of FAL/FAM. It was already published that FAL and FAM induce a blood pressure drop in rats, but only after inhibition of endogenous NO production [12]. However, our results demonstrated a dose-dependent blood pressure decrease without the need for NOS inhibition. Furthermore, this blood pressure decrease was significantly lower in the $sGC\alpha_1^{-/-}$ mice as compared to the wild-type control mice. This led us to conclude that the $sGC\alpha_1\beta_1$ isoform of the sGC enzyme is, at least in part, involved in the FAL/FAM effect. More importantly, for the first time, our in vivo study could prove a dose-dependent increase of intracavernosal pressure after intracavernosal administration of both FAL and FAM. The FAM-induced increase of intracavernosal pressure was almost completely abolished in the $sGC\alpha_1^{-/-}$ mice confirming the key role of the $sGC\alpha_1\beta_1$ isoform which is in accordance with our in vitro results in the CC. To our surprise, sGC was also involved in the FAL-induced rise of intracavernosal pressure even though sGC did not seem to participate in the in vitro corporal relaxation by FAL. This ambiguous observation demonstrates that, despite the fact that our in vitro and in vivo results are more or less comparable, in vitro experiments do not always completely correlate with in vivo experiments.

In the past, various other NO-donating compounds were already studied as potential treatment strategies for ED. Linsidomine chlorhydrate [41], GTN [42,43], SNP[44], and sodium nitrite [45] showed to induce penile erection. However, these compounds did not seem to present sufficient efficacy when compared to intracavernosal injection of papaverine or prostaglandin E_1, which were used as a control condition [46-50]. So, it would be interesting to test whether our oxime compounds turn out to be inferior or superior to papaverine and/or prostaglandin E_1. Besides poor efficacy, systemic hypotension is an undesired (side) effect that would limit the therapeutic value of NO donors in the treatment of ED. Since the oxime compounds are potent vasorelaxant agents with blood pressure lowering capacity, it is not unlikely that such a systemic hypotension could occur. However, local application of the oximes could possibly surpass the systemic blood pressure lowering

(side)effects. This local administration method was already tested for GTN in the form of an ointment or transcutaneous patch [42,43,48,51-54]. Quite recently, a new topical application method for erectogenic agents has been developed in which nanoparticles are used and applied as a gel to the glans and penile shaft [55]. This method has also been proven effective for the topical application of NO-releasing nanoparticles in a rat model of radical prostatectomy [56]. Maybe this method will make an excellent alternative or adjunct to oral medication in the future.

Why did we study NO-donating oxime derivatives ? Is it not just another group of NO donors which will possibly present tolerance and side effects as seen with current NO-delivery strategies ? First of all, the apparent resemblance between the vasorelaxant mechanisms of oximes and current NO-therapy does not necessarily imply identical pharmacokinetic and pharmacodynamic properties. Moreover, the underlying molecular pathways do not completely correlate, so it will be required to address efficacy, effectiveness, efficiency and safety for each group or even each compound individually. Secondly, it was already published that the effect of subsequent addition of FAL in aortic rings, which were previously exposed to a high concentration of FAL, was not affected, suggesting that FAL is not subjected to tolerance. Furthermore, the effect of FAL was not altered in aortic rings pre-exposed to GTN eliminating the possibility of cross tolerance [11]. Thirdly, oximes could offer some perspective for the creation of NO-hybrid molecules. Over the past few years, several NO-hybrid molecules were designed in which a NO donor moiety is coupled to a native well-known drug. The goal of such strategy is to develop a new compound which possesses NO activity in addition to the therapeutic effect of the original drug. In these molecules NO could improve the effect(s) of the native drug but could also present some complementary effects. Furthermore, the combination of NO and the original drug often results in a reduction of side effects [57]. Examples of such NO hybrids are a group of NO-releasing non-steroidal anti-inflammatory drugs (NO-NSAIDs), including NO-aspirin and NO-diclofenac. These NO-NSAIDs maintain their original anti-inflammatory effects but, as an additional property, they present a marked reduction of NSAID-mediated gastric damage through the action of NO [58]. Likewise, NO-NSAIDs have shown to be far more potent in inhibiting cancer growth as compared with their native NSAID compounds due to the combined inhibition of proliferation, induction of cell death and inhibition of cell-cycle-phase

transitions [59]. It has also been reported that incorporation of a NO-moiety into statins significantly potentiates their cardiovascular-protective properties by enhancement of their non-lipid-lowering anti-inflammatory and antiproliferative effects [60]. More in line with our research focus, a NO-hybrid of the PDE type 5 inhibitor sildenafil, namely sildenafil nitrate (NCX-911), was tested in the context of ED [61]. In the past, a large number of placebo-controlled, randomized, double blind trials had already shown that the native drug sildenafil can improve erections in men with ED, regardless of whether it originates form organic, psychogenic or mixed factors [62]. However, as stated before in the introduction, PDE type 5 inhibitors need a critical amount of NO in order to work properly and therefore seemed to be less effective in certain patient groups, such as long-term diabetics and non-nerve sparing prostatectomy patients [63,64]. Hence, a NO-hybrid of sildenafil was developed to supply the necessary minimal dose of NO. In contrast to sildenafil, it was proven that sildenafil nitrate maintains its potency in the absence of endogenous NO confirming the potential use of NO-hybrid PDE type 5 inhibitors in (pathological) NO-deficient conditions [61]. Our results with the oxime derivatives FAL and FAM show that the C=NOH functional group could also be a plausible candidate as NO donor moiety for the design of NO-hybrid molecules. After all, oximes are capable of releasing NO independent from intact endothelium. In the context of penile erection and ED, oximes act (partially) through the activation of sGC leading to the formation of the required minimal amount of cGMP for effective PDE type 5 inhibitor therapy. Moreover, they seem to be resistant to oxidative stress which adds to their therapeutic value in pathological conditions.

