
# The role of isoforms of soluble guanylyl cyclase in relaxation of vascular smooth muscle cells and corpora cavernosa

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## List of Abbreviations

| | |
|---|---|
| 5-HT | 5-hydroxytryptamine |
| 8-pCPT-cGMP | 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate sodium salt |
| α-MSH | alpha-melanocyte stimulating hormone |
| AAV | adeno-associated virus |
| ABH | 2(S)-amino-6-boronohexanoic acid |
| AC | adenylate cyclase |
| AC3056 | (2,6-de-t-butyl-4-((dimethyl-4-methoxyphenylsilyl)-methyloxy)phenol) |
| ACE | angiotensin converting enzyme |
| ACh | acetylcholine |
| ACTH | adenocorticotropin hormone |
| ALA | alpha lipoic acid |
| AMPA | alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| Ang | angiontensin |
| ANP | artial natriuretic peptide |
| ATP | adenosine triphosphate |
| BAY 41-2272 | 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-*b*] pyridine-3-yl]pyrimidin-4-ylamine |
| BAY 58-2667 | 4-[((4-carboxybutyl) {2-[(4-phenethyl-benzyl)oxy] phenethyl}amino)methyl] benzoic acid |
| BDNF | brain derived neurotrophic factor |
| BEC | S-(2-boronoethyl)-L-cysteine |
| $BH_4$ | tetrahydrobiopterin |
| BK | bradykinin |
| $BK_{Ca}$ | big-conductance calcium-dependent potassium channel |
| BNP | brain natriuretic peptide |
| BPH | benign prostatic hyperplasia |
| $Ca^{2+}$ | calcium |
| cAMP | adenosine 3',5'-cyclic monophosphate |
| CC | corpora cavernosa |
| cGMP | guanosine 3',5'-cyclic monophosphate |
| CGRP | calcitonin gene related peptide |
| CNP | C-type natriuretic peptide |
| CNS | central nervous system |
| CO | carbon monoxide |
| CORM | CO-releasing molecules |
| Cx | connexin |
| CYP3A4 | cytochrome P3A4 |
| $D_1/D_2$ receptor | dopamine 1/2 receptor |
| DAG | diacylglycerol |
| DEA-NO | diethylamine NONOate diethylammonium salt |

| | |
|---|---|
| EAC | endothelial antioxidant compounds |
| EC-SOD | extracellular superoxide dismutase |
| ED | erectile dysfunction |
| EDRF | endothelium-derived relaxing factor |
| EFS | electrical field stimulation |
| eNOS | endothelial nitric oxide synthase |
| ES | electrical stimulation |
| ESWL | extracorporeal shock wave lithotripsy |
| ET-1 | endothelin-1 |
| GC | guanylyl cyclase |
| GDNF | glial cell line derived neurotrophic factor |
| GH | growth hormone |
| GTP | guanosine triphosphate |
| HO | heme-oxygenase |
| ICP | intracavernosal pressure |
| IGF-1 | insulin-like growth factor-1 |
| iNOS | inducible nitric oxide synthase |
| $IP_3$ | inositol triphosphate |
| $K^+$ | potassium |
| $K_{ATP}$ | ATP-dependent potassium channel |
| $K_{Ca}$ | calcium-dependent potassium channel |
| $K_{IR}$ | inward-rectifying potassium channel |
| KRB | Krebs-Ringer bicarbonate |
| $K_V$ | voltage-dependent potassium channel |
| L-NAME | Nω-Nitro-L-arginine methyl ester hydrochloride |
| LUTS | lower urinary tract symptoms |
| MAP | mean arterial pressure |
| m-CPP | meta-chlorophenylpiperazine |
| MCR | melanocortin receptor |
| MLC | myosin light chain |
| MLCK | myosin light chain kinase |
| MLCP | myosin light chain phosphatase |
| MT-II | melanotan II |
| MUSE | medicated urethral system for erection |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NANC | non-adrenergic non-cholinergic |
| NE | norephinephrine |
| NGF | neuronal growth factor |
| NMDA | N-methyl-D-aspartic acid |
| nNOS | neuronal nitric oxide synthase |
| NO | nitric oxide |
| NOR | noradrenaline |
| NOS | nitric oxide synthase |

| | |
|---|---|
| NT-3 | neurotrophin-3 |
| ODQ | 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one |
| PACAP | pituitary adenylate cyclase activating polypeptide |
| PCR | polymerase chain reaction |
| PDE | phosphodiesterase |
| PDE-5 | phosphodiesterase type 5 |
| PDE-6 | phosphodiesterase type 6 |
| PDE-11 | phosphodiesterase type 11 |
| pGC | particulate guanylyl cyclase |
| PG | prostaglandin |
| $PGE_1$ | prostaglandin 1 |
| PIN | protein inhibitor of nitric oxide synthase |
| $PIP_2$ | phosphatidyl inositol 4,5 biphosphate |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PKG | cGMP dependent protein kinase |
| PLC | phospholipase C |
| PnNOS | penile neuronal nitric oxide synthase |
| PNS | peripheral nervous system |
| POMC | proopiomelanocortin |
| PSD | postsynaptic density protein |
| PT-141 | bremelanotide |
| PVN | paraventricular nucleus |
| RAS | renin-angiotensin system |
| RhoAGEF | RhoA guanosine exchange factor |
| RhoAGAP | RhoA GTPase activing proteins |
| RhoAGDI | RhoA guanosine dissociation inhibitor |
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |
| RT-PCR | reverse transcription polymerase chain reaction |
| sGC | soluble guanylate cyclase |
| SHR | spontaneous hypertensive rats |
| SIN-1 | linsidomine chlorhydrate |
| SNP | sodium nitropruside |
| SOD | superoxide dismutase |
| STZ | streptozotocin |
| TX | thromboxane |
| UGN | uroguanylin |
| VCD | vacuum constrictor device |
| VEGF | vascular endothelial growth factor |
| VIP | vasoactive intestinal peptide |
| YC-1 | 3-(5’-hydroxymethyl-2’-furyl)-1-benzyl indazole |

# Chapter I:

# General Introduction

The blood flow to the different tissues and organs is continuously regulated in order to meet their metabolic and functional needs. Several mechanisms are responsible for this blood flow regulation. Whereas the heart pump ensures appropriate blood pressure at the entrance of the organs, local mechanisms are responsible for the modulation of the blood flow within these organs. In the 1980s it has became increasingly clear that besides the nervous system, the endothelium, a monolayer of cells covering the luminal surface of all blood vessels, strongly participates in the regulation of tissue perfusion. In response to various mechanical and chemical stimuli, the endothelium releases vasoactive factors promoting either vasoconstriction or vasodilation. The resultant change in diameter will alter the vascular resistance and thus the blood flow within the tissue.

As yet nitric oxide (NO) is the most studied vasorelaxing factor released by both the endothelium and the non-adrenergic non-cholinergic (NANC) parasympathetic nitrergic nerves. Increased blood flow in response to NO and pharmacological NO-donors mainly occurs through the stimulation of soluble guanylyl cyclases (sGC) resulting in an intracellular cGMP accumulation. sGC thus has a prominent role in regulating tissue perfusion and blood pressure. In the last two decades scientific interest on the function of sGC and its therapeutic applications has grown tremendously. This chapter focuses on the role of sGC and its isoforms in smooth muscle relaxation of arteries and corpora cavernosa.

# I.1 Soluble guanylyl cyclase:

# structure and function

## I.1.1 Guanylyl cyclases

Guanylyl cyclases (GCs) are members of the family of nucleotide cyclising enzymes along with adenylyl cyclase (AC). They are widely distributed signal transduction enzymes that in response to various cellular stimuli catalyze the enzymatic conversion of guanosine triphosphate (GTP) to guanosine 3',5'-cyclic monophosphate (cGMP) and pyrophosphate. GCs are expressed in membrane/particulate (pGC) and cytosolic/soluble (sGC) forms and while they share similar structural characteristics, they differ in their mechanisms of physiological regulations. The known membrane-spanning forms are cell surface receptors for different peptide hormones such as atrial natriuretic factor and appear to exist as homodimers or other higher ordered structures. The cytoplasmic forms represent heme-containing heterodimers that are regulated by the gaseous messenger molecules NO and carbon monoxide (CO) [1].

## I.1.2 Structure of sGC

sGC has been purified from lung tissue as a multi-domain heterodimeric, heme-containing enzyme consisting of an α (~73-88 kDa) and a β (~70 kDa) subunit, with both units required for catalytic activity (figure I.1) [2;3]. sGC is a hemoprotein, although in contrast to various other hemoproteins which use the heme moiety as a catalyst in redox reactions or as an oxygen storage-transporting moiety, the heme group of sGC seems to have a unique role of being a receptor specific to various physiologically relevant gaseous activators such as NO and CO [4;5].

Domain structures for sGC have been proposed based on sequence similarity with other proteins, although a three-dimensional structure for the full protein is not available yet [6]. Analyses of the cDNA sequences revealed that α and β subunits, which both are involved in domain architecture, show a high degree in homology both in their amino (N)-terminal and their carboxyl (C)-terminal halves [7]. Each subunit can be divided into three functional domains, namely the heme-binding, dimerization and catalytic domain.

At their interface, the C-termini of each subunit build up the catalytic domain that exhibits a high degree of homology between membrane-bound GC and AC [8]. In contrast to pGC however, sGC activity demonstrates typical Michaelis-Menten kinetics, suggesting that sGC activity represents a single catalytic GTP site [9].

The N-terminal portion of both subunits, commonly referred to as the regulatory domains, constitutes the heme-binding domain [10]. The observation that heme-free enzyme exhibits basal activity but is not stimulated by NO led to the proposal that enzyme-bound heme acts as the receptor molecule for NO, transducing the binding signal to activation of the cyclase catalytic domain [11]. Each heterodimer contains a prosthetic heme moiety whose ferrous iron is coordinated by four nitrogens of the porphyrinic ring and another nitrogen provided by the invariant histidine residue 105 of the β subunit which acts as the axial ligand in the native state [12]. Mutation of this histidine located near the N-terminus of the β subunit results in the inability of sGC to bind heme and produces an enzyme that is unresponsive to NO [13]. The resting sGC thus has a five-coordinate high-spin $Fe^{II}$ heme and the histidine-bound heme is further stabilized through the interaction of its propionate side-chains with Tyr135, Ser137 and Arg139 (Y-x-S-x-R) of the β subunit [14;15]. It has recently been illustrated that the heme keeps the regulatory domain in a restricted basal conformation. This restricted basal conformation is released not only after binding of NO but also to various degrees by the chemical or genetic removal of the heme moiety from the regulatory domain [4].

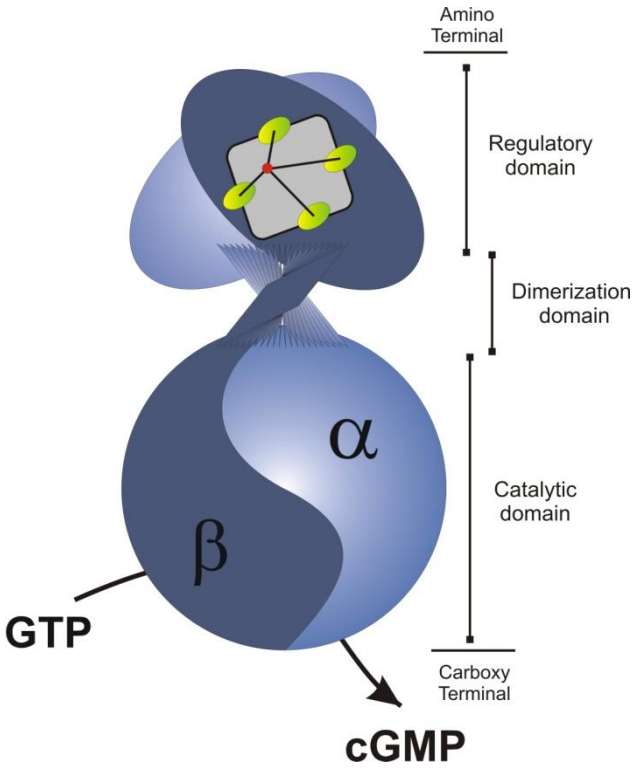


**Figure I.1** demonstrates the structure of the heterodimeric sGC enzyme.

Intervening between the heme binding and catalytic regions is a dimerization domain, which is obligatory for enzyme activation [16]. While for a long time this region preceding the catalytic domains was considered to be sufficient for dimerization, a recent report illustrated that the additional presence of N-terminal parts are mandatory for a stable subunit interaction [17].

## I.1.3 sGC isoforms

sGC is present in the cytosolic fraction of virtually all mammalian cells with the highest concentrations found in the lung and brain tissue [18]. Several isoforms have been cloned and characterized [19]. Originally, the $\alpha_1$ and $\beta_1$ subunits were the first subunits purified from bovine and rat lung. Interestingly, no enzyme activity was observed when the β subunit cDNA was transfected into L cells, an established cell line derived from mouse connective tissue. However, cells transfected with both subunit cDNAs showed significant sGC activity that was markedly activated by the NO-donor sodium nitroprusside (SNP), indicating that both subunits are essential for the enzyme activity as well as activation by NO [20]. Later, a novel sGC subunit was isolated from rat kidney using the catalytic domain sequence of sGC by polymerase chain reaction (PCR) and subsequently designated the $\beta_2$ subunit [21]. This subunit containing 86 additional amino acids in its C-terminal region compared to the $\beta_1$ subunit, is preferentially expressed in rat kidney and liver. In human, $\beta_2$ mRNA is present in kidney, gastric carcinoma and corpora cavernosa (CC) according to reverse transcription-PCR (RT-PCR) experiments [22]. Gupta et al. presented evidence for enzyme activity after co-expression of a $\beta_2$-GFP fusion construct with the $\alpha_1$ cDNA in COS cells [23]. However, in most laboratories, co-expression of the $\beta_2$ subunit revealed that $\alpha_1\beta_2$ heterodimers are inactive. Despite these controversies, it was suggested that this subunit may serve to regulate sGC$\alpha_1\beta_1$ activity. Recently, the $\beta_2$ cDNA was shown to exert NO-sensitive activity after expression in Sf9 or HEK293 cells in the absence of a second subunit, most likely as a $\beta_2\beta_2$ homodimer. It should however be noted that this only occurred using non-physiological concentrations of $Mn^{2+}$ ion [24]. Around the same time, Harteneck et al. isolated a cDNA coding for a second novel subunit of sGC from human fetal brain, which was designated the $\alpha_2$ subunit [7;25]. Co-expression of the $\alpha_2$ subunit with the $\beta_1$ subunit showed sGC activity indistinguishable from $\alpha_1\beta_1$ heterodimer, demonstrating the interchangeability of the subunit isoforms [26]. Giuli et al. also isolated cDNAs corresponding to both α and β subunits of sGC from human brain, and designated them $\alpha_3$ and $\beta_3$ [27]. However, Zabel et al. re-sequenced these cDNAs and

found that they were homologous to the bovine $\alpha_1$ and $\beta_1$ subunits, suggesting that they may simply be species variants [20].

Thus to date, only $\alpha_1$, $\alpha_2$, $\beta_1$ and $\beta_2$ have been cloned and sequenced. The $\alpha_1$ and $\alpha_2$ isoforms, with an overall similarity of about 48% identical amino acids, are fairly homologous in their middle- and C-terminus portions (87% identical amino acids), but the N-termini differ markedly with only 27% identical amino acids [2;7]. The $\beta_1$ and $\beta_2$ subunits differ markedly at both N- and C-termini, with less than 50% homology in their middle regions. Despite the existence of two isoforms for each subunit, only the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ heterodimers have been confirmed in vivo [26;28]. In mammals, the highest amount of the $\alpha_2\beta_1$ isoform was found in brain where the $\alpha_1\beta_1$ was present in comparable amounts. In all other tissues tested, $\alpha_1\beta_1$ enzyme was the prevailing isoform with particularly high concentrations found in lung, although low levels of the $\alpha_2\beta_1$ isoform was also identified in each of the tissues tested [29]. The differences in primary structure of both α subunits are contrasted by their functional similarity [7]. The apparent lack of difference in the catalytic activities and pharmacological properties between the different α subunits indicates that the α subunits might determine yet unknown properties of the resulting iso-enzymes.

It has been illustrated that RNA splicing also contributes to the heterogeneity of sGC subunits. Spliced variants of each α and β subunit isoforms have been reported, although the significance of these alternate transcripts largely remain unknown. $sGC\alpha_1$ subunit splice variants with N- and C- terminal truncations have been reported in brain, heart, arteries and B-lymphocytes [30;31]. An alternative splice-variant of the $\alpha_2$ subunit of sGC ($sGC\alpha_{2i}$) containing an in frame insert of 31 amino acids within the catalytic domain was identified in a number of cell lines and tissues. In co-expression experiments, the $\alpha_{2i}$ subunit competes with the $\alpha_2$ subunit for dimerization with the $\beta_1$ subunit, thereby reducing $sGC\alpha_2\beta_1$ catalyzed GC activity, although the heterodimer exhibited no GC or AC activity. These data show that the novel variant functions as a dominant negative protein and that post-transcriptional mRNA processing represents a potential mechanism for regulation of sGC activity.[32] In addition, a $\beta_1$ subunit that lacks 33 amino acids was found in human lung tissue and an alternative splice variant of the $\beta_2$ subunit was discovered in human CC [33;34]. Modulation of the level and diversity of splice forms may represent novel mechanisms modulating the function of sGC in different human tissues.

Interestingly, it has been reported that the C-terminal peptide of $sGC\alpha_2$ subunit associates with the postsynaptic density protein complex, PSD-95 [35]. As a consequence of this interaction, the $sGC\alpha_2\beta_1$ isoform is recruited to the synaptosomal membrane fraction locating this isoform in close proximity to the NO generating synthases. Thus, at least in brain, the $\alpha_2\beta_1$ isoform works as a sensor for NO formed by the PSD-95-associated neuronal NOS, allowing efficient signaling without raising second messenger concentrations throughout the cell [36]. The designation of sGC as a purely cytosolic enzyme was further challenged with the finding that the $\alpha_1\beta_1$ isoform was translocated to the plasma membrane in response to elevated calcium ($Ca^{2+}$) concentrations. The $Ca^{2+}$-dependent membrane association has been attributed to the interaction with heat shock protein 90 [37]. In the rat heart, approximately 1/5 of $sGC\alpha_1\beta_1$ is found in the membrane fraction [38]. Other tissues including the brain cortex, adrenal gland, skeletal muscle and colon also contain membrane-associated sGC [39]. This membrane-associated enzyme appeared to display a higher NO sensitivity.

### I.1.4 sGC, a receptor for the gaseous molecule NO

Whereas NO is probably an ancient simple molecule that may have participated as a messenger in evolution, before 1980 it was largely viewed as a pollutant present in automobile exhaust and cigarette smoke. However, this view of NO changed when Furchgott et al. showed that the endothelium released a labile factor, which he termed the endothelium-derived relaxing factor (EDRF), causing vascular relaxation when vascular smooth muscle preparations were stimulated with vasodilators such as acetylcholine, bradykinin and histamine [40]. Subsequently, it was proposed that EDRF was NO [41]. Despite the discovery of cGMP in urine in the late 1960s, the identification of the members of GC by the mid 1970s and the observation that NO both as a gas and released from nitrovasodilators activates sGC in 1977, research on sGC only increased after the finding that NO was the EDRF [42-45].

It is now established that the NO/sGC/cGMP system plays an important role in signal transduction and maintaining physiological function in both animals and plants [46]. In mammalian physiology, the NO/cGMP signal transduction cascade exerts diverse effects in the cardiovascular, neuronal, gastrointestinal as well as other systems [1;47]. Through the induced intracellular accumulation of cGMP, NO regulates numerous physiological processes, such as smooth muscle contractility, platelet reactivity, leukocyte adhesion, cell proliferation and migration, apoptosis, extracellular matrix production, gastrointestinal motility, intestinal

ion transport, fluid and electrolyte homeostasis, genital function, light transduction in the retina, memory formation, synaptic plasticity, host defence against pathogens and embryonic development [48]. The vital importance of sGC for mammalian physiology is directly confirmed by generation of sGC knockout mice [49].

The precise mechanism by which NO activates sGC to catalyze the conversion of GTP to cGMP is matter of scientific debate [18]. According to a kinetic model proposed by Marletta and coworkers, activation of sGC is biphasic [50;51]. Binding of NO to the sixth position in the ferrous heme yields an intermediate hexa-coordinate complex. This intermediate then undergoes a relatively slow transition triggering disengagement of the coordinated histidine-bond and displacement of iron from the protoporphyrin plane. Releasing the restrictions imposed by histidine-bond helps driving a subtle conformation change, favouring a more efficient GTP cyclising activity at the active site. The redundant catalytic domain near the C terminus of the α and β subunit is then thus activated resulting in a marked increase in $V_{max}$ and a decrease in the $K_m$ for GTP. However, it has recently been reported that the stoichiometric binding of NO to sGC generates a low level activity species, despite the cleavage of iron-histidine bond and that additional NO or an effector is necessary to fully activate sGC [52-58].

### I.1.5 CO: another physiological sGC activator?

CO is generated physiologically from lipid peroxidation or more importantly the oxidative destruction of heme catalysed by heme oxygenase (HO). HO is the initial and rate-limiting microsomal enzyme that in the presence of ferrous iron and stoichiometric amounts of nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen, catalyzes oxidation of heme at the alpha-methene bridge to form biliverdin IXa and liberates CO [59]. Interestingly, endogenous formation of HO-liberated CO was first described under both normal and pathophysiological conditions as early as in the 1950s [60]. Over the past decade many experimental studies have provided much support for the biological relevance of HO and CO in multiple organ systems. CO has been assumed to participate in many physiological functions such as long-term potentiation, olfactory signal transduction, blood pressure regulation, smooth muscle relaxation, platelet aggregation and others [61-67].

CO has been shown to exert a number of these functions through the activation of sGC. However its potency to activate sGC is significantly lower than that of NO [68]. Binding of CO stimulates cyclase activity only by 5-fold compared to 200-fold for NO. Despite a tremendous amount of data supporting CO activation of sGC in intact cells and tissues, the molecular mechanism underlying this effect has remained obscure because CO does not stimulate purified sGC [69;70]. It has been established that CO binding to sGC also involved the primary formation of a six-coordinate ligand enzyme complex, however, the iron-histidine bond remains intact following CO binding. This unique mechanism of enzyme activation by CO is thought to be the major limitation to the degree of CO-induced cyclase activation [71;72]. The proposal of CO as a physiological activator of sGC is thus opposed by the rather poor sGC stimulatory properties of CO. Moreover, the endogenous concentration of CO may not be enough to activate sGC as such and is consumed immediately after synthesis as it lacks vesicular storage. However, in the presence of certain exogenous compounds such as YC-1 and BAY 41-2272, CO activates the enzyme to the level of NO-bound sGC [71;73]. Obviously, YC-1 might mimic the effect of an unidentified endogenous factor that sensitizes sGC to activation by CO in certain tissues.

# I.2 Soluble guanylyl cyclase in the cardiovascular system

Far from being rigid tubes, blood vessels are dynamic structures and are the source of factors like NO which regulates vascular tone, platelet aggregation, leukocyte adhesion, cellular growth and proliferation and extracellular matrix deposition. The function of sGC, as the major target of NO, is thus fundamental to cardiovascular homeostasis and maintains an important cytoprotective and antithrombotic influence. The importance of sGC in the cardiovascular system is strengthened by the observation that mice deficient in sGC develop hypertension, show complete loss of NO-dependent aortic relaxation and inhibition of platelet aggregation [49].

## I.2.1 Vessel tone regulation

The endothelium controls the tone of the underlying vascular smooth muscle mainly through the production of vasoconstrictor and vasodilator mediators. Endothelial cells secrete these mediators in response to substances released from autonomic and sensory nerves, circulating hormones, coagulation derivatives and platelet products as well as agents produced by endothelial and vascular smooth muscle cells themselves. In addition, changes in shear stress elicit endothelium and flow-dependent vasodilatation. The endothelial mediators are also involved in the regulation by the endothelium of vascular architecture and the blood cell - vascular wall interactions [74-76].

### I.2.1.1 Smooth muscle contraction (Figure I.2)

One of the key pathways modulating vessel tone is the release of norepinephrine (NE) from the nerve endings. NE binds to the postjunctional $\alpha_1$ and $\alpha_2$ adrenergic receptors localized on the smooth muscle cells [77]. After activation of the serpentine $\alpha_1$-adrenergic receptors which are coupled to a heterotrimeric G-protein, a dissociation of the G-protein receptor subunits will occur, subsequently leading to activation of phospholipase C (PLC). This enzyme catalyses the hydrolysis of phosphatidylinositol 4,5 biphosphate ($PIP_2$) to form inositol trisphosphate ($IP_3$) and diacyl glycerol (DAG). $IP_3$ then binds to the sarcoplasmic reticulum (SR) receptors, causing the release of $Ca^{2+}$ into the cytosol. In the presence of elevated intracellular $Ca^{2+}$, DAG activates protein kinase C (PKC) to further sustain the increase in intracellular $Ca^{2+}$ through phosphorylation of L-type $Ca^{2+}$ channels and other proteins. The free $Ca^{2+}$ then binds to calmoduline, introducing a conformational change whereby the $Ca^{2+}$-calmodulin complex is then capable of exposing its sites of interaction with myosin light chain kinase (MLCK). Activation of MLCK results in phosphorylation of the myosin light

chains (MLC), which triggers the cycling of myosin cross bridges along actin filaments resulting in the development of force and smooth muscle contraction [78;79]. Activation of the $\alpha_2$-adrenergic receptor by NE inhibits AC activity via heterotrimeric $G_i$-proteins reducing intracellular adenosine 3',5'-cyclic monophosphate (cAMP). This reduction in cAMP synthesis leads to reduced protein kinase A (PKA) activation and increased intracellular $Ca^{2+}$ or $Ca^{2+}$/calmodulin sensitivity of MLCK. $\alpha_2$-adrenergic receptors have further been shown to modulate $Ca^{2+}$ influx and are implicated in activation of PLC, potassium ($K^+$) channels and $Na^+/H^+$ exchange [80;81]. The $\alpha_1$-adrenergic receptors however are the predominant adrenergic receptor subtypes in vascular tissues and therefore are estimated to play the major role in the physiology of vessel tone regulation [82;83].

Besides the phasic contraction mechanism which is dependent on an increase of intracellular $Ca^{2+}$, vascular smooth muscle contraction also involves a tonic contraction phase. This phase occurs after the $Ca^{2+}$ returns to basal levels and is independent of a rise in intracellular $Ca^{2+}$ levels [84;85]. This $Ca^{2+}$ sensitizing pathway enables vascular smooth muscle cells to maintain contractile force for an extended period of time at a low cost of energy. The $Ca^{2+}$ sensitizing pathway involves the activation of Rho-kinase by the monomeric G-protein RhoA. RhoA, a member of the Rho subfamily of Ras proteins, functions as a molecular switch, cycling between the inactive GDP bound state and the active GTP bound state. The activity of RhoA is tightly regulated by three proteins: RhoA guanosine exchange factor (RhoGEF), RhoA GTPase activing proteins (RhoGAP) and RhoA guanosine dissociation inhibitor (RhoGDI). In the inactive state, RhoGDI binds to RhoA-GDP to form a complex stabilizing RhoA-GDP and trapping the complex in the cytosol. Activation of RhoGEF facilitates the exchange of GTP for GDP on RhoA and the dissociation from RhoGDI. The active RhoA-GTP is then able to translocate from the cytosol to the plasma membrane, initiating signalling transduction. Finally RhoGAP accelerates the intrinsic GTPase activity of RhoA and promotes hydrolysis of GTP on RhoA-GTP thereby inactivating RhoA. Subsequently RhoA-GDP re-associates with RhoGDI and relocates to the cytosol [86;87]. Rho-kinase, a serine/threonine kinase, is one of the most studied RhoA effectors. Rho-kinase consists of two isoforms: α and β. Rho-kinase contains a RhoA binding site at the C terminus and a catalytic site at the N-terminus. Binding of RhoA causes a conformational change in Rho-kinase and its subsequent autophosphorylation increasing Rho-kinase activity. In smooth muscle cells, Rho-kinase induces vasoconstriction through the phosphorylation and subsequent inhibition of myosin light chain phosphatase (MLCP). MLCP is an enzyme catalyzing dephosphorylation

of MLC and thus promoting smooth muscle relaxation. Through its inhibition this favours the maintenance of phosphorylated MCL at low intracellular $Ca^{2+}$ concentrations, thus promoting the binding of actin and myosin for force generation [88-90].

Besides NE, other vasoconstrictor molecules such as endothelins, prostaglandins, neuropeptide Y and angiotensin II are also implicated in the physiology of smooth muscle contractility [76;91].

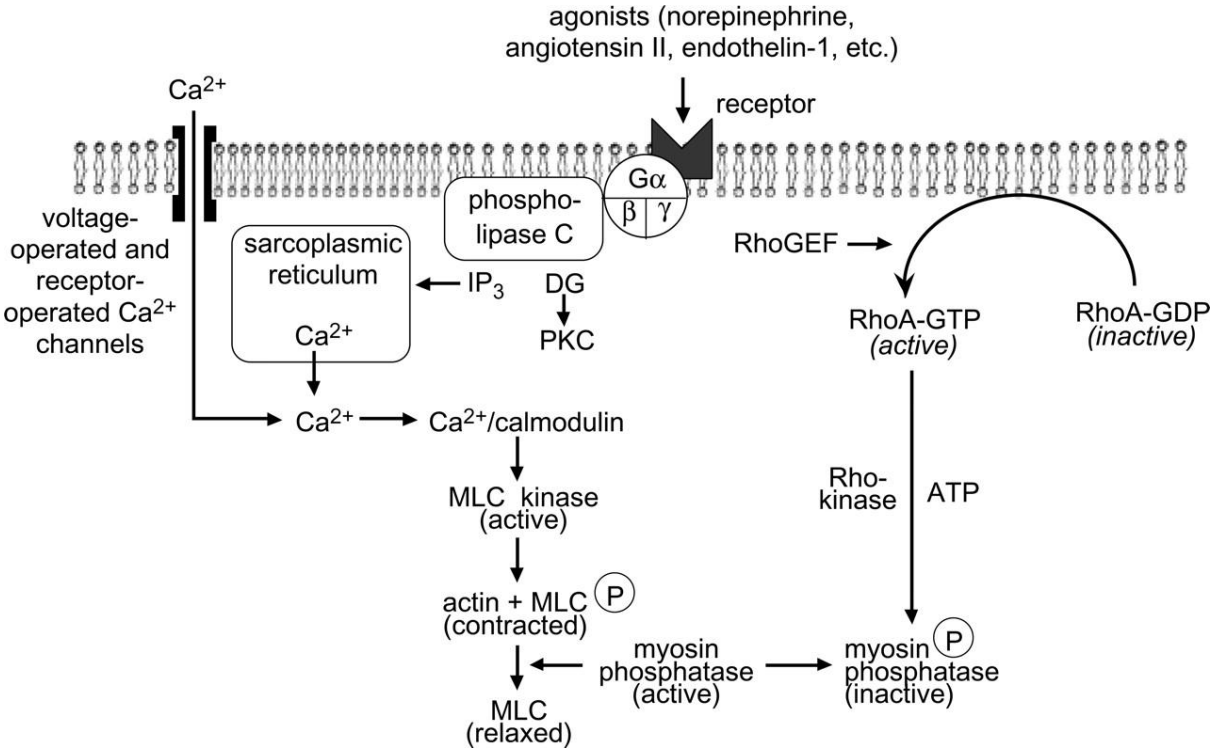


**Figure I.2** represents a schematic overview of the phasic and tonic smooth muscle contraction mechanism. The binding of various agonists to their specific receptors results in the activation of smooth muscle contraction. Activation of these receptors leads to increased PLC activity. Subsequently, PLC catalyses the hydrolysis of $PIP_2$ to form 2 potent second messengers: DAG and $IP_3$. The formation of $IP_3$ causes $Ca^{2+}$ release from the sarcoplasmic reticulum. Then DAG together with $Ca^{2+}$ activates PKC, which phosphorylates different target proteins in order to further sustain the increase in intracellular $Ca^{2+}$ levels. The free $Ca^{2+}$ ions bind calmodulin, resulting in the activation of MLC kinase and subsequent phosphorylation of the MLC, initiating shortening of the smooth muscle cells. However, the increase in $Ca^{2+}$ levels within the cell is transient. After the phasic contraction mechanism, the $Ca^{2+}$ sensitizing pathway takes over. This pathway, enabling smooth muscle cells to maintain contractile force for an extended period of time at a low cost of energy, involves the activation of the monomeric G-protein RhoA and its down-stream target Rho-kinase. This will result in the phosphorylation and inhibition of MLCP, favouring the maintenance of phosphorylated MCL. The figure is adapted from Webb R.C. Smooth muscle contraction and relaxation [79].

### I.2.1.2 Smooth muscle relaxation (Figure I.3)

Smooth muscle relaxation occurs either by removal of the contractile stimulus or by direct action of a substance that stimulates inhibition of the contractile mechanism. This means that smooth muscle relaxation requires a decrease of the intracellular $Ca^{2+}$ concentration and/or activation of the MLCP. Although many endogenous vasodilators have been shown to exert relaxant effects on different vascular beds, the main physiological pathway stimulating smooth muscle relaxation remains the NO/cGMP pathway.

NO is a gaseous messenger molecule with a short life-time, that is produced by NO synthases (NOS). The combination of molecular oxygen and the amino acid L-arginine in the presence of reduced NADPH and NOS yields L-citrulline and NO, through a 5 electron oxidation of the guanidine nitrogen of L-arginine. Three different isoforms of NOS are known, namely the endothelial NOS (eNOS) and neuronal NOS (nNOS) which are constitutively present and the inducible NOS (iNOS) isoform which is up-regulated in response to various stimuli [92]. The binding of acetylcholine (ACh) to its muscarinic receptors on the endothelial membrane of blood vessels results in an intracellular $Ca^{2+}$ increase, resulting in the formation of $Ca^{2+}$/calmodulin complexes. In endothelial cells the $Ca^{2+}$/calmodulin will activate eNOS [93]. The subsequent increase in blood flow causes shear stress, which further stimulates eNOS through phosphorylation by the $PI_3$-kinase/Akt pathway, resulting in sustained activation of eNOS [93;94]. In addition, ACh can also induce vasorelaxation by inhibiting the release of NE from the sympathetic nerve fibres [95]. NO, when released, will diffuse to the smooth muscle cell and activate sGC, resulting in an increase of the intracellular cGMP concentration. This latter second messenger conveys signals through activation of cGMP-dependent protein kinase (PKG), cGMP-regulated ion channels and cGMP-activated phosphodiesterase (PDE). The most important effector for cGMP in the mechanism of smooth muscle relaxation is PKG [96]. Activated PKG will phosphorylate numbers of targets with the final result of smooth muscle relaxation. The specific targets for PKG remain unclear till now and possible targets that have been suggested in literature are $K^+$ channels, different types of $Ca^{2+}$ channels (such as those located on the sarcoplasmic reticulum membrane, the voltage-dependent $Ca^{2+}$ channels and the L-type $Ca^{2+}$ channels), phospholamban, $IP_3$, sarcoplasmic reticulum $Ca^{2+}$/ATPase, RhoA and many others. The result is a net decrease of the intracellular $Ca^{2+}$ concentration, leading to a decrease in MLCK activity and/or an increase in the MLCP activity through inhibition of RhoA. Both mechanisms favour dephosphorylation of MLC and thus smooth muscle relaxation [97]. Additionally, the opening of the $K^+$ channels by PKG

will induce smooth muscle cell membrane hyperpolarization which will further oppose smooth muscle contraction [1;97-100].

Although NO is believed to be the principal stimulator of vasorelaxation, other vasodilators such as prostaglandin E1 ($PGE_1$), vasointestinal polypeptide (VIP), substance P, pituitary adenylate cyclase-activating polypeptide (PACAP) and others have also been suggested to underlay smooth muscle relaxation, however their contribution in vessel tone regulation remains rather vague. Many of the aforementioned agents exert their effects through (in)direct activation of AC which results in an intracellular accumulation of cAMP, a second messenger with downstream effects analogous to cGMP in smooth muscle relaxation [101;102].

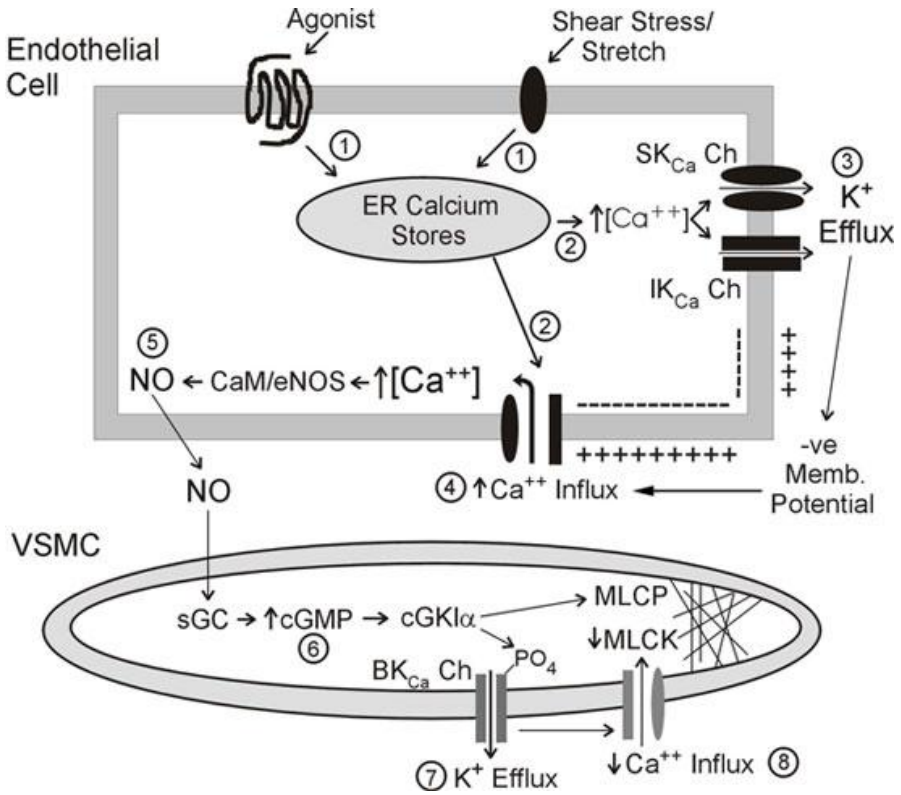


**Figure I.3:** Schematic overview of the molecular mechanism underlying NO-mediated vascular smooth muscle relaxation. Vasoactive agonists and fluid shear stress ① normally elevate intracellular $Ca^{2+}$ concentrations in endothelial cells ②, stimulating eNOS activity ⑤. Moreover, the increased $Ca^{2+}$ concentrations may activate $Ca^{2+}$-dependent $K^+$ channels ③ resulting in a hyperpolarization of the endothelial cells, an enhanced $Ca^{2+}$ influx ④ and thus a further increase in eNOS activity. In addition, fluid shear stress and other factors may cause $IP_3$-kinase/Akt-dependent phosphorylation of eNOS, resulting in a more sustained increase in catalytic activity. In the smooth muscle cell, NO causes relaxation by a myriad of actions, whereof increasing the intracellular cGMP concentration by activation of sGC ⑥ is one of the major mechanisms. By activating PKG, increased cGMP

concentrations will induce hyperpolarization of the smooth muscle cells through the opening of $K^+$ channels ⑦ and activation of MLCP ⑧. The ultimate result is a reduction in cytosolic $Ca^{2+}$ levels on the one hand and a reduction of the sensitivity of the contractile apparatus for $Ca^{2+}$ on the other hand. ER = endoplasmic reticulum; $SK_{Ca}$ Ch = small conductance calcium-dependent potassium channel; $IK_{Ca}$ Ch = intermediate conductance calcium-dependent potassium channel; CaM = calmodulin; VSMC = vascular smooth muscle cell.

## I.2.2 Vessel tone dysregulation

Abnormal increase in vascular smooth muscle tone due to an imbalance in vasodilatory and/or vasoconstrictive forces is involved in the pathogenesis of vascular diseases such as systemic and pulmonary hypertension, left heart failure, atherosclerosis and others. This imbalance, often referred to as endothelial dysfunction, is manifested by either a decreased secretion of vasodilator mediators, an increased production of endothelium-derived vasoconstrictors and/or resistance of vascular smooth muscle to endothelium-derived vasodilators [103]. Accumulating evidence from basic science and clinical research indicates that endothelial dysfunction is caused by impaired NO/cGMP signalling characterized by reduced bioavailiability and/or responsiveness to endogenously produced NO. In contrast, excessive vasodilation due to an overactivity of the pathway has been shown to contribute to anaphylactic shock and migraine [104;105].

## I.2.3 sGC as a therapeutic target in cardiovascular diseases

Due to its importance in the cardiovascular system, it is conceivable that an aberrant sGC function contributes to the pathophysiology of endothelial dysfunction and thus a variety of cardiovascular disorders including hypertension, coronary heart disease, thrombosis, atherosclerosis, myocardial infarction, stroke and angina pectoris. There are at least two mechanisms affecting sGC in vivo, namely down-regulation of mRNA/protein levels and desensitization [39]. It has been demonstrated that mRNA and protein levels of both the sGC $\alpha_1$ and $\beta_1$ subunits as well as sGC activity are reduced in arterial and pulmonary hypertension as well as in ageing [106-109]. Moreover, salt-sensitive hypertension in Dahl rats is associated with decreased and increased expression of the $\beta_1$ and $\beta_2$ subunit respectively, suggesting that exchange of $\beta$ subunits may be critical to blood pressure regulation [23;110]. Receptor desensitization is believed to occur due to increased oxidative stress, a risk factor of several cardiovascular disorders. Increased oxidative stress will result in the accumulation of oxidized and heme-free sGC [111]. Melichar et al. found a decreased sGC expression and

activity in neointimal layers of hypercholesterolemic rabbits, whereas Francois et al. actually found an overexpression of dysfunctional sGC in a similar hypercholesterolemic rabbit model [112;113]. The dysfunctional sGC seen in these models was later shown to be identical with heme-free sGC or sGC containing oxidized heme.[114;115] Increased formation of reactive oxygen species (ROS) further contributes to the vascular dysfunction by scavenging NO, resulting in reduced NO bioavailability, and the formation of the toxic agent peroxynitrite [116].

The pharmacological activation of sGC may thus have a broad clinical perspective for treatment of vascular diseases [117;118].