Although our results already suggest a significant effect of oxime derivatives in case of oxidative stress and in the absence of functional endothelium, their true therapeutic value should be further explored in the future, especially in the context of diseases. Just like various other pathologies, diabetes is associated with increased oxidative stress, especially in patients with poor glycemic control. Increased production of reactive oxygen species will affect vascular smooth muscle growth as well as endothelial function leading to a proinflammatory phenotype which contributes to the development of diabetic micro- and macrovascular complications [65]. Since diabetes is one of the biggest risk factors for the development of ED, as proven in numerous epidemiological studies, it would be interesting to study the efficacy of oximes in diabetic in vitro and in vivo models [66]. Though the

etiology of ED in diabetic patients is multifactorial, elevated oxidative stress levels seem to play an important role in its underlying pathophysiological mechanism [67]. It has been suggested that hyperglycemia-induced endothelial dysfunction, and therefore possibly also the associated vascular complications including ED, is most likely mediated by increased intracellular methylglyoxal levels [68]. Moreover, methylglyoxal, a highly reactive dicarbonyl metabolite produced during glucose metabolism, has been shown to impair endothelium-dependent vasorelaxation in a pathway dependent on oxidative stress [69]. Hence, it would be of interest to test whether our oxime derivatives are still effective in in vitro diabetic mimicking conditions using methylglyoxal, high concentrations of glucose or even better a combination of both. Nevertheless, other diabetes-related factors have also been proven to be involved in the endothelial impairment such as Amadori glycosylation adducts and advanced glycosylation end products [70,71]. So in a next step, it would be appropriate to use diabetic mouse models for the in vitro as well as in vivo evaluation of the oximes. Mice with streptozotocin-induced hyperglycemia have shown to be a good model for type I diabetes-induced ED by presenting decreased endothelium-dependent dilatation and lowered in vivo erectile reactivity [72]. However, over the last decades, the impact of type II diabetes, and related obesity, on public health has been gradually increasing in our ageing population. To study type II diabetes-induced ED, leptin receptor mutated obese db/db mice can be used which exhibit lowered overall vasorelaxation and altered cavernosal vasoreactivity [73]. If these oxime experiments in diabetic conditions would present some promising results, it would strongly strengthen the therapeutic value of oximes. Moreover, these future experiments could offer some more insight in the potential use of the oxime functional group as NO donor moiety in NO-hybrid compounds. After all, some NO-glibenclamide derivatives were already developed for the treatment of diabetes. In these molecules, the hypoglycemic activity, induced by the insulin secretagogue property of glibenclamide, is combined with the additional effects of NO including antiplatelet and antithrombic activities, vasorelaxation and cardioprotection [74]. Keeping this in mind, (a) beneficial effect(s) of oximes in our diabetic models could potentially imply a possible role in the future design of such antidiabetic NO-hybrids.

VII.2 Ruthenium-based metallocomplexes

As mentioned in the introduction, ruthenium has drawn the attention of drug designers only recently. Its ability to exchange ligands, the range of accessible oxidation states and its relatively low toxicity make ruthenium a good candidate for the development of NO- and CO-donating metallocomplexes [75]. These compounds have been designed as an alternative treatment strategy for current NO-delivery therapies in pathological conditions characterized by impaired NO-bioavailability. One of these new ruthenium-based NO donors is the nitrosyl polypyridine complex $[Ru(terpy)(bdq)NO]^{3+}$, which is abbreviated as TERPY. It has been published that the relaxation of endothelium-denuded vascular ring segments by TERPY relies on the release of both free radical NO and nitroxyl anions. Experiments on rat aorta and mesenteric resistance arteries have demonstrated that the TERPY-induced vasorelaxation mainly involves activation of sGC and K^+ channels but also the sarcoplasmic reticulum Ca^{2+}-ATPase can contribute to some extent [76,77]. Moreover, TERPY has shown to elicit an in vivo blood pressure lowering effect in normotensive as well as hypertensive rats [78,79]. Another NO-donating polypyridine ruthenium complex is the cis-$[Ru(bpy)_2(py)NO_2](PF_6)$ compound (= RuBPY) which is composed of ruthenium, bipyridine ligands and a nitrite group. RuBPY-induced vasorelaxation seems to require metabolization and depends on the presence of tissue [80]. It has been hypothesized that conversion of the nitrite group into radicalar NO activates sGC and induces hyperpolarization of the cell membrane subsequently leading to vasodilation [81]. As yet, neither TERPY nor RuBPY is tested on their ability to elicit relaxation of CC. Hence, we received a small amount of both compounds from the laboratory of prof. Roberto Santana da Silva (Professor of Chemistry, Department of Physics and Chemistry, São Paulo University, Brazil) to perform some preliminary experiments on mice CC. Unfortunately, both TERPY and RuBPY were unable to significantly relax phenylephrine-contracted mice CC as shown in supplementary figures 2 and 3. In contrast, TERPY and RuBPY did exhibit a pronounced vasorelaxant effect on mice aorta as well as femoral artery which is in line with the results on rat artery ring segments reported in literature [76,77].

Previous studies in our research department have tried to elucidate the underlying molecular pathways of the vasorelaxant and corporal relaxant effects induced by the CO-donating ruthenium compound CORM-2. Although CORM-2 was able to relax rat aorta,

mice aorta, mice femoral artery as well as mice CC, the mechanism(s) of effect seemed to differ. In rat aorta CORM-2 partially acted through activation of sGC and K^+ channels. This was in correspondence with our results on mice aorta which also proved the participation of sGC and K^+ channels, more specifically the voltage-dependent K^+ channels. However, in mice femoral artery sGC did not seem to play a role and only the involvement of voltage-dependent K^+ channels could be demonstrated [82]. In addition, the underlying mechanism of CORM-2-induced corporal relaxation remained even unclear [83]. Nevertheless, these CORM-2 experiments have revealed some interesting findings. Despite the anatomical and physiological differences between the studied tissues, it was unexpected to observe such tissue-specific variations in the molecular pathways of CORM-2. An even more remarkable observation was the discrepancy between the vasorelaxant effect of CORM-2 and the vasorelaxant effect of CO as such [82]. First of all, CORM-2 seemed to be more potent as compared to the administration of a saturated CO gas solution. Secondly, mice aorta and femoral artery ring segments do not relax in response to this CO gas solution whereas they do so in response CORM-2. On the contrary, in rat aorta both CO and CORM-2 are able to elicit vasorelaxation showing that besides tissue differences also species differences seem to take place [82,83]. Taken together, these enigmatic observations made us wonder if the ruthenium-core of CORM-2 could be held responsible as it is the most apparent difference between CORM-2 and CO as such.