### I.2.3.1 NO-donors and nitrovasodilators

The predominant therapeutic approach to endothelial dysfunction is a NO replacement therapy, as exemplified by organic nitrates which mimic the action of endogenous NO by bioconversion to NO or NO-related compounds. In 1879, William Murrell, a physician and practical therapist in London, introduced glyceryl trinitrate as a remedy for angina pectoris [117]. Since then NO donors have been used to treat a variety of disorders including angina pectoris, coronary artery disease, congestive heart failure and hypertension. Although nitrovasodilators were already introduced in 1879, it was only in the late 1970s that release of NO and activation of sGC was identified as the basis of glyceryl trinitrates effects [44]. In 1991, inhaled NO was shown to decrease pulmonary artery pressure and pulmonary vascular resistance in patients with primary pulmonary hypertension [119]. Later studies have confirmed the important role of inhaled NO in pediatric intensive care [120;121]. More recent evidence shows a beneficial effect of inhaled NO in patients with sickle cell disease [122]. Interestingly, in 1998, the Nobel Prize in Physiology or Medicine was awarded for NO as a signaling molecule in the cardiovascular system [123].

While the therapeutic value of nitrovasodilators in certain groups of patients is undisputed, there are several shortcomings with this approach. The development of tolerance upon prolonged use limits the therapeutic value of this class of compounds [124]. This is believed to be the result at least in part of decreased metabolic activation of nitrates or excessive superoxide, endothelin or angiontensin II levels [125-127]. Some patients may be(come) refractory to NO replacement therapy with organic nitrates due to a shift of the equilibrium of ferrous towards ferric heme in sGC as a result of increased oxidative stress. Moreover, NO or related compounds generated by the nitrovasodilators activate not only sGC but can also

affect the function of other proteins via nitrosation or interactions with metals, which may have beneficial or detrimental consequences. Therefore, an obvious need persisted for novel stimulators/activators of sGC that overcome these problems associated with organic nitrates.

### I.2.3.2 Novel sGC stimulators and activators

Recent advances in drug discovery provided a variety of other approaches to activate sGC, which may help to circumvent both the tolerance problem and some non-specific actions associated with NO-donor drugs [128].

#### I.2.3.2.1 sGC stimulators

In 1994 a new class of sGC modulators was discovered with the description of the antithrombotic properties of 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole (YC-1) in rabbits and mice [129]. Soon after the discovery of this pharmacological effect of YC-1 on platelets, YC-1 was found to be a modulator of sGC activity [130;131]. Interestingly, this agent could activate sGC in the absence of endogenous sGC ligands but highly potentiated the stimulatory effect of submaximal concentrations of NO and CO [70;132]. Spectroscopic studies indicated that, in contrast to NO, YC-1 binds to an allosteric heme-independent site, although identification of this site is a decade old puzzle [132-134]. One study has mapped the cysteine 238 and 243 region in the $\alpha_1$ subunit as the likely binding site of the YC-1 using photoaffinity labelling [135;136]. The finding that the sGC$\alpha_2\beta_1$ isoform exhibits virtually identical sensitivity to YC-1 as the sGC$\alpha_1\beta_1$ heterodimer is somehow controversial with the proposed YC-1 binding site as it has been established that the $\alpha_1$ and $\alpha_2$ subunits are quite diverse in their N-terminal region. Moreover, another study indicated that both cysteines including an additional 14 N-terminal residues can be deleted without loss of YC-1 sensitivity.[135] Also the mechanism of action of YC-1 is ambiguous. Like NO, YC-1 modulates the catalytic rate of the enzyme, increasing $V_{max}$ of cGMP formation by 40% and slightly decreasing the $K_m$ of sGC for GTP by 3-5 fold [137]. In addition, YC-1 appears to weaken the histidine-iron bond in the hexacoordinate NO and CO bound state and to decelerate the dissociation of the heme ligand [138;139]. Important to note is that next to activating sGC, YC-1 has been shown also to act as a non-specific PDE inhibitor and to stimulate NO synthesis and release [70;140;141].

Different experimental studies have shown that YC-1 induced a concentration-dependent relaxation in (endothelium denuded) rat and rabbit aortic rings pre-contracted with phenylephrine by increasing intracellular cGMP levels and this relaxant effect was potentiated

by a NO-donor [142;143]. Further studies demonstrated an in vivo effect of YC-1 on prevention of venous thrombosis in mice and a blood pressure lowering effect in both normotensive and hypertensive rats after intravenous administration of high doses [131;144]. Moreover, simultaneous intravenous application of YC-1 and SNP in rats induced a blood pressure reduction in both normotensive and hypertensive animals. In addition, YC-1 has been shown to inhibit vascular smooth muscle cell proliferation and markedly inhibited neointima formation after balloon-induced carotid artery injury in rats [145;146]. The finding that YC-1 stimulates sGC independently of NO-binding is a property of major importance for the treatment of endothelial dysfunction and other diseases wherein basal NO production is prejudiced. Moreover, the synergism observed using YC-1 in combination with nitrovasodilators may also be exploited in clinical practice, enabling a reduction in nitrovasodilator dose. Currently, YC-1 has already been proposed for the treatment of cardiovascular and thrombotic disorders [147].

That YC-1 activates sGC by binding to an allosteric site on the enzyme opened the possibility to discover a new class of compounds with a different pharmacological profile in comparison to NO-donors [136]. Moreover, the discovery of compounds binding to an allosteric site on sGC has raised the interesting question of putative endogenous analogues regulating cGMP signaling in mammalian tissues. Using YC-1 as a chemical lead structure, new NO-independent sGC activators were selected with a higher potency and devoid of non-specific actions which have been observed with the use of YC-1 [148]. Optimization of YC-1 led to the development of BAY 41-2272 and BAY 41-8343 as new allosteric sGC activators [148]. Benzydamine analogs like CFM-1571 are other examples of YC-1-related structural classes described as potent sGC activators [149]. Like YC-1 these new sGC activators bind sGC at a heme-independent site, show a strong synergism upon combination with NO and a loss of activation after removal or oxidation of the prosthetic heme moiety.

Similar to YC-1, BAY 41-2272 was effective in inhibiting aggregation of platelets induced by collagen and relaxes both endothelium intact and denuded rabbit aorta [150]. BAY 41-2272 was also shown to dilate ovine pulmonary arteries and dose-dependently inhibited the pressor response of acute hypoxia in the isolated perfused lung system [151]. Several studies evaluated the possible beneficial effects of BAY 41-2272 in various models of hypertension [152]. For instance, oral administration of BAY 41-2272 prevented systolic pressure increase and reduced the mortality in transgenic renin rats characterized with low NO availability

[136]. Treatment with BAY 41-2272 also abolished L-NAME-induced hypertension, brachycardia and cardiac hypertrophy [153]. Furthermore, BAY 41-2272 treatment significantly reduced acute and chronic pulmonary vascular resistance and thus pulmonary hypertension in lambs and mice respectively [151;154]. In a canine model of congestive heart failure, BAY 41-2272 acted as an arterial vasodilator and reduced the mean arterial and pulmonary pressure [155]. BAY 41-2272 also attenuated remodeling in models of systemic arterial hypertension, partially due to its antifibrotic, antiproliferative and antimigratory actions on vascular smooth muscle cells. Interestingly, cGMP-independent actions of BAY 41-2272 have been published such as a direct blockade of $Ca^{2+}$ influx and the inhibition of PDE-5 [156;157]. In addition, inhibition of superoxide anion formation and NADPH oxidase expression by BAY 41-2272 has been described in the CC [158].

Also BAY 41-8543 was shown to relax rabbit aortas as well as other vascular tissues and to produce a dose-dependent and long-lasting blood pressure lowering effect after intravenous and oral administration in anaesthetized normotensive rats as well as in conscious spontaneous hypertensive rats (SHRs) [159;160]. Even in a low NO, high renin rat model of hypertension, BAY 41-8543 was able to prevent the increase in blood pressure evoked by L-NAME. In anaesthetized dogs, the blood pressure lowering effect of intravenous injection of BAY 41-8543 was accompanied with decreased cardiac oxygen consumption as well as an increased coronary blood flow and heart rate. Moreover, BAY 41-8543 prolonged the rat tail bleeding time and reduced thrombosis [161;162].

The pharmacological profile of these compounds suggests that they possess the potential of being important research tools for in vivo investigation in the sGC/cGMP field and that they have clinical potential as treatments for cardiovascular diseases. Currently, BAY 63-2521, another heme-dependent sGC stimulator, is in clinical development as an oral therapy for patients suffering from moderate to severe pulmonary hypertension. It has demonstrated efficacy in a proof-of-concept study as the substance improved the main hemodynamic parameters, reducing pulmonary vascular resistance and increasing cardiac output from baseline. This study demonstrated that BAY 63-2521 improved the pulmonary hemodynamic parameters to a greater extend than inhaled NO and did not cause serious adverse events [163]. Phase II and III trials of BAY 63-2521 in pulmonary hypertension are ongoing [164-167].

Acrylamide derivatives such as A-350619, A-344905 and A-778935, are sGC stimulators that show no resemblance with the structure of YC-1 although act through a similar manner [168]. They bind sGC at a heme-independent site and synergistically activate sGC in the presence of submaximal concentrations of NO [169]. However, initial pharmacokinetic studies of A-350619 show limited oral bioavailability in rats and dogs [168].

I.2.3.2.2 sGC activators

Stasch and colleagues have presented provocative findings that clinical states of endothelial dysfunction can be associated with the accumulation of oxidized and heme-free sGC, which is insensitive to NO [114;115]. In the past couple of years a small but growing number of sGC activators were discovered which possess the unique ability to activate heme-free or oxidized sGC and thus selectively target the areas affected by the NO resistance syndrome [170]. The best known sGC activator is BAY 58-2667, also referred to as cinaciguat. This compound thus activates sGC even stronger when the enzyme has been oxidized by ODQ or rendered heme-deficient and its effects are additive rather than synergistic in combination with NO [128]. A publication illustrated that the heme-dependent sGC stimulators and heme-independent sGC activators address two different mechanisms of activating the enzyme. This was concluded by the observation that combination of BAY 41-8543 and BAY 58-2667 resulted in an additive effect on enzyme activation [170]. As BAY 58-2667 is based on the porphyrin structure, it has been hypothesized that its mechanism of action is through the binding to the heme binding domain of sGC, displacing the heme and freeing histidine 105. Like the previously discussed BAY compounds, BAY 58-2667 is orally available and has been proven to exert a long-lasting effect over 12 hours [128;136].

Several publications have illustrated that BAY 58-2667 is an effective cardioprotective agent and has hemodynamic effects similar to those of nitroglycerin. The compound relaxes rabbit saphenous artery rings, although it was shown that BAY 58-2667 has an increased potency in saphenous arteries of rabbits with atherosclerosis as compared to normal rabbits [128]. In addition, BAY 58-2667 was more potent in relaxing diabetic as compared to non-diabetic human mesocolon arteries [114]. These findings are consistent with the increased prevalence of the NO-insensitive oxidized or heme-free forms of sGC in various pathological animal models and cardiovascular diseases. In anesthetized dogs, BAY 58-2667 potently decreased mean arterial pressure (MAP), pulmonary artery pressure and mean right atrial pressure [151;171]. Moreover, BAY 58-2667 shows a potent dose-dependent and long-lasting

antihypertensive effect in conscious SHRs [128]. Cinaciguat has also been shown to reduce the coronary perfusion pressure and low doses of the compound dramatically decreased infarction in both isolated and in situ rabbit hearts as well as isolated rat hearts when administered just prior to reperfusion [172]. Furthermore, BAY 58-2667 reduced pulmonary vascular resistance and reversed pulmonary hypertension in chronically hypoxic mice and monocrotaline-injected rats [151]. In mongrel dogs with congestive heart failure and in a canine model of tachypacing-induced heart failure, administration of BAY 58-2667 improved cardiac output, which was accompanied by a significant drop in mean arterial pressure [173]. In addition, the compound has been illustrated to attenuate vascular atherosclerosis and restenosis, liver cirrhosis and renal fibrosis [166].

BAY 58-2667 is currently being evaluated for the treatment of heart failure in early clinical trials. Patients with acute decompensated heart failure were given BAY 58-2667 in two hour intervals for up to six hours. The substance induced potent arterial and venous vasodilatation and reduced both preload and afterload. BAY 58-2667 appeared to be well tolerated with only one out of 33 patients reporting an adverse event [117;174].

Two closely related derivatives, HMR1766 and S3448, are also members of the class of sGC activators, although it has been illustrated that they possess a lower potency compared to BAY 58-2667 [175]. Activation of sGC by these compounds was additive to the activation by NO donors and was potentiated by the heme iron oxidants ODQ and NS2028 [175;176]. Both compounds increased cGMP levels in cultured rat aortic smooth muscle cells and induced vasorelaxation of isolated endothelium-denuded rat aorta as well as porcine coronary arteries and human vascular tissues. Moreover, intravenous bolus injection with HMR1766 decreased arterial blood pressure in anesthetized rats and young pigs [177]. Recently, long-term oral treatment with HMR1766 was illustrated to attenuate monocrotaline-induced pulmonary arterial hypertension [178]. HMR1766 also significantly reduced plaque formation and improved the endothelium-dependent vasodilation in a mouse model of atherosclerosis [176]. Furthermore, SHRs showed improved endothelial function in the isolated aorta and a prolonged life span after HMR1766 treatment [175]. In addition, in a diabetic rat model as well as a rat model of myocardial infarction, treatment with HRM1766 reduced in vivo platelet adhesion [179;180]. Interestingly, Witte and collaborators showed that HMR1766 significantly activated sGC assayed in vitro in internal mammary artery species from patients with coronary artery diseases [181].

In conclusion, both sGC stimulators and activators have shown beneficial effects in different cardiovascular disorders. Moreover, these compounds are not prone to tolerance, suggesting advantages for these novel sGC stimulators/activators over the NO-based therapies [160;182].

### I.2.3.3 CO-releasing molecules and HO-inducers

Evidence supporting that vascular smooth muscle HO is a biologically significant system was initially based on the observation of enhanced expression of HO following experimental vascular injury [183]. Recently, a role for CO in mediating diverse cardiovascular functions is gaining popularity and has been the focus of several comprehensive review articles [184-188]. CO seems to play an important role in the prevention of atherosclerotic lesions, protects against ischemia/reperfusion injury in the heart, attenuates neointima formation after balloon angioplasty, inhibits apoptosis of vascular cells, regulates the blood vessel tone in some vascular beds and has an antiaggregatory effect on platelets [66;189-195]. It has been demonstrated that CO purports some of these cardiovascular functions via the activation of sGC. A rational extrapolation may thus be that by upregulating the HO/CO system, cardiovascular pathologies associated with an aberrant NO/cGMP signalling pathway may be corrected.

The number of ways to deliver CO is fairly limited. One such way is the administration of CO-releasing molecules (CORMs). The best known members of CO-releasing agents belong to a group of transition metal carbonyls such as CORM-1, CORM-2, CORM-3 and CORM-A1, which were reviewed recently [67;196]. These substances mainly differ in the mode and the speed of CO-release. Interestingly, CORM-3 has been shown to induce relaxation of rat aorta and this relaxation was accompanied by an increase in cGMP levels [197]. Application of YC-1 further enhanced the relaxing effect of this CORM compound, indicating that CORM-3 at least partially relaxes these vessels through the activation of sGC. Furthermore, CORM-3 significantly reduced the MAP of rats and this vasodilating effect was even stronger in the presence of YC-1. Also CORM-A1 was shown to cause a slow relaxation of rat aortic rings and to induce a slow decrease of MAP [198]. Boissiere et al. illustrated a relaxing effect of CORM-2 on aortas obtained from normotensive rats and from rats made hypertensive [199].

Acute application of pharmacological inducers to upregulate HO or the use of gene delivery method to overexpress HO-1 is a second way to locally enhance CO production. Pretreatment of vascular tissues with hemin enhanced the endogenous CO production and consequently

suppressed the phenylephrine-induced vasoconstriction in a time-and concentration-dependent manner [65]. Moreover, HO-inducers have been shown to decrease blood pressure in young SHRs and other models of hypertension and this effect was associated with enhanced the sGC concentration and cGMP content [200-202]. It should however be noted that also biliverdin and its derivative bilirubin are antioxidative agents and as such may contribute to blood pressure regulation by affecting oxidative processes and impacting on free radical stability [185;203;204]. In addition, the increased free iron availability after HO activation may also influence blood pressure [205].

# I.3 Soluble guanylyl cyclase and erection

**Based on the paper 'New therapeutic targets for the treatment of erectile dysfunction' from Decaluwé K, Pauwels B, Verpoest S, Van de Voorde J published in J Sex Med. 2011 Dec;8(12):3271-90.**

## I.3.1 Erectile function

### I.3.1.1 Anatomy of the corpora cavernosa (figure I.4)

The penis is comprised of soft tissue and functions as a blood filled capacitor of sufficient rigidity during erection for vaginal penetration. The CC, that are essential to this function are composed of a specialized vascular bed and are surrounded by thick connective tissue, called the tunica albuginea. The cavernous bodies consist of an interconnected network of sinusoidal spaces separated by a trabecular meshwork. The trabeculae are constructed by corporal smooth muscle cells, connective tissue and endothelium which lines the cavernosal spaces. The arterial blood supply arises from the resistance helicine arteries which branch from the deep penile cavernosal artery. The sinusoids are drained by sinusoidal venules which coalesce to form larger veins that pierce the tunica albuginea. The vascular system as well as the smooth muscles of the CC are controlled by nerves of the autonomic nervous system, namely sympathetic nerves originating from T12-L2 and parasympathetic nerves from S2-S4 [206;207].

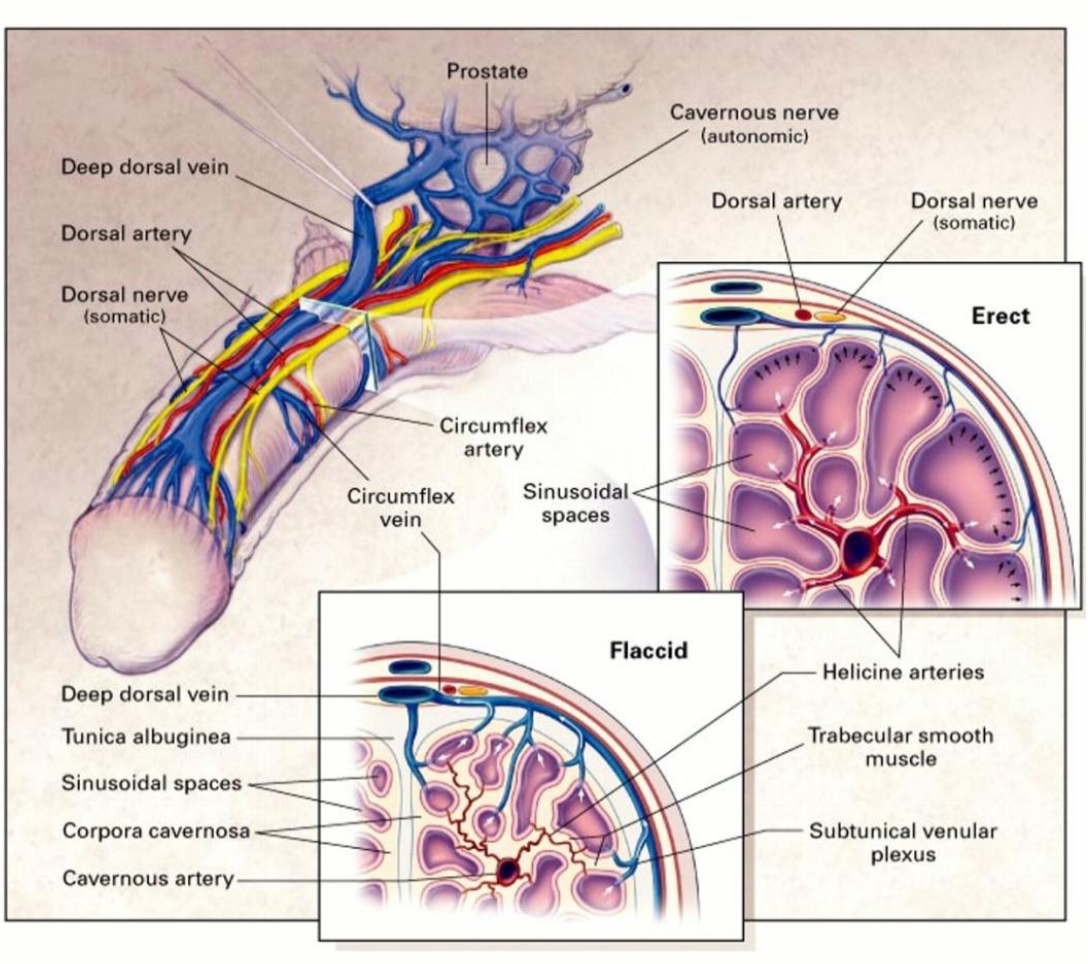


**Figure I.4.** Anatomy and hemodynamics of the corpora cavernosa. Adapted from Kandeel F.R. Male sexual function and its disorders: physiology [208].

### I.3.1.2 Hemodynamics of the erectile process

Human penile erection is a complex phenomenon requiring coordinated interaction between nervous, vascular and muscular systems. Under basal conditions, cavernosal vasoconstriction minimizes blood flow and intracavernosal pressure (ICP) remains low, maintaining the penis in the flaccid state. Upon sexual stimulation, the cavernous nerve terminals and the endothelium of the sinusoids and vessels in the penis produce and release neurotransmitters resulting in the relaxation of arterial and cavernosal smooth muscle cells. This leads to an increase of arterial blood flow to the cavernosal sinusoids whereby, due to the relaxation of the cavernosal smooth muscle cells, these cavities start to expand. As the CC are surrounded by the tunica albuginea which has a limited capability of stretching, the expansion of the cavernosal sinusoids will lead to compression of the subtunical venular plexuses against the overlying tunica albuginea, resulting in an inhibition of the venous outflow, impeding their ability to drain blood and trapping the blood into the cavernosal sinusoids. This mechanical compression of the emissary veins is called the veno-occlusive mechanism and results in a strong increase of the ICP leading to penile erection. The contraction of the ischiocavernous muscles is an adjuvant mechanism that further increases the ICP and penile rigidity, whereas contraction of the bulbocavernous muscle reinforces glans erection. When the erectogenic stimulus ceases, arterial and corporal smooth muscle contraction will occur under the influence of vasoconstrictors released by cavernous nerve terminals and the endothelium of the sinusoids and vessels in the penis. This leads to the narrowing of the arterial lumen and sinusoidal cavities, with reoccurrence of the venous outflow and a decrease in the ICP until detumescence is reached [206;207;209].

### I.3.1.3 Smooth muscle tone regulation

Normal erectile function is thus a complex neurovascular process that depends on a delicate balance between vasoconstrictor agents which cause cavernosal smooth muscles to contract limiting blood inflow, and vasodilators which relax cavernosal smooth muscles leading to increased blood inflow and erection. The control of CC smooth muscle tone is not only influenced by the endothelium but also subjected to strict regulation by both the central and peripheral nervous system. Relaxation of arteriolar and sinusoidal smooth muscles leading to erection appears to be mediated principally by the NO/cGMP pathway [207;209-211]. Other regulatory mechanisms and pathways, notably the VIP/cAMP pathway may also be involved in cavernosal smooth muscle relaxation although NO/cGMP is clearly the most important [212;213]. In contrast, the adrenergic neurotransmission has been reported as a promoter of

penile flaccidity through the activation of α-adrenergic receptors by neurotransmitters such as NE. Any change resulting in an imbalance of this complex physiological process may lead to erectile dysfunction (ED) [206].

### I.3.2 Erectile dysfunction

ED is defined as the inability to achieve and maintain an erection sufficient to permit satisfactory sexual intercourse. Although ED is non-life threatening, it remains a major health problem that seriously affects the patient's quality of life as well as that of their partners. The landmark Massachusetts Male Ageing study published epidemiological data in 1994 further highlighting this growing problem. This study reported that 52% of male participants in a group of 1290 males aged 40 – 70 had at least some degree of ED, with even 10% reporting severe ED. Risk factor adjustment analysis demonstrated that age, heart disease, hypertension, diabetes mellitus, hypercholesterolemia as well as lifestyle factors such as smoking, obesity, sedentary lifestyle and drug abuse are most associated with the condition [207;214].

### I.3.3 New therapeutic targets for treating ED

Currently different treatment options for ED are available, PDE-5 inhibitors such as sildenafil, vardenafil and tadalafil being the first choice. Several other PDE-5 inhibitors are also candidates to enter the market [215;216]. Although large multicenter clinical trials have shown the efficacy and tolerability of these drugs in ED with various etiologies and a broad range of severity, 30-35% of patients fail to respond [217]. Especially patients with severe neurological damage, diabetes mellitus or severe vascular disease may be resistant to PDE-5 inhibitors [218;219]. Prescribing another PDE-5 inhibitor to these patients is useless because when treatment with one PDE-5 inhibitor fails, the chance that another PDE-5 inhibitor will provide successful treatment is estimated at less than 5% [220;221]. The reason why some patients become refractory to the therapy is unclear. It has been suggested that tachyphylaxis to this therapy may develop, however worsening co-morbid illnesses (atherosclerosis and diabetes) seems to be a more likely explanation for the cause of diminished efficacy of PDE-5 inhibitors over time [222]. A major impediment to the use of PDE-5 inhibitors is also the contra-indication for their use in patients who take one of the many available formulations of nitrovasodilators. PDE-5 inhibitors may enhance the vasodilatory properties of nitrates and induce severe life-threatening hypotension. Other contra-indications for the use of PDE-5 inhibitors include patients suffering from severe cardiovascular complications, hemodynamic

instability as well as patients with a history of retinitis pigmentosa or diseases predisposing priapism such as leukaemia or multiple myeloma [223]. Caution should also be taken with concomitant use of a PDE-5 inhibitor and drugs interfering with the cytochrome P450 3A4 (CYP 3A4) isoenzyme, as this enzyme is strongly involved in metabolizing PDE-5 inhibitors. Patients may also discontinue PDE-5 inhibitor treatment because of the low efficacy but also because they suffer from the adverse effects associated with these agents [224;225]. These adverse effects are a consequence of their influence on the vasculature, upper gastro-intestinal tract, retina and other tissues and include headache, facial flushing, nasal congestion, dyspepsia and abnormal color vision or myalgia.

Besides PDE-5 inhibitors, apomorphine is another orally available drug for ED, which acts centrally as a nonselective dopamine receptor agonist. Although frequently used in the past, it is almost never prescribed anymore because of its low efficacy [226]. Intracavernous drug injections with papaverine, alprostadil and/or phentolamine are a second-line treatment option for patients unresponsive to oral drugs. Although this therapy is mostly efficacious, provoking a rapid, predictable, reliable erection, some patients are not comfortable with self-injection [215]. When drugs fail, non-pharmacological therapies can be applied, such as vacuum penile/constrictor devices and penile prosthesis implantation [222]. As with intracavernous injection therapies, non-pharmacological therapies are invasive and unappealing to many patients.

Conclusively, the current treatment options beyond PDE-5 inhibitors are limited, invasive, unappealing to many patients and sometimes ineffective and treatment failure with PDE-5 inhibitors has several important implications. Patients may perceive failure as a sign that they will never again achieve satisfactory sexual function and as a result, their intimate relationships may suffer. Patients clearly want an efficacious, safe, convenient medication with rapid onset. To meet these consumer demands, numerous new therapies are being developed. These include new oral medications, new intracavernosal pharmacotherapies and combination therapies. We are approaching an era where personalized medicine will replace a general treatment strategy and since it is unlikely that any single agent will ever provide a solution for all men with ED, an expanded armamentarium of treatment options will greatly enhance the chances that any given man will be able to find a therapy that is both acceptable and appropriate to him.

### I.3.3.1 Targets associated with vasorelaxation

I.3.3.1.1 Targets of the NO/cGMP pathway (Figure I.5)

*A) Nitric oxide (NOS)*

Because of its cardinal role in erection, NOS is one of the most attractive targets for ED treatment. A strategy to stimulate NOS activity is increasing the concentration of its substrate L-arginine. *L-arginine* induces slow, prolonged relaxations of CC isolated from animal and human origin [227]. Intrapenile delivery of L-arginine is implied in various experimental and clinical studies [210]. While one double-blind, placebo-controlled study showed some subjective improvement of the erectile function with L-arginine therapy over the placebo in men with ED, another randomized placebo-controlled cross-over study revealed that L-arginine is not better than placebo [228;229]. Even when effective, L-arginine cannot be applied to every patient, as L-arginine is contra-indicated in patients with a deficit of carbamyl-transferase (hyperammonemia). Furthermore, oral administration is hampered by extensive pre-systemic metabolism and high doses L-arginine are associated with several adverse effects. By using *L-citrulline* supplementation which is metabolised into L-arginine in the body, the adverse effects were attempted to be avoided. A preclinical study examining patients with mild ED showed some positive results, suggesting that it would be of interest to further explore the strategy of L-citrulline supplementation [230].

*Arginase* is a metallo-enzyme that in a number of cell types converts L-arginine to urea and L-ornithine [231]. Arginase exists in two isoforms: the hepatic type arginase I and the extrahepatic type arginase II. Although arginase I is traditionally thought to exist exclusively in the liver, it has recently been reported that significant expression of arginase exists in vascular endothelium and smooth muscle cells [232]. In endothelial cells, L-arginine is a common substrate for NOS and arginase, suggesting that arginase may down-regulate NO biosynthesis by competing with NOS for L-arginine. Thus NO production may be linked to the regulation of arginase activity [233]. Recently both arginase isoforms have been shown to exist in human CC [234]. Arginase activity has also been shown to be increased in human diabetic CC as well as in age-related ED [235-238]. A dietary L-arginine supplementation as well as acute infusion of L-arginine resulted in improved NO release and enhanced endothelium-dependent vasodilatation in the penis, underlining the importance of the bioavailability of the precursor L-arginine [227-229;239-243]. Furthermore, the arginase inhibitors 2(S)-amino-6-boronohexanoic acid (ABH) and S-(2-boronoethyl)-L-cysteine (BEC) are effective in enhancing neurogenic-mediated relaxation and restore both diabetic- and age-

related ED in different animal models [244-246]. In addition, both inhibitors enhanced the NO-dependent smooth muscle relaxation in human CC, implicating that arginase inhibition may have therapeutic potential for ED from different aetiology [234;235;244].

For its activity NOS requires co-enzymes and co-factors such as *tetrahydrobiopterin* ($BH_4$) [92]. Insufficient or impaired $BH_4$ metabolism increases superoxide anion formation and oxidative stress, resulting in reduced NO production. Administration of the $BH_4$ precursor sepiapterin to old rats improves their erectile responses and prevents the increased superoxide anion production [245]. Furthermore, a placebo-controlled, double-blind, randomized cross-over study with a single oral dose of $BH_4$ demonstrated that it is well tolerated and improves penile rigidity and tumescence in men with ED, indicating a potential clinical application for $BH_4$ in treating ED [247].

*NADPH* is another NOS co-factor. This co-factor is also used by NADPH oxidase. Up-regulation of NADPH oxidase leads to decreased NADPH levels resulting in superoxide anions formation and eNOS uncoupling, promoting vasculopathy [248]. Inhibiting NADPH oxidase by apocynin preserved the erectile function in hypercholesterolemic mice and hypertensive rats, demonstrating that NADPH oxidase inhibitors may have potential value as therapeutics for ED [249;250].

Oxidative stress occurs when cells are exposed to excessive levels of ROS as a result of an imbalance between pro-oxidants and the protective mechanisms conferred by antioxidants [251]. Superoxide radicals are the most important among the ROS with the vascular endothelium being the major source [252]. The interaction between NO and ROS is one of the important mechanisms implicated in the pathophysiological process of ED as well as other vascular diseases [248;252;253]. NO interacts with superoxide to form peroxynitrite, which has been reported to play a central role in atherogenesis resulting from its apoptotic effects. Oxidative damage to the vasculature due to oxidative stress caused by superoxide anion also plays a role in the natural ageing process and has also been implicated in diabetes [254-258]. Increased plasma reactive oxygen metabolites are observed in patients suffering from arteriogenic-related ED, supporting the use of *antioxidant therapy* as a prophylactic tool promoting erectile ability [259]. Antioxidant defences limit the indiscriminate damage caused by oxygen-free radicals and could therefore be beneficial for treatment of ED. Antioxidants can be either endogenous or exogenous and three basic types have been described being

antioxidant enzymes (superoxide dismutase (SOD) and catalase), chain breaking antioxidants (vitamine E, carotenoids and flavonoids), and transitional metal binding proteins (ferritin) [252]. One study investigated the effect of combined use of vitamin E and PDE-5 inhibitor sildenafil in their effect on erectile function. Vitamin E enhanced the therapeutic effect of the PDE-5 inhibitor in the animal model of diabetes [260]. Another study investigated the role of antioxidants such as alpha lipoic acid (ALA) and linolenic acid in the prevention of cavernosal dysfunction in a diabetic rat model [261;262]. Also the novel antioxidant AC3056 (2,6-de-t-butyl-4-((dimethyl-4-methoxyphenylsilyl)-methyloxy)phenol) partially reverses diabetes-related ED in rats. Interestingly, AC3056 also improves the NO-mediated corporal responses of diabetic patients, further supporting the potential of antioxidants to treat ED [263]. This study indicated that administration of ALA was associated with preservation of NANC cavernosal relaxation in diabetic models. These and other studies illustrate that the use of antioxidant therapy may enhance preservation of erectile tissue health [264-271]. However, the role of these antioxidants needs to be further quantified in both animal and human experiments before they can be used in humans. A clinical trial illustrated that combining endothelial antioxidant compounds (EAC) with sildenafil in patients with arteriogenic ED significantly improved the success rate to sildenafil from 45% to 68%, while the success rate for EAC alone was 32% [272]. Furthermore, Morano et al. (2007) documented a synergistic beneficial effect of propionyl L-carnitine and sildenafil on ED which could relate to reduced monocyte oxidative activity [273].

*Impaza*, a preparation containing ultralow doses of antibodies to eNOS, improves ED in a blind, placebo-controlled, randomized clinical trial [274;275]. The effect of impaza relates to restoring the NO production and thus elevating cGMP in the cavernous penile bodies. Impaza is well tolerated with headache being the only adverse effect reported. Although impaza is less efficacious than PDE-5 inhibitors, it may hold promise for patients for which PDE-5 inhibitors are contra-indicated. Another clinical study illustrated that combined administration of impaza and nitrates was highly efficacious and safe without sharp blood pressure drops [275].

As the predominant enzyme responsible for penile erection, NOS is a very attractive target for *gene therapy* [276]. The initial demonstration that gene therapy for ED is feasible and that the modulation of NOS expression is a valid target was published in 1997 [277]. This study reported that treatment of rats with a small amount of a construct containing the rat penile

iNOS enzyme coding region in a plasmid injected directly into the CC, improved ageing-related ED. This approach was able to correct the defective erectile response in the ageing rat for at least 10 days without any detectable side-effects. Attention for eNOS as a potential target for gene therapy quickly followed. Gene transfer of eNOS increased the expression of eNOS and the $Ca^{2+}$-dependent NOS activity in penile tissue [278]. Adenoviral gene transfer of eNOS to the aged CC was demonstrated to restore eNOS activity, mRNA expression and to increase corporal cGMP levels resulting in significant improvement in cavernous nerve stimulated erectile response as well as agonist-induced erectile response to the endothelium-dependent vasodilator ACh and the PDE-5 inhibitors [279-281]. Additionally, streptozotocin (STZ)-induced diabetic rats transfected with eNOS showed complete resolution of their erectile impairments as measured by cavernous nerve stimulation or intracavernous injection of ACh. Conclusively, gene transfer of eNOS has been described to correct ED in both aged and diabetic rats at least for 5 days [281]. nNOS is another good candidate for gene therapy of ED and in particular penile nNOS (PnNOS) because of the tissue-specific control of its enzyme activity [282]. Intracavernosal gene therapy with PnNOS cDNA rectified ageing-related ED in a rat model for at least 18 days when given by electroporation in a helper dependent adenovirus at low viral loads. This justifies the hypothesis that the therapeutic increase of PnNOS levels may ameliorate a deficient or insufficient NOS responsible for ED [283]. Although gene therapy with iNOS reversed ED, compared to eNOS and nNOS, iNOS is the least ideal gene candidate for therapeutic use in ED as it produces unlimited high concentrations of NO. Although sustained production of NO via iNOS may in the short term restore erectile function, long term production can be toxic to endothelial and smooth muscle cells of the penis. In contrast, both eNOS and nNOS are tightly regulated and produce physiological relevant levels of NO in the endothelial cells and cavernous nerves, which will translate to a more reliable therapeutic use [276].

Besides NOS isoforms, also NOS regulatory proteins are attractive candidates for gene therapy. Arginase receptor blockers have been shown to be effective in restoring age-related erectile function. The use of arginase inhibition as a new strategy for treating ED was further investigated by studying the effect of gene transfer of anti-arginase [246]. Adeno-associated virus (AAV) gene transfer of anti-arginase to the aged mouse penis decreased arginase I protein and mRNA and restored endothelial and erectile function in vivo as a direct result of an increase in penile constitutive NOS activity and elevated penile cGMP levels. These

studies further support the potential use of arginase inhibition in order to restore erectile function when the latter is impaired.

Over-expression of the endogenous protein inhibitor of NOS (PIN), which inhibits nNOS after binding, leads to a reduced erectile response. Conversely, silencing of PIN through gene therapy increased ICP in aged rats resulting in erection [284].

The antioxidant SOD, an enzyme that catalyzes the dismutation of superoxide into hydrogen peroxide and water, plays a pivotal role in protecting the endothelial cells against the free radicals and the prevention of peroxynitrite formation, which is extremely cytotoxic and contributes to tissue injury, vascular tone alteration and organ dysfunction. There are 3 known isoforms of SOD. The extracellular (EC)-SOD is thought to play a critical role in modulating the redox state of the vascular interstitium and prevents the pathophysiological effects of superoxide anions in the vasculature [285]. Oxidative stress, resulting from an imbalance between pro- and anti-oxidants is quite prominent in certain chronic disease states, including diabetes, hypercholesterolaemia and ageing. As antioxidants reverse ED, antioxidant-based gene therapy for both ageing- and diabetes-related ED may also improve endothelial and corporal function [286]. This hypothesis was confirmed by the finding that EC-SOD gene therapy led to decreased superoxide anions and increased cGMP levels in the aged penile vasculature and was found to enhance erectile response to cavernosal nerve stimulation and endothelium-dependent erectile responses [287]. In addition, in vivo adenoviral gene transfer of EC-SOD was able to reduce corporal superoxide anion levels and to raise cavernosal cGMP levels by increasing NO bioavailability and thus restoring erectile function in the STZ-induced diabetic rats [288]. Intracavernosal EC-SOD gene transfer has thus been demonstrated to restore both age-associated as well as diabetes-associated erectile function and may represent a novel therapeutic strategy for the treatment of ED.

*B) <u>Guanylyl cyclase (GC)</u>*

As cGMP is one of the most important second messengers involved in penile smooth muscle relaxation and erectile function, agents that elevate cGMP levels through direct stimulation of GC may offer benefit over the currently existing therapies by being independent of endogenous NO. Vast experimental and clinical evidence indicates that reduced bio-availability and/or responsiveness to endogenously produced NO contributes to the development of ED [289;290]. Impaired NO delivery to the smooth muscle cells upon sexual

stimulation has been put forward as the mean reason for PDE-5 inhibitor treatment failure [218;291]. Under such conditions the use of direct GC stimulators resulting in an NO-independent intracellular cGMP increase, might represent a promising alternative approach in the treatment of ED.

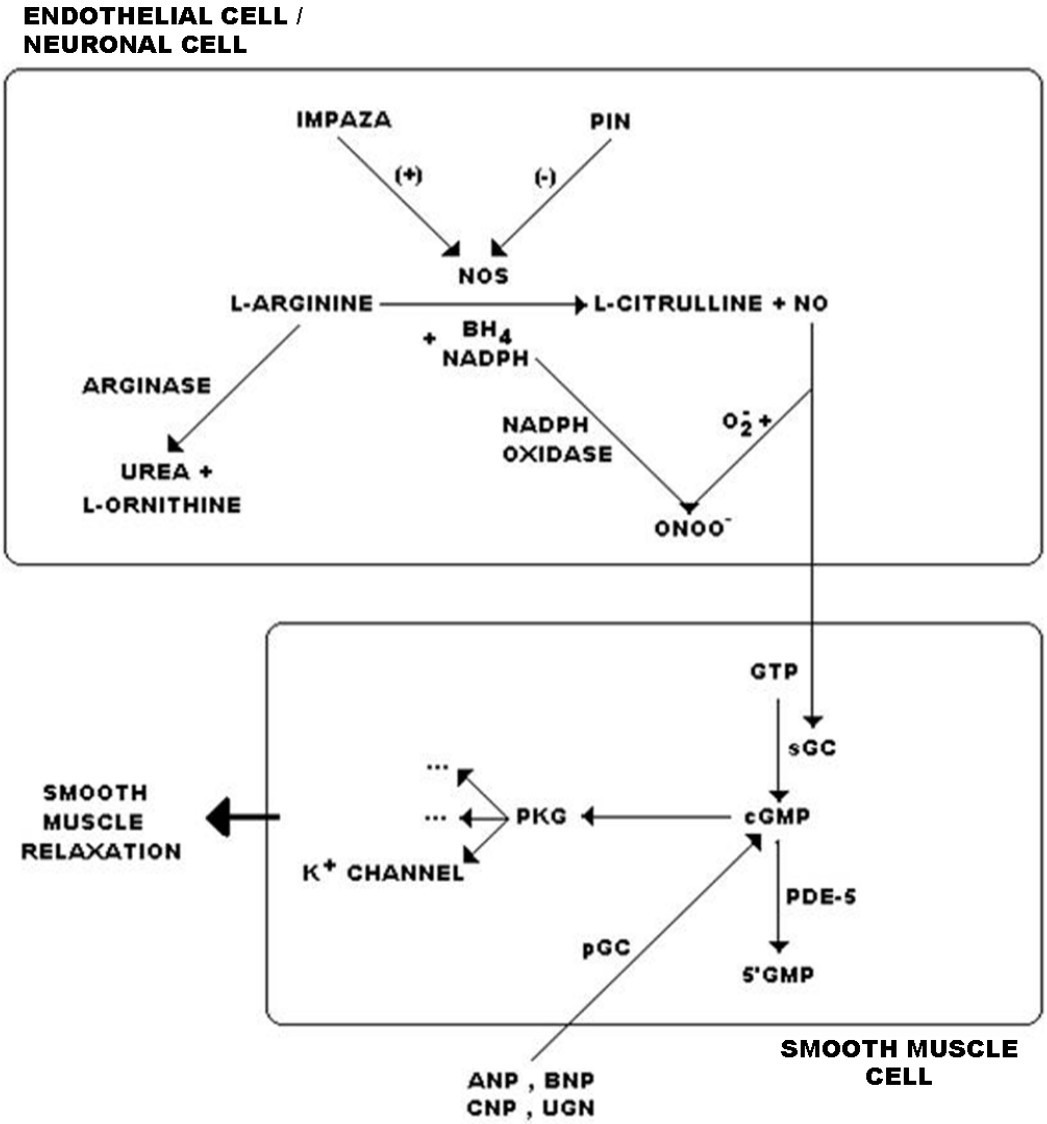


**Figure I.5** shows all potential targets for treating ED that are related somehow to the NO/sGC pathway.

- *Soluble guanylyl cyclase*

It is generally accepted that sGC, as the major effector molecule for NO, plays a very important role in penile smooth muscle cell relaxation. Because of its central role in this pathway, sGC represents an attractive and highly promising new therapeutic target for treating

ED [147;166;292;293]. Several sGC stimulators and activators have already been shown to exert pro-erectile effects in both physiological and pathophysiological conditions.