So far, the influence of ruthenium on vascular tone had never been studied. Therefore we tested the vasorelaxant capacity of some ruthenium compounds in vitro. $RuCl_3$, Ruthenium Red, CORM-2 and $RuCl_2(DMSO)_4$ all induced relaxation of mice aorta ring segments to some extent, though the potency of each compound varied greatly. Both $RuCl_3$ and Ruthenium Red showed a pronounced vasorelaxing effect whereas the effect of $RuCl_2(DMSO)_4$ was quite limited. Because of the surprising marked vasorelaxation by $RuCl_3$, we focused more on its effects in the following experiments. After all, only one other study had described a vasoactive effect of $RuCl_3$, but in contrast to our results they reported that $RuCl_3$ induced a limited contractile effect instead of a vasorelaxtion [84]. In the search for an explanation of these opposing observations, we noticed a difference in the experimental setup. Alshehri et al. used phenylephrine to contract the aortic ring segments whereas we used norepinephrine (NOR) in our in vitro model [84]. Although both vasoconstrictors are

α_1-adrenergic agonist, this difference in contractile agents could possibly clarify the conflicting results. The large relaxant effect of $RuCl_3$, which occurs when the tissues are contracted with NOR, appeared to be absent when contracting the mice aortic rings with phenylephrine. Moreover, the $RuCl_3$-induced relaxation was also abolished when contraction was evoked by serotonin, $PGF_{2\alpha}$, the thromboxane A2 mimetic U46619 or a high K^+ concentration. This specificity of the $RuCl_3$ effect towards NOR could not be explained by an alteration of the α_1-adrenergic pathways nor by cell death mechanisms. However, NOR is known for its sensitivity to oxidative degradation by heavy metals. Gradual decomposition of NOR will affect its contractile capacity and thus will lead to vasorelaxation. It has also been demonstrated that antioxidants can prevent this oxidative degradation and subsequent loss of contraction [85]. Our data have shown that ascorbic acid as well as N-acetyl L-cysteine, both powerful antioxidants, are able to counteract the $RuCl_3$ effect on NOR-contracted aorta ring segments confirming our hypothesis that $RuCl_3$ undermines the contractile activity of NOR by oxidative degradation. Any change in NOR activity will alter the tightly regulated balance of vascular tone as it is the most important neurotransmitter of the orthosympathetic nervous system. Consequently, the detrimental influence of ruthenium on NOR-induced tone should be taken into account when studying ruthenium-based compounds. Nevertheless, future research will have to address to which extent these ruthenium effects take part in the effects of the various ruthenium complexes that are still designed, produced and tested nowadays. Hence, stability experiments will be necessary to determine ìf ruthenium is actually liberated during solubilization, degradation, ligand exchange, etc. and to what degree.

As described before, various disease states are characterized by an impairment of NO-bioavailability. On the other hand, many other pathological conditions involve an overproduction of NO. High levels of NO can trigger both oxidative and nitrosative stress by formation of peroxynitrite and dinitrogen trioxide. Subsequently, these stress conditions will result in DNA damage and nitration of proteins which are responsible for the deleterious effects of excessive NO [86,87]. A number of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and Huntington's disease are believed to be caused in part by an increase in nitrosative stress in neurons [88]. Furthermore, an overproduction of NO has been associated with acute and chronic

inflammatory states such as septic shock [89,90]. While NOS inhibitors can be used to lower the synthesis of NO, an alternative therapeutic approach would be to remove or scavenge the excess NO [91]. An advantage of this strategy is that the rate of NO scavenging depends on both the concentration of NO and of the scavenger, meaning that for a given scavenger concentration scavenging occurs at a faster rate when the NO concentration is elevated. In contrast, NOS inhibitors alter NO production regardless of whether the NO production is high or low [92]. Ruthenium is able to form a stable interaction with NO creating nitrosyl complexes. Moreover, the broad coordination chemistry of ruthenium makes it perfectly suitable to modulate its properties to develop an effective NO scavenger. Some ruthenium polyaminocarboxylate complexes have already shown to be efficient and effective scavengers of NO [92-95]. Although NO removal is the goal in case of NO overproduction, this scavenging of NO would be an undesired side effect of therapeutics used in case of impaired NO-bioavailability, including ruthenium-based (NO- and) CO-donors. After all, these (NO- and) CO-donating molecules are applied to compensate the lost NO-bioavailability which means that scavenging of the remaining endogenous NO would seriously limit the therapeutic value of these drugs. It was already reported that CORM-2 lowers NO donor-induced rat aorta relaxations which was partially explained through quenching of NO by the transition metal complex [96]. CORM-2 as well as $RuCl_3$ have also shown to inhibit exogenous NO relaxations in murine gastric fundus, whereas endogenous NO responses by electrical field stimulation remained unaffected [97]. On the contrary, in rat aorta $RuCl_3$ seemed to attenuate both acetylcholine (ACh) and sodium nitroprusside(SNP) relaxations, while CORM-3 only reduced the ACh responses [84]. To get some more insight on the influence of ruthenium on NO-mediated relaxations, we examined the effect of $RuCl_3$, $RuCl_2(DMSO)_4$ and $[RuCl_2(CO)_3]_2$ (= CORM-2) on endogenous (ACh) as well as exogenous (SNP) NO-induced responses. Incubation with CORM-2 diminished both ACh- and SNP-induced relaxations. In contrast, $RuCl_3$ only altered the SNP effect, whereas $RuCl_2(DMSO)_4$ had even no effect on the NO responses. Neither scavenging of NO by ruthenium nor other hypotheses such as partial agonism by CO and quenching of NO by formation of reactive oxygen species could fully explain these inconclusive results regarding the effect of ruthenium compounds on NO-induced relaxation. Nevertheless, it must be noted that in future research on ruthenium-based therapeutics this potentially unwanted side effect should be taken into account.

In general one could say that ruthenium seems to be a good candidate for the design of drugs, including NO- and CO-delivering agents. A wide range of ruthenium-based NO- and CO-containing compounds have already shown to exert a substantial (vaso)relaxant effect and therefore could be viewed as a possible therapeutic strategy for the treatment of cardiovascular diseases and/or ED. Although the results are promising, the path towards ruthenium-based NO and/or CO- therapy is long and many obstacles need to be overcome. Besides their proven efficacy, the effectiveness, safety and feasibility of these compounds need to be addressed. As can be concluded from our data, in future studies on ruthenium-containing molecules, the influence on sympathetic vascular tone and the possible NO-scavenging (side) effects should always be kept in mind. Luckily, the unique physico-chemical features of ruthenium-based metallocomplexes offer more control of their pharmacokinetic and pharmacodynamic properties. Moreover, due to their great structural variety, rational design of these compounds could potentially result in drugs which aim for specific (molecular) targets. This can be of interest for instance in the development of new treatments of ED. From compounds which relax corporal smooth muscles, it can be expected that they exert undesired cardiovascular (side) effects as a result of a generalized relaxation of vascular smooth muscles. Hence, modulation of these compounds in a way that they more specifically target erectile tissue would have a positive impact on efficacy as well as safety. Anyhow, to date ruthenium-based metallocomplexes still remain a hot topic. The search for new alternatives is still ongoing as each day more and more compounds are produced and tested.

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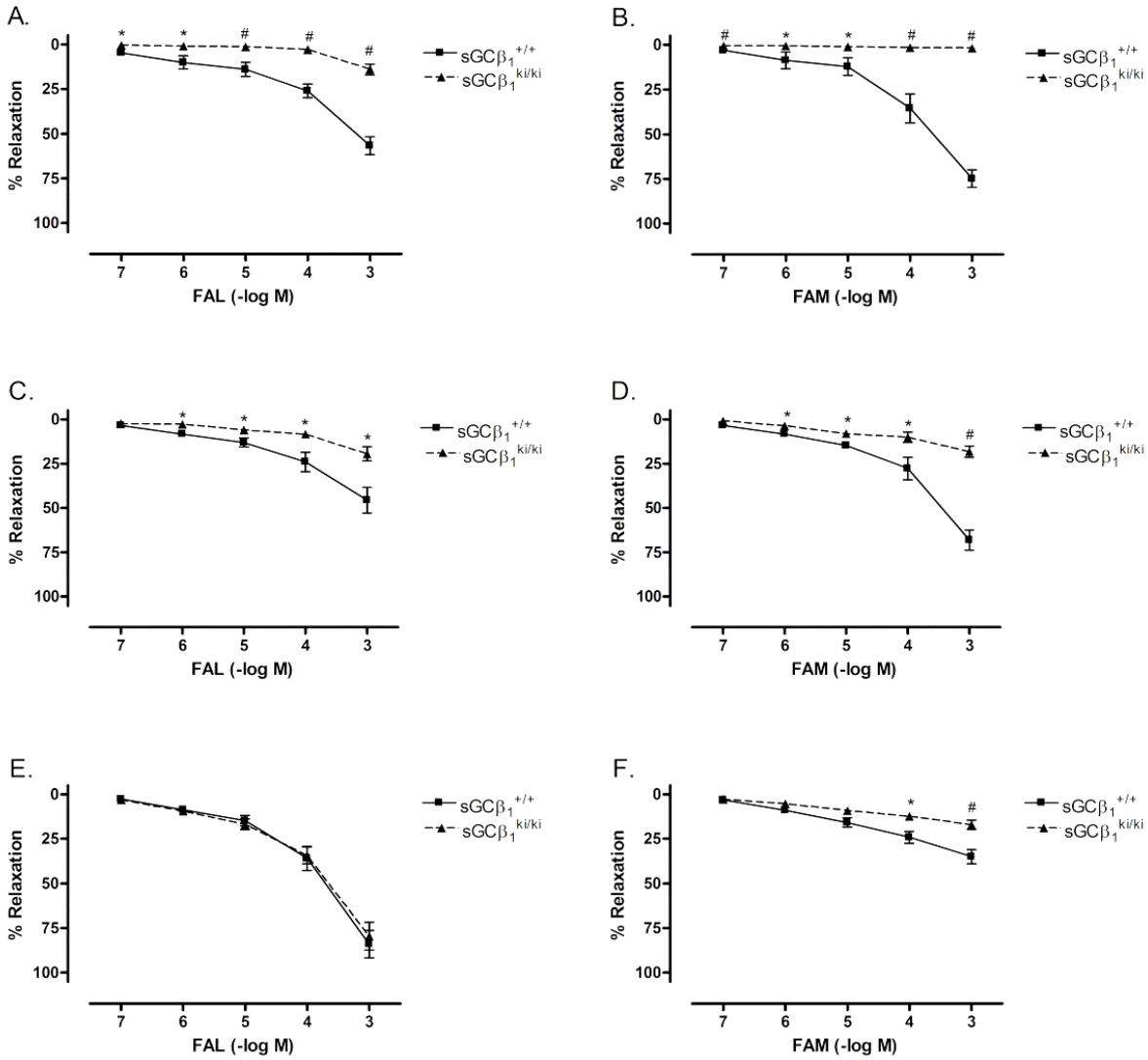
Chapter VIII