NO and NO-donors are the best studied sGC stimulators. In isolated CC strips and penile arteries NO, nitrosothiols like S-nitrosocysteine, S-nitroso-N-acetylcysteine, S-nitrosogluthathione and NO-donors like nitroglycerine and isosorbide dinitrate cause relaxation and mimic the vasodilatation induced by electrical field stimulation (EFS) [294-301]. Despite NO is the main neurotransmitter involved in erection, therapeutic application of NO-donors like nitroglycerine, SNP and linsidomine chlorhydrate (SIN-1) for the treatment of ED has been less promising due to the poor efficacy and adverse effects secondary to non-specific interactions of NO with other biological molecules [294;302-304]. Another major drawback of this therapy is the development of tolerance after prolonged use [305].

In vitro studies using the sGC stimulator YC-1 have demonstrated that this compound is effective in relaxing CC tissue isolated from different species such as rat, rabbit [306;307]. YC-1 caused concentration-dependent relaxant responses in NE-contracted CC preparations and enhanced the relaxing effects of the NO-donor SNP, supporting the synergism between NO and YC-1 in CC tissue strips [307;308]. YC-1 also enhanced NO- and cGMP-dependent nerve-mediated relaxant responses elicited by EFS of NE-contracted CC [306]. ODQ, a known potent inhibitor of sGC [309], partially antagonised the relaxing effect of YC-1 in pre-contracted CC strips, confirming the mechanism of action of the compound. Interestingly, incubation of CC cells with YC-1, but not with PDE-5 inhibitor sildenafil, led to a concentration-dependent increase in cGMP. This increase was even further potentiated in the presence of SNP [307]. In vivo pharmacological studies illustrating that YC-1 induces penile erection in a conscious rat model, supports these in vitro findings. Upon intracavernous injection, YC-1 elicited dose-dependent erectile responses. The compound also enhanced erections induced by cavernous nerve stimulation and by subcutaneously administered apomorphine when given systemically [306]. Moreover, intraperitoneal administration of YC-1 evoked penile erection in rats with 70% incidence [307]. These data suggest that stimulation of the sGC activity by YC-1 may activate pro-erectile pathways and thus facilitate penile erection in vivo. Although YC-1 has already been proposed for the treatment of cardiovascular and thrombotic disorders, it may thus also be beneficial for the treatment of ED. However, the cardiovascular effects of YC-1 require further investigation as co-

administration of YC-1 and SNP elicited hypotension because of sGC-activation in vascular tissues other than CC [144].

In vitro studies indicate the pro-erectile effect of another sGC stimulator, BAY 41-2272, as it induces concentration-dependent relaxation of rabbit, mouse and human CC [310;311]. The compound was 32 times more potent in lowering NE-induced corporal tone than YC-1 and twice as potent as the NO-donor spermine-NONOate. Importantly BAY 41-2272 also enhanced nitrergic relaxations induced by EFS, further supporting the concept that BAY 41-2272 synergizes with NO. The sGC-dependency of BAY 41-2272 was indicated by the significant inhibition of the relaxant response by ODQ [312]. Furthermore, the concentration-response curve to BAY 41-2272 was shifted to the right in the presence of a NOS inhibitor, suggesting that endogenous basal NO levels could potentiate the effect of this sGC stimulator [310]. Moreover, in vivo studies using rabbits have shown that BAY 41-2272 given either intravenously or orally, causes penile erection and produces a synergistic effect when combined with SNP. In the absence of NO, BAY 41-2272 induced only a very weak erection in these rabbits [313]. The efficacy of the sGC stimulator in the rabbit model seems comparable to that of sildenafil. The dependency of the compound's efficacy on the presence of NO suggests that BAY 41-2272 may enhance erections induced by sexual stimulation, without major systemic side-effects. In a diabetic rat model, BAY 41-2272 potentiated the remaining nitrergic relaxation responses, suggesting that NO-independent sGC stimulators could be more useful than PDE-5 inhibitors in the treatment of ED in patients with long-term diabetes. In contrast to sildenafil, BAY 41-2272 was able to enhance the residual nitrergic relaxation of the anococcygeus muscle isolated from STZ-induced diabetic rats [314]. This suggests that sGC activation may be more efficacious than PDE-5 inhibitors in the treatment of diabetes-induced ED and possibly also in ED related to cavernous nerve injury during radical pelvic surgery.

Both in vitro and in vivo studies have been performed to evaluate the potency of A-344905 and A-350169 to induce or enhance erection. After pre-treatment with a NOS inhibitor, A-350619 relaxed CC tissue strips in a concentration-dependent manner whereas A-344905 did not cause relaxation. However, when A-344905 and A-350619 were tested in the presence of SNP, both compounds relaxed the strips more potently and A-344905 then showed a similar efficacy as A-350619 in inducing CC tissue strip relaxation in spite of the lower potency in enzyme assay. In addition, injections of A-344905 induced penile erection in rats and when a

lower dose of A-344905 was tested in combination with a non-effective dose of apomorphine, this induced penile erections comparable to the optimal dose of apomorphine, suggesting a synergy with endogenous NO production. Moreover, systemic administration of A-350619 induced penile erection in a conscious rat model. Interestingly, both compounds did not elicit other cardiovascular effects [168;293].

In addition, S3448 and HMR-1766 have been shown to stimulate the activity of purified sGC from human CC homogenate in a concentration-dependent and reversible fashion. Moreover, S3448 elicited a concentration-dependent relaxation in pre-constricted CC strips [175].

Taken together, this experimental evidence indicates that the use of sGC stimulators might represent a novel promising strategy for the therapy of ED. In the CC, local and transient high levels of NO would be expected by the release of NO as a neurotransmitter from NANC nerve terminals upon sexual stimulation. For the treatment of ED, it would be very useful to develop an sGC stimulator that binds to the allosteric non-heme site and that in itself has little or no effect on increasing the basal activity to minimize the cardiovascular side-effects, but that in the presence of released NO during sexual excitation strongly amplifies the erectile effects of NO [293]. This description is consistent with the above mentioned characteristics of the sGC stimulators, which advance them as efficacious drugs for treatment of ED from different aetiologies. However, the currently existing sGC stimulators have been shown to activate both subunits of the enzyme [315]. Because of the abundance of sGC in other tissues and organs besides CC, one would expect these drugs to exert comparable side-effects as PDE-5 inhibitors, such as for instance headache and dyspepsia through sGC activation in the vasculature and upper gastro-intestinal tract. Considering the significant systemic effects of these sGC stimulators, the possibility of life-threatening hypotension cannot be ignored, especially if they are used in combination with nitrovasodilators or PDE-5 inhibitors [144;160]. The presence of two different isoforms of the enzyme offers a potentially more selective therapeutic approach [308]. By performing RT-PCR, it was demonstrated that the main sGC-isoform expressed in human CC is the $sGC\alpha_1\beta_1$ isoform [34;308]. When wanting to selectively target penile tissue in order to have as little as possible side-effects, it would be favourable to activate only the sGC isoform with the least effect in the systemic arterial system. Therefore, information on the physiological importance of the sGC isoforms in penile erection as well as in other physiological systems is necessary to validate sGC subunits as

therapeutic targets which could lead to the development of isoform-specific activators with restricted adverse effects [311;316].

Besides NO, CO has been described as another physiological activator of sGC, although CO is far less potent than NO in activating sGC [317;318]. The gaseous molecule is formed during the degradation of heme to biliverdin by the enzyme HO. Two isoforms of the enzyme have been characterised, designated as the inducible HO-1 and the constitutive HO-2 [319]. Immunocytochemical studies have demonstrated immunoreactivity for HO-1 and HO-2 in perivascular nerves of human erectile tissue [320]. Moreover, CO has been shown to relax isolated CC tissue, an effect that was even further enhanced by the application of the NO-independent sGC stimulator YC-1 [71]. Some authors suggested that impaired CO-mediated vasodilatation could be implicated in ED [290;321]. In addition, gene therapy using HO-1 cDNA-liposome complex transfer increased intracavernosal cGMP concentration in rats with subsequent sinusoidal relaxation [322]. Furthermore, an HO-1 inducer restored HO-1 gene expression and enzymatic activity to normal control levels, significantly improving erectile function in diabetic rats [323;324]. These data suggest that CO-releasing molecules or enhancers of HO-1 and/or HO-2 could be beneficial in treating ED, although this has to be examined in more detail.

- *Particulate guanylyl cyclase*

As mentioned membrane-bound pGC also produces cGMP, thus stimulating pGC is another potential approach enhancing the NO/cGMP pathway. This membrane-bound enzyme is the target for atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) as well as guanylin peptides such as uroguanylin (UGN) [1]. The presence of GC-B has been demonstrated in CC and helical artery smooth muscle cells and the production of cGMP by pGC was shown in rabbit and rat CC membranes stimulated by CNP, ANP and BNP [325]. Furthermore, CNP was able to relax rabbit, rat as well as human CC strips [326]. ANP and UGN have also been studied in vitro for their human CC relaxant capacities. Both agents were able to enhance relaxation of human CC strips in organ bath experiments. The relaxant capacity of UGN was mediated in large part by a rise in intracellular cGMP and not influenced by the addition of either a NOS inhibitor or ODQ [327]. These results may hold promise for the future treatment of ED with pGC stimulators, especially in patients lacking endogenous NO supply. However, only a limited number of animal studies have focused on this target. More research will need to be done to fully

understand the role of pGC in erectile function and its potential as a new molecular target for treatment of ED [328].

*C) Cyclic GMP-dependent protein kinase (PKG)*

Enhancing the activity of cGMP's most important target, PKG, by increasing the cGMP concentration was the direct goal of PDE-5 inhibitors. Due to non-selective PDE inhibitory activity, caffeine consumption improved the erectile function of diabetic rats [329]. Furthermore, intracavernous cGMP administration in patients with ED resulted in penile erection [330]. Moreover, gene therapy using PKG restored the enzymes activity improving the erectile function in diabetic animals [331;332]. Conclusively, agents that increase PKG activity could be further explored for there potential as treatment of ED.

*D) Potassium ($K^+$) channels*

$K^+$ channels have been shown to play a fundamental role in both the physiological and pathophysiological regulation of smooth muscle tone in various tissues, including the CC [333]. Among the several subtypes of $K^+$ channels described in human CC, the metabolically regulated adenosine triphosphate (ATP)-dependent $K^+$ ($K_{ATP}$) channel and the big-conductance $Ca^{2+}$-dependent $K^+$ ($BK_{Ca}$) channel are thought to be the most physiologically relevant [333-336]. Emerging evidence suggests that impairment of $K^+$ channel activity in cavernosal and arterial smooth muscle cells or reduced passive conductance of electrical signals can lead to ED [337-339].

Several $K^+$ channel openers have been shown to cause effective relaxation of isolated corporal preparations and to produce functional erections offering clinically therapeutic interests as a treatment modality for ED [340]. The opening of $K^+$ channels leads to the efflux of $K^+$ following its electrochemical gradient out of the corporal smooth muscle cell. As positive charge moves out, the corporal smooth muscle cell hyperpolarizes. This reduces excitability, primarily by inhibition of $Ca^{2+}$ influx through L-type voltage-dependent $Ca^{2+}$ channels [341]. Interestingly, blocking the $BK_{Ca}$ channel in vitro led to an increase in NE-induced contractions of human CC strips, whereas the $BK_{Ca}$ channel opener NS1619 was able to reduce these contractions [340;342-344]. Furthermore, the observation that NS11021 relaxes rat and also human CC and facilitates erectile responses in anesthetized rats [345]. In addition to the observation that several other $BK_{Ca}$ openers elicited pro-erectile responses in animal models, these data suggest the potential clinical use of $BK_{Ca}$ openers as new treatment

modality for ED [346;347]. Besides $BK_{Ca}$ openers, $K_{ATP}$-channel openers such as pinacidil and levcromakalim/cromakalim also efficaciously relax rat penile erectile tissue and dose-dependently increase the ICP, supporting a potential role for $K_{ATP}$-channel openers in treating patients suffering from ED [348;349]. These results strongly support the idea that the $K^+$ channel could become a very important target in treating patients suffering from ED.

However, as pinacidil however reduced systemic arterial blood pressure, hemodynamic side-effects compromise the use of these drugs. Intracavernosal application of $K^+$ channels through the use of gene therapy may be a way to overcome this obstacle. Gene transfer of the $K_{ATP}$-channels has already been shown to restore ageing-induced ED in rats, supporting the potential of gene therapy targeting $K^+$ channels in the treatment of ED [350]. In addition, BKCa channels have also been subject to gene therapeutic strategies. In elegant experiments, it was demonstrated that injection of hSlo cDNA, which encodes for the α-subunit of $BK_{Ca}$ channels, into the rat CC could increase gap junction formation and enhance erectile responses to nerve stimulation in aged and diabetic rats [351-354]. The hSlo cDNA transfection was sustained for at least 2 months. Furthermore gene therapy with the $BK_{Ca}$ gene has been shown to restore erectile function in atherosclerotic monkeys [355].

There is also some, albeit limited clinical experience with $K^+$ channel modulators for the treatment of ED. An orally available $BK_{Ca}$ channel opener BMS 223131 has been evaluated in phase II study and PNU-83757, another $K^+$ channel opener developed for intracavernosal therapy, efficaciously improved erectile function in men with ED of vascular etiology [356;357]. Interestingly, gene transfer of the $BK_{Ca}$ channel for the treatment of ED was the first phase I trial of human gene therapy approved by the US FDA [358]. The final conclusion of this phase I trial was that in a single dose escalation study, ion channel gene transfer with $BK_{Ca}$, can be administered safely to men with ED without adverse effects. These data support the potential utility of gene therapy in the treatment of ED. Further data demonstrating long-term efficacy of this ion-based gene therapy for ED are warranted.

I.3.3.1.2 Targets associated with other vasodilatory pathways (Figure I.6)

*A) Histamine receptors*

Histamine is a vasoactive amine which is endogenously produced in many organs, including the penis. In the erectile physiology, histamine has been suggested as a neurotransmitter involved in the peripheral mechanism of nerve-induced penile erection. Furthermore, the

widespread clinical use of $H_2$ receptor antagonists for the treatment of peptic ulcer disease has been associated with reports of erectile failure, supporting the idea that histamine is involved in normal erectile function [359-361]. Importantly, histamine operates in both central and peripheral nervous system implying that the contribution of this pathway is not straightforward. Many discrepancies have already been published regarding the effect of histamine and its different subtype receptors on penile erection [362].

Histamine has been shown to induce smooth muscle contraction, relaxation or a contraction followed by relaxation in the human CC [363]. However, intracavernosal injection of histamine in men caused erection in all patients and this strong relaxing effect of histamine was indicated to be mediated by activation of $H_2$ receptors [364]. Nimmegeers et al. also found the involvement of $H_2$ receptors in the histamine-induced corporal relaxation and illustrated that this response was independent upon NO [365]. In contrast, Kim et al. found no direct effect of $H_2$ receptor antagonists on the cavernosal smooth muscle cells and suggested that any clinically observed response may be through effects on central nervous system (CNS) function [366]. Along with other research groups they demonstrated the involvement of the $H_1$ receptor in corporal smooth muscle contraction and indicated that $H_1$ receptor antagonism enhanced nerve-induced corporal relaxation [363;366]. Since $H_1$ receptor antagonism and $H_2$ receptor agonism increased NANC-mediated corporal relaxation, these receptors possess potential as an intracavernosal pharmacotherapeutic targets for the treatment of ED [365;366].

Research on the efficacy of interference with histamine receptors is as yet limited, possibly due to the complexity and poorly understood physiological role of histamine in erection. Additional studies need to be performed in order to evaluate its potential use as a therapeutic target for ED.

*B) Peptide neurotransmitters*

The peptide neurotransmitters VIP and calcitonin gene related peptide (CGRP) are localized in the CC. Already two decades ago, these neurotransmitters have been approached as targets for ED [367-370]. In analogy with VIP, CGRP relaxes the CC smooth muscle by activation of AC with subsequent increases in intracellular cAMP, leading to erection. Studies in animals and humans collectively demonstrated that VIP related fibres in the penis are attenuated in diabetic ED [371]. Also CGRP has been shown to be down-regulated in the ageing rat penis [372].

Despite positive results obtained with VIP in vitro, in vivo animal studies provide inconsistent functional effects in response to intracavernosal VIP administration. However, a recently developed VIP analogue specifically for ED treatment, stearyl-norleucine-VIP, enhances both sexual activity and erection in different rat models suffering from ED [373]. Although these results suggest that stearyl-VIP may be considered useful, no further animal/human studies have been performed using this stearyl-VIP. In addition, intracavernous injection of VIP cDNA, which easily incorporates into CC and is expressed for more than 2 weeks, improves erectile response in diabetic rats [374]. Despite some benefit described in a clinical trial, no patients, suffering from ED of various aetiology, experienced sufficient efficacy after intracavernosal VIP injection [375]. However, combined intracavernosal injection of VIP with phentolamine mesylate is already authorized in some European countries [376]. This combination, resulting in a response rate of 75.1% versus 12% for the placebo, was shown to be safe with transient facial flushing as the main adverse effect [377].

Intracavernosal CGRP administration in dogs increased the cavernous arterial flow, inducing cavernous smooth muscle relaxation and venous outflow occlusion [368]. Additionally, gene transfer of prepro-CGRP increased the corporal CGRP concentration and thus cAMP levels in ageing rats, resulting in restoration of the erectile response to ES of cavernosal nerves [378]. Similar positive results were obtained with AAV-mediated over-expression of CGRP in diabetic rats [379]. Comparable to data obtained in dogs, intracavernous CGRP administration in patients dose-dependently increased the penile arterial inflow, cavernous smooth muscle relaxation and cavernous outflow occlusion [369]. Furthermore, intracavernosal CGRP combined with $PGE_1$ showed a high success rate in improving erectile function of patients selected for penile implants [380]. All these studies suggest that in vivo CGRP gene transfer or CGRP up-regulation is an effective approach for improving ED.

*C) Prostaglandin receptors*

Prostaglandin (PG) and thromboxane (TX) derivatives of the arachidonic acid metabolism exert paracrine effects on corporal smooth muscle. The effects of prostanoids are mediated through prostanoid receptors that are G-protein-coupled with different transduction systems. In human CC, cDNAs encoding representatives for $PGD_2$, PGEs, $PGF_{2\alpha}$, $PGI_2$ and $TXA_2$ receptors have been cloned, although the role of all these different receptors is not well established yet [381-383]. $PGE_1$ and $PGE_2$ are the most abundant prostanoids synthesised by the corporal smooth muscle cells and cause relaxation by activating the AC/cAMP pathway

and by decreasing NE release from sympathetic nerves [384;385]. Intracavernosal injection as well as intraurethral insertion of $PGE_1$ enhancing the erectile function is already used clinically [384]. $PGE_1$ and $PGE_2$ act on specific prostanoid receptor subtypes designated $EP_1$-$EP_4$. Prostanoid receptor subtypes $EP_2$ and $EP_4$ are expressed in human corporal smooth muscles and relax CC through AC activation. Specifically targeting these receptors could offer an alternative therapeutically valuable approach for the treatment of ED [382]. In this regard the $PGD_1$-selective agonist AS702224 more effectively relaxed human cavernosal tissue than $PGE_1$ and $PGD_2$, a finding that was further confirmed in vivo using rabbits and rats [386]. Besides these vasorelaxing prostanoids, several constrictor prostanoids such as $PGH_2$, $PGF_{2\alpha}$ and $TXA_2$ are synthesised by human CC [387]. Although their role as penile constrictors is not well established yet, impaired relaxation in human diabetic tissue was shown to be normalized by thromboxane receptor inhibition. Furthermore, diabetic patients show enhanced sensitivity to U46619, a thromboxane receptor mimetic. These experiments support the potential use of thromboxane inhibitors for treating ED.

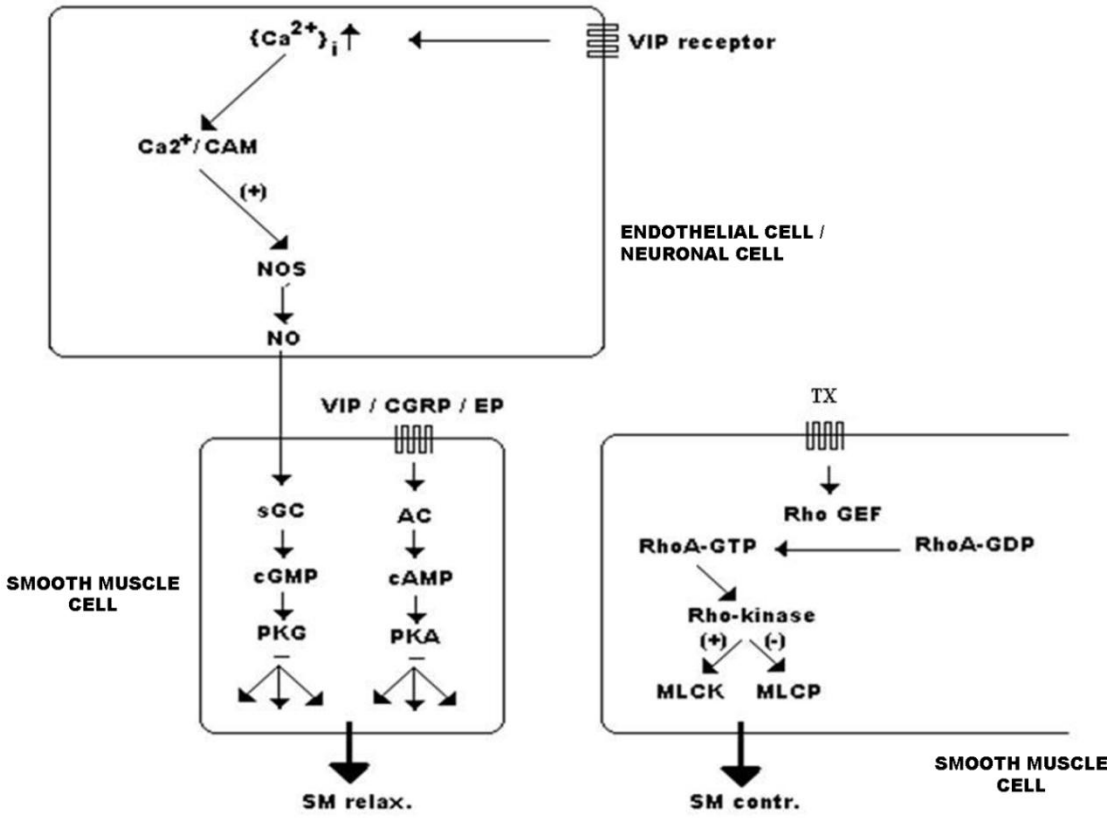


**Figure I.6** illustrates the molecular mechanisms underlying VIP and CGRP as well as prostanoid-induced relaxation or contraction.

**I.3.3.2 Targets associated with vasoconstriction (Figure I.7)**

A potential new approach for the treatment of ED may be based on directly inhibiting biochemical pathways that control smooth muscle contraction [388;389].

*A) Rho-kinase*

Of particular interest is the activity of the RhoA/Rho-kinase-mediated $Ca^{2+}$-sensitization pathway which is believed to be significantly involved in maintaining basal noradrenergic tone in the penile flaccid state [390-392]. Western blotting analysis has demonstrated the presence of Rho-kinase in both human and rabbit penile corporal tissue [393;394]. Abundant evidence has shown that elevated RhoA/Rho-kinase activity contributes to the pathogenesis of diseases such as diabetes and hypertension and multiple epidemiological studies have suggested that ED is strongly associated with these chronic diseases [395-401]. Under such conditions, inhibitors of the RhoA/Rho-kinase pathway could offer a potential alternative for the treatment of ED associated with these chronic diseases [390]. In addition, Rho-kinase inhibition may be explored as an alternative therapy when PDE-5 inhibitors are contra-indicated for instance because of concomitant use of nitrovasodilators.

Based on the hypothesis that antagonism of Rho-kinase results in increased CC pressure, initiating the erectile response independently of NO, the effect on cavernosal tone of drugs interfering with Rho-kinase activity was examined. Y-27632 is a pyridine derivative that has been shown to be 200 times more specific for Rho-kinase than for other protein kinases. It exerts its effect by competing for the ATP binding site on Rho-kinase preventing Rho-kinase mediated phosphorylation of MLCP [402;403]. Another family of compounds derived from isoquinoline sulfonamide shows selectivity for Rho-kinase. HA-1077 (fasudil hydrochloride) is an orally available Rho-kinase inhibitor and like Y-27632 competes for the ATP binding site on Rho-kinase. However, HA-1077 is also a potent inhibitor of PKA and PKC, which makes it difficult to separate activity of the Rho-kinase-mediated pathway from PK-mediated $Ca^{2+}$ sensitization using this reagent [404;405]. H-1152-P is another recently developed Rho-kinase inhibitor that is similar in structure to HA-1077 except that it has two extra methyl groups which significantly enhance its inhibitory effect and selectivity for Rho-kinase [406]. SAR407889 is a Rho-kinase inhibitor under development which has been shown to be eightfold more active than fasudil in the treatment of hypertension [407].

Y-27632 has been shown to inhibit contraction of human, rat and rabbit smooth muscles in penile CC strips in a concentration-dependent way [408-410]. Y-27632 had no effect on

intracellular $Ca^{2+}$ concentrations but attenuated the contractions caused by NE. In a rat model, intracavernous administration and also topical application of Y-27632 increased ICP/MAP in a dose-dependent manner and this effect was proven to be NO-independent [408;411]. Moreover, Y-27632 potentiated the increases in ICP/MAP to cavernous nerve stimulation at minimal voltage, suggesting that RhoA/Rho-kinase is constantly active and plays an important role in maintaining the penis in the flaccid state [392;408]. Furthermore, Y-27632 also potentiated the rise in ICP/MAP resulting from intracorporal injection of the NO-donor NOR-1 [409].

Additionally, inhibition of the RhoA/Rho-kinase pathway using Y-27632 and other Rho-kinase inhibitors has been shown to improve erectile function in rat models associated with ED risk factors such as ageing, diabetes mellitus, hypertension and hypogonadism. An age-related increase in RhoA expression has been documented in rat vascular tissues and has been held responsible for age-associated vascular disorders [397]. In old rats, the impact of Rho-kinase inhibition following intracavernosal Y-27632 could be seen immediately and a significant improvement in erectile response was observed at all voltage levels of stimulation [412]. Y-27632 administration has been shown to attenuate the ageing-related changes in male erectile function seen in rats [396;413].

The RhoA/Rho-kinase pathway may be a major molecular mechanism involved in the pathogenesis of diabetes-related ED [398;399;401]. Corporal RhoA and Rho-kinase protein levels were shown to be significantly higher in the STZ-induced diabetic rats [414]. In addition, western blot analysis showed that the inhibitory MYPT-1 phosphorylation of MLCP was increased in corporal tissue isolated from the STZ-induced diabetic rats. The up-regulation of the RhoA/Rho-kinase pathway results in heightened corporal smooth muscle tone in diabetic rats by inhibiting MLCP and suppressing eNOS activity which leads to impaired erectile function. Both Y-27632 and HA-1077 have been investigated in vitro in CC isolated from diabetic mice [415]. Diabetic mouse CC exhibited relaxations in response to the Rho-kinase inhibitors that were similar to those observed in CC isolated from non-diabetic mice. Direct intracavernosal injection of the Rho-kinase inhibitor Y-27632 caused a dose-dependent increase in ICP/MAP in both the control and STZ-induced diabetic rats. The increase in ICP/MAP in response to Y-27632 in the diabetic rats was however significantly greater at all doses studied, further supporting that Rho-kinase activity is increased in the diabetic CC and that Rho-kinase inhibitors may be useful for the treatment of ED [414;416]. Very recently, it has been illustrated that SAR407899 dose-dependently relaxed the pre-

contracted rat, rabbit as well as human CC and that the compound was also effective in vivo. Moreover, SAR407899 has a similar potency and efficacy in healthy versus diabetic animals and possessed a greater potency and longer duration of action compared to sildenafil [417].

Since the first demonstration that enhanced RhoA/Rho-kinase activity contributes to the increased peripheral vascular resistance in several models of hypertension, accumulating evidence over the years further underlined the importance of RhoA/Rho-kinase signalling in the pathogenesis of hypertension and hypertension-related ED [395;418-420]. In stroke-prone SHR, a well-recognized genetic model of hypertension and DOCA salt-induced hypertensive rats, ICP/MAP was significantly lower that that of control rats in response to electrical stimulation (ES) of the major pelvic ganglion [418]. Intracavernosal administration of Y-27632 improved the erectile function in these hypertensive rats, although to a lesser extent compared to normotensive rats [421]. Furthermore, HA-1077 was used to investigate the role of chronic Rho-kinase inhibition in the prevention of pelvic atherosclerosis and the resulting vasculogenic ED in rats. Treatment with HA-1077 partially but significantly ameliorated the development of pelvic atherosclerosis in addition to normalizing erectile function [420]. In addition, also H-1152-P was demonstrated to possess pro-erectile effects in healthy rats both in vitro and in vivo. The sustained corporal relaxations induced by this powerful Rho-kinase inhibitor were not affected by the inhibition of the NO-signalling pathway [406]. The observation that Rho-kinase antagonism stimulates penile erection in rats independently of the NO pathway introduces a potential alternative approach for the treatment of ED.

Studies in the rat model of castration have suggested that up-regulation of the RhoA/Rho-kinase pathway in the penis could be an underlying mechanism in hypogonadism-associated ED [422]. Contractile responses to NE are increased in CC muscle strips isolated from castrated rats in parallel with increased sensitivity to Y-27632 and increased protein expression of RhoA and Rho-kinase in penises. In addition, Rho-kinase inhibition was effective in reversing ED in these castrated hypogonadal rats.

Gene transfer of AAV encoding a dominant negative RhoA mutant to the penis of aged rats markedly improved erectile function [396;423]. Moreover, AAV gene transfer of the dominant negative RhoA mutant to the diabetic penis decreased RhoA/Rho-kinase protein expression and restored erectile function in vivo through two distinct mechanisms: a reduction in the increase in penile vascular tone and improvement in endothelial-derived NO [414].

Interestingly, the Rho-kinase inhibitor SAR407899 tested in patients with mild to moderate ED, significantly increased the duration of penile rigidity. The compound is safe and well tolerated with as major adverse effects headache and orthostatic hypotension. (ClinicalTrials.gov identifier: NCT00914277) This clinical trial strongly indicates that Rho-kinase inhibition presents an interesting feature to approach ED therapy.

Besides the previously discussed Rho-kinase inhibitors, statins - although described as risk factors for ED - are reported to possess pro-erectile effects by counteracting RhoA/Rho-kinase activity. Rosuvastatin partially restored the erectile response in obese-diabetic rats by lowering RhoA/Rho-kinase expression levels [424]. Atorvastatin also ameliorated the erectile response to ES of the cavernous nerve in diabetic rabbits and restored sildenafil responsiveness in these animals [425]. Similarly, atorvastatin improved sildenafil responsiveness in SHRs [426]. Improvement of sexual function and increased sildenafil responsiveness by atorvastatin is also reported in men suffering from moderate-to-severe ED [427]. Moreover, postoperative statin use in patients that underwent radical retropubic prostatectomy contributes to earlier recovery of the erectile function. Potency of patients 6 months after the surgery was 26.1% for the group taking only sildenafil and 55% for the group taking sildenafil plus atorvastatin [428].

As to the role in managing ED, the NO-independent mechanism of Rho-kinase in potentiating penile erection presents a unique manner to approach ED therapy [211]. However, further investigation is necessary to determine clinical effectiveness and safety. Great concerns are raised about the profound systemic effects of Rho-kinase inhibitors, suggesting that this pharmacological therapy has to be restricted to local delivery. So the primary question is whether these compounds can be developed to target the RhoA/Rho-kinase pathway in the vasculature of the penis while minimizing unwanted effects on other vascular beds. It has been demonstrated that both Rho-kinase isoforms α and β are present in corporal tissue [393;394]. Keeping this in mind, it would be interesting to identify the physiological roles of these two isoforms in the mechanism of erection and eventually manipulate the isoform that has a pronounced effect in the penile vasculature and preferably a low effect in other vascular beds. Alternatively, increasing RhoGDI and RhoGAP activity, which suppress RhoA activity, or suppressing RhoGEF activity could also offer benefit [89;429;430].

*B) Adrenergic receptors*

The α-adrenergic nervous system and its conventional neurotransmitter NE provide the principal regulatory control of anti-erectile contraction in the CC and penile vessels. Current knowledge indicates that NE acts principally on post-junctional $\alpha_1$-adrenoceptors, given their predominance over pre- and post-junctional $\alpha_2$-adrenoceptors in human CC tissue and seemingly also the penile vasculature [431;432]. Since the early 1980s α-adrenergic blockade was first used and represented a major breakthrough in the treatment of ED [433]. Nevertheless, this approach has yet to be fully exploited in part because of limited understanding of the mechanism of adrenergic receptor function in erectile tissue. Several α-adrenoceptor antagonists have been tested in animals and human for their ability to facilitate the erectile function. α-adrenergic blockade is also one of the most widely used treatments for lower urinary tract symptoms (LUTS) suggestive of benign prostatic hyperplasia (BPH) [434]. As these pathologies are strongly associated with ED and α-adrenoceptor antagonists improve ED, the benefit of using these antagonist in patients suffering from both LUTS/BPH and ED has been evaluated in many clinical trials [435;436].

Several adrenoceptor antagonists have been shown to possess pro-erectile effects and some are already used therapeutically to treat ED. Yohimbine is an indole alkaloid that has been widely used for treatment of psychogenic ED [437;438]. Yohimbine's aphrodisiac activity may be mediated through a combination of CNS effects and peripheral effects, including the blockade of pre- and postsynaptic $\alpha_2$-adrenoreceptors resulting in enhanced blood flow and decreased blood outflow from erectile tissue as well as enhanced libido [439]. It should however be mentioned that presynaptic $\alpha_2$-adrenoreceptor antagonism also results in an increased release of catecholamines from the peripheral sympathetic nerve terminals, which would be detrimental for the erectile function. This might be compensated by the concomitant application of an $\alpha_1$-adrenoreceptor antagonist. Nevertheless, it has been shown that increasing concentrations of yohimbine relax rabbit and human NE and endothelin-1 (ET-1) pre-contracted CC preparations almost completely [440;441]. However the relaxant effect of yohimbine is both endothelium- and androgen-dependent. This might explain the lack of efficacy of this drug in the treatment of some forms of organic ED [440]. Phentolamine mesylate, an $\alpha_1$- and $\alpha_2$-adrenoceptor antagonist caused concentration-dependent relaxations in pre-contracted corporal tissue strips isolated from rabbits and humans [442;443]. Intracavernosal phentolamine alone or in combination with other vasoactive agents is currently used to treat mild-to-moderate ED [444]. Also oral phentolamine mesylate is

available and facilitates penile erection in animal and human models [445;446]. Prazosin hydrochloride, a selective $\alpha_1$-adrenoceptor antagonist that was primarily introduced as an antihypertensive agent, has also been shown to produce concentration-dependent relaxations in pre-contracted CC tissue strips [447]. The compound is already used to treat psychogenic ED [448]. Doxazosin mesylate is a selective $\alpha_{1A}$- and $\alpha_{1D}$-adrenergic blocker that is clinically used in the treatment of hypertension and BPH [449]. Interestingly, a study on the treatment of mild hypertension illustrated that patients taking doxazosin reported a lower incidence of ED [449-451]. Terazosin hydrochloride, an $\alpha_1$-adrenergic selective antagonist with high affinity for $\alpha_{1D}$-receptor subtype, has been reported in a single case study to cause prolonged erection in a spinal cord injury patient [451;452]. Delequamine is a selective $\alpha_2$-adrenergic receptor antagonist which shifts the contractile dose-response curve of UK-14304 to the right in human and rabbit CC [453]. It was suggested that delequamine may reverse the inhibition of the general arousal response in young men with psychogenic impotence, as $\alpha_2$-adrenoceptors are the predominant NE receptors in the CNS [454]. In addition, trazodone hydrochloride, an oral serotonergic antidepressant with α-adrenergic receptor antagonistic activity, was noted to improve erectile function in impotent men and to cause prolonged erection and priapism in potent men [455-457]. However, while intracavernosal injection of trazodone in the rabbit penis produced full penile erection in 76%-84% of animals [458], clinical studies reported controversial results [459;460]. While in one study trazodone is not more effective than placebo in improving erections in patients suffering from severe ED, another clinical trial showed some efficacy in psychogenic ED.

All these data indicate that inhibition of α-adrenergic receptors may have advantage in treating ED, although most of the above mentioned antagonists show low efficacy when treating patients with organic ED [435]. The presence of different $\alpha_1$-adrenoceptor subtypes in corporal tissue, with $\alpha_{1A}$ being predominant in human CC [461], may conceivably lead to the development of selective acting antagonists that could provide increasingly potent pharmacological effects in the treatment of ED [462] Unfortunately, Ro70-0004/03, a selective, orally active $\alpha_{1A}$-adrenoceptor antagonist, had no effect on erectile function [463].

In contrast to α-adrenoceptors, β-adrenoceptor stimulation is associated with vascular and corporal relaxation, suggesting that β-adrenoceptor agonists might be useful in the treatment of ED [464]. Activation of the $\beta_3$-adrenoceptor, playing a major role in lipolysis, relaxes CC in a cGMP-dependent but NO-independent manner. Moreover, human CC exert basal $\beta_3$-

adrenoceptor activity which is linked to RhoA/Rho-kinase pathway inhibition [465]. All these observations lead to the assumption that $\beta_3$-adrenoceptors represent new potential therapeutic targets for ED treatment.

*C) Angiotensin receptors*

There is increasing evidence that a local renin-angiotensin system (RAS) exists within the CC [466]. A component of the RAS is the octapeptide hormone angiotensin-II (Ang-II). In mammalian cells, Ang-II is formed from Ang-I by the angiotensin-converting enzyme (ACE) and mediates its effects via at least 2 high-affinity plasma-membrane receptors, $AT_1$ and $AT_2$ [467;468]. Human CC have been shown to produce and secrete physiologically relevant amounts of Ang-II [469]. Furthermore, Ang-II has been shown to contract corporal tissue strips, primarily through activation of the $AT_1$ receptor [468]. Moreover, intracavernosal administration of Ang-II elicits erectile tissue contraction and terminates spontaneous erections in dogs [469]. In addition, Ang-II levels have been demonstrated to be up-regulated in disease states such as diabetes [235]. As Ang-II seems to be associated with penile detumescence, drugs that reduce the formation or action of Ang-II such as ACE inhibitors or angiotensin-receptor blockers have been suggested to improve erectile responses [470;471].

Based on this hypothesis, intracavernosal injection of losartan which selectively blocks $AT_1$ receptor, resulted in smooth muscle relaxation and erection [472]. Furthermore, losartan has been shown to improve ageing and diabetes-induced impotence in rat models [470;473]. In addition, valsartan - another Ang-II receptor blocker - has been demonstrated to markedly reduce ED in hypertensive males [474]. Comparably, ACE inhibitors such as enalapril and captopril also ameliorated blood inflow to the CC of hypertensive rats [475]. This effect was indicated to be due to structural remodelling of the penile vasculature, suggesting that Ang-II may also be involved in the regulation of collagen content [476]. These results illustrate that Ang-II receptor antagonists may be worth to be considered as a clinical treatment for ED. However, clinical studies are controversial. A pilot study illustrated that an ACE inhibitor did not improve cavernosal perfusion after 6 months administration in patients suffering from severe atherosclerotic ED [471]. On the other hand, irbesartan improved erectile function in patients with metabolic syndrome, supporting a beneficial role of Ang-II receptor antagonists [477]. Moreover, valsartan and losartan strongly decreased blood pressure and markedly improved ED in hypertensive men [478;479]. These controversial results illustrate that further

investigation is required to understand the role of RAS in penile erection and the clinical value of angiotensin-receptor blockers and ACE inhibitors in ED treatment.

Nowadays, it is generally accepted that Ang-II is not the only active peptide of the RAS. Other members, including Ang-(2-8), Ang-(3-8) and Ang-(1-7) may also mediate the actions of this system [480]. Of these, Ang-(1-7) has been demonstrated to induce CC relaxations [481]. Even CC strips isolated from older as well as diabetic rabbits showed relaxations towards Ang-(1-7) [482]. It is illustrated that pre-incubation of CC with Ang-(1-7) effectively attenuated Ang-II induced contraction. Moreover, infusion of Ang-(1-7) into CC potentiated the elevation of the ICP induced by ES of the major pelvic ganglion in rats. The vasodilator effects of Ang-(1-7) are mediated by a non-$AT_1$ or $-AT_2$ receptor that is called the Mas receptor. Genetic deletion of Mas resulted in compromised erectile function and severely depressed response to ES of the major pelvic ganglion. Furthermore, the attenuated erectile function in hypertensive rats was fully restored by Ang-(1-7) administration [481]. These observations suggest that Ang-(1-7) may have therapeutic value in treatment of ED since it can antagonize the actions of Ang-II in the CC. Activation of the Mas receptor mediated signalling may be particularly beneficial in disease states where Ang-II activity is elevated.

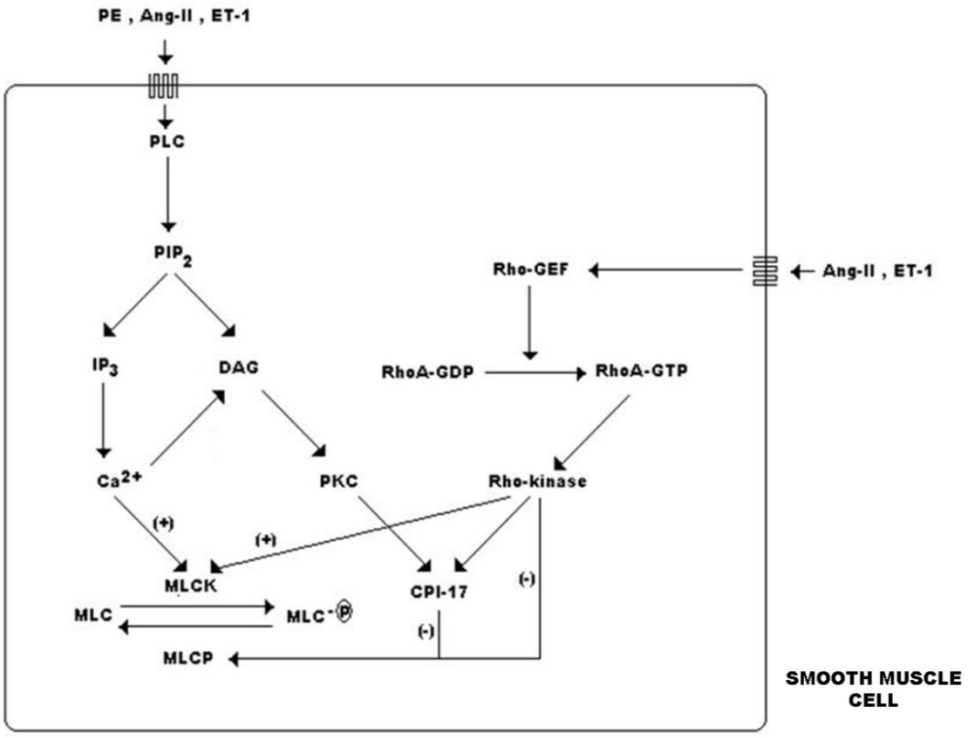


**Figure I.7** demonstrates the molecular mechanisms behind vasoconstriction induced by different peptides such as Ang-II, ET-1 and PE.