Supplementary data

VIII.1 Supplementary data of oximes in sGCβ$_1$ knock-in mice



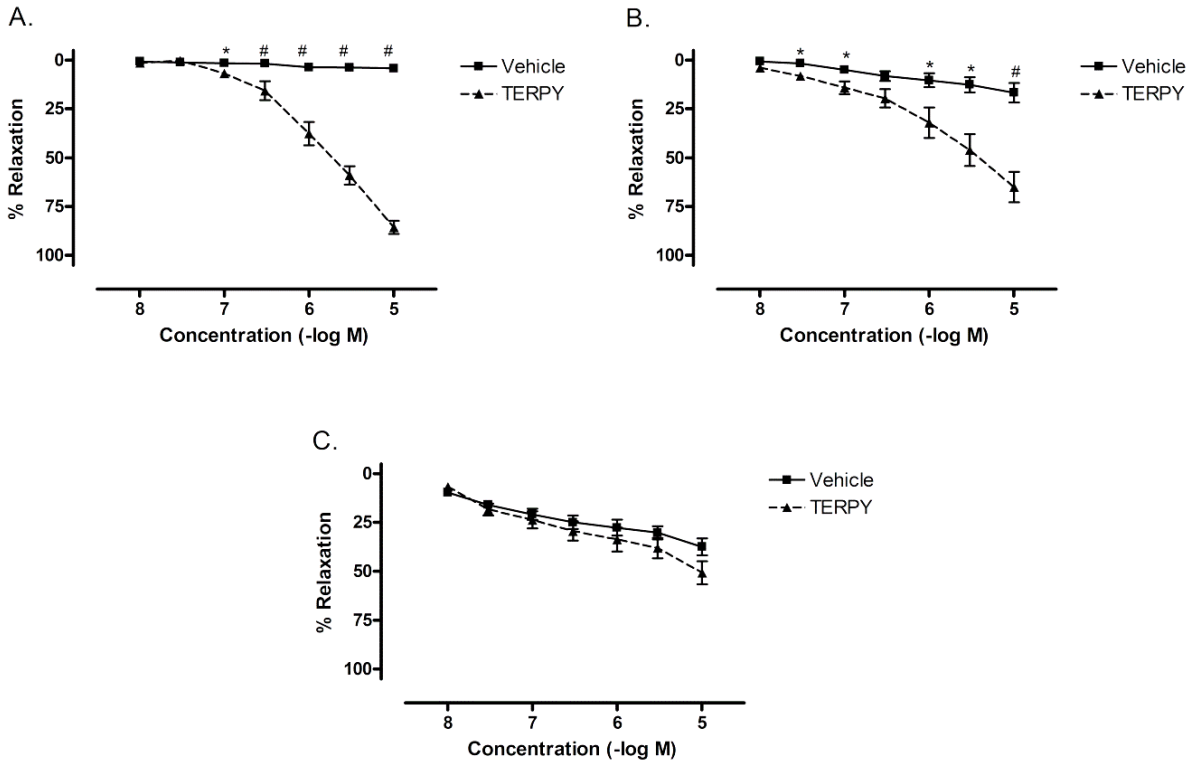
Supplementary figure 1: The effect of formaldoxime (FAL) and formamidoxime (FAM) in thoracic aorta (A and B); in femoral artery (C and D) and in corpora cavernosa (E and F) from wild-type control (sGCβ$_1^{+/+}$) (■) and genetically modified knock-in (sGCβ$_1^{ki/ki}$) (▲) mice. Data are expressed as % relaxation of the norepinephrine (NOR)-induced tone (N = 5-6); *P<0.05 and #P<0.01

In the artery ring segments of sGCβ$_1^{ki/ki}$ mice, the FAL response is significantly reduced, whereas the FAM effect is even completely abolished (Suppl. fig. 1 A-D). The FAM effect is also diminished in sGCβ$_1^{ki/ki}$ corpora cavernosa but no difference was observed in the FAL relaxations of sGCβ$_1^{ki/ki}$ and sGCβ$_1^{+/+}$ corpora cavernosa (Suppl. fig. 1 E-F). When comparing the result of the sGCβ$_1^{ki/ki}$ mice with our results in the genetically modified sGCα$_1^{-/-}$ mice, the FAL response is significantly lower in the sGCβ$_1^{ki/ki}$ mice both in aorta (maximal effect

$sGC\alpha_1^{-/-}$: 52.16 % ± 1.87 vs $sGC\beta_1^{ki/ki}$: 14.00 % ± 2.95; p = 0.011) and femoral artery (maximal effect $sGC\alpha_1^{-/-}$: 44.14 % ± 8.60 vs $sGC\beta_1^{ki/ki}$: 19.49 % ± 3.98; p = 0.033). Furthermore, also a significantly lower FAM effect is seen in aorta (maximal effect $sGC\alpha_1^{-/-}$: 21.87 % ± 2.20 vs $sGC\beta_1^{ki/ki}$: 1.79 % ± 0.63; p = 0.004) as well as femoral artery (maximal effect $sGC\alpha_1^{-/-}$: 41.25 % ± 2.74 vs $sGC\beta_1^{ki/ki}$: 18.29 % ± 3.13; p = 0.004) of the $sGC\beta_1^{ki/ki}$ mice. In contrast, the FAL- and FAM-induced relaxations in the corpora cavernosa do not differ between $sGC\alpha_1^{-/-}$ and $sGC\beta_1^{ki/ki}$ mice.

VIII.2 Supplementary data of ruthenium-based NO-donating molecules

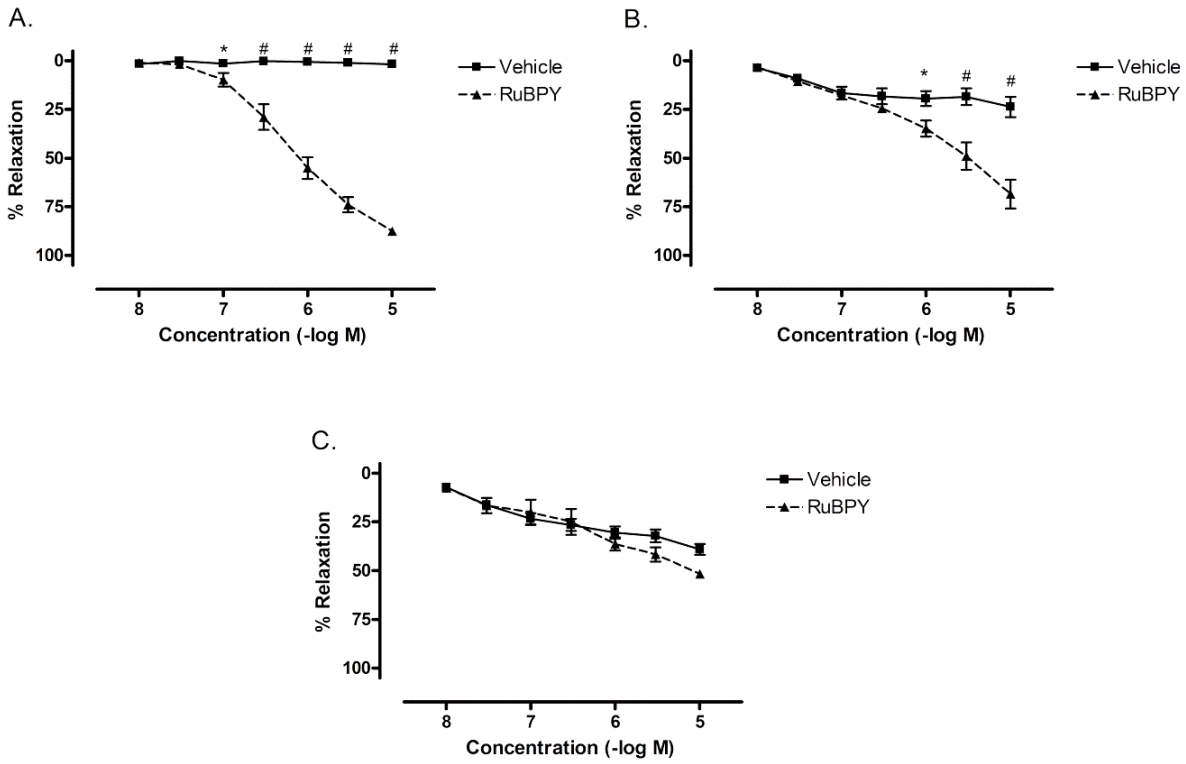
VIII.2.1 $[Ru(terpy)(bdq)NO]^{3+}$



Supplementary figure 2: The effect of $[Ru(terpy)(bdq)NO]^{3+}$ (=TERPY) (▲) versus vehicle control (■) in thoracic aorta (A); in femoral artery (B) and in corpora cavernosa (C) from Swiss mice. Data are expressed as % relaxation of the phenylephrine-induced tone (N = 4-7); *P<0.05 and #P<0.01

TERPY strongly induces vasorelaxation of mice aorta ring segments with a maximal effect of 85.71 % ± 3,36 at 10 µmol/L. Although less potent, TERPY also significantly relaxes mice femoral artery ring segments with a maximal effect of 65.09 % ± 7,82. In contrast, the effect of TERPY in the mice corpora cavernosa does not significantly differ from the vehicle control condition.

VIII.2.2 5 Cis-[$Ru(bpy)_2(py)NO_2$](PF_6)



Supplementary figure 3: The effect of cis-[$Ru(bpy)_2(py)NO_2$](PF_6) (= RuBPY) (▲) versus vehicle control (■) in thoracic aorta (A); in femoral artery (B) and in corpora cavernosa (C) from Swiss mice. Data are expressed as % relaxation of the phenylephrine-induced tone (N = 4-7); *P<0.05 and #P<0.01

As with TERPY, RuBPY induces a significant vasorelaxant effect in mice aorta ring segments with a maximal effect of 87.58 % ± 1,50 at 10 µmol/L. Furthermore, RuBPY elicits a pronounced vasorelaxant of mice femoral artery ring segments with a maximal effect of 68.42 % ± 7,34. Surprisingly, in the mice corpora cavernosa also the effect of RuPBY does not significantly differ from the vehicle control condition.

Chapter IX

Summary

Ever since nitric oxide (NO) was discovered as being the vascular endothelial-derived relaxing factor in the 1980s, an enormous amount of research has been done to enlarge our knowledge on this vital signaling molecule. Given the importance of NO in the normal human physiology, it is not surprising that several pathological conditions are characterized by an impairment of NO-bioavailability. A better understanding of the molecular pathways underlying the various physiological effects of NO has led to the unraveling of new therapeutic targets and the subsequent development of diverse treatment strategies. For instance, nitrates are being used as NO donating compounds for many years now to compensate the loss of NO functioning in some cardiovascular disorders. Likewise, phosphodiesterase (PDE) type 5 inhibitors are used as a treatment for erectile dysfunction (ED) to slow down the degradation of cGMP further downstream in the NO signal transduction cascade. This enables sufficient penile erection in case of lowered NO availability. Although in theory these therapies seem perfect, in reality their therapeutic value is limited by a few drawbacks. After long term usage of nitrates, a well-known attenuation of their effect occurs due to the development of tolerance. Moreover, even though efficacy and safety of PDE type 5 inhibitors have been proven in large clinical trials, up to 35 % of the patients fail to respond adequately. In this light, the urge for new and better alternatives still remains.

Because of their NO-releasing capacities, oxime derivatives have been presented as a new group of vasodilatory compounds. As a first aim of this thesis we wanted to find out if these compounds were also able to elicit relaxation of corpora cavernosa (CC) besides their known vasorelaxing effect on arterial ring segments (**Chapter IV**). Formaldoxime (FAL) and formamidoxime (FAM), both non-aromatic oximes, concentration-dependently relaxed mice corporal tissue. In the search for the underlying mechanism(s), it became evident that the molecular pathways activated by FAL and FAM in the arteries clearly differ from those in the CC. For the FAL-/FAM-induced relaxation of mice aorta and femoral artery, we suggested the involvement of (a) NO synthase (NOS)- and endothelium-independent, but NADPH-CYP450 reductase-dependent mechanism(s) with subsequent release of NO or a NO-related compound and activation of soluble guanylyl cyclase (sGC). However, in the CC only independence of NOS and the endothelium as well as a limited contribution of sGC was proven.

To further explore the role of sGC as well as the relative importance of the two active sGC isoforms in the FAL-/FAM-induced relaxations, we used genetically modified mice (**Chapter V and supplementary data**). A deletion in the catalytic domain of the $sGC\alpha_1$ subunit in the $sGC\alpha_1^{-/-}$ knock-out mice selectively inactivates the $sGC\alpha_1\beta_1$ isoform. In these mice, the effect of FAL and FAM was significantly lower as compared to their wild-type controls. The FAL and FAM response was even further diminished in the $sGC\beta_1^{ki/ki}$ knock-in mice, in which a mutation of the histidine 105 residue alters both sGC isoforms. Thus, in addition to the $sGC\alpha_1\beta_1$ isoform also the $sGC\alpha_2\beta_1$ isoform seems to be involved in FAL-/FAM-induced relaxations.

Besides their effect in normal physiological conditions, we also tested the effect of FAL and FAM in case of oxidative stress as it is often associated with various disease states including ED (**Chapter V**). In contrast to neuronal, endogenous and other exogenous NO-induced corporal relaxations, both FAL and FAM did not seem to be susceptible to oxidative stress. Taken together with the NOS- and endothelium-independence of their effect, FAL and FAM offer some promising perspectives as alternative ED treatment strategy.

Although in vitro experiments try to mimic 'real life' conditions, in vitro efficacy does not necessarily imply effects in vivo. Therefore, we performed an in vivo study in which blood pressure and intracavernosal pressure were monitored after intravenous or intracavernosal injection of FAL/FAM (**Chapter V**). Our results showed that FAL and FAM dose-dependently decrease blood pressure. Moreover, both compounds elicit a distinct increase of intracavernosal pressure after intracavernosal injection. Furthermore, experiments with the $sGC\alpha_1^{-/-}$ knock-out mice indicated that the effects of FAL/FAM on blood pressure and intracavernosal pressure are (partly) mediated by the $sGC\alpha_1\beta_1$ isoform.