*D) Calcium-activated chloride channels*

Recently, an excitatory $Ca^{2+}$-activated $Cl^-$-current has been demonstrated in rat, rabbit and human corporal myocytes [483;484]. This current is activated by agonist-induced $Ca^{2+}$-release from stores and also occurs as spontaneous transient currents, which are typically caused by $Ca^{2+}$-sparks. This current is thus activated by $\alpha_1$-adrenergic agonists such as NE [485]. Both $Ca^{2+}$-activated $Cl^-$-channel blockers and $Cl^-$-channel transport inhibitors have shown to increase ICP and prolong nerve-evoked erections, thus promote vasodilatation by modulating the excitatory current. These experiments reveal the $Ca^{2+}$-activated $Cl^-$-current as a new therapeutic target for ED treatment that merits further research [486].

*E) Endothelin receptors*

Endothelins are a family of endogenous peptides mainly secreted by endothelial cells. Three distinct genes that encode three isopeptides (ET-1, ET-2 and ET-3) of the family were found in human and other mammalian genomes [487]. It has been shown that human penile CC endothelium has the ability to synthesize and release ET-1 [488]. ET-1 may induce both contraction and relaxation depending on the receptor subtype activated: $ET_A$ or $ET_B$. When ET-1 binds to $ET_A$ receptors in smooth muscle cells, intracellular $Ca^{2+}$ levels are elevated and/or the RhoA/Rho-kinase pathway is activated resulting in vasoconstriction. However, the binding of ET-1 to $ET_B$ receptors in endothelial cells activates eNOS resulting in NO release and vasorelaxation [488]. Several laboratories have demonstrated the presence of both $ET_A$ and $ET_B$ receptor subtypes in human erectile tissue. The $ET_A$-mediated cavernous vasoconstriction however seems to be predominant in vivo [489]. Hence, the vasoconstrictor actions of ET-1 in CC tissue strips can be blocked by prior treatment with an $ET_A$ antagonist [487]. In addition, $ET_A$ receptor antagonists have been shown to induce a rise in ICP [490]. As elevated ET-1 levels may induce ED, pharmacological $ET_A$ antagonists could be useful as an alternative treatment. The $ET_A$ receptor antagonist BMS-193884 was tested in men with mild-to-moderate ED. Although the drug was well tolerated, BMS-193884 did not significantly improve erectile function [491]. Despite the inefficacy of this compound, antagonism of ET-1-induced vasoconstriction still can be considered as a potential new treatment strategy for ED.

Conversely, activating the $ET_B$ receptor might also be worth considered for ED treatment. As yet no experiments have been performed studying the influence of $ET_B$ receptor agonists on erectile function. However, $ET_B$ receptor changes are described in animal models of BPH,

hypercholesterolaemia and diabetes [492]. Further research on the (patho)physiological importance of endothelins in CC is warranted to validate endothelin receptors as potential therapeutic targets.

### I.3.3.3 Other targets

*A) Cannabinoid and vanilloid receptors*

It has been reported that the endogenous cannabinoid anandamide might play a peripheral role in penile erection through a potentiating effect on neurogenic relaxation in the rat CC. This effect could be antagonized by either cannabinoid $CB_1$ or vanilloid $VR_1$ receptor specific antagonists. Using immunoblotting, the presence of both receptors was demonstrated within the rat CC [493]. One study has investigated the effect of acute administration of anandamide on CC tissue strips from STZ-induced diabetic rats. It was concluded that endocannabinoids such as anandamide could, probably by enhancing NO production in nitrergic nerves, enhance the NANC-mediated relaxation. This is the first study suggesting a potential for using $CB_1$ and/or $VR_1$ receptor agonists as new targets for treating ED [494]. Importantly, $CB_1$ receptors are not only present in CC but also centrally, in the hypothalamic paraventricular nucleus (PVN), a brain area involved in penile erection [495]. Paraventricular injection of the $CB_1$ receptor antagonist SR 141716A dose-dependently induces penile erection. This effect is mediated by inhibition of GABA release, stimulating glutamatergic neurotransmission and activating central oxytocinergic neurons. Although no clinical studies have yet been undertaken, modulation of $CB_1$ and/or $VR_1$ receptor activity may be considered for treating ED [495-497].

*B) Connexins*

A series of publications have documented the presence and physiological relevance of gap junctions in the coordination of contraction and relaxation responses among the corporal smooth muscle cells [498]. Although there are 16 known mammalian gap junction proteins, the dominant one in human corporal smooth muscle cells is undoubtedly connexin 43 (Cx 43) [499-501]. The presence of these aqueous intracellular channels provides partial cytoplasmic continuity between coupled smooth muscle cells and ensures the intercellular transit of most of the known second messenger molecules and/or ions that regulate corporal smooth muscle cell tone. Cx 43 gap junctions thus coordinate uniform smooth muscle tone through the establishment of a syncytial cellular network so that only a fraction of corporal smooth muscle cells needs to be directly activated in order to elicit a rapid relaxing or contractile

response [502-504]. Interestingly, Cx 43 expression and permeability are significantly decreased in ageing, hypertension and diabetes [505;506]. Their (patho)physiological importance make the Cx 43 gap junctions attractive therapeutic targets for the treatment of ED. Testing of drugs that are able to control Cx 43 expression is thus very attractive but also difficult as Cx 43 is ubiquitous expressed, so that these drugs will most probably exert numerous side-effects. Studies on the CC specific expression of Cx proteins could help solve this difficulty. Otherwise examining gene therapeutic application of Cx 43 for restoring ED could also be a valuable option.

*C) Growth factors*

Vascular corporal maintenance depends on angiogenic balance, suggesting that angiogenic growth factors such as vascular endothelial growth factor (VEGF), angiopoietins and insulin-like growth factor-1 (IGF-1) may be important for normal erectile function [507;508]. Studies have demonstrated that reduction of VEGF and its receptors is markedly associated with a number of pathophysiological changes in the CC [509]. The reason for this is probably that any decrease in the activity of VEGF contributes to the loss of expression of eNOS and cavernosal smooth muscle cells by apoptosis. Pro-erectile effects of VEGF administration are illustrated in diabetic, hypercholesterolemic, arteriogenic, venogenic, castrated and even ageing-related ED in rats [510-513]. Interestingly, VEGF in combination with brain derived neurothrophic factor (BDNF), also enhances cavernosal nerve regeneration, further supporting an important role for VEGF in restoring ED from various aetiologies [514]. In addition, repeated intracavernous injections of the angiopoietin $Ang_1$ variant, COMP-$Ang_1$, enhances VEGF-induced angiogenesis, completely restoring erectile function in diabetic mice [515]. Furthermore, combined AAV-$Ang_1$ and VEGF gene transfer promoted angiogenesis cooperatively in a rat model of hypercholesterolemic ED, fully restoring its erectile function [516]. Moreover, IGF-1 gene transfer also restored erectile function in aged rats [517]. These studies show pro-erectile effects of angiogenic growth factors, suggesting their potential as targets for treating ED from various aetiologies.

BDNF is a member of the neurotrophin family and plays a distinct role in neuron regeneration in both the CNS and peripheral nervous system (PNS) [518]. Several investigators have examined the ability of BDNF gene transfer to restore ED due to cavernous nerve injury [519;520]. Intracavernous injection of a construct for BDNF in rats prevented degeneration of nNOS-containing neurons in the pelvic ganglion after bilateral cavernous nerve freezing and

stimulated nerve regeneration, resulting in improved erectile responses [514;521]. These results are very promising for the recovery of erectile function after bilateral cavernous injury such as after radical prostatectomy. As mentioned before, combined intracavernosal VEGF and BDNF administration synergistically facilitates neural regeneration and promotes neurite sprouting from the major pelvic ganglion. This combination avoided neurological and vascular changes in hyperlipidemia-induced ED [514]. Other neurotrophins such as glial cell line-derived neurotrophic factor (GDNF), neuronal growth factor (NGF) and neurotrophin-3 (NT-3), also protected nerves from mechanical and metabolic changes and recovered erectile function in rat models of neurogenic-ED [520;522;523]. Furthermore, (neuro)immunophilins improved the erectile function in animal models of cavernosal nerve injury [524]. Neuroimmunophilin ligands FK506, rapamycin and GPI-1485 prevent cavernous nerve degeneration, preserving erectile function in rats subjected to partial nerve crushing. These data suggest that neurotrophins/neuroimmunophilins are potentially useful to maintain erectile function in men following radical prostatectomy.

### I.3.3.4 Targets involved in central control of penile erection (Figure I.8)

The introduction of the centrally acting non-selective dopamine agonist apomorphine as a treatment option for ED has directed interest to the central mechanisms as targets for pharmacological interventions [439]. Central mechanisms influencing penile erection may involve regulatory functions of different neurotransmitters at supraspinal sites, such as the PVN and medial preoptic area of the hypothalamus [525;526]. There are clearly significant interactions between oxytocin, dopamine and NO driven CNS pathways in penile erection [527;528]. However, the physiological role of these transmitters in the erectile process has yet to be determined.

*A) Melanocortin receptors*

The melanocortins are widely distributed and involved in various activities. The precursor of all melanocortins is proopiomelanocortin (POMC), a polyhormone that may generate as many as eight peptides, depending on the cleavage sites [529]. Some 40 years ago it was already discovered that intraventricular injection of adenocorticotropin hormone (ACTH) and alpha-melanocyte stimulating hormone (α-MSH) triggered erection, grooming behaviour, stretching and yawning in several animal species [530]. A role for the melanocortins in human erectile function was discovered accidentally in 1996 among men enrolled in a clinical dermatology study. They received melanotan-II (MT-II), a synthetic analog of α-MSH, which was

designed to trigger tanning and it was noted that the peptide invariably induced an erection in nearly all male participants [531;532]. Most of the effects of ACTH, α-MSH and the peptide analogues are mediated via specific subtypes of melanocortin receptors (MCRs). Currently five MCRs have been characterized and all are coupled to a G-protein. The MCRs increase target cell cAMP levels when activated [533].

There have been several attempts to characterize the mechanisms by which melanocortins facilitate erections and many of these mechanisms remain poorly understood. Bremelanotide, an active metabolite of MT-II was shown to be an agonist at the melanocortin-3-receptor ($MC_3R$) and melanocortin-4-receptor ($MC_4R$). $MC_3R$ and $MC_4R$ are located in the hypothalamus, more specifically in areas that play a pivotal role in erectile function such as the PVN [534]. Additionally, $MC_4R$ has also been localized to the penis and major pelvic ganglion [535]. Because of this finding, the MCRs were postulated to have local effects as well. Several studies however indicated that MCRs play no physiological role in the peripheral erectile mechanism, as intracavernosal injection of MT-II had no effect on erections in rats [535-538]. These findings support previously documented studies showing the inability of MT-II to elicit smooth muscle relaxation in rabbit cavernosal strips [539].

MCR agonists have shown promise not only in animal models, but also in preliminary studies in humans [533;540;541]. Subcutaneous administration of MT-II and bremelanotide (PT-141) as well as intranasal administration of bremelanotide caused a statistically significant erectile response and were able to induce penile erections in patients with psychogenic and/or organic ED [542;543]. The compounds were generally well tolerated, with nausea, yawning, stretching and flushing as the most common adverse effects reported. The ability of PT-141 to cause erections in patients unresponsive to PDE-5 inhibitors, suggests that interfering with the melanocortinergic system may provide an alternative ED treatment with a potentially broad patient base [544]. However, the blood pressure increase associated with this treatment raises some concerns regarding its safety.

When studies were undertaken to distinguish the roles of $MC_3R$ and $MC_4R$ from each other, it was demonstrated that using a selective $MC_4R$ antagonist, HS-014, activities such as grooming and stretching can be elicited in animals by ACTH or α-MSH, but not penile erections [545;546]. Based on these results it was hypothesized that $MC_3R$ may be the key receptor in mediating the pro-erectile effects of melanocortins. Nevertheless, data supporting this notion using selective $MC_3R$ agonists are still not substantiated. In contrast data have

been published demonstrating that THIQ, a molecule known as a $MC_4R$ specific agonist, facilitates erections in rats. The role of $MC_4R$ was further verified with experiments involving $MC_4R$ knockout mice [535]. These mice showed diminished copulatory behaviour which could be an additional argument in favour of a central $MC_4R$ function. A remark should however be made about the latter experiments. Given that the $MC_4R$ knockout mice are obese, it is difficult to make a definitive conclusion regarding whether the reduced copulatory behaviour in these mice is due to the absence of the $MC_4R$ or due to the presence of obesity, which is known to decrease sexual activity as well [547]. Conclusively, the principle of subtype selective MCR agonism is a new and potentially useful therapeutic option and in terms of this potential therapeutic value, new agonists are under investigation at present. MK-0493, a selective $MC_4R$ agonist, did not produce erectogenic activity different from placebo in patients with ED [545]. Recently, a new selective $MC_4R$ agonist, compound 40a, demonstrated similar efficacy as sildenafil in patients with ED [548]. Further studies will provide information regarding the potential of melanocortins for ED treatment.

*B) Oxytocin*

Oxytocin has been implicated in sexual behaviour and is demonstrated to be released from the hypothalamic supraoptic area and paraventricular nuclei that project to spinal centres involved in the control of erectile function [549;550]. Despite some preliminary data in old clinical trials, current research is limited to animal models [551-554]. Oxytocin has been shown to be a potent stimulator of penile erection when injected into the lateral cerebral ventricle, the PVN, the hippocampus and intrathecally in rats, an effect that was blocked by administration of an oxytocin antagonist [555]. Furthermore, oxytocin reduction is correlated with sexual impotence [556]. Since many of the centrally active agents that can stimulate erection seem to act via oxytocinergic mechanisms, it would be rational to explore the potential of oxytocin and/or analogues in ED treatment [557].

*C) Dopamine receptors*

During sexual stimulation, the hypothalamus is exposed to input of various substances of which dopamine appears to be the primary erectogenic neurotransmitter [558]. Dopamine has been implicated to exert its erectogenic effect by stimulation of $D_2$ receptors, as is supported by the erectile functions of apomorphine [559]. Several types of dopamine receptors however are key components of the autonomic and somatic erection arc [560]. In the medial pre-optic area, primarily $D_1$ receptors seem to be involved in erectile function, while in the

paraventricular nucleus rather $D_2$ receptors [561] Up till now five subtypes of dopamine receptors, denoted $D_1$-$D_5$, can be distinguished. Although it was formerly believed that the effects were mediated in large part by $D_2$ receptors, recent evidence points toward the $D_4$ receptor subtype as a mediator for the pro-erectogenic effects of dopamine [357;562].

There has been recent interest in using specific dopamine receptor subtype analogs as specific targeted therapies for ED. In this respect, cabergoline, a selective $D_2$ receptor agonist improves the erectile function of men suffering from psychogenic ED [563]. In addition, research data in animal models showed that also dopamine $D_1$ receptor agonists (eg A 77636) can induce erections and may represent a possible alternative to apomorphine in the treatment of ED, avoiding the typical adverse effects related to the stimulation of $D_2$ receptors, such as nausea [564]. Moreover, ABT-724, a novel compound that is currently under preclinical investigation for its potential to selectively activate the $D_4$ receptor, induces erections dose-dependently, without affecting MAP [565;566]. When the drug was administered subcutaneously in a rodent study, the compound facilitated erectile responses. ABT-670 is more recent developed $D_4$ selective agonist with superior oral bioavailability compared to ABT-724 [567]. Other murine research using $D_4$ agonists, including the new agents PD-168077, PIP3EA and CP-226269, showed promising results but further research is required to elucidate their potential clinical usefulness [568-570].

*D) Growth hormone releasing peptide receptors*

A new class of peptide molecules that release growth-hormone (GH), with an efficacy higher than that of the endogenous GH-releasing hormone, has recently been characterized being hexarelin and hexarelin analogue peptides [571-573]. Hexarelin analogues such as EP 60761, EP 80661 and EP 50885 have been demonstrated to induce erections not only when injected in the PVN but also when given systemically in rats, although to a lesser extent [572;574]. They induced penile erection by increasing central oxytocin transmission, possibly by activating NOS in the cell bodies of oxytocinergic neurons located in the PVN that control penile erection. With the exception of one in vitro study, there is no further indication whether hexarelin analogues can influence human erectile function [575].

*E) 5-Hydroxytryptamine receptors*

The involvement of 5-hydroxytryptamine (5-HT) or serotonin in sexual function is complex, involving effects on erection, ejaculation and behaviour [576]. Many 5-HT receptor subtypes

have been implicated in sexual function and although 5-HT exerts a general inhibitory effect on male sexual behaviour, it may act to facilitate or inhibit sexual responses depending upon the action at different sites and at different subtypes of the 5-HT receptors within the CNS [560]. An inhibitory role of 5-HT on erectile function is further evidenced by increased ED incidence in men taking serotonin specific re-uptake inhibitors [577;578].

It has been established that the 5-$HT_{1A}$ , 5-$HT_{1B}$, 5-$HT_{2A}$ and 5-$HT_4$ receptor subtypes are responsible for the inhibitory effect on erectile function, while the 5-$HT_{1C}$ and 5-$HT_{2C}$ receptors subtypes facilitate erectile function [579;580]. Developments of new and specific 5-$HT_{2C}$ receptor agonists may thus result in therapeutically useful drugs. Surprisingly, trazodone which is known as an inhibitor of 5-$HT_2$ receptors, has been reported to improve erectile function perhaps by displaying agonist activity at the 5-$HT_{2C}$ receptor, although this response can also be due to its $\alpha_1$-adrenoceptor antagonistic actions [455;456;581;582]. 1-(3-chlorophenyl)-piperazine, a trazodone metabolite and N-trifluoromethylphenyl-piperazine are considered partial agonists at 5-$HT_{2C}$ receptors and usually display 5-$HT_{2A}$ receptor antagonistic actions. Both compounds have been shown to induce erections but they also significantly inhibit ejaculation and sexual behaviour [580;583-585]. Furthermore, RSD 992, RO-60-0175 and YM348 induce penile erections by (partially) agonizing 5-$HT_{2C}$ receptors [560;586;587]. In addition, LY237733, a potent 5-HT receptor antagonist with preferential affinity for 5-HT1c and 5-HT2 receptors augmented the sexual responses of male rats [588]. These findings may give rationale for the use of 5-HT agonists/antagonists in the pharmacotherapy of ED.

*F) <u>Glutamate receptor</u>*

Hippocampal glutamate ionotropic receptors present in the hypothalamic PVN as well as in the hippocampus may also play a significant role in the initiation of penile erection [589]. Injections of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) were associated with an increase in maximal ICP and with prolonged erections in rats [589-591]. Both NMDA and AMPA receptor antagonists block reflexive erections [592]. Furthermore, the excitatory amino acid concentration increases in the PVN when penile erection occurs in physiological contexts, further supporting a role for glutamate participation in the erectile function [593;594]. More research however is required in order to explore the possible application of glutamate receptors as new therapeutic targets in the treatment of ED.

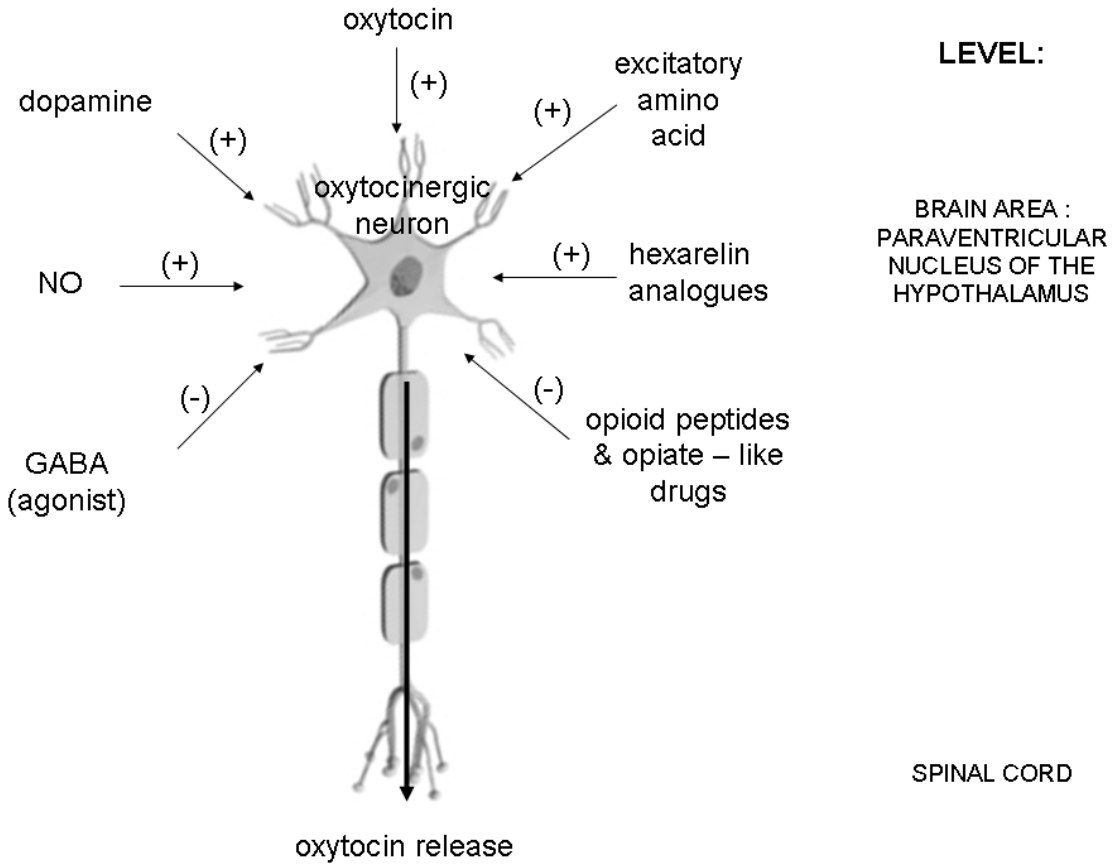


**Figure I.8** represents a simplified scheme of central control of penile erection. In brain areas such as the PVN of the hypothalamus different peptides can either stimulate or inhibit oxytocinergic neurons that project to the spinal cord. NO = nitric oxide and GABA = gamma-aminobutyric acid.

### I.3.3.5 Emerging therapies

#### I.3.3.5.1 Combination therapies

Combination therapy aims to increase efficacy and/or decrease adverse effects. Combining a centrally acting agent with a drug having a peripheral site of action seems attractive but other combinations may also be interesting [595]. Several combinations of the above mentioned treatment modalities are possible and have already been explored. In addition, some molecules have been developed for ED treatment that posses an intrinsic combination mechanism.

*A) Nitrosylated α-adrenergic antagonists*

Moxisylyte has a competitive and relatively selective blocking action on the $\alpha_1$-adrenoceptor and in vitro moxisylyte relaxed NE-contracted human CC preparations [596]. Intracavernosal injection of moxisylyte also resulted in facilitation of penile erection [597]. Nitrosylation of

moxisylyte and also yohimbine resulted in compounds that were significantly more potent in relaxing NE-contracted CC preparations than their parent compounds [598]. Moreover, intracavernous administration of the nitrosylated compounds produced greater and more long-lasting increases in ICP than the non-nitrosylated compounds without effect on blood pressure. These nitrosylated compounds release an NO-donor and a parent molecule that antagonizes adrenergic receptors thus having the dual function of both inducing corporal smooth muscle relaxation while inhibiting sympathetic tone. It was suggested that SNO-moxisylyte and SNO-yohimbine may be useful therapeutic agents for the local pharmacological treatment of ED.

*B) NO-releasing PDE- 5 inhibitors*

NCX is a NO-releasing PDE-5 inhibitor that has a more potent relaxant effect on pre-contracted human CC strips compared to sildenafil citrate [599]. By directly activating sGC and inhibiting PDE-5 it causes an increase in the intracellular cGMP concentration without the need for endogenous NO supply [314;600;601]. The compound has shown to be efficacious in diabetic rats as well as in hypercholesterolemic rabbits [314;602]. Administration of such agents may be promising for patients with comorbidities which are associated with impaired NO bioavailability such as diabetes, hyperlipidemia and ED after radical prostatectomy.

*C) NO-releasing $K^+$ channel openers*

Nicorandil, a $K^+$ channel opener and NO-donor, relaxes rabbit cavernosal tissue by a mechanism that involves activation of $K_{ATP}$ channels and stimulation of sGC [603;604]. Further evaluation of this compound could reveal promising possibilities in the treatment of ED.

I.3.3.5.2 Gene therapy

The application of gene therapy for ED represent an exciting new field. Gene therapy may effectively restore or supplement defective functions and/or antagonize the expression of a mutant gene in order to correct the dysfunction that leads to a particular disease. The goal of gene therapy for organic impotence is to allow the patient to sustain physiologically elicited erections without resorting to pharmacological treatment immediately prior to the sexual act [605]. Gene therapy for the treatment of ED is associated with a number of advantages as the penis seems to be a suitable organ for the use of gene therapy because of its external location,

easy accessibility and the low turnover rate of vascular smooth muscle cells, which allow a desired gene to be expressed for long periods without affecting the systemic circulation [606]. Gene therapy approaches have focused on a number of signalling pathways that are crucial for penile erection, such as NO/cGMP, RhoA/Rho-kinase, growth factors, ion channels, peptides and the control of oxidative stress. Gene transfer of therapeutic genes has been shown to be highly efficacious in improving the diminished erectile responses in a number of experimental animal models [607]. All targets already tested in gene therapeutic experiments are summarized in Table 1.

I.3.3.5.3 Tissue engineering

Tissue engineering aims to reconstruct penile tissue in order to treat ED [608]. Cultured human corporal smooth muscle cells may be used in conjunction with biodegradable polymer scaffolds to create CC smooth muscle tissue in vitro and in vivo [609]. Also endothelial cells can be seeded on either biodegradable polyglycolic acid polymer or acellular corporal collagen matrices scaffolds [610]. Animal experiments showed that engineered CC achieved adequate structural and even functional characteristics. Currently tissue engineering belongs to the field of basic research but may evolve quickly to a clinically applicable technique for ED treatment [611].

I.3.3.5.4 Low-intensity extracorporeal shock wave lithotripsy (Low intensity ESWL)

Low-intensity ESWL has the ability to promote neovascularisation in different organs. Because of this ability, low-intensity ESWL has been proposed to possess efficacy in treating ED, especially arteriogenic-related ED. Low-intensity ESWL significantly increased the duration of erection as well as the penile rigidity in patients with organic ED and as expected improved the penile endothelial function. The therapy showed no adverse effects during the follow-up and was not associated with pain [612]. The current available short-term results are very promising and demand further evaluation.

| **The gene therapeutic target** | **The animal model used** |
|---|---|
| iNOS | Age-related ED in rat [277] |
| eNOS | Age- and diabetic-ED in rat [278] |
| PnNOS | Age-related ED in rat [283] |

| | |
|---|---|
| Arginase (inhibition) | Age-related ED in mouse [246] |
| Protein NOS inhibitor (inhibition) | Age-related ED in rat [284] |
| EC-SOD | Age- and diabetic-ED in rat [286] |
| HO-1 | Induced sinusoidal relaxation in rat [322] |
| PKG | Diabetic-ED in rat [331] |
| $BK_{Ca}$ channels | Age- and diabetic-ED in rat AND atherosclerotic monkeys [351-353;355] |
| $BK_{Ca}$ channels | Patients with mild to moderate ED [358] |
| KATP channels | Age-related ED in rat [350] |
| VIP | Diabetic-related ED in rat [374] |
| CGRP | Age- and diabetic-ED in rat [378;379] |
| Rho-kinase (inhibition) | Age- and diabetic-ED in rat [423] |
| VEGF | Age- and diabetic ED and cholesterolemic, arteriogenic, venogenic, castration-induced ED in rat [510-512;519] |
| $Ang_1$ | Hypercholesterolemic-induced ED in rats [515] |
| BDNF | Neurogenic ED in rats [519;521] |
| GDNF | Neurogenic ED in rats [520] |
| NT-3 | Diabetic-ED in rats [520] |
| IGF-1 | Age-related ED in rat [517] |

**Table I.1** summarizes all the targets that already have been tested in gene therapeutic experiments.

## I.3.4 Conclusion

The future treatment of ED will inevitably incorporate molecules influencing the NO/cGMP cascade, but certainly also other targets of both central and peripheral pathways involved in erection. At present, the only orally available agents used for the treatment of ED interfere with the NO/cGMP pathway. Although this pathway still possesses interesting targets, many targets described in this review go beyond NO signalling. Incorporation of new drugs and therapies into the urologists armamentarium will eventually be coupled to the increasing knowledge of the pathophysiology of ED, which will help to tailor an appropriate individual treatment to the particular patient.

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# Chapter II:

# Aims of the study

## II.1 General Aims

Blood flow regulation as well as erectile function depends on the ability of blood vessels to dilate. The physiological importance of the NO-sGC-cGMP pathway within (corporal) blood vessels dilation is widely appreciated [1;2]. Dysregulation of the NO-sGC-cGMP pathway, such as decreased NO bio-availability, is known to trigger the development of cardiovascular diseases as well as ED [3]. Agents interfering with the NO-sGC-cGMP pathway are already used to treat angina pectoris, hypertension as well as ED, namely nitrovasodilators and PDE-5 inhibitors [4;5]. While the first drugs deliver exogenous NO solving the lack of NO availability, the latter inhibit the cGMP breakdown by PDE-5, increasing the intracellular cGMP concentration. Both groups of drugs result in the resumption of smooth muscle relaxation, leading to blood vessel and corporal dilation, illustrating that interference with the NO-sGC-cGMP pathway is efficient. However, like any other drug, these agents also exert unwanted side-effects. Research is thus still ongoing to deliver drugs that preferably have a higher efficacy and a lower adverse-effect profile. Due to its central role, the sGC enzyme is a very promising target and exists in two different active isoforms, being $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$ [6]. The presence of 2 different isoforms creates the possibility to develop an isoform-specific therapeutic agent which possesses a high efficacy while exerting less adverse effects. Therefore knowledge on the functional role of both isoforms is required. Up till now, no sGC inhibitor has been developed acting isoform-specific [7]. The group of prof. Brouckaert (Flanders Interuniversity Institute for Biotechnology (VIB), Ghent, Belgium) succeeded to develop $sGC\alpha_1^{-/-}$mice. These mice make it possible to examine the functional role of the $sGC\alpha_1\beta_1$ isoform [7]. More recently, they also were able to create $sGC\beta_1^{ki/ki}$ mice, whereby the sGC enzyme lacks the ability to be stimulated by its physiological regulators NO and CO [8]. A comparison of results obtained with the $sGC\alpha_1^{-/-}$ mice and the $sGC\beta_1^{ki/ki}$ mice will provide indirect information on the functional role of the $sGC\alpha_2\beta_1$ isoform.

Besides NO, CO has been shown to possess vasodilatory properties and some authors have illustrated that activation of sGC is the underlying molecular mechanism [9]. However, the toxicity associated with the use of CO-gas has led to the development of CORMs, releasing CO in a more local, safe and controllable manner [10]. These CORMs have shown positive outcomes in cardiovascular studies [11]. Despite these positive results, more knowledge on the (patho)physiological functions of CO and CORMs is required.

## II.2 Specific Aims

### II.2.1 NO-induced corporal relaxation in sGC$\alpha_1$ knockout mice

Our first study, described in **chapter IV.1**, aimed to determine the importance of the sGC$\alpha_1\beta_1$ isoform in NO-induced erectile function *in vitro*. This was established by studying isometric tension changes of CC isolated from sGC$\alpha_1^{-/-}$ mice. However, *in vitro* results not always fit with *in vivo* results, as the vascular tone *in vivo* is constantly regulated by neuronal, humoral and hemodynamic factors, whereas the influence of these factors mostly disappears under the *in vitro* conditions. Therefore the aim of the second study, described in **chapter IV.2**, was to evaluate whether the previous *in vitro* data could be confirmed in the *in vivo* conditions using sGC$\alpha_1^{-/-}$ mice. The main advantage of *in vivo* studies is that, in general, the results have a high physiological relevance.

### II.2.2 NO-induced corporal relaxation in sGC$\beta_1$ knock-in mice

The previous experiments showed significantly reduced corporal responses to exogenously applied NO and NO-independent sGC activators in CC strips isolated from the sGC$\alpha_1^{-/-}$ mice. However a substantial relaxant effect still remained, suggesting that besides the predominant sGC$\alpha_1\beta_1$ also the sGC$\alpha_2\beta_1$ and/or sGC-independent mechanisms are involved. Using sGC$\beta_1^{ki/ki}$ mice, experiments were performed characterizing the mechanism(s) responsible for the remaining responses observed in sGC$\alpha_1^{-/-}$ mice. **Chapter IV.3** presents the results of the *in vitro* and *in vivo* studies exploring the corporal responsiveness towards NO-donors and sGC stimulators/activators of sGC$\beta_1^{ki/ki}$ mice.

### II.2.3 Exploring the functional role of sGC in CO- and CORM-2-induced vascular relaxation

The aim of a following study was to examine the molecular mechanisms underlying the vasodilatory properties of the CO and CORM-2 in vitro. CORM-2 is a compound frequently used to evaluate (patho)physiological effects of CO, although the question remains whether it resembles the molecular mechanism behind CO-induced vasorelaxation. Focusing on pharmaceutical potential, knowledge on the specific mechanisms involved in the vasodilatory properties of CORM-2 as well as other CORMs is demanded. Data obtained from this study are described in **chapter IV.4**.

### II.2.4 Exploring the functional role of sGC in CO- and CORM-2-induced corporal relaxation

The aims of the study presented in **chapter IV.5** were to assess the effect of CO and the CO-donor CORM-2 on the corporal tension and the molecular mechanisms underlying their responses in CC. Research on CO and/or CORMs in the field of erectile function remains scarce, with only a small number of publications indicating the HO/CO pathway as a therapeutic target to treat ED [12]. Further research to understand the role of the HO/CO pathway in erectile (dys)function could ultimately lead to new therapeutic strategies.

## II.3 Reference List


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# Chapter III:

# Materials and Methods

## III.1 The *in vitro* technique

An in vitro technique as measure for vascular reactivity has the advantage that it allows analysis of the local sensitivity of the tissue in the preferred experimental conditions, independent of complicated interferences coming from other parts of the organism. Moreover, the concentrations of the substances and drugs administered to the tissues are accurately known. The *in vitro* technique used to study the mechanical properties of ring segments of isolated aorta and femoral artery as well as CC was the wire myograph. Two different types of myographs (based on the automated dual channel vessel myograph, model 500A, JP trading, Aarhus, Denmark) were constructed by the technical staff of our laboratory. The differences between the two different myographs are the tissue holders within the organ baths. (Figure III.1) While ring segments of vessels are mounted in the organ chambers through mounting them onto two thin wires clamped at two holders; CC were longitudinally clamped between two holders. One of these holders is fixed to a micrometer, used to adjust the distance between the two wires or two holders, for vessels and CC respectively. The other holder is connected to a force transducer allowing us to measure isometric tension changes in vessels and CC. The length of the tissue is thus maintained constant, while the change in force is recorded. The force transducer transforms the tension in an electrical signal. This signal is then being amplified and registered by a reader system. The strength of this technique is that the tension measurements are very sensitive and confounding regulatory mechanisms active *in vivo* do not interfere in the *in vitro* studies.

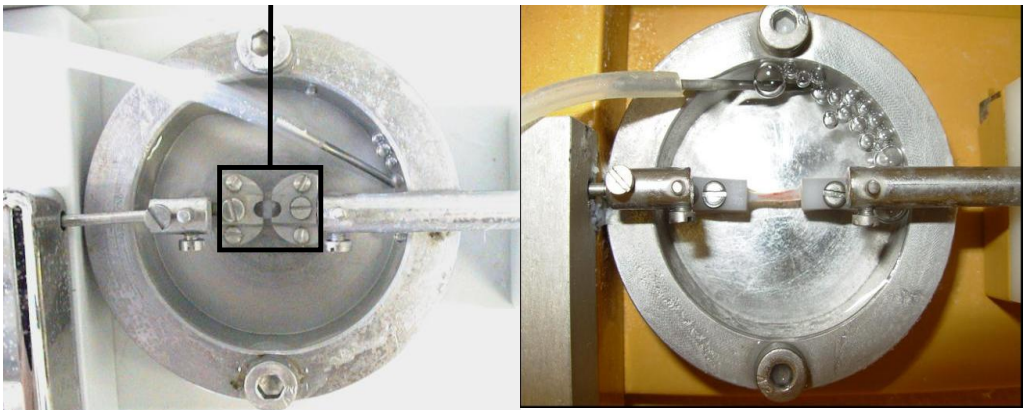

**Figure III.1** The two different types of myographs used in the present study. The myograph depicted on the left is the vessel myograph, while at the right side the myograph used for CC tissue strips is depicted.

### III.1.1 Calibration of the myograph

Before starting the experiments, the force transducer needs to be calibrated. Calibration of the myograph is established by using a special calibration balance and is necessary before starting the experiments. The half circular opening of the left holder attached to the transducer is obstructed by a wire fixed to both the distal and proximal screws of the holder. The free arm of the calibration balance is placed in the middle opening of the holder. The calibration balance exists of the free arm and two little bowls. A small weight of 2 grams is put in the left bowl, causing a deflection of the free arm which will exert a force on the wire fixed on the holder. (Figure III.2) Based on the principle of a lever, this force is consistent with 1 gram (= 9.81 mN) as the length of the free arm of the balance is twice the distance from the fulcrum to the bowl.

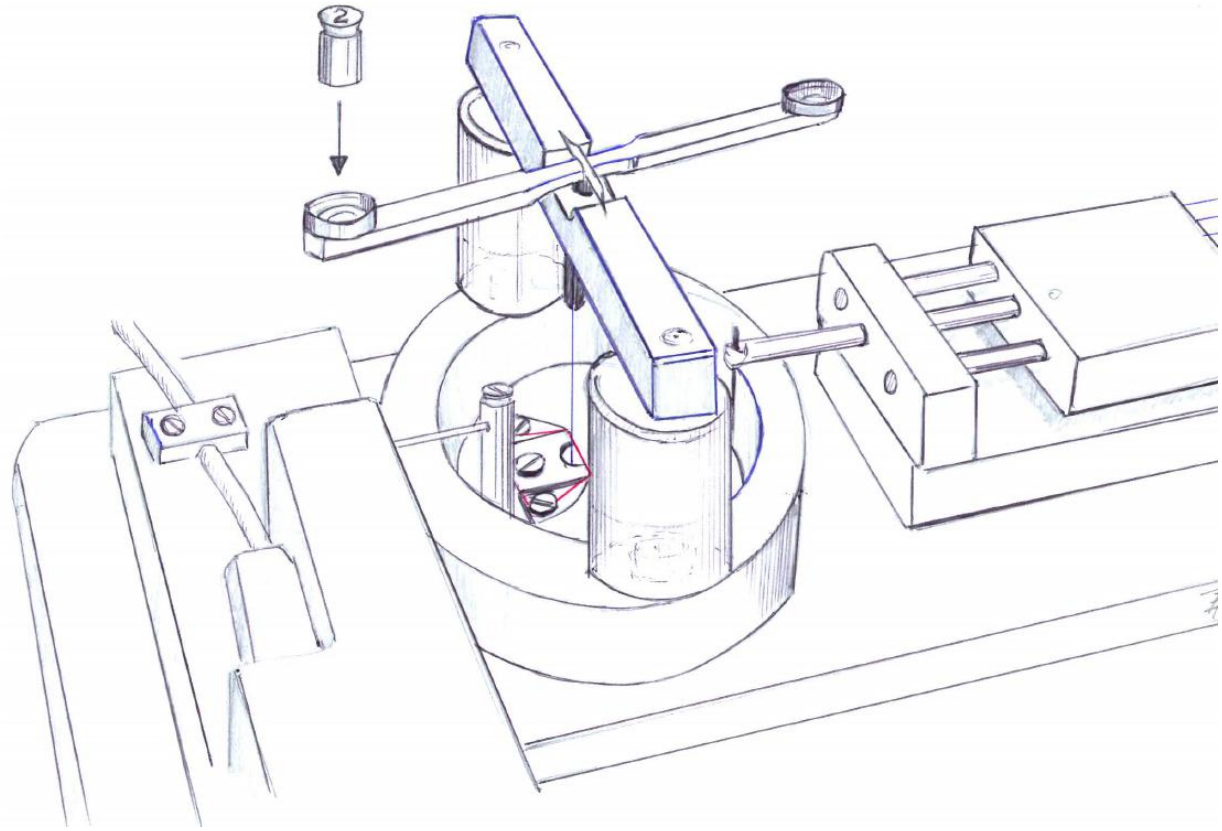


**Figure III.2** illustrates how the calibration of the myograph is performed.

### III.1.2 Transgenic sGC$\alpha_1^{-/-}$ mice and sGC$\beta_1^{KI/KI}$ mice

As mentioned in chapter II, we made use of transgenic sGC$\alpha_1^{-/-}$ mice and sGC$\beta_1^{ki/ki}$ mice and their controls. These mice were generated, genotyped and bred in the Department of Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology, Ghent, Belgium. The genotyping of the mice was done prior to the experiments by PCR and Southern blot analysis.

In sGC$\alpha_1^{-/-}$ mice with a 129SvJ background, exon 6 of the $\alpha_1$ gene, encoding an essential part of the conserved catalytic domain, is deleted. This deletion resulted in a selective loss of activity of the sGC$\alpha_1\beta_1$ isoform. The targeting vector was constructed using 4.3 kb 5' and 2.5 kb 3' sGC$\alpha_1$ homology fragments, isolated from a 129SvJ lambda FIXII mouse genomic library (Stratagene), a neomycine (neo)-resistance cassette (for positive selection) and a thymidine kinase cassette (for negative selection). The construct was engineered in that way that exon 6 of the sGC$\alpha_1$ gene and the neomycine-resistance cassette are flanked by loxP-sites. Embryonic stem cells, derived from a 129/SvJ strain of mice were electroporated with the linearized targeting vector and subjected to positive and negative selection with G418 and gancyclovir. To excise the floxed neo cassette (conditional knockout) ad/or the floxed exon 6 (full knockout), targeted embryonic stem cell clones were transiently transfected with a Cre-expressed plasmid. Chimeras were generated by aggregating embryonic stem cell clones, carrying the desired deletion with morula stage embryos. The heterozygous offspring were then mated to one another to produce homozygous recombinants.

The sGC$\beta_1$ knockin mice (sGC$\beta_1^{ki/ki}$ mice) were developed by converting residue 105, the codon of which is located in exon 5, from histidine (His) to a phenylalanine (Phe). Through this mutation, the sGC enzyme can no longer be activated as the mutation region lies in the binding region, however, its basal activity is retained. The targeting construct contains 4.1 kb 5' and 3 kb 3' flanking regions of homology cloned from a 129SvJ lambda FIXII mouse genomic library (Stratagene), a positive and negative selection marker (a floxed neo cassette and a thymidine kinase cassette respectively). In addition to the three mutations which place the His with a Phe residue, 5 other silent mutations were introduced to develop a means to genotype for the inclusion of Phe during homologous recombination with amplification of a PCR fragment making use of primers spanning this regions, followed by a restriction digest of the fragment with the restriction enzyme SmaI, specifically cleaving only the mutant fragment. Also Southern blot (mutation specific restriction) was used to confirm homologous

recombination. The $sGC\beta_1^{ki/ki}$ mice were generated following the same procedure as the $sGC\alpha_1^{-/-}$ mice. But, the recepient blastocyst was derived from a C57BL6/J strain. Genotyping of the mutant mice happened by a PCR spanning the region where one LoxP site remains after excision of the neo cassette, resulting in 73 extra base pairs in the mutant fragment.

**III.1.3 Dissection of the tissues**

At the day of the experiment, mice aged between 8 and 15 weeks, died after cervical dislocation, while rats aged 6 till 9 weeks were decapitated after sedation with $CO_2$-gas. From both mice and rats the thoracic part of the aorta was isolated and transferred to a petri-dish containing a cold, oxygenated (95% $O_2$ / 5% $CO_2$) physiological Krebs-Ringer Bicarbonate (KRB) solution. Any surrounding adipose/connective tissue was removed as much as possible in situ. From the mice, the femoral artery was also collected by dissecting the artery lose from the femoral vein and nerve. In addition, CC were obtained from mice. All connective, adventitial, fibrous and adipose tissues were removed together with the dorsal arteries, dorsal vein, corpus spongiosum and the urethra. After removal of the glans penis, CC were separated and excised at the base. After obtaining the nicely cleaned femoral artery and CC, the tissues were transferred to a petri-dish containing cold, oxygenated KRB solution. During the dissection one needs to be very careful not to stretch or damage the tissue and to keep the endothelium as intact as possible.

**III.1.4 Mounting of the tissues on the myograph**

Each vessel preparation is mounted using a microscope by means of two thin stainless steel wires (40 µm diameter) that are clamped at a set of two holders in the organ chamber of the myograph. Arterial segments of about 2.5 mm in length are cut in order to be ready for mounting. First, a thin wire (40 µm) is cut to length and clamped between the two holders in the organ bath. (Figure III.3B) Then, the far end of the wire is fixed on the left holder by wrapping it around the fixing screw, whereas the near end is kept free, pointing towards the operator. (Figure III.3C) Using forceps, the ring segment is held as close as possible to the proximal end and is pulled over the free end of the wire. (Figure III.3D) Once the vessel is pulled over the wire, the holders are separated and the ring segment is drawn further over the wire until the segment is situated in the opening between the holders. Subsequently also the near end of the wire is fixed with a screw. Tightening the screw will also tighten the wire. (Figure III.3E and F) Once again the holders are separated and now the second wire is very carefully guided through the lumen of the ring segment, avoiding to damage the endothelium

of the vessel. (Figure III.3G) The holders were brought together and both ends of the second wire are then fixed on the right holder. Finally, the wires are levelled so that both wires are in a single horizontal plane and the length of the segment (l) is measured with a micrometer eyepiece (Zeiss, Germany). Lose wire ends were removed to avoid interference with the experiments. (Figure III.3H) The mounted vessel segments are then equilibrated in a KRB solution at 37°C, pH 7.4 and oxygenated, for 30 minutes in order to simulate physiological circumstances, before a preload is imposed on the vessel.

The mounting of the CC resembles that of the arteries but is easier. Corporal tissues are mounted as a whole. Both the left and right end of the tissue is placed between the clasping parts of the holders. Then by tightening the screw, both tips of a CC are fixed between the holders.

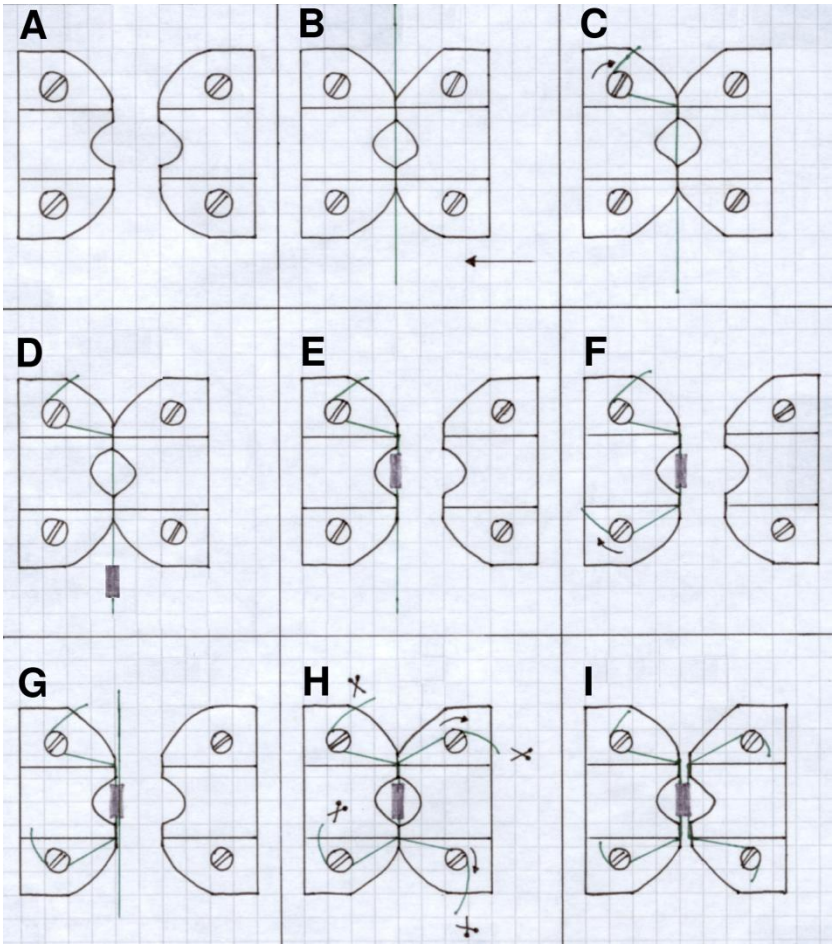


**Figure III.3** represents a schematic overview of the different steps of the mounting procedure of an arterial segment.

## III.1.5 Preparation of the ring segment before the experiment

### III.1.5.1 Preparation of the aortic and corporal segments

After mounting, the tissues are allowed to rest during 30 minutes before they are gradually stretched until a fixed stable preload is obtained. In mouse and rat aorta a preload of 0.5 g and 1 g respectively is used, while for murine CC a preload of 0.45 g is applied. This preload offers a nearly maximal reactivity in these tissues. The equilibration period takes about 60 minutes as in the beginning the tension always spontaneously declines partly after stretching, needing regular adjustment until a fixed stable tension is obtained.

### III.1.5.2 Preparation of the femoral artery segments

Active force development of a muscle cell depends on its length [1;2]. Stretching a vessel to a certain diameter will influence the length of the vascular smooth muscle cells and also the active response since they are circumferentially oriented in small arteries. It is a general finding that the active force development in vascular tissue peaks at a certain internal circumference or diameter, which is equivalent to a particular length of the smooth muscle cell. Aiming for optimal experimental conditions, it is recommended to set the vessels to an internal circumference which gives a maximum response at the beginning of each experiment. Because the vessel wall is elastic, the diameter of a vessel will depend on the transmural pressure. This entails that the conditions should clearly be defined before the vessel diameter is determined. Conventionally, the size of a vessel is defined as being the size when the vessel is fully relaxed and under a transmural pressure of 100 mmHg. It has been found that the active force production is maximal when the internal circumference (IC) is 0.9 times the internal diameter a vessel would have in situ when relaxed and under a transmural pressure of 100 mmHg [1;3-5]. The aim of the normalization procedure is therefore to determine the internal circumference for a vessel mounted in the myograph, denoted $IC_{100}$, which the vessel would have when relaxed and under a transmural pressure of 100 mmHg.

#### *III.1.5.2.1 The normalization procedure*

Before starting the normalization procedure, the vessel is brought to a resting position corresponding to a force of 0 mN. The holders in the organ bath are placed so that both mounting wires touch, resulting in the registration of a negative force. Subsequently the holders are slightly pulled back using the micrometer enabling us to accurately position the holders, until the force registered by the transducers returns to baseline. At this point, the

force registered by the transducer ($y_0$) is 0 mN and the distance between the two wires ($x_0$) is 0 µm.

During the normalization procedure the vessel is distended in steps using the micrometer. One minute after each step (i), the corresponding micrometer setting ($x_i$) and force ($y_i$) are registered. The stepwise distension is stopped when the calculated corresponding pressure exceeds 100 mmHg (13.3 kPa). From the measurements the internal circumference ($IC_i$) and the wall tension ($T_i$) can be calculated. With these values it is possible to calculate the transmural pressure ($P_i$) that would yield this particular $IC_i$ in the ring segment. Equations used to calculate the internal circumference, the wall tension and the transmural pressure are explained below:

- The $IC_i$ is calculated from the distance between the wires ($x_i - x_0$) and the known diameter of the mounting wires (40 µm):
  $\mathbf{IC_i} = 2 \times ((2\pi \times 20\ \mu m) / 2) + 4 \times 20\ \mu m + 2 \times (x_i - x_0) = 205.66\ \mu m + 2 \times (x_i - x_0)$

- The wall tension ($T_i$) is the measured force ($y_i$) divided by the wall length. The wall length is two times the length of the vessel, since there is both an upper and a lower wall. The length of the vessel (l) is measured using a calibrated eyepiece mounted on the dissecting microscope.
  $\mathbf{T_i} = y_i / (2 \times l)$

- The transmural pressure ($P_i$) can be calculated based on Laplace's law, stating that in a cylinder, wall tension ($T_i$) is proportional to the pressure times the radius of the cylinder. The radius of a cylinder can be computed from the internal circumference ($IC_i = 2\pi r$). Therefore, $P_i$ can be determined with the following formula:
  $\mathbf{P_i} = T_i / (IC_i / 2\pi)$

From the calculation of the effective pressure corresponding to each distention, an exponential curve is fitted representing the relation between the internal circumference and pressure data. The internal circumference on the curve corresponding to 100 mmHg is determined and denoted $IC_{100}$. Having found this point, the ideal position of the holder is set to $IC_{90} = 0{,}9 \times IC_{100}$ since it has been shown that at this internal circumference, the active force production is

maximal. The normalized lumen diameter is taken as $L_{90} = IC_{90} / \pi$. It should however be noted that the pressure calculated is only an estimate for the intraluminal pressure necessary to extend the vessel to the measured internal circumference.

III.1.5.3 Applying precontractions

After establishing a stable preload for larger tissues or after the normalization procedure used for smaller vessels, the tissues are ready for experimental use. However, before starting the actual experiment, precontractions are applied to the different tissue until the contractions are more stable and reproducible. For the vessels, the maximum contractile capacity is assessed by stimulating the arteries with $K^+$ 30 or 120 nM and 1 or 5 µM NOR for rat and murine vessels respectively. Using CC, precontractions using 5 µM NOR are used as high $K^+$ concentrations do not result in a stable contraction. Following stabilization, the actual experiment can start. As most isolated vascular tissues do not have an intrinsic tone, it is necessary to apply precontraction to the tissues in order to study relaxations.

III.1.5.4 Control of the endothelium

During most of the experiments it is of great importance that the endothelium remains sufficiently intact not creating deceiving results. In order to evaluate the functionality of the endothelium, a concentration-response curve is made for ACh (1 nM – 10 µM) after the tissues were precontracted with NOR. In some experiments however the endothelium is removed deliberately in order to examine the role of the endothelium itself. Removal of the endothelium was done by rubbing the luminal surface of the vessel with a poly-ethylene (PE) tube for 30 seconds or by carefully bubbling the vessel with carbogen gas during 2 till 3 minutes. Important in this technique is to be as careful as possible not to damage the vascular smooth muscle cells, in order to maintain the contractility of the vessel. The lack of endothelium was functionally confirmed by the failure of ACh (1 nM – 10 µM) to relax the vessel investigated.

## III.2 In vivo techniques

The technique for measuring penile hemodynamics enables to evaluate even subtle erectile responses by analyzing intracavernosal pressure (ICP) and systemic blood pressure [6]. The mice were anaesthetized intraperitoneally with either a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) or an isoflurane/oxygen breathing mixture. For the latter, 10 % isoflurane

was applied for induction, whereas during the preparation and measurements, the concentration of isoflurane was set at 3.5%. Before starting each experiment, pressure transducers were calibrated in mmHg. The anaesthetized mice were placed in a supine position on a thermally isolated blanket and they breathed spontaneously.

### III.2.1 Blood pressure measurements

To evaluate the penile hemodynamics, the systemic blood pressure needs to be assessed simultaneously. Therefore, a PE-10 catheter filled with heparinized saline (100 U/mL) was inserted into the left carotid artery and connected to a pressure transducer to monitor the MAP using the powerlab recorder.

### III.2.2 Intracavernosal pressure measurements

The penis was denuded and both CC were exposed by blunt dissection of overlying ischiocavernous muscle. To monitor the ICP, a 30-gauge needle attached to a PE-10 tube, filled with heparinized saline (100 U/mL), was inserted into the left CC. The PE-10 tube is further connected to a pressure transducer and the data were recorded on the computer using powerlab software.

### III.2.3 Intravenous injections

The jugular vein was catheterized for intravenous bolus injections with a PE-10 tube which is connected to a needle in order to inject small volumes (10 µl). Also this catheter is filled with heparinized saline (100 U/ml) in order to avoid blood clotting in the tubes. Different pharmacological agents were administered intravenously, resulting in dose-response curves. Between every dose injected the catheter is flushed a couple of times with saline, to remove residual concentrations of the pharmacological agents used.

### III.2.4 Intracorporal injections

Different pharmacological agents can be administered intracavernosally via a separate cannula consisting of a 30-gauge needle which is attached to PE-10 tube that is connected to a 25 µl syringe. This cannula is inserted into the right CC. Because the corporeal bodies communicate in the mouse penis, physiologic effects resulting from the administration of a drug into one corporal body can successfully be monitored via a separate apparatus applied to the other one [7]. Injection volumes were standardized to 2 µl to ensure minimal volume related changes on ICP. After injection of a certain dose of a pharmacological agent the

cannula is flushed a number of times with saline, removing residual pharmacological substances.

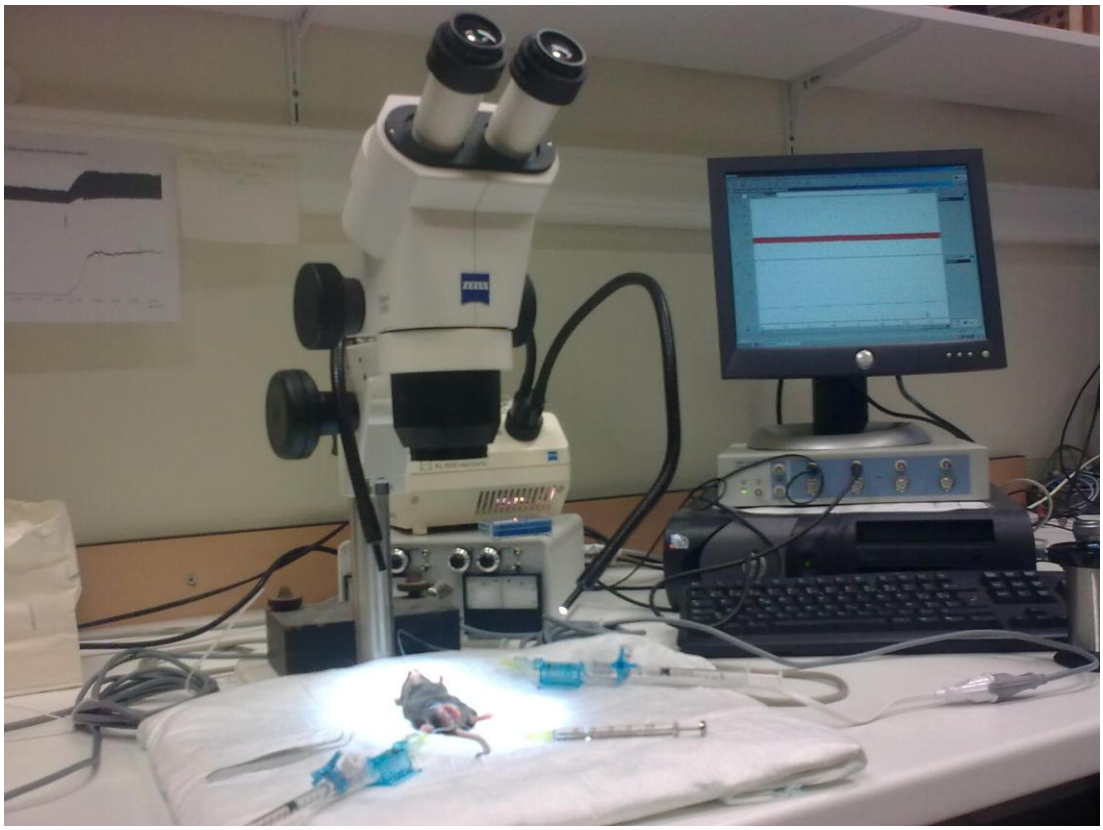

**Figure III.4** is a photograph illustrating the in vivo technique whereby both the MAP (upper channel PC) and ICP (lower channel PC) of an anaesthetized mouse are simultaneously recorded. The signals are first transduced to the digital scale by the signal transducer and then amplified using the powerlab.

### III.2.5 Electrical stimulation of the cavernous nerve

For some experiments electrical stimulation (ES) of the cavernous nerves was performed to elicit erectile response. Bladder and prostate were exposed via a midline suprapubic incision and testes and epididymis were repositioned into the abdomen after they were divided from their scrotal attachments. The bilateral cavernosal nerves were located and isolated lateral to the urethra, at the lower lateral portion of the prostate [8]. A stainless steel bipolar electrode with parallel hooks was placed around the isolated nerve. The electrode was attached to a Grass S88 stimulator and the following stimulation parameters were used: 5, 10 and 15 Hz, duration of 5 ms, 8 V. Each stimulation period lasted 60 seconds and a resting interval of 15 minutes was applied.

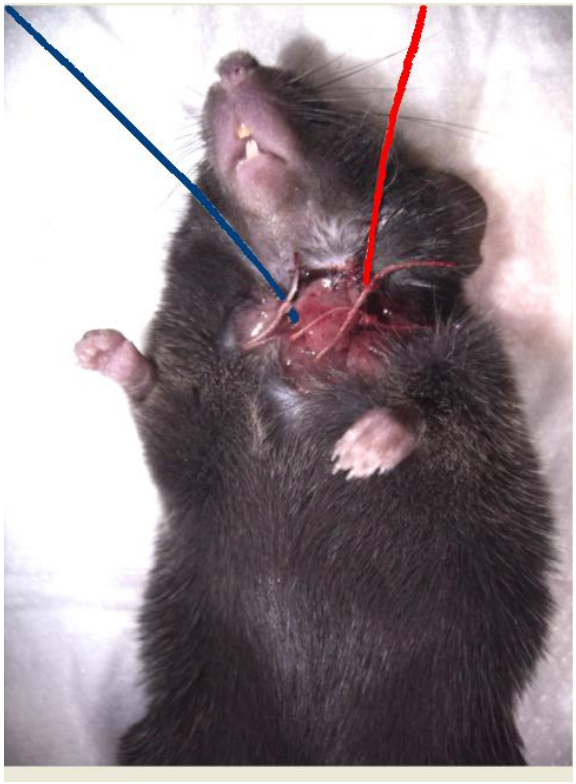

**Figure III.5** is a photograph taken of a mouse whereby a catheter is inserted into the carotid artery (red colored catheter) to measure MAP and another catheter is inserted into the jugular vein (bleu colored catheter) to deliver different agents.

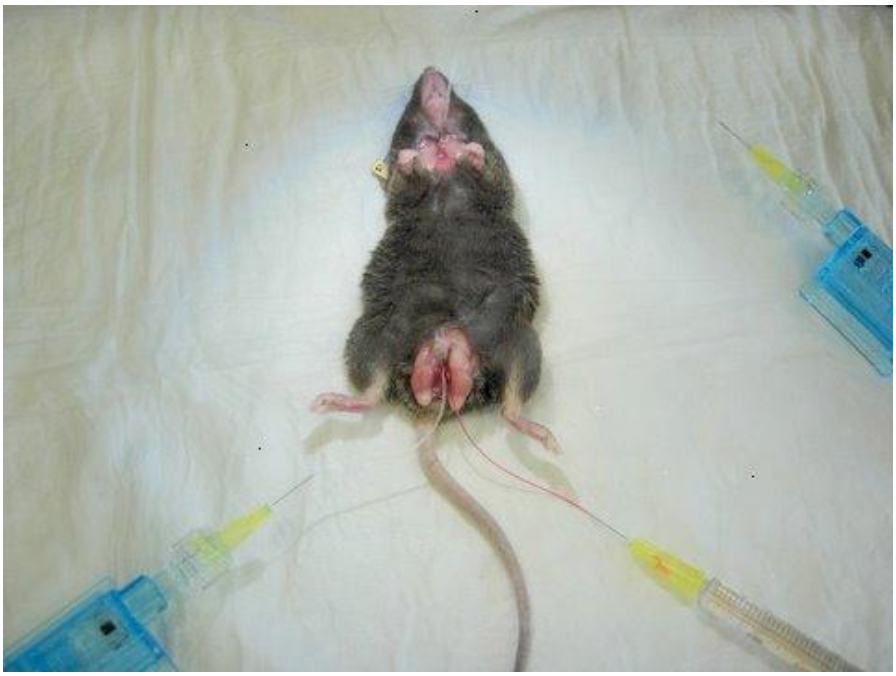

**Figure III.6** illustrates a mouse in which 2 cannula were inserted, one into the left CC for intracavernosal injections and one into the right CC for ICP measurement. An extra catheter is inserted into the carotid artery for the measurement of the MAP.

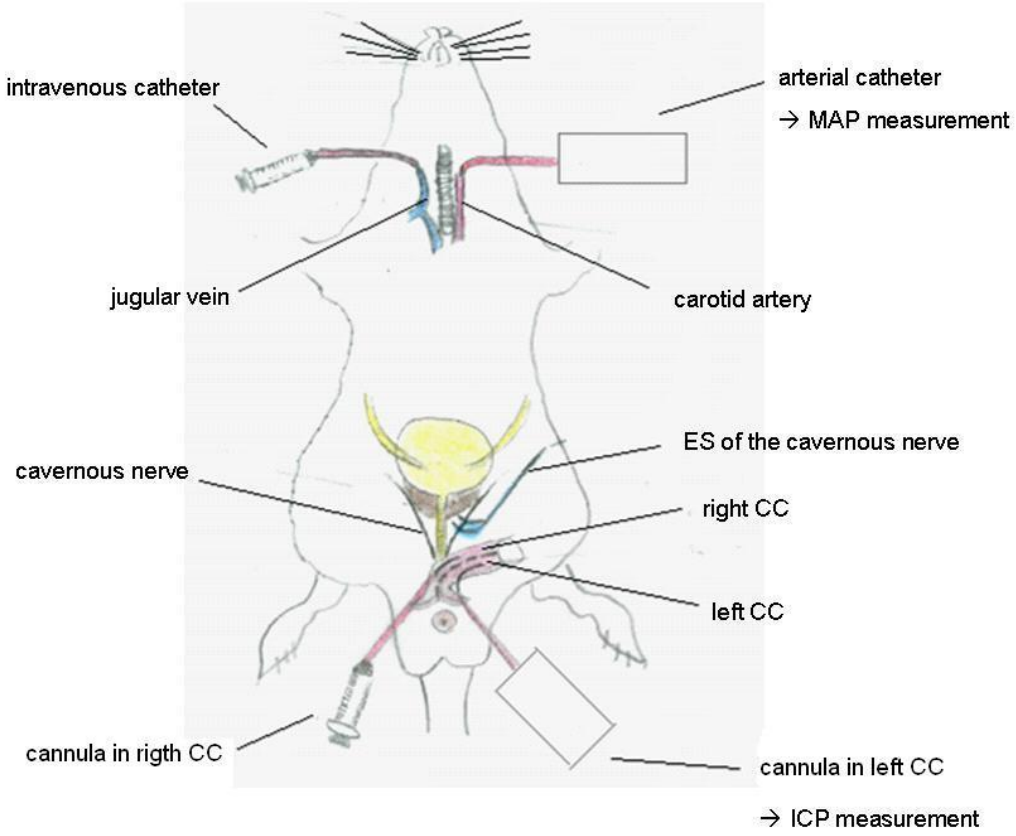


**Figure III.7** is a simplified drawing of the different techniques/handlings used for the in vivo studies.

## III.3 Data processing and statistical analysis

Data are presented as mean values ± SEM; *n* represents the number of animals. In the in vitro studies relaxations are expressed as the percentage change of the pre-contracted tone developed by the addition of NOR or the percentage relaxation. For the in vivo study, results are calculated as the ICP, adjusted for the MAP, expressed in percentage (ICP/MAP x 100). Statistical significance was evaluated by using Student's *t*-test for paired and unpaired observations (SPSS, version 12) or with two-way ANOVA with Bonferroni post hoc test (GraphPad Prism, version 4), when appropriate. Additional to the Student's t-test for unpaired observation, non-parametric tests and the Mann-Whitney U test were performed to evaluate the statistics. $P<0.05$ was considered as statistically significant.

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# Chapter IV:

# Results

# Manuscript 1

# Role of the soluble guanylyl cyclase $\alpha_1$ subunit in mice corpus cavernosum smooth muscle relaxation

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## IV.1.1 Abstract

sGC is the major effector molecule for NO, and as such an interesting therapeutic target for the treatment of ED. To assess the functional importance of the $sGC\alpha_1\beta_1$ isoform in CC relaxation, CC from male $sGC\alpha_1^{-/-}$ and wild-type mice were mounted in organ baths for isometric tension recording. The relaxation to endogenous NO (from ACh, bradykinin and EFS) was nearly abolished in the $sGC\alpha_1^{-/-}$ CC. In the $sGC\alpha_1^{-/-}$ mice, the relaxing influence of exogenous NO (from SNP and NO-gas), BAY 41-2272 and T-1032 (PDE-5 inhibitor) were also significantly decreased. The remaining exogenous NO-induced relaxation seen in the $sGC\alpha_1^{-/-}$ mice was further significantly decreased by the sGC inhibitor ODQ. The specificity of the impairment of the sGC related responses was demonstrated by the unaltered relaxations seen with forskolin (AC activator) and 8-pCPT-cGMP (cGMP-analogue). In conclusion, the $sGC\alpha_1\beta_1$ isoform is involved in corporal smooth muscle relaxation in response to NO-dependent and -independent sGC stimulators. The fact that there is still some effect of exogenous NO in the $sGC\alpha_1^{-/-}$ mice, suggests the contribution of (an) additional pathway(s).

## IV.1.2. Introduction

Penile erection is a complex, neurally regulated physiologic event that involves increased blood filling of the corporal tissue and restricted venous outflow, both resulting from corporal smooth muscle relaxation [1]. NO is widely accepted as the principal mediator of the erectile response. It is produced by nNOS in NANC nerves, innervating the penis[2]. Although also sinusoidal and vascular endothelial cells release NO in response to mechanical [3] and chemical stimuli [1;4], neurogenic NO is generally considered as the primary source required for penile erection. However, the importance of NO produced by eNOS for penile erection is becoming increasingly recognized [5]. Regardless of the source, NO binds to the heme component of sGC, leading to a 300-fold increase in the catalytic conversion of GTP to cGMP and pyrophosphate [6]. This high amount of cGMP conveys signals through activation of PKG-I, eventually leading to smooth muscle relaxation [7;8]. sGC is a heterodimer composed of two subunits, α and β [9], both essential for catalytic activity [10]. Two isoforms for each subunit (α1/α2 and β1/β2) have been described [11-13], but only the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ heterodimers are found active [14]. $sGC\alpha_1\beta_1$ is the predominantly expressed isoform in most tissues except in the brain, in which the levels of both isoforms are comparable [15]. Various diseases, including hypertension [16;17], hypercholesterolemia [18], diabetes mellitus [19] and renal failure [20], that cause ED are highly associated with impairments of the NO/cGMP

signaling pathway. The central role of this pathway is demonstrated by the PDE-5 inhibitor sildenafil as today's most successful therapy for the treatment of ED. However, since some side-effects and limitations for use have been reported [21;22], there is an increasing interest for alternative therapeutic measures. sGC is, as the predominant intracellular receptor of NO, a promising therapeutic target. The aim of the present study was therefore to analyze the functional importance of the $sGC\alpha_1\beta_1$ isoform in penile smooth muscle relaxation using $sGC\alpha_1^{-/-}$ mice.

## IV.1.3. Materials and methods

### IV.1.3.1. Animals

All experiments were performed on male homozygous $sGC\alpha_1^{-/-}$ (n= 6-9) mice and $sGC\alpha_1^{+/+}$ (n=6-11) mice (genetic background: mixed Swiss-129) [23], bred in the Department of Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology, Ghent, Belgium. The animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). On the day of experiment, the mice were sexually mature (age: 10-15 weeks) and euthanized by cervical dislocation.

### IV.1.3.2. Tissue collection

The penile tissue was dissected free by removal of connective and adventitial tissues along the shaft of the penis, the dorsal arteries, dorsal vein, corpus spongiosum, urethra and glans penis. Then, the CC were separated by cutting the fibrous septum between them and were excised at the base. They were kept in cooled KRB solution until mounting.

### IV.1.3.3. Tension measurements

Of each mouse, one CC was mounted horizontally in a myograph with one end fixed to a force-displacement transducer and the other to a micrometer. The tissue chambers contained 10 ml KRB solution at 37 °C (pH 7.4) equilibrated with 95% $O_2$ - 5% $CO_2$. The preparations were preloaded with 0.45 g of tension and allowed to equilibrate for 60 minutes in bath fluid that was frequently replaced with fresh KRB solution. The preparations were 3 times contracted with 5 µmol/L NOR, washed and allowed to relax to resting tension before starting the protocol. When the pre-contraction response reached a stable level, EFS (train duration 20 s or 40 s; frequency: 1, 2, 4 and 8 Hz; pulse duration: 5 ms; 80 V), delivered by a Grass

stimulator via two parallel platinum electrodes, was applied to the tissue or various vasodilating substances were added to the bath medium. In some experiments, increasing concentrations of NOR were added at a stable resting tension to analyze the contractile response. EFS was repeated after incubation with atropine (1 µmol/L) and guanethidine (4 µmol/L) for 30 minutes to eliminate responses mediated by cholinergic and noradrenergic nerves, respectively. In addition, the influence of the sGC inhibitor ODQ (1 µmol/L, 20 min preincubation) was investigated on EFS and drug-induced effects. Between response-curves, the CC were washed and allowed to recover for 20-30 min. At the end of the experiments, tissues were lightly patted dry and weighed.

**IV.1.3.4. Drugs**

The experiments were performed in a KRB solution of 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 $H_2O$. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), acetylcholine chloride (ACh), bradykinin acetate (BK), Nω-nitro-L-arginine, forskolin, 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate (8-pCPT-cGMP), atroprine, guanethidine and norepinephrine bitartrate (NOR) were obtained from Sigma-Aldrich (St.Louis, MO), BAY 41-2272 from Alexis (San Diego, USA) and sodium nitroprusside (SNP) from Merck (Darmstadt, Germany). ODQ and BAY 41-2272 were dissolved in dimethylsulfoxide and ACh in 50 mmol/L potassium hydrogen phtalate buffer, pH 4.0. The other drugs were dissolved in distilled water. Saturated NO solution was prepared from gas (Air liquide, Belgium) as described by Kelm & Schrader [24]. All concentrations are expressed as final molar concentrations in the organ bath. The final concentration of dimethylsulfoxide in the organ bath never surpassed 0.1%.

**IV.1.3.5. Calculations and statistics**

Data are presented as mean values ± SEM; n represents the number of CC (each obtained from a different mouse). Relaxations are expressed as a percentage of the tone developed by the addition of NOR. Contractions are expressed in mN. Statistical significance was evaluated using Student's t-test for paired and unpaired observations (SPSS, version 18) or with two-way ANOVA with Bonferroni post hoc test (GraphPad Prism, version 4) when appropriate. $P < 0.05$ was considered as significant.

## IV.1.4. Results

The weight of the CC preparations did not significantly differ between $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ mice (13.54 mg ± 0.80 (n=9) vs. 12.77 mg ± 0.77 (n=11, P > 0.05)).

In response to increasing concentrations of NOR (10 nmol/L-10 µmol/L), the penile tissue isolated from $sGC\alpha_1^{-/-}$ mice, developed an equal force per mg tissue compared with the $sGC\alpha_1^{+/+}$ preparations (figure IV.1.1).

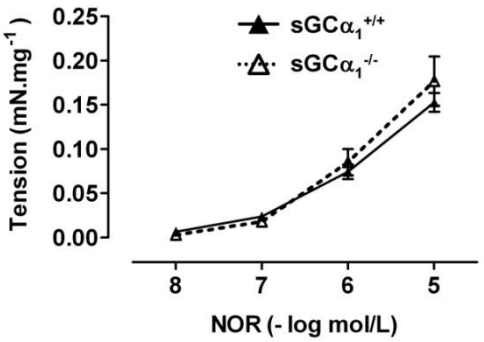


**Figure IV.1.1** Cumulative concentration-contraction curve to NOR in CC from $sGC\alpha_1^{+/+}$ (■; n = 11) and $sGC\alpha_1^{-/-}$ (▲; n = 9) mice.

The ability to relax NOR-contracted CC preparations through release of endothelial NO was tested by addition of ACh (10 µmol/L) and BK (50 µmol/L). ACh relaxed the $sGC\alpha_1^{+/+}$ preparations, whereas it contracted the tissues of $sGC\alpha_1^{-/-}$ mice (figure IV.1.2). Inhibition of sGC by ODQ, resulted in a contractile effect of ACh in both $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ CC tissues (figure IV.1.2). BK had a relaxant effect in the CC of both $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ mice, though the response in the $sGC\alpha_1^{-/-}$ preparations was significantly reduced (4.55% ± 2.80 in $sGC\alpha_1^{-/-}$ vs. 26.82% ± 2.86 in $sGC\alpha_1^{+/+}$ (n = 6 each, P < 0.05)).

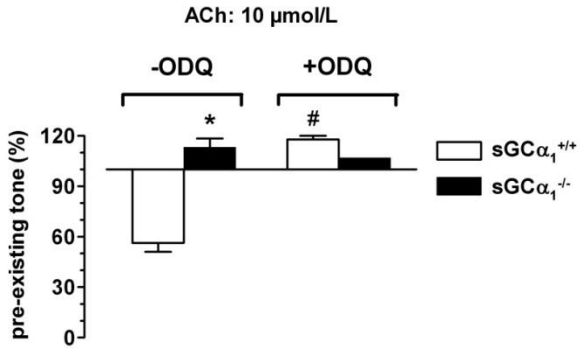


**Figure IV.1.2** Relaxation effect of ACh on precontracted (5 µmol/L NOR) CC from $sGC\alpha_1^{+/+}$ (n = 7) and $sGC\alpha_1^{-/-}$ (n = 7) mice in control conditions (-ODQ) and in the presence of ODQ (+ODQ). * $sGC\alpha_1^{+/+}$ vs. $sGC\alpha_1^{-/-}$, # -ODQ vs. +ODQ: P < 0.05.

The effect of neuronal NO was examined by stimulating the intrinsic nerves with EFS. EFS relaxed the tissues of $sGC\alpha_1^{+/+}$ mice in a frequency-dependent manner (figure IV.1.3A), whereas the response in the $sGC\alpha_1^{-/-}$ preparations was nearly abolished (figures IV.1.3B and IV.1.4A). Following preincubation with ODQ, the relaxations induced by EFS in the $sGC\alpha_1^{+/+}$ preparations were completely blocked and resulted even in a small contractile response of EFS at 8 Hz (figure IV.1.4A). As the response to EFS in the CC of the $sGC\alpha_1^{-/-}$ mice was very small, the influence of ODQ was negligible. The presence of guanethidine and atropine, significantly reduced relaxation by EFS on the $sGC\alpha_1^{+/+}$ CC (figure IV.1.4B). The very limited EFS-induced response in the $sGC\alpha_1^{-/-}$ preparations was unaltered by guanethidine and atropine, even after increasing the stimulation period from 20 s to 40 s (data not shown).

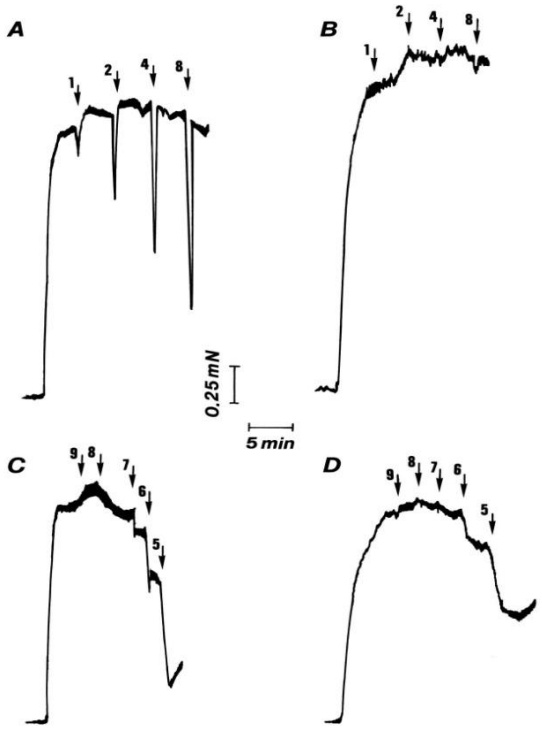


**Figure IV.1.3** Original tracing showing a response curve to EFS (Hz) (A, B) and to SNP (-log mol/L) (C, D) in CC from a $sGC\alpha_1^{+/+}$ (A, C) and a $sGC\alpha_1^{-/-}$ (B, D) mouse.

Administration of increasing concentrations of the endothelium-independent NO-donor compound SNP (1 nmol/L – 10 µmol/L) resulted in a concentration-dependent relaxation of the $sGC\alpha_1^{+/+}$ (figure IV.1.3C) and $sGC\alpha_1^{-/-}$ (figure IV.1.3D) CC preparations, that was significantly reduced in the $sGC\alpha_1^{-/-}$ mice as compared to $sGC\alpha_1^{+/+}$ mice (IV.1.5A). The maximal relaxation to SNP in the CC from $sGC\alpha_1^{-/-}$ mice was decreased by approximately 38%. Preincubation of the CC tissues with ODQ strongly inhibited the SNP-induced responses in both $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice (figure IV.1.5A).

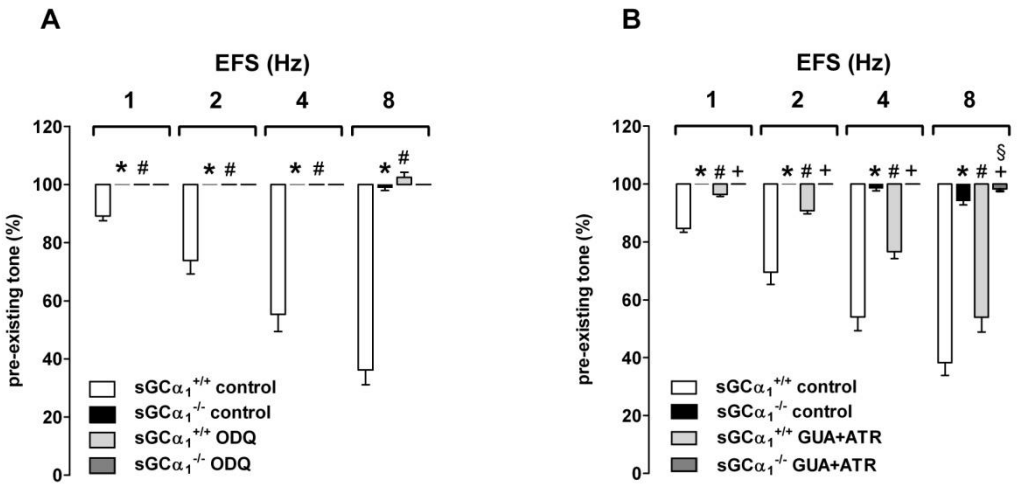


**Figure IV.1.4** Effect of EFS on precontracted (5 µmol/L NOR) CC from $sGC\alpha_1^{+/+}$ (n = 6) and $sGC\alpha_1^{-/-}$ (n = 6) mice in control conditions and in the presence of ODQ (A) or guanethidine (GUA) and atropine (ATR) (B). * $sGC\alpha_1^{+/+}$ vs. $sGC\alpha_1^{-/-}$, + $sGC\alpha_1^{+/+}$ vs. $sGC\alpha_1^{-/-}$ both in the presence of GUA+ATR, # -ODQ or -GUA+ATR vs. +ODQ or +GUA+ATR for $sGC\alpha_1^{+/+}$ mice, § -GUA+ATR vs. +GUA+ATR for $sGC\alpha_1^{-/-}$ mice: $P < 0.05$.

Exogenous NO delivered as gas (1 µmol/L – 100 µmol/L) and added non-cumulatively, was able to relax the CC preparations of both $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ mice in a concentration-dependent way. However, the response to NO-gas was significantly reduced in the penile tissues of the $sGC\alpha_1^{-/-}$ mice as compared to those of $sGC\alpha_1^{+/+}$ mice (figure IV.1.5B). The maximum response to NO-gas was significantly diminished in $sGC\alpha_1^{-/-}$ CC by approximately 36% compared to control. Treatment with ODQ significantly reduced the relaxant effect of NO-gas in both $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ CC preparations (figure IV.1.5B).