Multiple studies have already suggested that ruthenium based NO- and carbon monoxide (CO)-releasing molecules (CORMs) could be suitable alternatives for current NO-related therapy. Because of its favorable features, ruthenium seemed to be a perfect candidate for the design of such NO- and CO-delivery agents. To date, the effect of ruthenium as such on vascular tone was not tested yet. Therefore we examined the influence of various ruthenium-containing compounds on contraction and NO-induced relaxation of aortic ring segments (**Chapter VI**). Of these ruthenium based molecules, $RuCl_3$ presented the most

marked vasorelaxing effect. However, this effect was limited to tissues contracted with norepinephrine (NOR) as $RuCl_3$ did not alter contraction evoked by serotonin, $PGF_{2\alpha}$, U46619 or a high K^+ concentration. The specificity towards NOR was explained by the sensitivity of NOR for $RuCl_3$-induced oxidative degradation which undermines its contractile activity. Furthermore we demonstrated that some ruthenium based compounds are able to diminish NO-mediated relaxations which can possibly be viewed as an undesired side effect of NO- and CO-releasing therapeutics.

Conclusions. Although the exact mechanism(s) of the FAL-/FAM-induced effects in penile tissue remain(s) elusive, both our in vitro and in vivo experiments showed that FAL and FAM could be useful as alternative treatment for ED. However, future research will be necessary to fully explore their therapeutic potential. Furthermore we can conclude that ruthenium seems ideal for the design of drugs, including NO- and CO-releasing molecules. Nevertheless, it should always be kept in mind that ruthenium as such can exert an influence on sympathetic vascular tone and can present NO-scavenging (side) effects.

Chapter X

Samenvatting

Sinds stikstofmonoxide (NO) ontdekt werd in de jaren ‘80 als de vasculaire relaxerende factor die door het endotheel wordt vrijgesteld, is reeds zeer veel onderzoek verricht om onze kennis omtrent deze essentiële signaalmolecule te vergroten. Gezien het belang van NO in de normale fysiologie van de mens, is het niet verwonderlijk dat verschillende pathologieën gekenmerkt worden door een verstoring in de biologische beschikbaarheid van NO. Een beter inzicht in de moleculaire mechanismen die aan de basis liggen van de talrijke fysiologische NO effecten heeft geleid tot het ontrafelen van nieuwe therapeutische doelen en de daaropvolgende ontwikkeling van diverse behandelingsstrategieën. Nitraten worden bijvoorbeeld al vele jaren gebruikt als NO-donerende componenten om het verlies aan NO functie in bepaalde cardiovasculaire aandoeningen te compenseren. Evenzo worden fosfodiësterase (PDE) type 5 inhibitoren gebruikt voor de behandeling van erectiele disfunctie (ED). Deze PDE type 5 inhibitoren remmen de afbraak van cGMP verderop in de NO signaal transductiecascade en zorgen op die manier voor voldoende erectie van de penis in geval van een verlaagde NO beschikbaarheid. Hoewel in theorie deze therapieën perfect lijken, is er in realiteit ook een keerzijde aan de medaille die hun therapeutische waarde limiteert. Na langdurig gebruik van nitraten treedt er immers vermindering op van hun effect door het ontwikkelen van tolerantie. Bovendien reageert tot 35 % van de patiënten onvoldoende op PDE type 5 inhibitoren, hoewel hun effect en veiligheid reeds aangetoond werd in grote klinische studies. In dit opzicht blijft het noodzakelijk om op zoek te gaan naar nieuwere en betere alternatieven.

Omwille van hun vermogen om NO vrij te stellen, werden oxime derivaten voorgesteld als een nieuwe groep van vasodilaterende stoffen. Als eerste doel van deze thesis onderzochten we of deze moleculen, naast hun gekende vasorelaxerende effect op arteriële ringsegmenten, ook in staat zijn om relaxatie van de corpora cavernosa (CC) teweeg te brengen (**Hoofdstuk IV**). Formaldoxime (FAL) en formamidoxime (FAM), beide niet-aromatische oximes, relaxeerden corporaal weefsel van de muis op een concentratie-afhankelijke manier. In de zoektocht naar de onderliggende mechanismen werd het duidelijk dat de moleculaire mechanismen die geactiveerd worden door FAL en FAM in de arteriën in sterke mate verschillen van deze in de CC. Voor de FAL-/FAM-geïnduceerde relaxatie van muis aorta en femorale arterie suggereerden we de betrokkenheid van een NO synthase (NOS)- en endotheel-onafhankelijk, maar NADPH-CYP450 reductase-afhankelijk mechanisme

met daaropvolgend de vrijstelling van NO of een NO-gerelateerde component en de activatie van het oplosbaar guanylaat cyclase (sGC). In de CC daarentegen werd enkel onafhankelijkheid van NOS en van het endotheel aangetoond alsook een beperkte bijdrage van het sGC.

Om verder de rol van het sGC alsook het relatieve belang van de twee actieve sGC isovormen in de FAL-/FAM-geïnduceerde relaxaties te onderzoeken, maakten we gebruik van genetisch gemodificeerde muizen (**Hoofdstuk V en supplementaire data**). Een deletie in het katalytische domein van de α_1 subeenheid van sGC in de $sGC\alpha_1^{-/-}$ knock-out muizen inactiveert selectief de $sGC\alpha_1\beta_1$ isovorm. In deze muizen was het effect van FAL en FAM significant lager in vergelijking met hun wild-type controles. De FAL en FAM respons was bovendien in nog sterkere mate verminderd in de $sGC\beta_1^{ki/ki}$ knock-in muizen bij dewelke beide isovormen beïnvloed worden door een mutatie van het histidine 105 residu. Bijgevolg lijkt niet alleen de $sGC\alpha_1\beta_1$ isovorm maar ook de $sGC\alpha_2\beta_1$ isovorm betrokken bij de FAL-/FAM-geïnduceerde relaxaties.