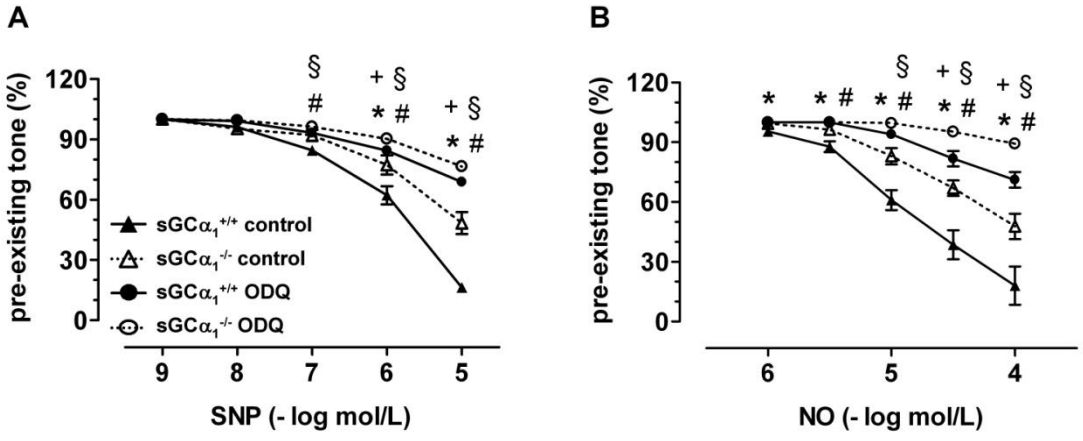


**Figure IV.1.5** Relaxation effect of SNP (A) and NO-gas (B) on precontracted (5 µmol/L NOR) CC from $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice in control conditions (▲ and Δ) and in the presence of ODQ (● and ○). * $sGC\alpha_1^{+/+}$ vs. $sGC\alpha_1^{-/-}$, + $sGC\alpha_1^{+/+}$ ODQ vs. $sGC\alpha_1^{-/-}$ ODQ: $P < 0.05$, (n = 6-7).

The inhibition of PDE-5 by T-1032 [25] (1 nmol/L – 10 µmol/L), resulted in a concentration-dependent relaxant response in the penile tissue from both $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ mice. This response was however significantly smaller in the $sGC\alpha_1^{-/-}$ mice than in $sGC\alpha_1^{+/+}$ mice (figure IV.1.6A).

Addition of BAY 41-2272, an NO-independent sGC stimulator [26] (1 nmol/L – 10 µmol/L), produced a concentration-dependent relaxation in the CC preparations of both $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ mice. However BAY 41-2272 had a significantly smaller effect in the $sGC\alpha_1^{-/-}$ preparations compared to $sGC\alpha_1^{+/+}$ penile tissue (figure IV.1.6B).

There was no difference in the concentration-dependent response to the cell membrane permeable cGMP-analogue, 8-pCPT-cGMP (100 nmol/L – 10 mmol/L) between the $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ preparations (figure IV.1.6C). Also forskolin, an AC-stimulator, induced an identical concentration-dependent responses in the $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ preparations (figure IV.1.6D).

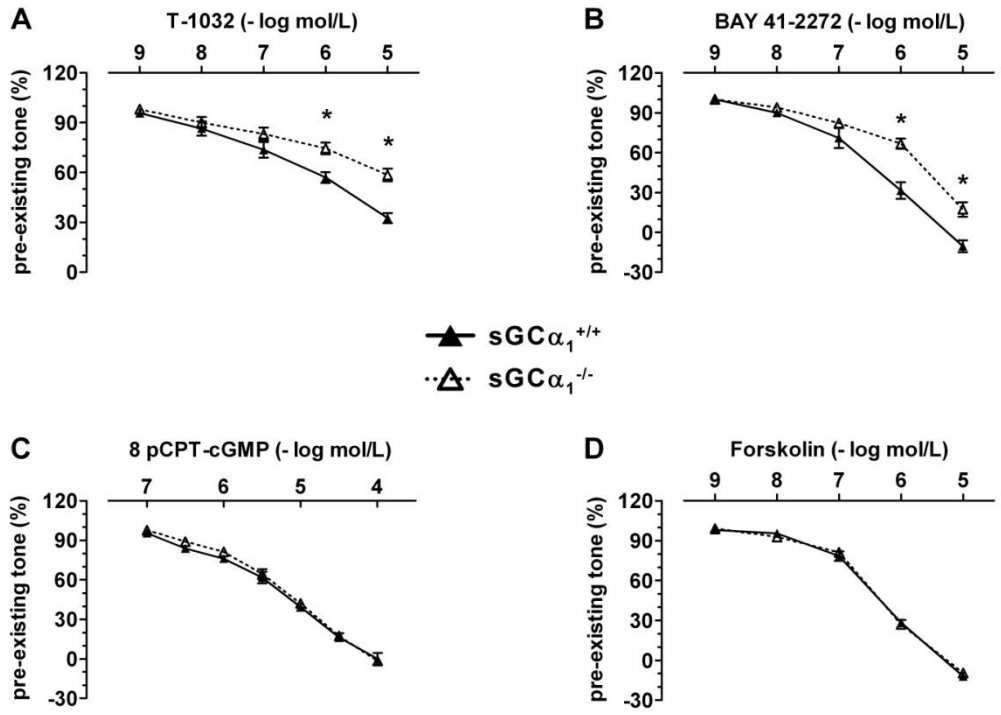


**Figure IV.1.6** Relaxation effect of T-1032 (A), BAY 41-2272 (B), 8-pCPT-cGMP (C) and forskolin (D) on precontracted (5 µmol/L NOR) CC from $sGC\alpha_1^{+/+}$ (▲; n = 6) and $sGC\alpha_1^{-/-}$ (Δ; n = 7) mice. * $sGC\alpha_1^{+/+}$ vs. $sGC\alpha_1^{-/-}$: $P < 0.05$.

## IV.1.5. Discussion

It is generally accepted that sGC, as major effector molecule for NO, plays a very important role in penile smooth muscle cell relaxation. An understanding of the functional importance of the sGC isoforms in penile erection is necessary to validate the sGC subunits as therapeutic targets for the treatment of ED. It has been shown that the main isoform of sGC expressed in the CC is $sGC\alpha_1\beta_1$ [27]. By the present study, this notion is translated in its functional importance, as the response to endogenous NO from endothelial origin is nearly abolished in the CC preparations of $sGC\alpha_1^{-/-}$ mice compared to the $sGC\alpha_1^{+/+}$ CC tissues. CC from $sGC\alpha_1^{-/-}$ mice developed even contractions in response to ACh and showed significantly less relaxation in response to BK. This indicates that endothelium-derived NO exerts its effect through activation of the $sGC\alpha_1$ subunit. These observations are in line with our observations on aorta and femoral arteries [28]. This is an interesting finding for the development of new therapies for ED, as the role of endothelial NO in penile erection is becoming more significant than originally thought [5]. Moreover, our data suggest the involvement of endothelium-derived NO induced by ACh released from parasympathetic nerve fibers [29], as atropine inhibited the EFS-induced relaxation in the CC of $sGC\alpha_1^{+/+}$ mice. Those EFS-induced relaxations are completely mediated by NO/sGC, as they were completely abolished by the sGC inhibitor ODQ. In the CC preparations of the $sGC\alpha_1^{-/-}$ mice, the response to EFS was completely abolished, even after prolonged stimulation (40 s), indicating that $sGC\alpha_1\beta_1$ also functions as the predominant target for neuronal NO. Furthermore, this finding does not support the putative role of VIP as inhibitory neurotransmitter in penile erection [30].VIP, which is present in the nerves of murine CC, as well as other species, stimulates AC and subsequently elevates the PKA [31]. As it has been shown that there are cross-modulatory functions between the sGC/cGMP- and AC/cAMP-signaling pathways [32], one could suggest that the latter has a complementary role in the control of cavernous smooth muscle tone. However, we show that the AC activator, forskolin, relaxes the CC preparations of both $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ mice to a similar extent. Therefore, there is no evidence for an upregulation and possible compensatory effect of the AC/cAMP transduction pathway in the $sGC\alpha_1^{-/-}$ mice. Furthermore, this unaltered forskolin-induced response in the $sGC\alpha_1^{-/-}$ mice demonstrates that the reduced sGC related responses in this study are not due to an aspecific impairment of relaxation related to structural damage.

Our data not only illustrate the functional importance of the $sGC\alpha_1\beta_1$ isoform in vasorelaxations induced by endogenous NO but also by exogenous NO, since the response to SNP and NO-gas were significantly reduced in the CC of the $sGC\alpha_1^{-/-}$ mice. However, in the $sGC\alpha_1^{-/-}$ preparations, SNP (release of exogenous NO upon biotransformation) and NO-gas (represents exogenous NO as such) still elicit a relaxing effect, indicating that $sGC\alpha_1\beta_1$ is not the sole mechanism responsible for those relaxations. ODQ, which inhibits both sGC isoforms, had a strong inhibitory effect on the exogenous NO-induced relaxations observed in the $sGC\alpha_1^{-/-}$ mice. Therefore, we suggest that also the minor $sGC\alpha_2\beta_1$ isoform participates in the responses to SNP and NO-gas. Additionally, we suggest that also (an) sGC-independent mechanism(s) may be involved, as the relaxing effect of exogenous NO in the $sGC\alpha_1^{-/-}$ CC is not completely abolished by ODQ. It has been shown that, by stimulation of $Na^+/K^+$-ATPase activity, NO (derived from SNP) relaxes human CC smooth muscle cells independently of its ability to increase the intracellular cGMP concentration [33] The ability of NO to directly activate $Ca^{2+}$-dependent $K^+$ channels or sarcoplasmic/endoplasmic reticulum $Ca^{2+}$-ATPase, as described for vascular smooth muscle cells [34], might be involved.

The administration of T-1032, which blocks the hydrolysis of cGMP by PDE-5, resulted in a relaxation that is significantly reduced in the $sGC\alpha_1^{-/-}$ penile tissue as compare to $sGC\alpha_1^{+/+}$ penile tissue. This observation suggests a smaller basal sGC activity in the $sGC\alpha_1^{-/-}$ mice. Molecules such as BAY 41-2272, that stimulate sGC without the need of NO [26], are of particular interest for ED-patients who respond less well to PDE-5 inhibitors because of severe endothelial and/or nerve dysfunction [35]. The finding that the response to BAY-41-2272 was significantly diminished but not completely abolished in the $sGC\alpha_1^{-/-}$preparations, implies that besides $sGC\alpha_1\beta_1$, also $sGC\alpha_2\beta_1$ and/or (an) sGC-independent mechanism(s) participate(s) in those relaxation. BAY 41-2272 has been shown to activate both sGC isoforms [36] and also to exert some cGMP-independent actions [37].

In the $sGC\alpha_1^{-/-}$ mice, the signaling cascade downstream of sGC functions normal because the cGMP-analogue 8-pCPT-cGMP relaxed CC of the $sGC\alpha_1^{-/-}$ mice to the same extent as the $sGC\alpha_1^{+/+}$ preparations.

## IV.1.6. Conclusions

The present study demonstrates the involvement of the predominantly expressed isoform, $sGC\alpha_1\beta_1$, in murine CC smooth muscle relaxation in response to NO and NO-independent sGC stimulator. However, as some responsiveness to exogenous NO (SNP and NO-gas) and

an sGC stimulator (BAY 41-2272) remains in the $sGC\alpha_1^{-/-}$ mice, also the less abundantly expressed isoform, $sGC\alpha_2\beta_1$ and/or (an) sGC-independent mechanism(s) are suggested to participate.

## IV.1.7. Acknowledgements

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# Manuscript 2

# In vitro and in vivo studies on the importance of the soluble guanylyl cyclase $\alpha_1$ subunit in penile erection

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## IV.2.1 Abstract

**PURPOSE:** sGC, which plays a pivotal role in penile erection, is a heterodimer build up by an α and a β subunit. For both subunits two isoforms have been characterized, but only the $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$ isoforms seem to be functionally active. To elucidate the functional role of the $sGC\alpha_1\beta_1$ heterodimer in the mechanism of erection, experiments were performed in vivo and on isolated CC using $sGC\alpha_1^{-/-}$ mice.

**MATERIALS AND METHODS:** For the *in vivo* study sGC-dependent and -independent vasorelaxing agents were injected intracavernosally in $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ mice and the rise in intracavernosal pressure was recorded. For the *in vitro* study, isolated CC tissues from $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ mice were mounted in organ baths for isometric tension recording and concentration-dependent curves were obtained for sGC-dependent and -independent vasorelaxing agents. These experiments were performed on 2 different mice strains (129SvJ and C57BL6/J) to determine potential strain differences.

**RESULTS:** The responses in $sGC\alpha_1^{-/-}$ after administration of the NO-donors, SNP and spermine-NO, and to ES are significantly reduced although not completely abolished. Responses to sGC-independent vasorelaxing agents are similar in $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ mice from both strains, suggesting that the decreased potential of smooth muscle relaxation is not related to structural changes or changes in the pathway downstream sGC.

**CONCLUSION:** This study illustrates the strain-independent importance of the $sGC\alpha_1\beta_1$ heterodimer, although remaining vasorelaxing responses in the $sGC\alpha_1^{-/-}$ mice suggest a complementary role for the $sGC\alpha_2\beta_1$ isoform or (an) sGC-independent mechanism(s).

## IV.2.2 Introduction

Penile erection is a hemodynamic process coordinated by smooth muscle relaxation which is established by activation of the NO/cGMP pathway [1;2]. Because of its central role in this pathway, sGC represents an attractive new therapeutic target for treating ED [3]. sGC is an obligatory αβ heterodimer of which two isoforms for each subunit have been characterized ($\alpha_1/\alpha_2$ and $\beta_1/\beta_2$), although only the $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$ heterodimers are catalytically active [4;5]. To date few information is available on the physiological relevance of these two isoforms. Therefore the aim of this study was to investigate the functional importance of the $sGC\alpha_1\beta_1$ in penile erection in order to determine the possible value of this isoform as a

pharmacological target. As studies have already demonstrated that $sGC\alpha_1\beta_1$ is the predominantly expressed isoform in the CC [6], we hypothesized that this isoform physiologically also plays the dominant role in penile erection. Experiments were performed *in vitro* and *in vivo* on sGC alpha1 knockout ($sGC\alpha_1^{-/-}$) mice [7], using two different pure bred strains, namely 129SvJ and C57BL6/J, to detect potential strain differences.

## IV.2.3 Materials and methods

### IV.2.3.1 Animals

Mature (13-16 weeks) male $sGC\alpha_1^{-/-}$ and $sGC\alpha_1^{+/+}$ mice (C57BL6/J or 129SvJ) bred in the SPF facility of the Department of Molecular Biomedical Research, VIB, Ghent, Belgium were used [7]. The animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication no. 85-23, revised 1996). The experiments were approved by the local Ethical Committee for Animal Experiments, Faculty of Medicine and Health Sciences, Ghent University, Belgium.

### IV.2.3.2 *In vitro* study

The CC were dissected free from the surrounding structures, separated from each other and excised at the base. Of each mouse, one CC was mounted in a myograph for isometric tension recording with one end fixed to a transducer and the other to a micrometer. The tissue chambers were filled with 10 mL KRB solution at 37°C (pH 7.4) equilibrated with 95% $O_2$ – 5% $CO_2$. The preparations were preloaded (0.45g) and allowed to equilibrate for one hour in KRB that was frequently replaced. The preparations were 3 times contracted with 5 µmol/L NOR, washed and allowed to relax to resting tension before starting the protocol. When the next precontraction with 5 µmol/L NOR reached a stable level, EFS (parameters: train duration 20 s; 1, 2, 4 and 8 Hz; pulse duration 5 ms and 80 V), delivered by a Grass stimulator via two parallel platinum electrodes, was applied to the tissue or various vasodilating substances were added to the bath medium. Between the response-curves, the CC were washed and allowed to recover for 20-30 min.

### IV.2.3.3 *In vivo* study

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). During the experiment the mice breathed spontaneously and their body temperature was maintained at 37°C by means of a heated blanket. A PE-10 tube was introduced in the left carotid artery and

a 30-gauge needle attached to a PE-10 tube was inserted into the right CC. Both PE-10 tubes were connected to a pressure transducer to simultaneously monitor the MAP and ICP respectively and filled with heparinised saline (100 U/mL). In a first set of experiments the response to sGC-dependent and –independent vasodilators were investigated through intracavernosal administration via a separate cannula (30-gauge needle attached to PE-10 tube and 25 µl syringe) inserted into the left CC (injection volume was standardized to 2 µL). In a second set of experiments ES of the cavernous nerves was performed. This nerve is found lateral to the urethra, at the lower lateral portion of the prostate. A platinum electrode with parallel hooks (0.7-1 mm) was placed around the nerve and the following stimulation parameters were used: 5, 10 and 15 Hz, duration 5 ms, 8 V. Each stimulation had a duration of 60 s and a resting interval of 15 minutes.

#### IV.2.3.4 Drugs and Chemicals

The KRB solution for the *in vitro* studies had 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 $H_2O$. Sodium nitroprusside (SNP) was obtained from Merck (Darmstadt, Germany). Norepinephrine (NOR), N-[4-[1-(3- Aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine (Spermine-NO), forskolin, 8-(4-Chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate sodium salt (8-pCPT-cGMP), and $N^{\omega}$-Nitro-L-arginine methyl ester hydrochloride (L-NAME) from Sigma-Aldrich (St Louis, MO, USA). Forskolin was dissolved in ethanol. The other drugs were dissolved in water (*in vitro*) or heparinised saline (100 U/mL) (*in vivo*). All concentrations are expressed as final molar concentrations in the organ bath studies, while for the *in vivo* studies the amount of agent injected is given as µg/kg.

#### IV.2.3.5 Calculations and statistics

Data are presented as mean values ± SEM; n represents the number of mice. For the *in vitro* study relaxations are expressed as a percentage of the pre-existing tone developed by NOR. For the *in vivo* study, results are calculated as the ICP adjusted for the MAP, expressed in percentage (ICP/MAP x 100). Statistical significance was evaluated using Student's t-test for unpaired observations (SPSS, version 19).

## IV.2.4 Results

### IV.2.4.1 *In vitro* studies on 129SvJ background (Figure IV.2.1)

SNP, spermine-NO and EFS relaxed the tissues of $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice in a concentration- or frequency-dependent manner, however the response in the $sGC\alpha_1^{-/-}$ preparations was significantly reduced, although not completely abolished. In contrast, 8-pCPT-cGMP and forskolin induced identical concentration-dependent relaxations of both $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ CC.

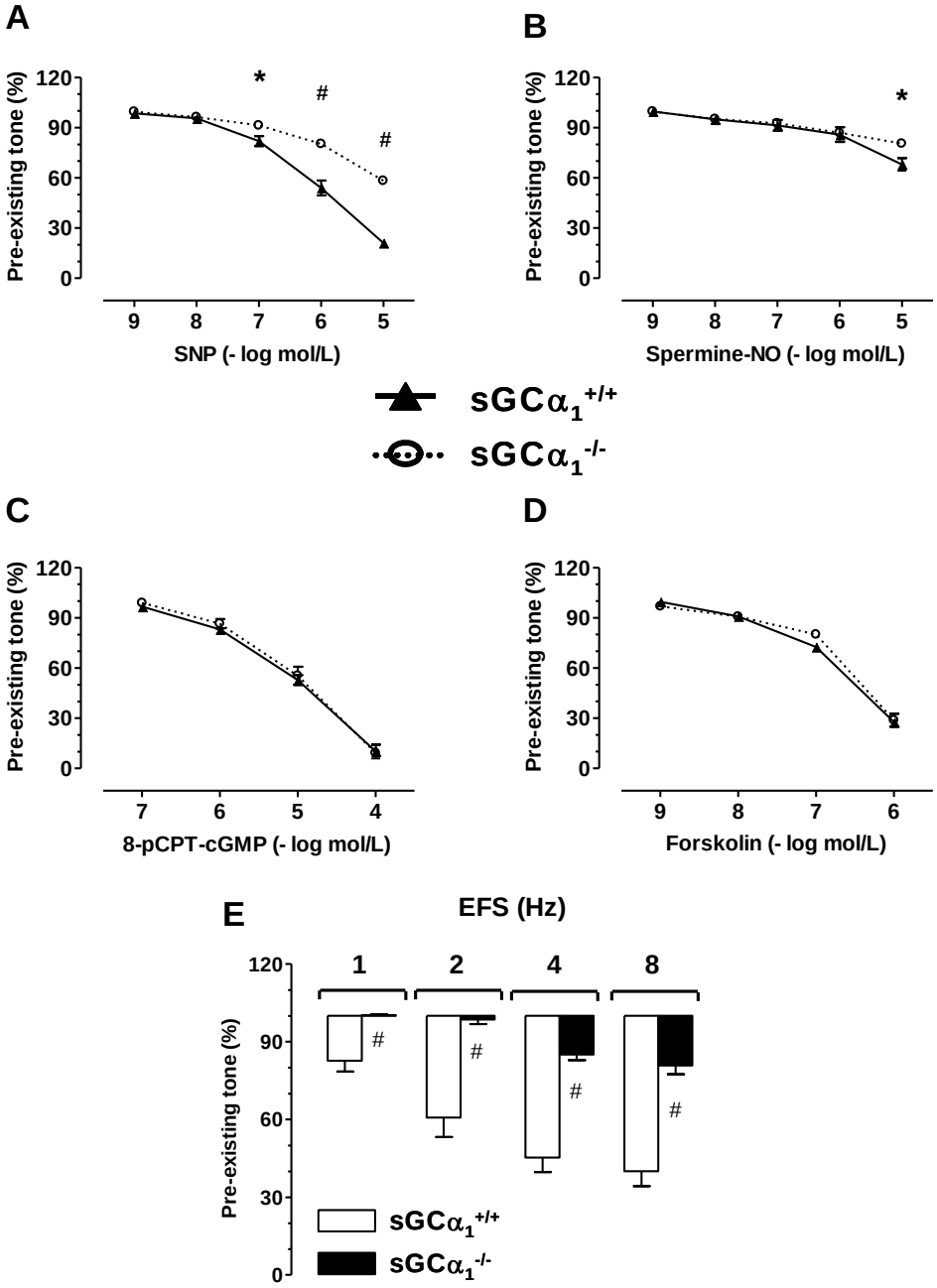


**Figure IV.2.1** Relaxation induced by SNP (A), Spermine-NO (B), 8-pCPT-cGMP (C), Forskolin (D) and EFS (E) in precontracted (5 µmol/L NOR) CC from $sGC\alpha_1^{+/+}$ (▲) and $sGC\alpha_1^{-/-}$ mice (○) with a 129SvJ background. *($sGC\alpha_1^{+/+}$ vs. $sGC\alpha_1^{-/-}$): $P < 0.05$, (n = 4), # ($sGC\alpha_1^{+/+}$ vs. $sGC\alpha_1^{-/-}$): $P < 0.001$, (n = 4).

**IV.2.4.2 *In vitro* studies on C57BL6/J background (Figure IV.2.2)**

SNP, spermine-NO and EFS elicited concentration- or frequency-dependent relaxations of both $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ CC preparations that are strongly reduced for the CC of $sGC\alpha_1^{-/-}$ mice. Similar concentration-dependent relaxations for $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ CC were however induced by 8-pCPT-cGMP and forskolin.

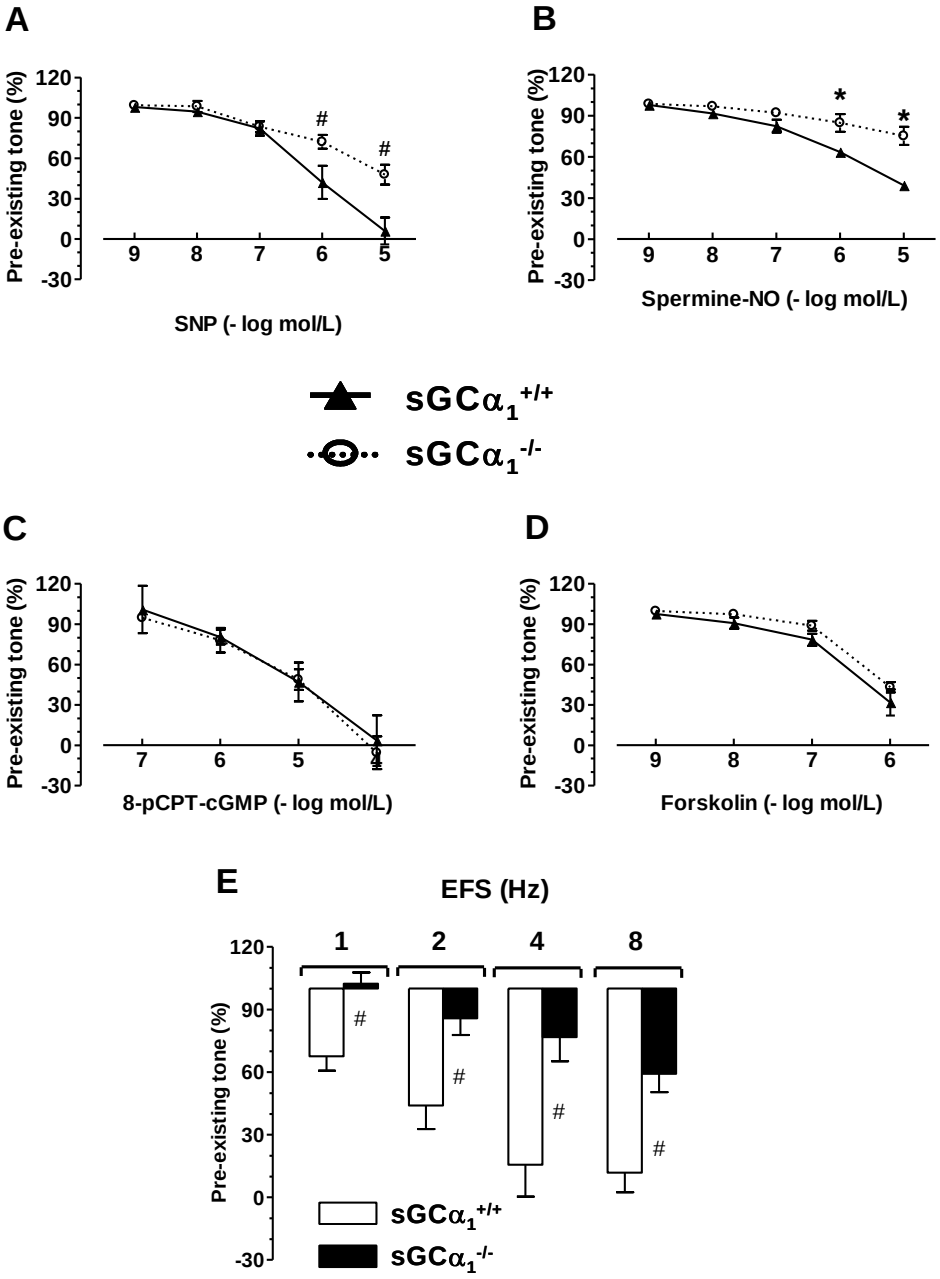


**Figure IV.2.2** Relaxation induced by SNP (A), Spermine-NO (B), 8-pCPT-cGMP (C), Forskolin (D) and EFS (E) in precontracted (5 µmol/L NOR) CC from $sGC\alpha_1^{+/+}$ (▲) and $sGC\alpha_1^{-/-}$ mice (○) with a C57BL6/J background. *($sGC\alpha_1^{+/+}$ vs. $sGC\alpha_1^{-/-}$): $P < 0.05$, (n = 4), # ($sGC\alpha_1^{+/+}$ vs. $sGC\alpha_1^{-/-}$): $P < 0.001$, (n = 4).

**IV.2.4.3 *In vivo* studies on 129SvJ background (Figure IV.2.3)**

Basal ICP, when adjusted for the MAP, did not differ significantly between $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice (8.2 % ± 0.7 for $sGC\alpha_1^{+/+}$ (n = 20) vs 7.1 % ±0.6 for $sGC\alpha_1^{-/-}$ (n = 20, P > 0.05)).

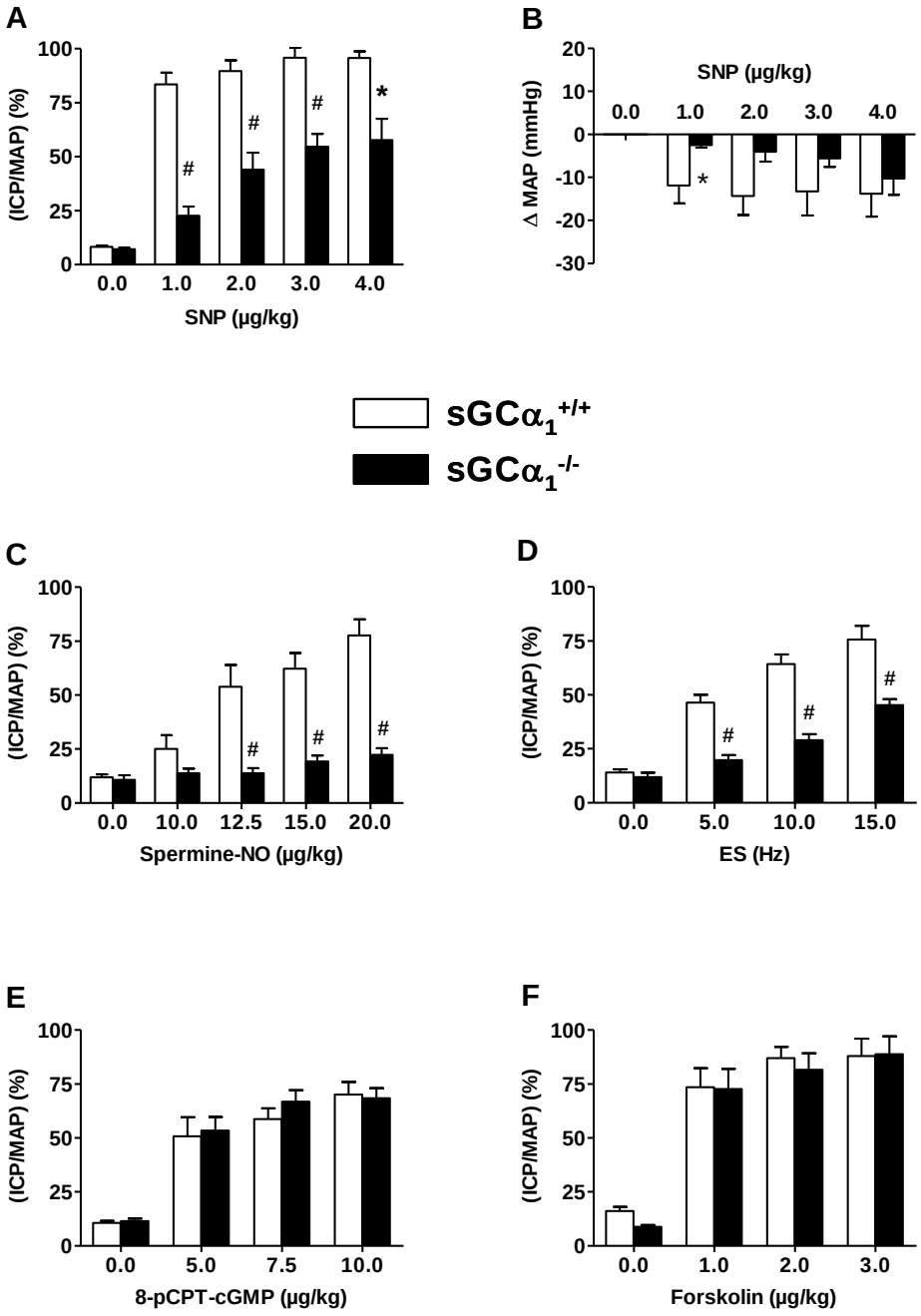


**Figure IV.2.3** The ICP/MAP ratio (in %) in response to SNP (A), Spermine-NO (C), ES (D), 8-pCPT-cGMP (E) and Forskolin (F) of $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice with a 129SvJ background. (B): effect of intracavernosal administrated SNP on the MAP of $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice with a 129SvJ background ($\Delta MAP = MAP_{after} - MAP_{before}$). *($sGC\alpha_1^{+/+}$ vs. $sGC\alpha_1^{-/-}$): P < 0.05, (n = 4), # ($sGC\alpha_1^{+/+}$ vs. $sGC\alpha_1^{-/-}$): P < 0.001, (n = 4).

IV.2.4.3.1 Intracavernosal injection of SNP, spermine-NO and stimulation of the cavernous nerve.

Injection of increasing amounts of SNP and spermine-NO as well as stimulation of the cavernous nerve resulted in a concentration- or frequency-dependent increase in ICP/MAP in both $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice, although the rise in ICP/MAP was significantly lower in $sGC\alpha_1^{-/-}$ mice compared to $sGC\alpha_1^{+/+}$ mice. SNP was the only agent that elicited a very transient decrease of the MAP and this decrease was somewhat lower in $sGC\alpha_1^{-/-}$ mice as compared with $sGC\alpha_1^{+/+}$ mice but not significantly different except for the lowest concentration.

IV.2.4.3.2 Intracavernosal injection of 8-pCPT-cGMP and forskolin

Injection of 8-pCPT-cGMP and forskolin resulted in an increase in the ICP/MAP that did not differ between $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice.

**IV.2.4.4 *In vivo* studies on C57BL6/J background (Figure IV.2.4)**

Basal ICP/MAP did not differ between $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice (15.4 % ± 1.9 for $sGC\alpha_1^{+/+}$ (n = 20) vs 13.2 % ± 0.6 for $sGC\alpha_1^{-/-}$ (n = 20, $P > 0.05$)).

IV.2.4.4.1 Intracavernosal injection of SNP, spermine-NO and stimulation of the cavernous nerve

Injection of increasing amounts of SNP, spermine-NO as well as the stimulation of the cavernous nerve resulted in a concentration- or frequency-dependent increase in ICP/MAP in both $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice which was significantly reduced in $sGC\alpha_1^{-/-}$ mice as compared to $sGC\alpha_1^{+/+}$ mice. The MAP only showed a transient decrease in response to the intracavernosal injection of SNP and this decline did not differ significantly between $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice.

IV.2.4.4.2 Intracavernosal injection of 8-pCPT-cGMP and forskolin

Injection of 8-pCPT-cGMP and forskolin resulted in similar increases in ICP/MAP for $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice.

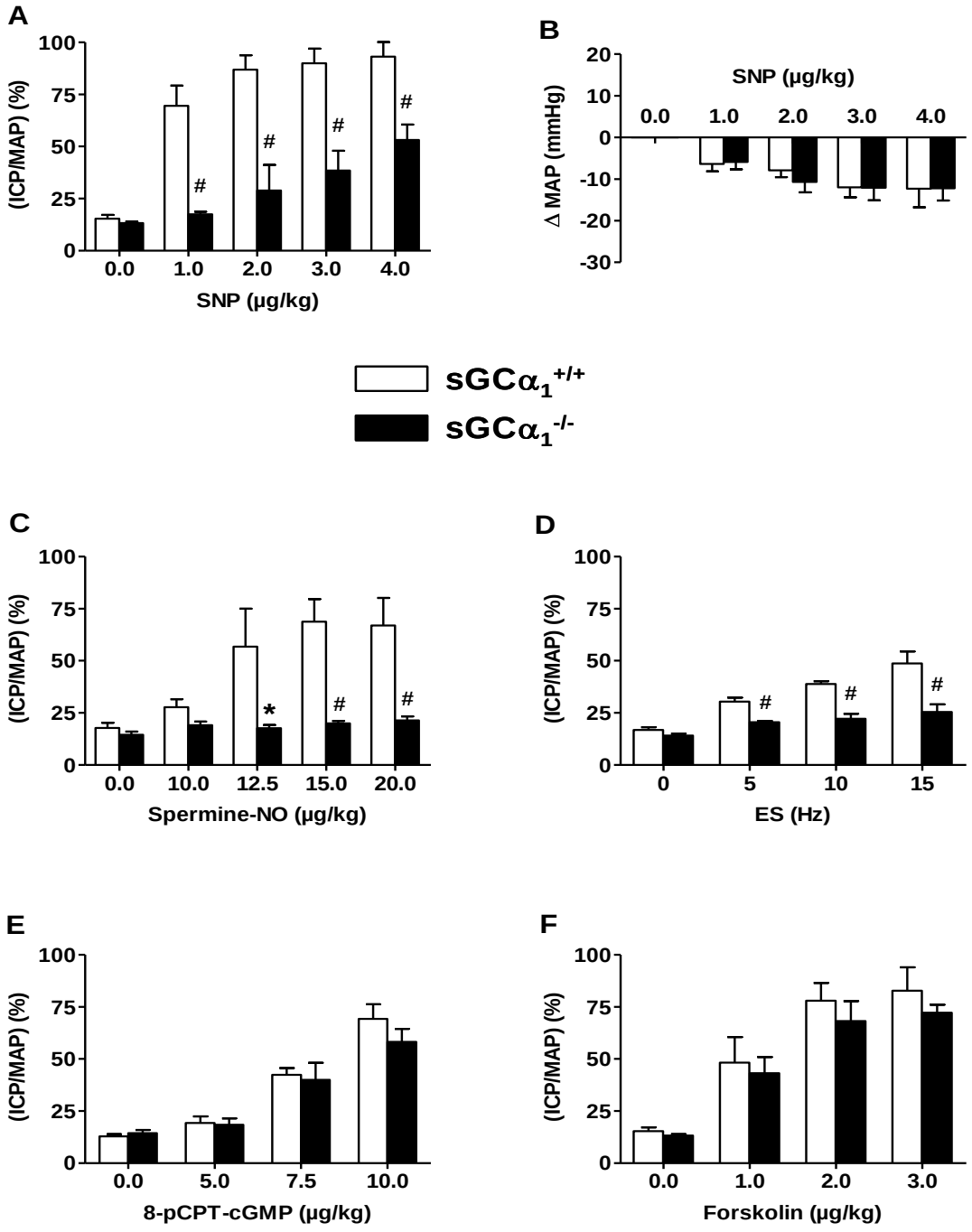


**Figure IV.2.4** The ICP/MAP ratio (in %) in response to SNP (A), Spermine-NO (C), ES (D), 8-pCPT-cGMP (E) and Forskolin (F) of $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice with a C57BL6/J background. (B): effect of intracavernosal administrated SNP on the MAP of $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice with a C57BL6/J background ($\Delta MAP = MAP_{after} - MAP_{before}$). *($sGC\alpha_1^{+/+}$ vs. $sGC\alpha_1^{-/-}$): $P < 0.05$, (n = 4), # ($sGC\alpha_1^{+/+}$ vs. $sGC\alpha_1^{-/-}$): $P < 0.001$, (n = 4).

## IV.2.5 Discussion

According to the Massachusetts Male Aging Study 52% of men over 40 experience ED, with the prevalence still increasing due to the aging of the population and other factors [8]. Current

first-line ED treatment often involves the use of PDE-5 inhibitors. These drugs have, despite their efficacy, some drawbacks and limitations in use [9-11]. First, they exert adverse effects such as headache, dyspepsia and visual disturbances, which are related to their effects in the vasculature, upper gastro-intestinal tract and retina, respectively [9]. Second, PDE-5 inhibitors are contra-indicated for patients taking nitrovasodilators due to the high risk of life-threatening hypotension [9]. Third, nearly 30% of the patients do not respond to the therapy, especially patients of postradical prostatectomy and diabetics, implying that endogenous NO production is impaired to such an extent that inhibition of cGMP degradation provides no significant benefit [10]. The alternative therapies nowadays available for patients refractory to PDE-5 inhibitors are all invasive and remain unsatisfactory [10;11], increasing the need for new therapeutic targets.

sGC, due to its central role in the mechanism of erection, lends itself as a highly promising new therapeutic target and agents directly activating sGC may represent a novel approach for treating ED [2;12]. Recently, NO-independent sGC stimulators as YC-1 and BAY 41-2272 have gained special attention as these drugs are able to relax CC *in vitro* and induce penile erection *in vivo* [6;13-15].

However as sGC is also abundant in the vasculature, upper gastro-intestinal tract and retina, one would expect these drugs to exert comparable side-effects as PDE-5 inhibitors. Therefore, information on the physiological importance of the sGC isoforms in penile erection is necessary to validate sGC subunits as therapeutic targets which could lead to the development of isoform specific activators with restricted adverse effects.

As it has been shown that the $sGC\alpha_1\beta_1$ heterodimer is the predominantly expressed isoform in CC [6], one would expect it also to be physiologically the most important isoform. The present study strongly supports this hypothesis, the importance of the $sGC\alpha_1$ subunit is illustrated by the reduced responses to SNP, spermine-NO and EFS or stimulation of the cavernous nerves in $sGC\alpha_1^{-/-}$ mice. However this study also provides very convincing evidence that $sGC\alpha_1\beta_1$ is not the sole mediator for induction of penile erection as we still observe a substantial relaxation to both exogenous and endogenous NO in the $sGC\alpha_1^{-/-}$ mice.

Intracavernosal injection of L-NAME (50 mg/kg) completely blocked the response to the cavernous nerve stimulation, indicating that solely NO is responsible for the remaining

response seen in $sGC\alpha_1^{-/-}$ mice (data not shown). Moreover, the identical responses in $sGC\alpha_1^{+/+}$ and $sGC\alpha_1^{-/-}$ mice to forskolin and 8-pCPT-cGMP indicate that the substantial responses observed in $sGC\alpha_1^{-/-}$ mice are not due to an aspecific impairment of relaxation related to structural damage or a compensatory effect of the AC/cAMP pathway [16], neither to changes in the pathway downstream sGC which could have been induced by the genetic modification carried out [17]. Taking together, these observations strengthen the idea that besides $sGC\alpha_1\beta_1$ (an)other mechanism(s) mediate(s) erectile function. Activation of $sGC\alpha_2\beta_1$ is the most likely explanation, however sGC-independent mechanisms such as direct activation of $Na^+/K^+$-ATPase by NO or a combination of sGC-dependent and -independent mechanisms should also be considered [18]. Further research will be necessary to elucidate the origin of the remaining response of NO in $sGC\alpha_1^{-/-}$ mice.

The fact that hypertension was observed in 129SvJ $sGC\alpha_1^{-/-}$ mice [7], but not in C57BL6/J $sGC\alpha_1^{-/-}$ mice [19], underlines potential strain-dependent differences in the importance of the $sGC\alpha_1$ subunit in the cardiovascular system. As penile erection is highly dependent on cardiovascular reactivity, two different pure bred strains were studied and compared with the results of a previous study [19]. While the response of CC to EFS *in vitro* was abolished in mixed Swiss/129SvJ $sGC\alpha_1^{-/-}$ mice, CC from pure 129SvJ $sGC\alpha_1^{-/-}$ strains and especially from the C57BL6/J strain were still able to relax, however not to a similar extent as the $sGC\alpha_1^{+/+}$ mice, illustrating that studying different mice strains is necessary to draw final conclusions [19].

## IV.2.6 Conclusion

This study illustrates the functional importance of the $sGC\alpha_1$ subunit in penile erection, suggesting that selective targeting of $sGC\alpha_1\beta_1$ might offer a therapeutic approach compensating the depressed NO/cGMP pathway. However, as endogenous and exogenous NO still elicits a relaxing effect in $sGC\alpha_1^{-/-}$ mice, a role for the $sGC\alpha_2\beta_1$ isoform or an sGC-independent mechanism cannot be excluded.

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# Manuscript 3

# Corpora cavernosa smooth muscle responsiveness in soluble guanylyl cyclase $\beta_1$ His 105 Phe mutant mice

**In preparation**

## IV.3.1 Abstract

**PURPOSE:** to assess the relative contribution of the sGC isoforms and/or a(n) sGC-independent targets(s) in the mechanism of penile erection.

**MATERIALS AND METHODS:** mutant mice ($sGC\beta_1^{ki/ki}$) were used, whereby an inserted mutation yields a sGC enzyme that retains basal activity but fails to respond to NO. During *in vitro* experiments, isolated CC from $sGC\beta_1^{ki/ki}$ and wild-type mice were mounted in organ baths for isometric tension recordings in response to various sGC-dependent and -independent vasorelaxing agents. During *in vivo* experiments these agents were injected intracavernosally while recording the ICP.

**RESULTS:** NO-induced responses were strongly abolished in $sGC\beta_1^{ki/ki}$ mice both *in vitro* and *in vivo*. The heme-dependent, NO-independent sGC stimulator BAY 41-2272 relaxed CC of mutant and wild-type mice, although this response is strongly attenuated in $sGC\beta_1^{ki/ki}$ mice. Interestingly the response to the heme- and NO-independent sGC activator BAY 58-2667 was significantly increased in comparison to wild-type mice. Specificity of the impaired responses found in $sGC\beta_1^{+/+}$ mice was illustrated by unaltered responses in wild-type and mutant mice to sGC-independent vasorelaxing agents.

**CONCLUSIONS:** the relaxing effects normally observed in CC in response to NO are completely abrogated in $sGC\beta_1^{ki/ki}$ mice, suggesting that NO induces penile erection solely through sGC activation. The heme- and NO-independent sGC activator BAY 58-2667 strongly relaxes penile tissue of $sGC\beta_1^{ki/ki}$ mice indicating that this compound may offer value in treating ED. Furthermore, these experiments provide indirect evidence that besides $sGC\alpha_1\beta_1$ also $sGC\alpha_2\beta_1$ is of importance in the erectile function.

## IV.3.2 Introduction

ED, defined as the inability to achieve/maintain an erection sufficient for sexual intercourse, is currently treated by PDE-5 inhibitors. Despite their high efficacy, these drugs have some limitations in use. Moreover, not all patients respond to this therapy and other strategies to treat ED are all invasive and/or unsatisfactory for patients, necessitating the search for new therapeutic alternatives [1].

Disorders in the NO/cGMP signalling cascade have been implicated in the development of endothelial dysfunction, which is strongly associated with ED [2;3]. A substantial decrease in NO-bioavailability and/or sensitivity, frequently observed in patients with severe ED, might lead to PDE-5 inhibitor resistance as inhibition of cGMP degradation has no longer significant therapeutic advantage [4]. For these patients, direct activation of sGC using stimulators or activators of the enzyme could offer a valuable alternative. Interestingly, multiple studies illustrated the existence of 2 sGC isoforms, $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$ [5]. Therefore potential tissue-dependent differences in the expression and/or activity of both isoforms might offer a more tissue-selective therapeutic approach, minimizing side-effects.

While biochemical studies already revealed that the $sGC\alpha_1\beta_1$ isoform is predominant in the CC [6], only a few studies investigated the physiological relevance of the sGC isoforms [4;7]. In a previous study, we illustrated that besides $sGC\alpha_1\beta_1$ activation also another mechanism must be involved in NO-induced erectile response. The less abundantly expressed $sGC\alpha_2\beta_1$ isoform and/or a(n) sGC-independent mechanism(s) may be involved [4;7]. To assess the relative contribution of the $sGC\alpha_2\beta_1$ isoform and/or a(n) sGC-independent mechanism(s), *in vitro* and *in vivo* studies were conducted on mice lacking NO-sensitive sGC, namely $sGC\beta_1^{ki/ki}$ mice [8].

## IV.3.3 Materials and methods

### IV.3.3.1 Animals

All experiments were performed on male homozygous $sGC\beta_1^{ki/ki}$ and $sGC\beta_1^{+/+}$ mice (genetic background : mixed 129SvJ-C57BL6/J), developed and bred in the Department of Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology, Ghent, Belgium [8]. The animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication no. 85-23, revised 1996). The studies were approved by the local Ethical Committee for Animal Experiments, Faculty of Medicine and Health Sciences, Ghent University, Belgium. On the day of the experiment, the mice were sexually mature (age 10-16 weeks).

### IV.3.3.2 *In vitro* study

After cervical dislocation, the penile tissue was isolated as described before [7]. The CC were separated by cutting the fibrous septum between them and excised at the base. Of each mouse, one CC was mounted horizontally in a myograph with one end fixed to a force-displacement

transducer and the other to a micrometer. The tissue chambers contained 10 mL KRB solution at 37°C (pH 7.4) equilibrated with 95% $O_2$ – 5% $CO_2$. The preparations were preloaded with 0.45 g of tension and allowed to equilibrate for 60 minutes in bath fluid that was frequently replaced by fresh KRB solution. The preparations were 3 times contracted with 5 µmol/l NOR, washed and allowed to relax to resting tension before starting the protocol. When the pre-contraction response reached a stable level, EFS (train duration 20 s, frequency: 1, 2, 4 and 8 Hz, pulse duration: 5 ms and voltage: 80 V), delivered by a Grass stimulator via two parallel electrodes, was applied to the tissue. In other experiments various vasodilating substances were added at a stable tension, achieved with 5 µmol/l NOR, to analyse the relaxing response of the CC. Between the response-curves, the CC were washed and allowed to recover for 20-30 min.

#### IV.3.3.3 *In vivo* study

Mice were anesthetized with an isoflurane/oxygen breathing mixture. To induce anaesthesia, 10% isoflurane was applied, whereas during the preparation and measurements, the concentration of isoflurane was set at 3.5%. During the experiment mice breathed spontaneously and body temperature was maintained at 37°C by means of a heated blanket. Surgical dissection was performed exposing the left carotid artery and the CC. A PE-10 tube was introduced in the left carotid artery and a 30-gauge needle attached to a PE-10 tube was inserted into the right CC. These were connected to a pressure transducer and a recorder (Powerlab 4/30) for the simultaneous monitoring of ICP and MAP. For intracavernosal drug administration a separate cannula (30-gauge needle attached to PE-10 tube and 25 µl syringe) was inserted into the left CC. All cannula were filled with heparinised saline (100 U/mL). Injection volume was standardized to 2 µL to ensure minimal volume related changes on the ICP. For ES of the cavernous nerve, the bladder and prostate were exposed via a midline suprapubic incision and testes and epididymides were repositioned into the abdomen after they were divided from their scrotal attachments. The bilateral cavernosal nerves were isolated lateral to the urethra, at the lower lateral portion of the prostate. An electrode with parallel hooks (0.7-1 mm), attached to a Grass S88 stimulator, was placed around the isolated nerve. The following stimulation parameters were used: 5, 10 and 15 Hz, duration 5 ms, 8 V. Each stimulation had a duration of 60 s and a resting interval of 15 min.

### IV.3.3.4 Measurement of sGC activity

After 30 minutes of equilibration in a KRB solution at 37°C, bubbled with 95% $O_2$-5% $CO_2$ (pH 7.4), the corporal tissues were either incubated with DEA-NO (10 µmol/l), BAY 58-2667 (10 µmol/l) or used as control. This reaction was stopped after 1 minute of incubation by snap freezing the tissue in liquid nitrogen. The collected segments were then kept at -80°C until further processing. sGC enzyme activity, measured as described by Bloch et al. [9], is expressed as pmol of cGMP produced per minute per milligram of protein in corporal extract supernatant.

### IV.3.3.5 Drugs and Chemicals

The *in vitro* experiments were performed in KRB solution of 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 $H_2O$. Acetylcholine chloride (ACh), N-[4-[1-(3-Aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine (Spermine-NO), diethylamine NO-NOate diethylammonium salt (DEA-NO), forskolin, 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate sodium salt (8-pCPT-cGMP) and norepinephrine bitartrate (NOR) were obtained from Sigma-Aldrich (St Louis, MO, USA), BAY 41-2272 from Alexis (San Diego, USA) and sodium nitroprusside (SNP) from Merck (Darmstadt, Germany). BAY 58-2667 (4-({(4-carboxybutyl)[2-(2-{[4-(2-phenylethyl)benzyl]oxy}phenyl)ethyl]amino}methyl) benzoic acid) was kindly provided by prof. Stasch (Bayer Schering, Wuppertal, Germany). BAY 58-2667 was dissolved in a solution of PBS, DGME and Cremophor EL (60%/20%/20%), BAY 41-2272 was dissolved in dimethylsulfoxide, ACh in 50 mmol/l potassium hydrogen phthalate buffer, pH 4.0 and forskolin in ethanol. The other drugs were dissolved in distilled water for *in vitro* experiments or heparinised saline (100 U/ml) for *in vivo* experiments. Saturated NO solution was prepared from gas (Air liquide, Belgium) as described by Kelm & Schrader [10]. All concentrations are expressed as final molar concentrations in the organ bath studies while for the *in vivo* studies the amount of agent injected is given as µg/kg. The final concentration of dimethylsulfoxide or ethanol in the organ bath never surpassed 0.1%.

### IV.3.3.6 Calculations and statistics

Data are presented as mean values ± SEM.; n represents the number of mice. In the *in vitro* studies relaxations are expressed as a percentage of the relaxation. For the *in vivo* study,

results are calculated as the ICP, adjusted for the MAP, expressed in percentage (ICP/MAP x 100). Statistical significance was evaluated using Student's t-test for paired and unpaired observations (SPSS, version 20). P<0.05 was considered as significant.

## IV.3.4 Results

### IV.3.4.1 *In vitro* results

IV.3.4.1.1 NO– and sGC–dependent

The vasorelaxing influence of endothelium-derived NO and neuronal-derived NO was examined using ACh and by EFS of the intrinsic nerves on CC isolated from $sGC\beta_1^{+/+}$ and $sGC\beta_1^{ki/ki}$ mice. ACh (10 µmol/l) substantially relaxes NOR pre-contracted CC tissues from the $sGC\beta_1^{+/+}$ mice, while it further contracts CC from the $sGC\beta_1^{ki/ki}$ mice. (Figure IV.3.1A)
Applying EFS relaxes NOR-contracted CC of $sGC\beta_1^{+/+}$ mice in a frequency-dependent manner and this effect is abolished in tissues of $sGC\beta_1^{ki/ki}$ mice. Moreover, CC of $sGC\beta_1^{ki/ki}$ even showed a tendency to contract with EFS at higher frequencies. (Figure IV.3.1B)

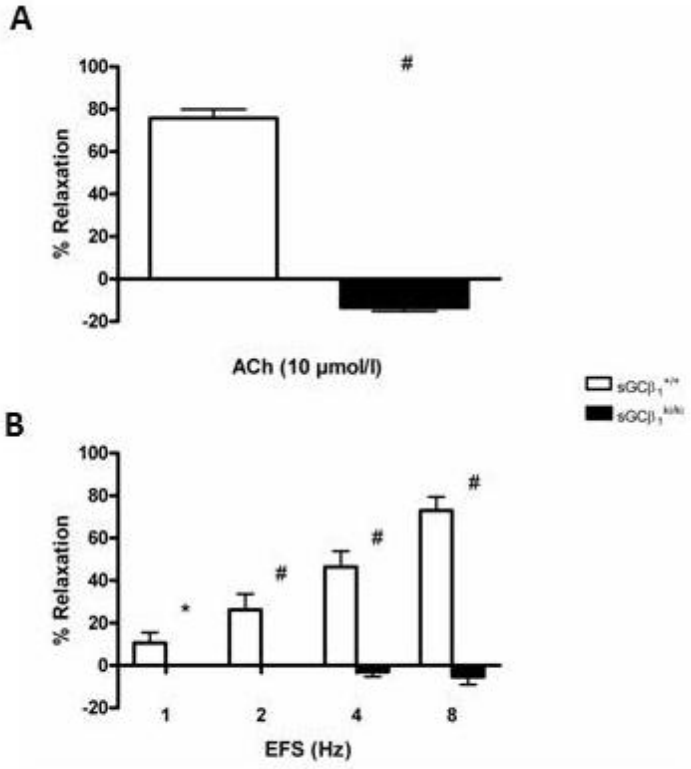


**Figure IV.3.1** Relaxation effect of endogenous NO on precontracted (50 µmol/l NOR) CC from $sGC\beta_1^{+/+}$ and $sGC\beta_1^{ki/ki}$ mice, evoked by **A**. ACh (n = 8); **B**. EFS (n = 8); * ($sGC\beta_1^{+/+}$ vs $sGC\beta_1^{ki/ki}$): $P < 0.05$, # ($sGC\beta_1^{+/+}$ vs $sGC\beta_1^{ki/ki}$): $P < 0.01$.

Besides endogenous NO, also exogenous NO was investigated using three different NO-donors, SNP (1 nmol/l – 10 µmol/l), spermine-NO (1 nmol/l – 10 µmol/l), and NO-gas (1 µmol/l – 0.1 mmol/l). (Figure IV.3.2) All NO-donors relax the pre-contracted corporal tissues from $sGC\beta_1^{+/+}$ mice in a concentration-dependent manner, a response that is significantly impaired in $sGC\beta_1^{ki/ki}$ mice. It should be noted that SNP and NO-gas still induce a small relaxation in $sGC\beta_1^{ki/ki}$ mice.

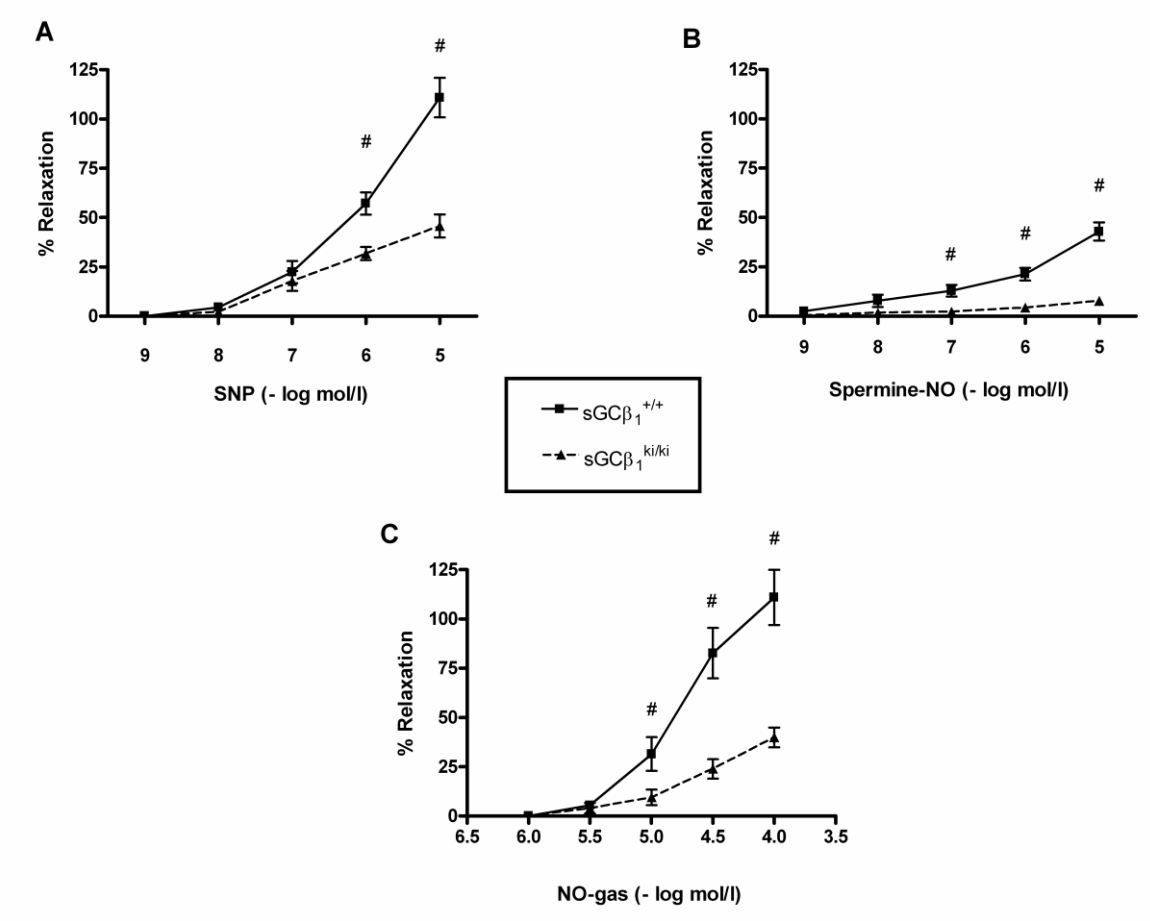


**Figure IV.3.2** Relaxation effect of exogenous NO on precontracted (50 µmol/l NOR) CC from $sGC\beta_1^{+/+}$ and $sGC\beta_1^{ki/ki}$ mice, evoked by **A**. SNP (n = 8); **B**. Spermine-NO (n = 4); **C**. NO-gas (n = 8); # ($sGC\beta_1^{+/+}$ vs $sGC\beta_1^{ki/ki}$): $P < 0.01$.

VI.3.4.1.2 NO– and sGC–independent

Addition of increasing concentrations of the cell membrane permeable cGMP-analog, 8-pCPT-cGMP (100 nmol/l to 0.1 µmol/l) and the AC stimulator forskolin (1 nmol/l to 1 µmol/l), elicited identical concentration-dependent relaxations in CC preparations from both $sGC\beta_1^{+/+}$ and $sGC\beta_1^{ki/ki}$ mice. (Figure IV.3.3)

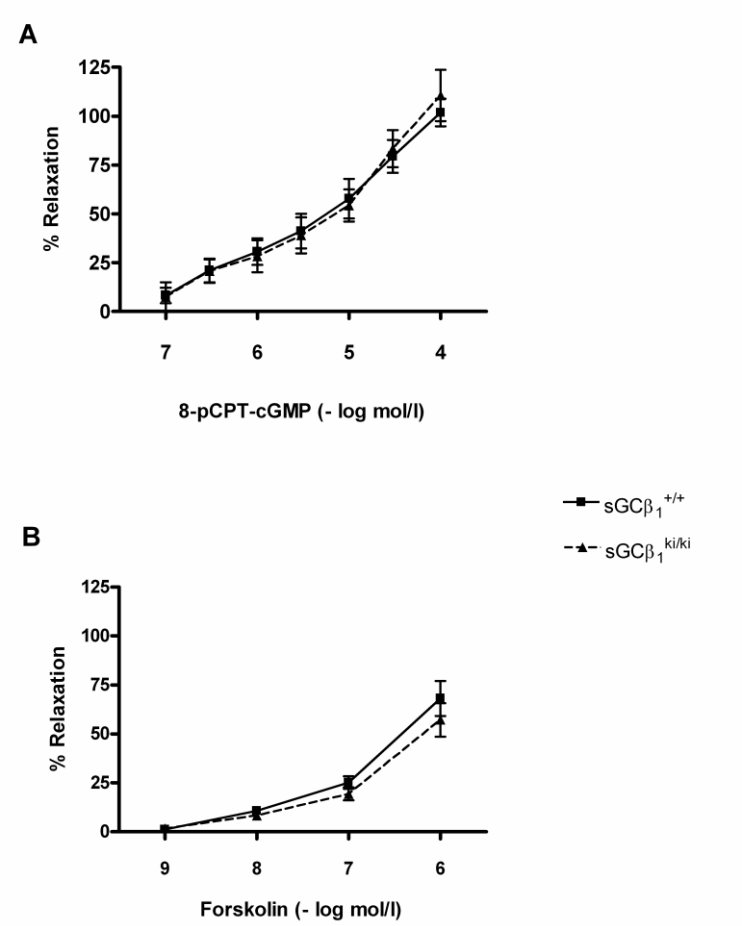


**Figure IV.3.3** Relaxation effect of sGC-independent vasodilators on precontracted (50 µmol/l NOR) CC from $sGC\beta_1^{+/+}$ and $sGC\beta_1^{ki/ki}$ mice, evoked by **A**. 8-pCPT-cGMP (n = 5); **B**. forskolin (n = 4).

IV.3.4.1.3 NO–independent but sGC–dependent

Administration of BAY 41-2272 (1 nmol/l – 10 µmol/l), a NO-independent although heme-dependent sGC stimulator, produced concentration-dependent relaxations in the $sGC\beta_1^{+/+}$ and $sGC\beta_1^{ki/ki}$ CC tissues. In $sGC\beta_1^{ki/ki}$ CC, the relaxant effect of BAY 41-2272 is slightly but significantly attenuated. (Figure IV.3.4A)

Addition of BAY 58-2262 (1 nmol/L – 10 µmol/L), a sGC activator acting heme- and NO-independent, resulted in concentration-dependent relaxations in the $sGC\beta_1^{+/+}$ and $sGC\beta_1^{ki/ki}$ CC tissues. However, the relaxant effect of BAY 58-2667 is significantly increased in $sGC\beta_1^{ki/ki}$ CC preparations. (Figure IV.3.4B)

## IV.3.4.2 Measurement of sGC activity

As to be expected, the baseline activity of sGC did not differ in CC of $sGC\beta_1^{+/+}$ and $sGC\beta_1^{ki/ki}$ mice. Our data illustrate that while NO increases the sGC activity in wild-type mice, no up-regulation of the enzyme's activity is observed in penile tissue of $sGC\beta_1^{ki/ki}$ mice. Stimulation

with BAY 58-2667 resulted in an up-regulation of sGC activity in $sGC\beta_1^{+/+}$ mice, but surprisingly this response is absent in corporal tissues of $sGC\beta_1^{ki/ki}$ mice. (Figure VI.5)

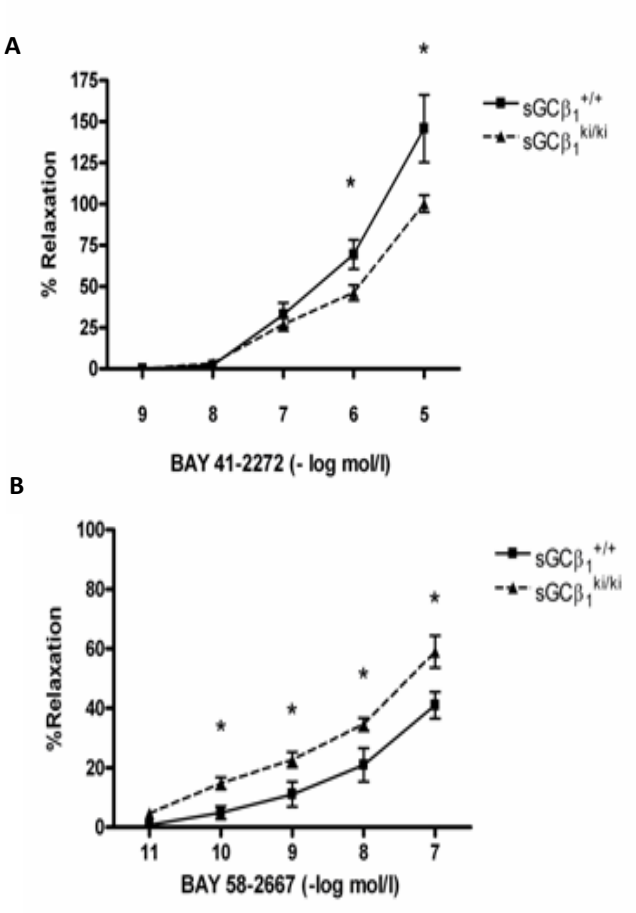


**Figure IV.3.4** Relaxation effect of the NO-independent sGC-activator BAY 41-2272 (n = 7) and the NO- and heme -independent sGC-activator BAY 58-2667 (n = 8) on precontracted (50 µmol/l NOR) CC from $sGC\beta_1^{+/+}$ and $sGC\beta_1^{ki/ki}$ mice. * ($sGC\beta_1^{+/+}$ vs $sGC\beta_1^{ki/ki}$): $P < 0.05$.

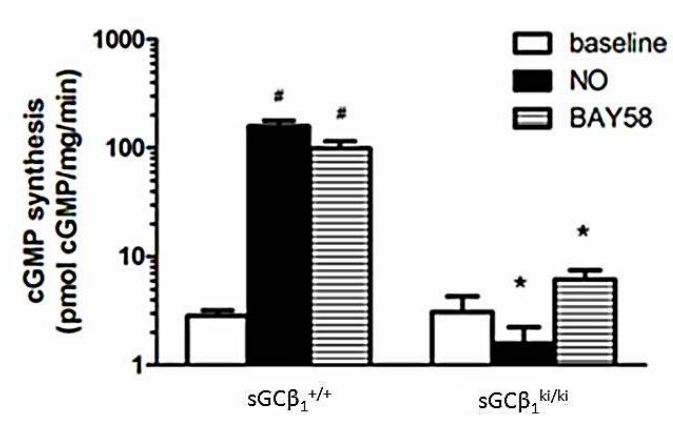


**Figure IV.3.5** sGC activity measurements in penile tissues isolated from $sGC\beta_1^{+/+}$ and $sGC\beta_1^{ki/ki}$ mice. (n = 6) * ($sGC\beta_1^{+/+}$ vs $sGC\beta_1^{ki/ki}$): $P < 0.05$; # (baseline sGC activity in $sGC\beta_1^{+/+}$ vs sGC activity after addition of DEA-NO/BAY 58-2667 in $sGC\beta_1^{ki/ki}$): $P < 0.05$.

## IV.3.4.3 *In vivo* studies

IV.3.4.3.1 Basal ICP and MAP

The basal ICP, when adjusted for the MAP, did not differ significantly between $sGC\beta_1^{+/+}$ and $sGC\beta_1^{ki/ki}$ mice (15.7 % ± 1.1 for $sGC\beta_1^{+/+}$ (n = 18) vs 11.0 % ± 2.4 for $sGC\beta_1^{ki/ki}$ (n = 18, P > 0.05)). (Figure IV.3.6)


**Figure VI.6** The baseline values for ICP/MAP ratio (in %) of $sGC\beta_1^{+/+}$ and $sGC\beta_1^{ki/ki}$ mice (n=18).

IV.3.4.3.2 NO– and sGC–dependent

The *in vivo* effect of endogenous NO was examined by ES of the cavernosal nerve at different frequencies (8 V, 1 ms, 60 s, 5 – 15 Hz). A frequency-dependent increase in ICP/MAP was observed in $sGC\beta_1^{+/+}$ mice after ES, while ES of the cavernosal nerve in the $sGC\beta_1^{ki/ki}$ mice did not induce any changes in ICP/MAP. (Figure IV.3.7A) No vasodilatory substances other than NO were released during ES, as L-NAME administered intracavernosally before ES of the cavernosal nerve, abolished the increase in ICP/MAP normally observed with ES in the $sGC\beta_1^{+/+}$ mice (own unpublished observations). The intracavernosal administration of increasing amounts of exogenous NO using SNP (1 – 4 µg/kg) and spermine-NO (10 – 20 µg/kg) results in a concentration-dependent increases in ICP/MAP in $sGC\beta_1^{+/+}$ mice, while in the $sGC\beta_1^{ki/ki}$ mice responses induced by the NO-donors were completely abolished. (Figure IV.3.7B and C)

VI.3.4.3.3 NO– and sGC–independent

The intracavernosal administration of 8-pCPT-cGMP (5 – 10 µg/kg) and forskolin (1 – 4 µg/kg) increases the ICP/MAP equally in $sGC\beta_1^{+/+}$ and $sGC\beta_1^{ki/ki}$ mice. (Figure IV.3.8)

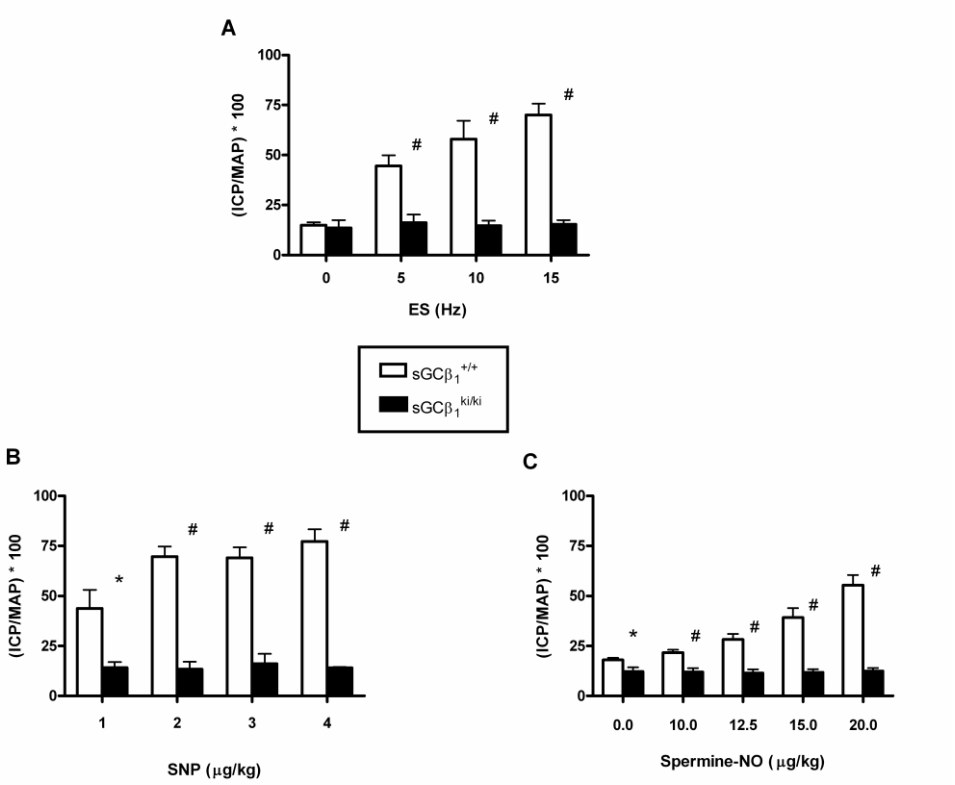


**Figure VI.7** The ICP/MAP ratio (in %) in the response to **A**. ES (n = 4); **B.** SNP (n = 5); **C**. Spermine-NO (n = 5); * ($sGC\beta_1^{+/+}$ vs $sGC\beta_1^{ki/ki}$): $P < 0.05$, # ($sGC\beta_1^{+/+}$ vs $sGC\beta_1^{ki/ki}$): $P < 0.01$.

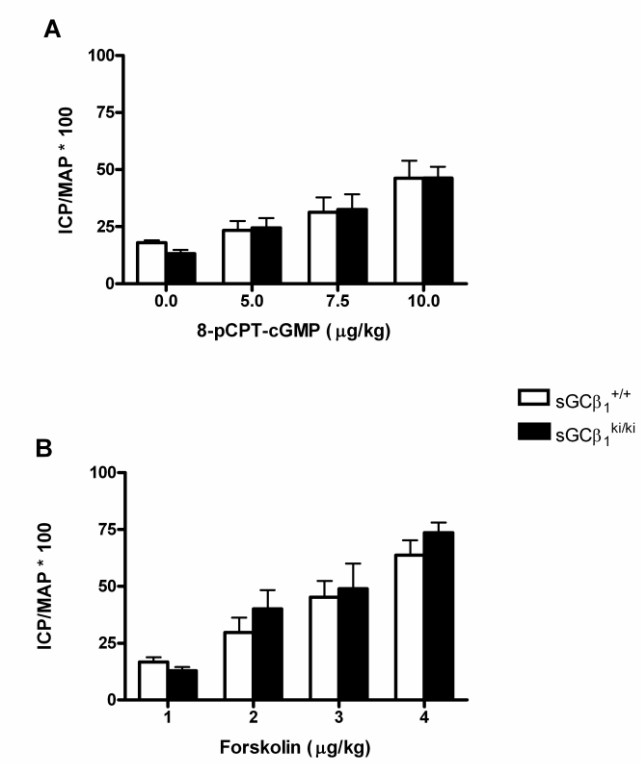


**Figure VI.8** The ICP/MAP ratio (in %) in the response to **A**. 8-pCPT-cGMP (n=4); **B**. forskolin (n=5) * ($sGC\beta_1^{+/+}$ vs $sGC\beta_1^{ki/ki}$): $P<0.05$, # ($sGC\beta_1^{+/+}$ vs $sGC\beta_1^{ki/ki}$): $P<0.01$.

## IV.3.5 Discussion

ED is an important male health problem affecting, to some degree, 35% - 54% of men over 40 years of age [11]. Convincing evidence exists for the NO/cGMP axis, with sGC as the major effector molecule for NO, to play a pivotal role in mediating cavernosal relaxation and penile erection [12]. The use of PDE-5 inhibitors, producing a NO-dependent increase in cGMP concentrations, is a successful approach which further emphasizes the importance of the cGMP system in the erectile response [13].

Epidemiological studies showed that approximately 30% of patients do not respond to PDE-5 inhibitors.[11;13] The unresponsiveness towards PDE-5 inhibitors might suggest that the NO bioavailability is impaired to such an extent that inhibition of cGMP degradation produces no significant therapeutic advantages. For these patients, direct sGC stimulators/activators might be more effective. In many pathological conditions, including ED, it is illustrated that the iron-containing heme group of sGC is oxidized, resulting in desensitization of sGC to compounds binding the heme [11]. Recently, heme and NO-independent sGC activators have been produced [14]. While promising, none of these sGC activators have yet been examined for their effects on penile tissue. The strong hemodynamic effects of sGC stimulators/activators might limit their clinical usefulness [15;16]. The presence of two physiologically active isoforms of sGC offers a potentially more selective therapeutical approach, limiting side-effects. Therefore, knowledge of the relative physiological importance of both sGC isoforms is of substantial interest. Despite having similar biochemical and kinetic properties, the two sGC isoforms may differ in their subcellular and tissue localization [17].

Previous studies, using $sGC\alpha_1^{-/-}$ mice, illustrated that in penile smooth muscle relaxation the predominant $sGC\alpha_1\beta_1$ isoform is of great importance, though it is not the only target of known sGC stimulators. Involvement of the less abundantly expressed $sGC\alpha_2\beta_1$ isoform and/or (an) sGC-independent mechanism(s) could be argued [4;7]. The issue whether $sGC\alpha_2\beta_1$ and/or other mechanism(s) participate in penile smooth muscle relaxation, was unravelled using a mice model expressing a mutated sGC. In $sGC\beta_1^{ki/ki}$ mice, the important histidine 105 is converted to phenylalanine in the $sGC\beta_1$ subunit, yielding a heterodimer that retains basal cyclase activity but fails to respond to NO due to failure of binding the heme [8;18]. That this genetic transformation renders all active sGC isoforms insensitive to NO is illustrated by our data obtained from measuring the sGC activity. (Figure IV.3.5)

One of the main findings in our study is the absolute failure of NO to influence erectile function in $sGC\beta_1^{ki/ki}$ mice, revealing the unique role of sGC as receptor for NO in our mice. While activation of $Ca^{2+}$-and voltage-dependent $K^+$-channels, sarcoplasmic reticulum $Ca^{2+}$-ATPase and $Na^+/K^+$-ATPase have been described as sGC-independent vasorelaxing actions of NO in vascular smooth muscle cells [19-22], these cGMP-independent NO-induced mechanisms do not contribute to the erectile function. (Figure IV.3.1, IV.3.2 and IV.3.7). Corporal relaxations in response to the NO-donors SNP and NO-gas during *in vitro* experiments were not completely abolished in CC tissues isolated from $sGC\beta_1^{ki/ki}$. However *in vivo* experiments argue against the role of sGC-independent mechanisms, as intracavernosal injection of SNP did not increase the ICP in $sGC\beta_1^{ki/ki}$ mice in contrast to $sGC\beta_1^{+/+}$ mice. The observation that SNP and NO-gas relax CC can be explained by the fact that the high NO concentrations used likely act through non-physiological and even toxic mechanisms [17;23;24].

Comparable to our results, another report illustrated that CC of transgenic mice lacking the cGMP target, PKG-I, fail to relax upon activation of the NO/cGMP signalling cascade [24]. Previously we reported that the remaining exogenous NO-induced responses observed in $sGC\alpha_1^{-/-}$ mice were abrogated in the presence of the sGC inhibitor ODQ [4]. The lack of response in CC preparations isolated from $sGC\beta_1^{ki/ki}$ mice provides indirect evidence that the remaining response to NO previously observed in $sGC\alpha_1^{-/-}$ mice is due to activation of $sGC\alpha_2\beta_1$.

Although the best studied sGC stimulators are the organic nitrates, they suffer from development of tolerance [11]. BAY 41-2272 is an NO-independent sGC stimulator, binding to an allosteric site of the enzyme. It is suggested that BAY 41-2272 is able to activate both sGC isoforms [25]. Previously we reported that the relaxing effect of BAY 41-2272 is significantly decreased in the CC of $sGC\alpha_1^{-/-}$ mice. In the present study no further inhibition of the BAY 41-2272 vasodilatory response is observed in the CC isolated from $sGC\beta_1^{ki/ki}$ mice (10 µM BAY 41-2272 in $sGC\alpha_1^{-/-}$ vs. $sGC\beta_1^{ki/ki}$ mice: 17.2 % ± 5.5 vs. 2.5 % ± 5.2). (Figure IV.3.4) Despite the fact that BAY 41-2272 is dependent on the presence of a reduced prosthetic heme moiety of sGC [11;15], BAY 41-2272 could still elicit a substantial response in the CC isolated from the $sGC\beta_1^{ki/ki}$ mice. This suggests that in penile tissue, BAY 41-2272, next to $sGC\alpha_1\beta_1$, partly exerts its relaxant effect through (an) sGC-independent mechanism(s). BAY 41-2272 has already been reported to induce a cGMP-independent vasorelaxation through activation of the $Na^+$-$K^+$-ATPase and inhibition of the $Ca^{2+}$-entry [16].

Another interesting finding in this study is that the NO- and heme-independent sGC activator BAY 58-2667 relaxes CC in a concentration-dependent manner. (Figure IV.3.4) Moreover, the response to BAY 58-2667 is significantly increased in CC of $sGC\beta_1^{ki/ki}$ mice compared to wild-type mice. BAY 58-2667 is a heme- and NO-independent sGC activator. In $sGC\beta_1^{ki/ki}$ mutant mice, the histidine 105 is altered to a phenylalanine. As mentioned in the introduction, the prosthetic heme moiety is coordinated by four nitrogens of the porphyrinic ring and another nitrogen provided by this histidine 105 residue. Thus by altering the histidine, the prosthetic heme moiety is unable to bind sGC. The subsequent loss of heme renders the enzyme NO-resistant, providing us a model for the situation in oxidative stress where sGC also looses its NO-sensitivity. As BAY 58-2667 is more potent in binding and activating heme-free sGC than does BAY 41-2272, which is heme-dependent, the outcome of our study was to be expected. From clinical point of view, this finding is of major importance. Many diseases associated with ED, such as hypertension and diabetes mellitus, are characterized with reduced NO-sensitivity due to oxidation of sGC. For these patients, PDE-5 inhibitors will offer low therapeutic advantage in treating ED as only a low concentration of cGMP is produced. As shown by this study, sGC stimulators as NO fail to activate oxidized sGC and thus provide no advantage over PDE-5 inhibitors in restoring erectile function. In addition, also the responses to BAY 41-2272 were significantly reduced. Surprisingly, although BAY 58-2667 significantly up-regulated the sGC activity in wild-type mice, no significant increase in its activity could be observed in $sGC\beta_1^{ki/ki}$ mice, an observation that is in contrast with our functional data. (Figure IV.3.5) A potential explanation for this discrepancy could be that the expression levels of one or multiple sGC subunits is lower in the $sGC\beta_1^{ki/ki}$ mice. Another potential explanation is that BAY 58-2667 exerts also sGC-independent effects, resulting in vascular and corporal smooth muscle relaxation. Despite the discrepancy, the functional study clearly illustrates that the erectile function of patients might ameliorate after administration of BAY 58-2667.

That the pathway downstream sGC is not affected by the mutation is shown by the similar responses of CC in $sGC\beta_1^{+/+}$ and $sGC\beta_1^{ki/ki}$ mice to 8-pCPT-cGMP and forskolin. (Figure IV.3.3 and IV.3.8) In addition, these results also illustrate that the abolished sGC-related responses observed are certainly not due to an aspecific impairment of relaxation related to structural damage and that smooth muscle cells of $sGC\beta_1^{ki/ki}$ mice are still intact and capable to relax. Furthermore, a compensatory role for the AC/cAMP signalling pathway can be ruled

out [26]. This observation is in line with the study using PKG-I deficient mice, in which forskolin relaxes CC to a similar extent as the wild-type mice [24].

## IV.3.6 Conclusion

This is the first study demonstrating that NO does not influence the erectile function through mediators that are sGC/cGMP-independent. Another important finding in this study is that the NO and heme-independent sGC activator BAY 58-2667 offers therapeutic value for ED, in conditions in which reactive oxygen species are elevated such as diabetes and hypertension. Furthermore, the present study confirms that the $sGC\alpha_2\beta_1$ isoform contributes to the mechanism of penile erection. As the $sGC\alpha_2\beta_1$ isoform is less expressed in most tissues, development of agents specifically acting on this isoform might be of therapeutic interest. These new insights may help develop future pharmacological strategies for treatment of ED.

## IV.3.7 Acknowledgements

This work was supported by a grant of FWO-Vlaanderen, the Bijzonder Onderzoeksfonds (BOF) of Ghent University and Geconcerteerde Onderzoeks Actie (GOA) of Ghent University and Interuniversity Attraction Poles P6/30 (Belgian government). The authors would also like to thank the DMBR animal caretakers for maintaining the animal facility.

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# Manuscript 4

# Divergent mechanisms involved in CO- and CORM-2-induced vasorelaxation

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## IV.4.1 Abstract

CO may play an important physiological role in regulation of the vascular tone. CORM-2 is frequently used as a CO-donor to evaluate (patho)physiological properties of CO and its potential therapeutic applications. The aim of this study was to examine the molecular mechanisms underlying the vasodilatory properties of CORM-2 as this has not yet been extensively explored. Isometric tension recordings were performed using mice and rat isolated aortic ring segments as well as mice femoral artery ring segments. Responses to CO (10 μmol/l - 300 μmol/l) and CORM-2 (30 μmol/l - 600 μmol/l) were evaluated in the presence/absence of activators/inhibitors of different molecular pathways. CO was unable to relax mice blood vessels, whereas it induced concentration-dependent relaxations in rat aorta. The response to CO was inhibited by both the sGC inhibitor ODQ (10 μmol/l) and $K^+$ channel blocker tetraethyl-ammonium chloride (3 mmol/l). CORM-2 relaxed both mice and rat isolated blood vessels in a concentration-dependent manner, however this response was only partially blocked by ODQ and tetraethyl-ammonium chloride. Interestingly, 4-aminopyridine (3 mmol/l) inhibited the CORM-2-induced vasodilatation whereas iberiotoxin (100 nmol/l) had no influence. The molecular mechanisms underlying CORM-2-induced relaxation differ from those of CO-induced relaxation. While CO relaxes vessels through activation of sGC and/or calcium-activated $K^+$-channels, CORM-2 exerts its vasodilatory properties only partially through sGC or $K^+$-channels activation. CORM-2-induced vasodilatation seems to involve voltage-dependent rather than calcium-activated $K^+$-channels.