Naast hun effect onder normale fysiologische omstandigheden hebben we ook het effect van FAL en FAM getest in geval van oxidatieve stress aangezien dit vaak geassocieerd wordt met verschillende ziektetoestanden waaronder ED (**Hoofdstuk V**). In tegenstelling tot de neuronale, endogene en andere exogene NO-geïnduceerde corporale relaxaties, leken zowel FAL als FAM niet gevoelig voor oxidatieve stress. In combinatie met de NOS- en endotheelonafhankelijkheid van hun effect bieden FAL en FAM enkele veelbelovende perspectieven als alternatieve behandelingsstrategie voor ED.

Hoewel in vitro experimenten zoveel mogelijk de fysiologische omstandigheden trachten na te bootsen, impliceert werkzaamheid in vitro niet noodzakelijk effecten in vivo. Daarom voerden we een in vivo studie uit in dewelke bloeddruk en intracaverneuze druk werden gemeten na intraveneuze of intracaverneuze injectie van FAL/FAM (**Hoofdstuk V**). Onze resultaten wezen uit dat FAL en FAM de bloeddruk verlagen op een dosis-afhankelijke manier. Bovendien lokken beide componenten een uitgesproken toename uit van de intracaverneuze druk na intracaverneuze injectie. Verder toonden experimenten met de $sGC\alpha_1^{-/-}$ knock-out muizen aan dat de effecten van FAL/FAM op bloeddruk en intracaverneuze druk (gedeeltelijk) gemedieerd zijn door de $sGC\alpha_1\beta_1$ isovorm.

Meerdere studies hebben reeds aangegeven dat NO- en koolstofmonoxide (CO)-vrijstellende moleculen op basis van ruthenium geschikt kunnen zijn als alternatieve behandelingsstrategie voor huidige NO-gerelateerde therapie. Omwille van zijn gunstige eigenschappen bleek ruthenium een geschikte kandidaat te zijn voor het ontwikkelen van dergelijke NO- en CO-vrijstellende middelen. Tot op de dag van vandaag werd echter het effect van ruthenium zelf op de vasculaire tonus nog niet getest. Daarom bestudeerden we de invloed van verschillende componenten die ruthenium bevatten op de contractie en NO-geïnduceerde relaxatie van aorta ringsegmenten (**Hoofdstuk VI**). Van deze moleculen op basis van ruthenium vertoonde $RuCl_3$ het meest uitgesproken vasorelaxerende effect. Dit effect was echter beperkt tot weefsels die gecontraheerd werden met norepinefrine (NOR), daar $RuCl_3$ de contractie uitgelokt door serotonine, $PGF_{2\alpha}$, U46619 of een hoge K^+ concentratie niet beïnvloedde. Deze specificiteit ten opzichte van NOR werd verklaard door de gevoeligheid van NOR voor $RuCl_3$-geïnduceerde oxidatieve degradatie dewelke zijn contractiecapaciteit ondermijnt. Daarnaast toonden we aan dat enkele stoffen gebaseerd op ruthenium in staat zijn om NO-gemedieerde relaxaties te verminderen wat mogelijks beschouwd kan worden als een ongewenst neveneffect van therapeutica die NO of CO vrijstellen.

Conclusies. Ondanks het feit dat de exacte mechanismen van de FAL-/FAM-geïnduceerde effecten in de penis nog onbekend blijven, toonden zowel onze in vitro als in vivo experimenten aan dat FAL en FAM mogelijks nuttig kunnen zijn als alternatieve behandeling van ED. Toekomstig onderzoek zal echter noodzakelijk zijn om hun therapeutisch potentieel volledig op te helderen. Daarnaast kunnen we besluiten dat ruthenium geschikt lijkt voor het ontwikkelen van geneesmiddelen waaronder moleculen die NO of CO vrijstellen. Desalniettemin moet men er altijd rekening mee houden dat ruthenium zelf een invloed kan uitoefenen op de sympathische vasculaire tonus alsook NO-capterende (neven)werkingen kan vertonen.

Curriculum Vitae

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Research Foundation Flanders (FWO Vlaanderen)

V. Scientific publications in peer-reviewed journals

Decaluwé K, **Pauwels B**, Verpoest S, Van de Voorde J. New therapeutic targets for the treatment of erectile dysfunction. *Journal of Sexual Medicine*. 2011;8(12):3271–90.

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Vanden Daele L, Boydens C, **Pauwels B**, Van de Voorde J. Vasorelaxing effect of resveratrol on bovine retinal arteries. *Investigative Ophthalmology & Visual Science*. 2016;57(4):1655–61.

VI. Abstracts and presentations at national/international meetings

Pauwels B, Boydens C, Decaluwé K, Van de Voorde J. NO-donating oximes induce erection through mechanisms other than those involved in arterial vasodilation. Bullet presentation at the *Science Day Ghent University*, Gent, Belgium (2014)

Pauwels B, Boydens C, Decaluwé K, Van de Voorde J. NO-donating oximes induce erection through mechanisms other than those involved in arterial vasodilation. Oral presentation at the *2nd Benelux congress on Physiology and Pharmacology* (Physphar), Maastricht, The Netherlands (2014)

Pauwels B, Boydens C, Decaluwé K, Van de Voorde J. NO-donating oximes relax corpora cavernosa through mechanisms other than those involved in arterial relaxation. *Acta Physiologica.* Poster presentation at the *Joint meeting of the Federation of European Physiological Societies (FEPS) and the Hungarian Physiological Society*, Budapest, Hungary (2014)

Pauwels B, Boydens C, Decaluwé K, Van de Voorde J. NO-donating oximes relax corpora cavernosa through mechanisms other than those involved in arterial relaxation. Poster presentation at the *European Council for Cardiovascular Research* (ECCR), 18th Annual meeting, Garda, Italy (2014)

VII. Student supervision and training

"CORM-2 and its relation to CO- and NO-induced vasorelaxation"
Graduation thesis of ***Eva Naert***
(student Master of Science in Pharmaceutical Care, 2012)

"Mechanisms involved in the Formaldoxime and Formamidoxime-induced smooth muscle relaxation"
Graduation thesis of ***Dana De Saegher***
(student Master of Science in Pharmaceutical Care, 2013)

"The effect of ruthenium-containing molecules on vascular tone"
Graduation thesis of ***Enya Kerkhove***
(student Master of Science in Pharmaceutical Care, 2014