## IV.4.2 Introduction

CO, endogenously produced as a side-product in the catabolism of heme by HO enzymes has been shown to exert vasodilatation independent of its capability to induce hypoxia [1]. Moreover, CO has already been shown to regulate the vascular tone and to control the blood pressure [2]. Because of its vasodilating effects, the HO/CO pathway seems a very attractive new target for treating pathologies such as coronary disease and hypertension, which are characterized by an increased vascular contractility [3]. The development of CORMs, which are moleculaes that release CO locally in a safe and controlled way, has been crucial to unravel the physiological and pathophysiological properties of CO and its potential therapeutic applications [4]. Although the vasodilatory properties of CORM-2 have been illustrated [5], the molecular mechanisms mediating CORM-2-induced vasorelaxation remain rather obscure. Activation of sGC as well as direct activation of $K^+$ channels, in particular the

KCa channels have been shown to be involved in CO-induced vasodilatation [6;7]. However, whether these molecular targets are also relevant for CORM-2-induced vasorelaxatin is still a matter of debate. Knowledge on the specific mechanisms involved in the vasodilatory properties of CORM-2 is however essential when using it as a pharmacological tool. To gain better insight in the mechanisms of CORM-2-induced vasodilation, isometric tension recordings were performed on isolated thoracic aorta and the femoral artery from mice. Besides exploring potential regional heterogeneity, potential species differences were also investigated. Therefore the CO and CORM-2 responses were compared on thoracic aortas of mice and rats.

## IV.4.3 Materials and methods

### IV.4.3.1 Animals

Male or female mice with a 129SvJ genetic background were used and male Wistar rats purchased from Janvier, Le Genest St-Isle, France. Both mice and rats had ad libitum access to food and water and were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). On the day of the experiment mice aged between 8 and 12 weeks were killed by cervical dislocation while rats aged 6 to 9 weeks were killed by decapitation after sedation with carbon dioxide gas.

### IV.4.3.2 Tissue preparations and mounting

The mice/rat thoracic aorta as well as mice femoral artery were carefully isolated and transferred to cooled KRB solution. Ring segments of the arteries were mounted in a small-vessel myograph with a tissue chamber filled with 10 mL of KRB solution and were prepared free from the adhering tissues. Two stainless steel wires (40 µm diameter) were guided through the lumen of the segments. One wire was fixed to a force-displacement transducer and the other was connected to a micrometer. After mounting, the preparations were allowed to equilibrate for 30 min in the KRB solution bubbled with 95% $O_2$-5% $CO_2$ (pH 7.4) at 37° C. The aortic rings were gradually stretched until a stable preload was obtained of 0.5g and 1g for mice and rat thoracic aorta respectively. The isolated femoral arteries from mice were set to their normalized internal diameter, by stretching the arteries in progressive steps. From the passive wall tension-internal circumference relationship obtained by these measurements, the

artery was stretched to a diameter corresponding to 90% of the diameter the vessel would have under a transmural pressure of 100 mmHg [8].

**IV.4.3.3 Experimental design**

After applying the optimal resting tension, the preparations were allowed to equilibrate for 30 min with rinsing every 10 min. After the equilibration period all ring segments were precontracted 1 to 3 times with either a KRB solution containing 120 mmol/L $K^+$ for mice isolated arteries or a KRB solution containing 30 mmol/L $K^+$ for rat isolated thoracic aorta. Thereafter the arteries were washed and allowed to relax to basal tension before starting the actual protocol. Contraction was elicited with 5 µmol/L NOR for mice thoracic aorta, 1 µmol/L NOR for rat thoracic aorta and 10 µmol/L NOR for mice femoral artery segments. When a stable contraction plateau was obtained, a concentration-response curve for ACh (1 nmol/L – 10 µmol/L) was obtained in order to analyse the functionality of the endothelium. After having established the concentration-response curve for ACh, the ring segments were rinsed thoroughly until they reached their basal tension again.

As preliminary experiments have shown that responses to CORM-2 were not reproducible in the same preparation, the influence of all interfering agents was tested using parallel ring segments from the same animal. The influence of the CORM-2 solvent dimethylsulfoxide (DMSO) was also tested. Thus, for the experiments one of the ring segments was used as a control strip, while the other was incubated with specific blockers/activators. In one series of experiments the role of intact endothelium was explored. Therefore, the endothelium was removed in one of the vascular rings by passing bubbles of carbogen gas through the lumen of the vessels for 2 till 3 minutes. The lack of endothelium was confirmed by the failure of ACh (10 µmol/L) to induce relaxation. After the incubation period with the drugs or solvent, the ring segments were precontracted with NOR and concentration-response curves for either CO (10 µmol/L – 300 µmol/L) or CORM-2 (30 µmol/L – 600 µmol/L) were established. The influence of the following inhibitors/activators as well as some combinations of these agents was examined: the sGC blocker ODQ (10 µmol/L), the sGC sensitizer YC-1 (1 µmol/L), the NOS inhibitors L-NAME (100 µmol/L) and L-NNA (100 µmol/L), the non-specific $K^+$ channel blocker TEA (3 mmol/L), the large-conductance $K_{Ca}$ channel blocker iberiotoxin (100 nmol/L), the small-conductance $K_{Ca}$ channel blocker apamin (500 nmol/L), the large- and intermediate-conductance $K_{Ca}$ channel blocker charybdotoxin (100 nmol/L), the voltage-dependent $K^+$ ($K_v$) channel blocker 4-aminopyridine (4-AP) (3 mmol/L), the inward rectifying

$K^+$ ($K_{ir}$) channel blocker $Ba^{2+}$ (10 µmol/L) and the $K_{ATP}$ channel blocker glibenclamide (3 µmol/L). While the incubation time for ODQ and YC-1 was set at 10 minutes, all other agents were incubated for a 20 minute period.

#### IV.4.3.4 Data analysis and statistical procedures

Data are presented as mean values ± SEM; *n* represents the number of preparations (each obtained from a different mouse/rat). Relaxations are expressed as the percentage decrease of the precontraction tone developed by the addition of NOR. Statistical significance was evaluated by using Student's *t*-test for paired and unpaired observations (SPSS, version 20) or with two-way ANOVA with Bonferroni post hoc test (GraphPad Prism, version 4), when appropriate. $P < 0.05$ was considered as significant.

#### IV.4.3.5 Drugs, chemicals, reagents and other materials

The experiments were performed in a KRB solution of 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 $H_2O$. Dimetylsulfoxide (DMSO), acetylcholine chloride (ACh), 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1), Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), Nω-Nitro-L-arginine (L-NNA), norepinephrine bitartrate (NOR), tricarbonyldichlororuthenium(II)dimer (CORM-2), apamin, charybdotoxin, tetraethyl-ammonium chloride (TEA), glibenclamide, barium chloride ($Ba^{2+}$), 4-aminopyridin (4-AP) were obtained from Sigma-Aldrich (St.Louis, MO). Iberiotoxin was obtained from Alomone labs (Jerusalem, Israel). ODQ was dissolved in ethanol, YC-1, glibenclamide and CORM-2 were dissolved in DMSO and ACh in 50 mmol/L potassium hydrogen phthalate buffer, pH 4.0. The other drugs were dissolved in distilled water. A saturated CO solution (1 mmol/L) was prepared from CO gas (Air liquide, Belgium) as described before [9]. Investigation of the negative control solution was also included in this study. CORM-2 was always freshly prepared before administration into the organ baths. All concentrations are expressed as final molar concentrations in the organ bath.

### IV.4.4 Results

#### IV.4.4.1 CO versus CORM-2

CO relaxes rat aorta in a concentration-dependent manner but exerts no effect on the mice vessels, namely the mice aorta (Figure IV.4.1A) and femoral artery (unpublished

observations). As evidence exists for an inhibitory role of endogenous NO on CO-induced vasodilatation, the effect of CO in the presence of the endogenous NO inhibitor L-NAME (100 µmol/L) was examined [10]. However no relaxation was observed in the presence of L-NAME (100 µmol/L), refuting the previous hypothesis (unpublished observations).

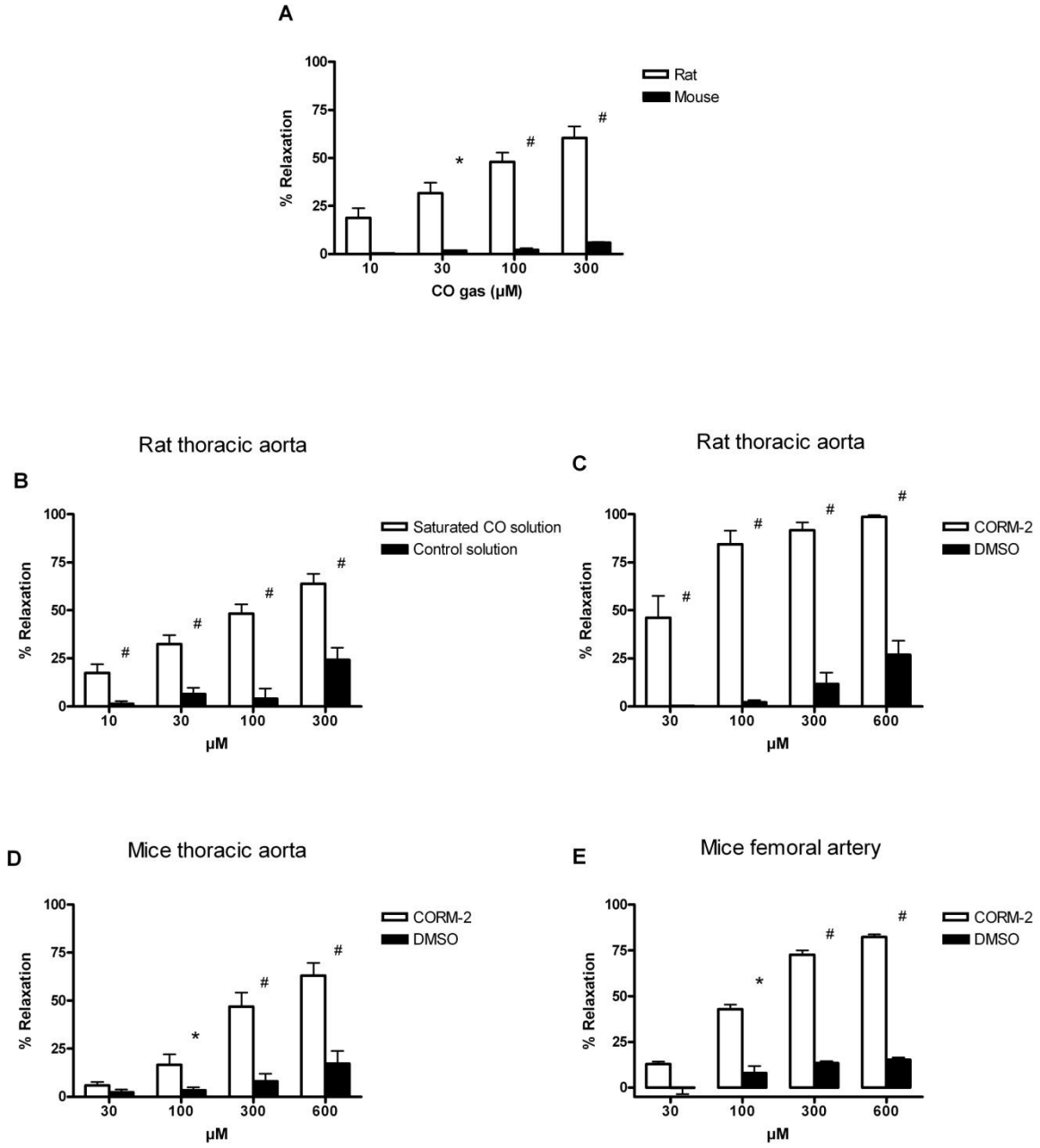


**Figure IV.4.1** (A) Effect of exogenously applied CO in rat (□) and mice (■) thoracic aorta precontracted with respectively 1 and 5 µmol/L NOR (n = 6) (B) Relaxation induced by saturated CO solution (□) versus solvent (■) in rat thoracic aorta precontracted with 1 µmol/L NOR (n = 6). (C - E) Concentration-response curves induced by CORM-2 (□) and its solvent DMSO (■) in (C) rat thoracic aorta precontracted with 1 µmol/L NOR (n = 6), (D) mice thoracic aorta precontracted with 5 µmol/L NOR (n = 6) and (E) mice femoral artery precontracted with 10 µmol/L NOR (n = 6). Data are expressed as % relaxation of the NOR-induced tone; * $P < 0.05$, # $P < 0.001$.

It should be noted that the solvent solution of CO relaxes rat aortic ring segments at the highest concentration (Figure IV.4.1B). As the solvent solution contains the same concentration of NOR, dilution of this contractile agent can be excluded as being the trigger for vasodilatation. Furthermore, the solution was warmed up to 37°C, ruling out cooling effects as well. The relaxing effect of the solvent solution must probably be explained by a volume-effect because adding 2.5 mL to an 8 mL organ bath which was necessary to obtain the final concentration of 300 µmol/L CO.

In contrast to CO, CORM-2 relaxes all rat and mice arteries studied in a concentration-dependent manner (Figure IV.4.1C – 1E). It should be kept in mind that DMSO used as solvent for CORM-2 also relaxes all blood vessels investigated, although to a lesser extent than CORM-2.

### IV.4.4.2 Involvement of sGC

To examine the potential involvement of sGC in both CO- and CORM-2-induced vasorelaxation, rat aortic segments were pre-incubated with the sGC inhibitor ODQ (10 µmol/L) or the sGC sensitizer YC-1 (1 µmol/L) during 10 min before precontraction with 1 µmol/L NOR. Preliminary experiments illustrated that 1 µmol/L YC-1 had no influence on the precontraction level (unpublished observations). Figure IV.4.2A and IV.4.2B show that ODQ strongly inhibits the CO-induced relaxations (Figure IV.4.2A), while YC-1 accelerates and even potentiates the CO-induced relaxation response (Figure IV.4.2B). These data suggest that CO-induced vasorelaxation strongly depends upon sGC activation. Surprisingly, ODQ only partially blocked CORM-2-induced relaxations in both rat and mice thoracic aortas (Figure IV.4.2C and IV.4.2E). Moreover, ODQ had no influence on the CORM-2-induced response in mice femoral arteries (Figure IV.4.2G). Likewise, YC-1 slightly but significantly potentiated the effect of CORM-2 in both rat and mice thoracic aortas but had no influence on the CORM-2-induced relaxation of isolated mice femoral arteries (Figure IV.4.2D, IV.4.2F and IV.4.2H). Taken together these results indicate that CORM-2-induced relaxation is only partially dependent or even completely independent upon sGC activation.

### IV.4.4.3 Interaction with the NOS/NO pathway

Prior incubation with the NOS inhibitor L-NNA did not significantly alter the CO-induced relaxation (Figure IV.4.3A). Surprisingly, incubation of rat aorta with L-NNA strongly and significantly decreased the relaxation response elicited by CORM-2 (Figure IV.4.3B). While

L-NNA exhibited an inhibitory effect on CORM-2 vasodilatory properties in rat aortas, L-NNA did not influence the CORM-2-induced vasorelaxation in the mice isolated vessels (Figure IV.4.3C and IV.4.3D).

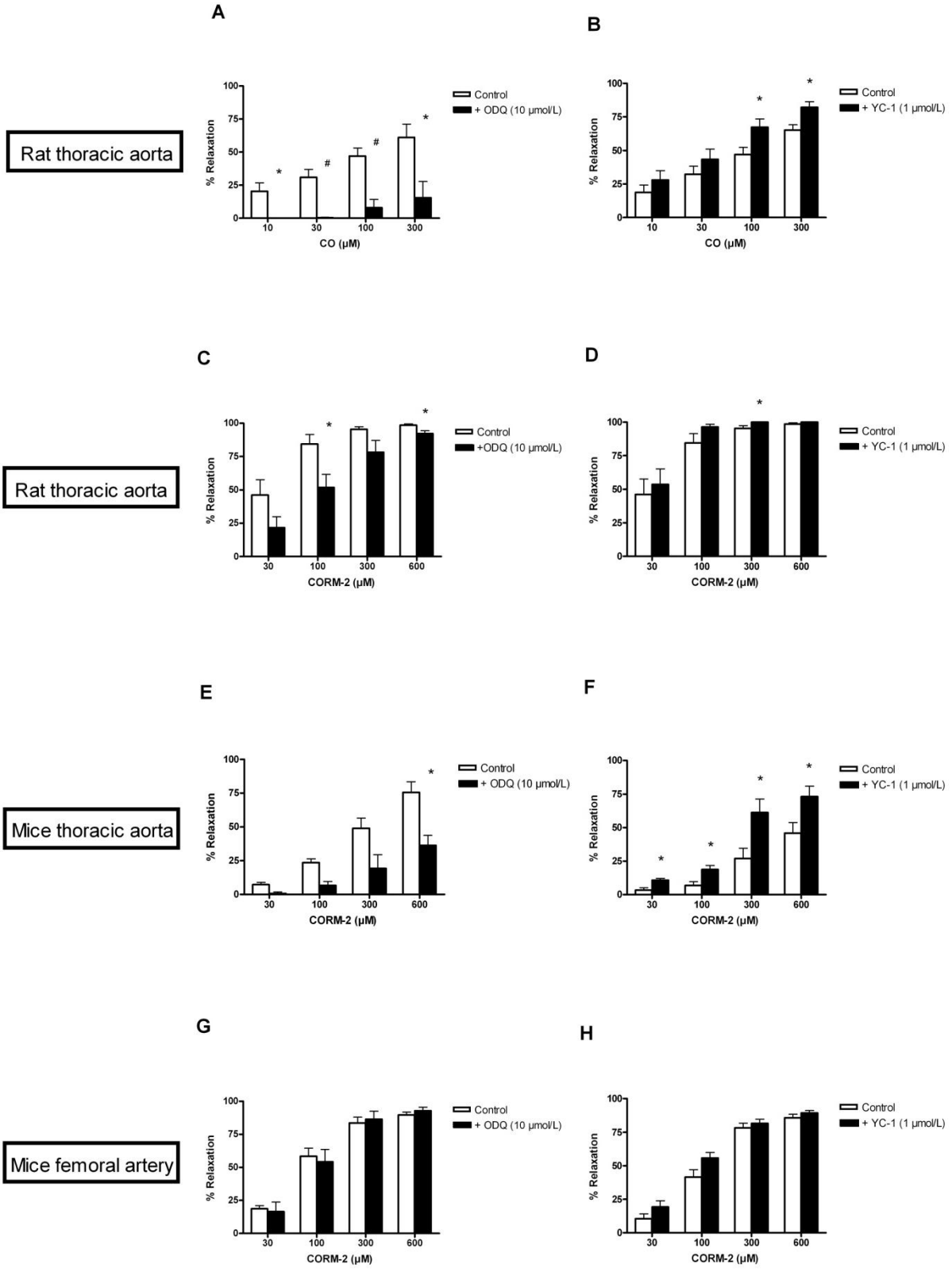

**Figure IV.4.2** Effect of exogenously applied CO in the absence (□) or presence (■) of (A) ODQ (n = 6), (B) YC-1 (n = 6) on rat thoracic aorta precontracted with 1 µmol/L NOR. (C - H) Concentration-response curves obtained by CORM-2 in the absence (□) or presence of ODQ (10 µmol/L) or YC-1 (1 µmol/L) (■). (C) Rat thoracic aorta incubated with ODQ for 10 min before contraction with 1 µmol/L NOR (n = 6), (D) rat thoracic aorta incubated with YC-1 for 10 min before contraction with 1 µmol/L NOR (n = 6), (E) mice thoracic aorta ring incubated with ODQ for 10 min before contraction with 5 µmol/L NOR (n = 7), (F) mice thoracic aorta incubated for YC-1 during 10 min before contraction with 5 µmol/L NOR (n = 7), (G) mice femoral artery incubated with YC-1 for 10 min before contraction with 10 µmol/L NOR (n = 6), (H) mice femoral artery incubated with YC-1 for 10 min before contraction with 10 µmol/L NOR (n = 6). Data are expressed as % relaxation of the NOR-induced tone; * $P < 0.05$, # $P < 0.001$.

The endothelium is a source of endogenous NO but also releases other vasodilator and vasoconstrictor agents after stimulation. To analyse whether CO- and/or CORM-2-induced vasorelaxation is dependent on one of these endothelial substances, we compared CO- and CORM-2-induced responses in vessels with and without intact endothelium. Our data show that removal of the endothelium had no effect on the relaxation elicited by CO and CORM-2, a finding that was observed in all vessels investigated. These results thus demonstrate that CO and CORM-2-induced responses are endothelium-independent (unpublished observations).

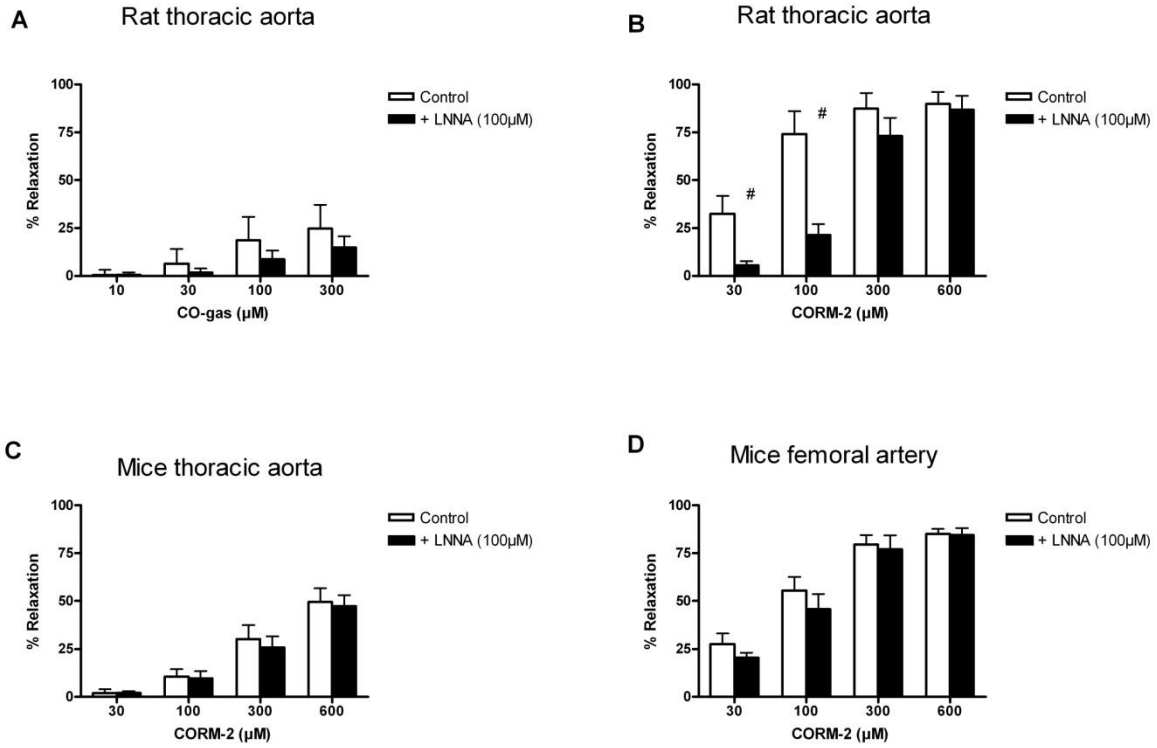


**Figure IV.4.3** (A) Effect of CO in the absence (□) or presence (■) of L-NNA on rat thoracic aorta precontracted with 1 µmol/L NOR (n = 6). (B - D) Effect of CORM-2 in the absence (□) and presence of L-NNA (■) in (B) rat thoracic aorta precontracted with 1 µmol/L NOR (n = 6), (C) mice thoracic

aorta precontracted with 5 µmol/L NOR (n = 6) and (D) mice femoral arteries precontracted with 10 µmol/L NOR (n = 6). Data are expressed as % relaxation of the NOR-induced tone; # P < 0.001.

### IV.4.4.4 Involvement of $K^+$ channels

Comparable to the results obtained with ODQ, CO-induced relaxations in rat aortic ring segments were almost completely abolished in the presence of the non-specific $K^+$ channel blocker TEA (3 mmol/L) (Figure IV.4.4A). Furthermore, TEA significantly, although not completely reduced the responses to CORM-2 in all arteries studied (Figure IV.4.4B – IV.4.4D).

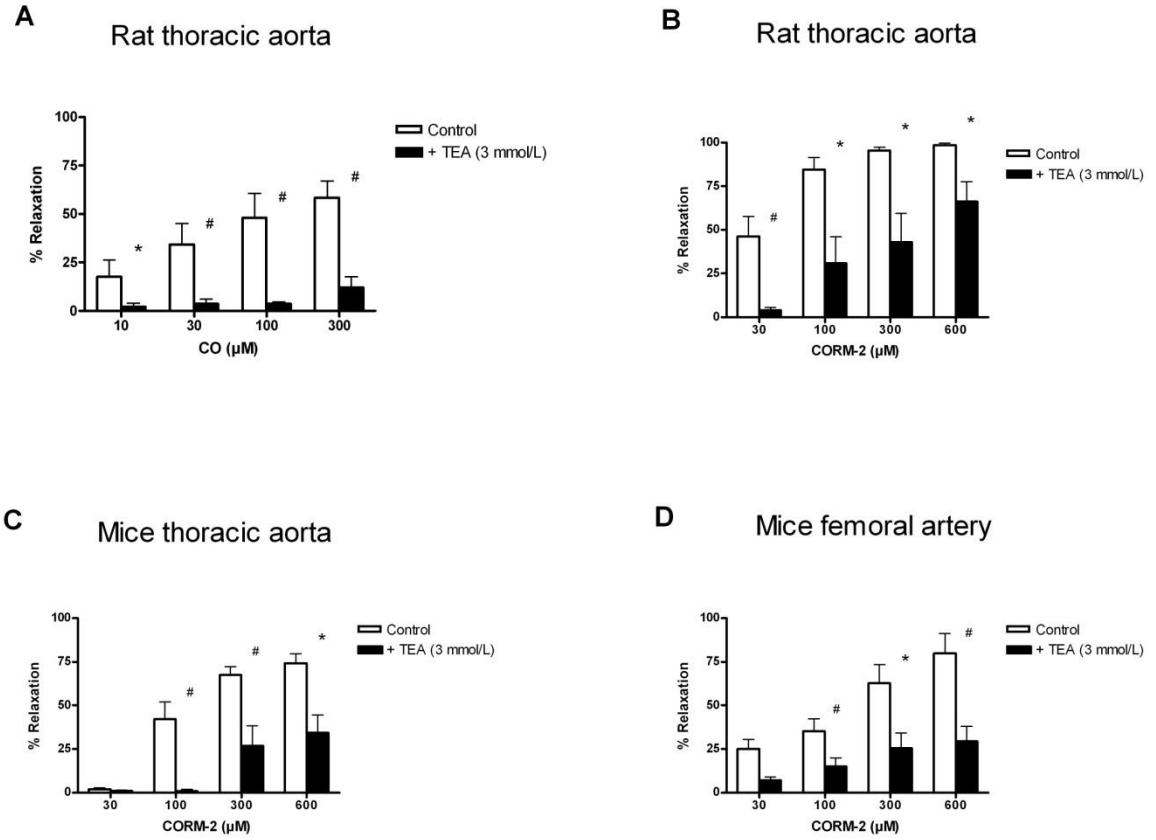


**Figure VII.4** (A) Effect of CO in the absence (□) or presence (■) of TEA (3 mmol/L) in rat thoracic aorta precontracted with 1 µmol/L NOR (n = 6). (B - D) Concentration-response curves of CORM-2 in the absence (□) or presence of TEA (3 mmol/L, 20 minutes incubation time) (■).(B) in rat thoracic aorta precontracted with 1 µmol/L NOR (n = 6), (C) in mice thoracic aorta precontracted with 5 µmol/L NOR (n = 6), (D) in mice femoral arteries precontracted with 10 µmol/L NOR (n = 8). Data are expressed as % relaxation of the NOR-induced tone; * P < 0.05, # P < 0.001.

We further investigated which type(s) of $K^+$ channels are involved in CORM-2-induced vasodilatation. A role for $K_{Ca}$ channels was first studied using the intermediate- and large-conductance $K_{Ca}$ channel blocker charybdotoxin (100 nmol/L). Although charybdotoxin did not significantly inhibit the vasorelaxation elicited by CORM-2 in mice aorta, an inhibitory

trend cannot be ignored (Figure IV.4.5D). The potential involvement of $K_{Ca}$ channels was explored more thoroughly using the small-conductance $K_{Ca}$ channel blocker apamin (500 nmol/L) and the big-conductance $K_{Ca}$ channel blocker iberiotoxin (100 nmol/L) (Figure IV.4.5A, IV.4.5B and IV.4.5C). Comparable to the results obtained with charybdotoxin, these inhibitors did not significantly reduce the relaxation responses to CORM-2. Furthermore simultaneous administration of apamin plus charybdotoxin also failed to change the vasorelaxing effects of CORM-2 in both vessels (unpublished observations), excluding the contribution of $K_{Ca}$ channels in CORM-2-induced vasodilatation.

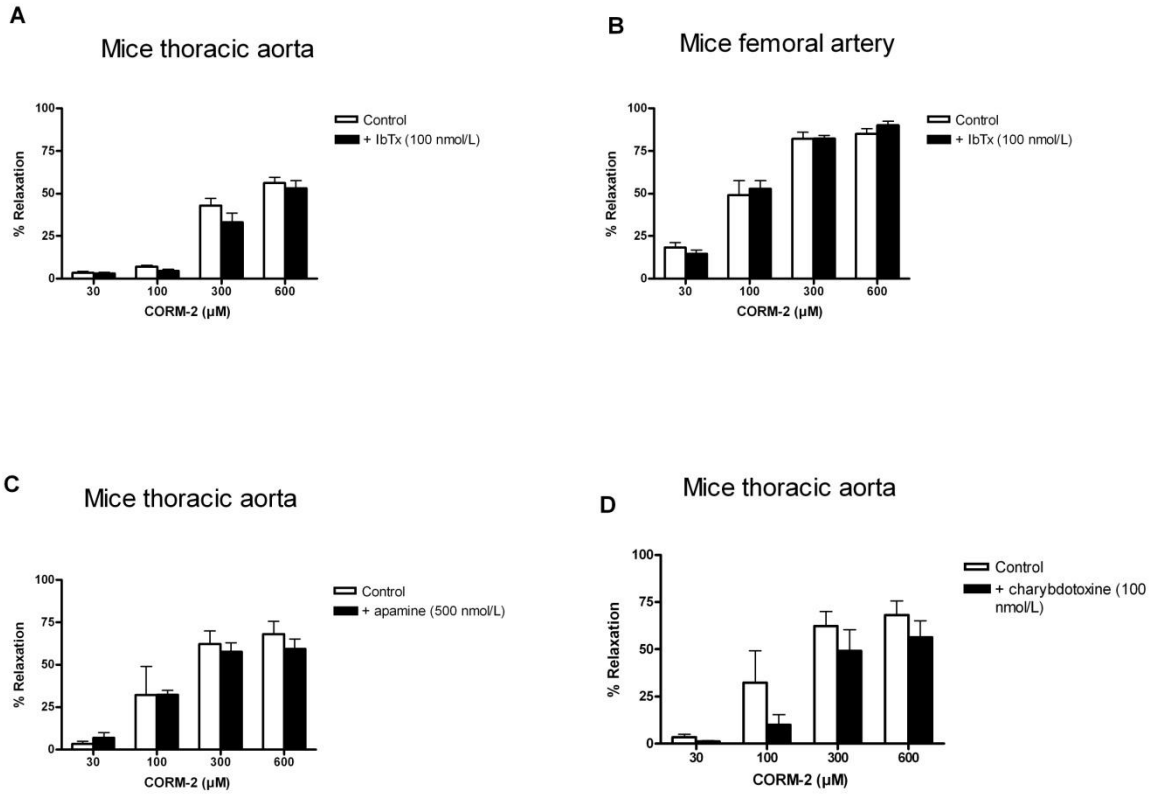


**Figure IV.4.5** Concentration-response curves of CORM-2 in the absence (□) or presence of iberiotoxin/apamin/charybdotoxin (■). Influence on the CORM-2 induced response of incubation with (A) iberiotoxin (100 nmol/L) for 30 min in mice thoracic aorta precontracted with 5 µmol/L NOR (n = 7), (B) iberiotoxin (100 nmol/L) for 30 min in mice femoral arteries precontracted with 10 µmol/L NOR (n = 7), (C) apamin (500 nmol/L) for 20 min in mice thoracic aorta precontracted with 5 µmol/L NOR (n = 6), (D) charybdotoxin (100 nmol/L) for 20 minutes in mice thoracic aorta precontracted with 5 µmol/L NOR (n = 6). Data are expressed as % relaxation of the NOR-induced tone; * $P < 0.05$, # $P < 0.001$.

Vasorelaxation elicited by CORM-2 also remained unaltered in the presence of the $K_{ATP}$ channel blocker glibenclamide (unpublished observations). Furthermore, incubation of the mice arteries with the $K_{ir}$ blocker $Ba^{2+}$ also failed to significantly change the responses to

CORM-2, except in the aortic ring segments at 100 µM of CORM-2 (Figure IV.4.6A and IV.4.6B). Interestingly, the use of 4-AP (3 mmol/L) partially but significantly decreased the relaxations elicited by CORM-2 in both aorta and femoral artery (Figure IV.4.6C and IV.4.6D), suggesting that CORM-2 induces relaxation through the opening of $K_v$ channels.

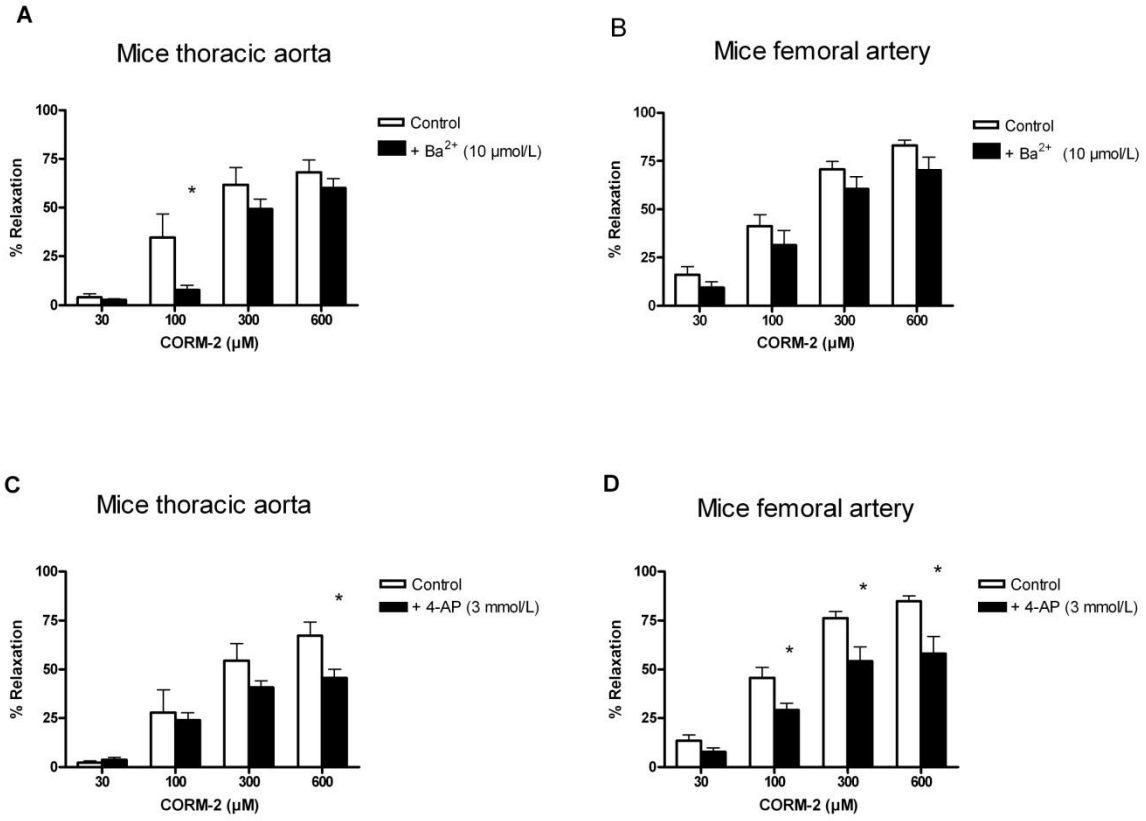


**Figure IV.4.6** Concentration-response curves of CORM-2 in the absence (□) or presence of specific $K^+$ channel blocker (■). Influence on the CORM-2 induced response of incubation of (A) mice thoracic aorta segments with $Ba^{2+}$ (10 µmol/L) for 20 min prior to contraction with 5 µmol/L NOR (n = 8), (B) mice femoral arteries with $Ba^{2+}$ (10 µmol/L) for 20 min prior to contraction with 10 µmol/L NOR (n = 6), (C) mice thoracic aorta with 4-AP (3 mmol/L) for 20 min prior to contraction with 5 µmol/L NOR (n = 7), (D) mice femoral arteries with 4-AP (3 mmol/L) for 20 min prior to contraction with 10 µmol/L NOR (n = 7). Data are expressed as % relaxation of the NOR-induced tone; * $P < 0.05$, # $P < 0.001$.

## IV.4.5 Discussion

CO is a well known vasodilator. As the delivery of CO as such is not a safe therapeutic strategy, CORMs have been developed. CORM-2 is one of the first developed CO-releasing molecules and has already been extensively used to evaluate the (patho)physiological properties of CO. Contact with heme-containing proteins triggers a fast CO release from CORM-2 and albeit CORM-2 has been shown to elicit vasorelaxation, the basic mechanism

underlying this action still remains to be elucidated [5]. It is tempting to presume that CORM-2 induces vasorelaxation through similar mechanisms as CO.

The present study highlights that the mechanisms behind CORM-2 induced vasodilatation differ from the mechanisms underlying CO-induced vasodilatation. That CORMs do not always behave as authentic CO, has been previously illustrated in vascular tissues as well as gastro-intestinal tissues [5;9;11;12]. However, this study is the first illustrating the different mechanisms responsible for the vasorelaxing effect of CO and CORM-2.

In the present study, we unexpectedly found that mice isolated vessels are unresponsive to CO. This is apparently in contrast with a previous report showing CO-induced vasodilatation in rat aorta [1;6;7]. To ensure that the lack of response is not due to technical defaults, we also investigated rat aortic ring segments. In rat aorta, we indeed observed a concentration-dependent relaxation with CO. That the vasorelaxation caused by CO is not universal to all vascular tissues has been illustrated by Brian et al. [13]. They found that the vascular tone of basilar and middle cerebral arteries from rabbits was not affected by CO whereas that of the rabbit aorta was. While this group showed that the CO-induced response was tissue-dependent, the present study clearly illustrates that this response is also species-dependent.

Evidence is accumulating supporting an interaction between the NOS/NO and HO/CO pathways. This interaction seems to be very dynamic and complex [10;14]. A potential inhibition of CO-induced vasodilatation by endogenous NO has been reported and could explain the lack of response to CO in the murine vessels. However in the presence of the NOS inhibitor L-NAME, CO was still unable to induce vasodilatation [10]. Thus an explanation for the heterogeneity of response remains unknown.

In contrast to CO, CORM-2 relaxed all arteries investigated. However, rat aorta seems to be more sensitive to CORM-2 compared to mice aorta and mice femoral artery. This may further help explain species-related differences for CO. Possibly a higher CO sensitivity in some vascular tissues relies on a greater availability of its target molecule(s) [15]. The observation that CORM-2 elicits vasorelaxation in murine isolated vessels whereas CO does not, suggests that CORM-2 acts at least in part through molecular mechanism(s) that differ from those of CO.

While CO relaxes vascular strips through the activation of sGC, CORM-2-induced responses are only partially dependent or even completely independent on sGC activation in mice/rat aorta and mice femoral artery respectively. Our observations are in line with previous reports that illustrated the sGC-independency of CO-induced smooth muscle relaxation and/or that CORM-2-induced relaxation only partially involves the activation of sGC [1;5;9;16;17]. The reason for the difference in sGC-dependency is not clear, but it further supports the idea that CORM-2 (partially) acts through different mechanisms compared to CO.

As mentioned before, the NOS/NO and HO/CO pathways interact with each other in a very complex way. In order to better understand the role of endogenous NO within CO- and CORM-2-induced vasorelaxation, experiments were performed using the NOS inhibitor L-NNA. It was found that the responses to CO and CORM-2 are not affected by endogenous NO. The only exception was observed in rat aorta, in which the NOS inhibitor L-NNA strongly inhibited the CORM-2-induced vasorelaxation. These results suggest that in rat aorta endogenous NO potentiates the vasodilatory effects of CO derived from CORM-2.

That the NOS/NO and HO/CO pathway may positively affect each other corresponds with other studies [9;18]. Why L-NNA inhibits the CORM-2-induced response only in rat aorta and not in mice aorta remains enigmatic. However, it should be noted that complete inhibition of NO production is difficult. A substantial level of endogenous NO might explain the lack of influence of L-NNA in the other vessels [19]. Although it should be noted that the precontraction levels elicited by NOR are enhanced in the presence of L-NNA, which might be responsible for the smaller relaxation of CORM-2 in rat aorta in the presence of L-NNA. The observation that L-NNA inhibits the CORM-2-induced vasodilatation in rat aorta, but has no influence on the vasodilatory properties of CO delivered as a gas, further highlights the differences in molecular mechanisms underlying CO- and CORM-2-induced responses.

As a source of endogenous NO and other vasodilator as well as vasoconstrictor agents, the endothelium could have a role in CO- and/or CORM-2-induced responses. However removal of the endothelium had no effect on the relaxation elicited by CO and CORM-2, suggesting that CO- and CORM-2-induced responses act endothelium-independently. This observation further indicates that the inhibitory effect seen with CORM-2 in the presence of L-NNA in the rat aorta is most likely the consequence of the higher level of tonus. That CO and CORM-2

act independently of the endothelium is in line with other studies showing that the vasorelaxation induced by CO is endothelium-independent [6;16].

In line with previous studies, our results indicate that $K^+$ channels are responsible for CO-induced vasorelaxation [1] and also contribute to CORM-2-induced vasodilatation. However, only a partial inhibition of CORM-2-induced relaxation was observed in the presence of a high concentration of TEA, indicating that other mechanisms are involved as well. Co-administration of ODQ and TEA did not produce a more pronounced inhibition of CORM-2 relaxation (unpublished observations). These data provide indirect evidence for the involvement of a molecular mechanism other than the activation of sGC and/or $K^+$ channels in CORM-2-induced relaxation. These results with CORM-2 are in contrast with the results obtained with CO, which indicated that CO relaxes through sGC activation and $K^+$ channel activation.

It has been repeatedly demonstrated that activation of $K_{Ca}$ channels mediates CO-induced vasorelaxation [6;7]. Our study indicates that this is not the case for CORM-2-induced responses as neither iberiotoxin, charybdotoxin nor apamin exerted an influence on the CORM-2-induced vasodilatation. Conclusively, vasodilatation in response to CORM-2 is dependent on $K^+$ channels other than $K_{Ca}$ channels. Besides $K_{Ca}$ channels, the contribution of $K_{ATP}$ channels and possibly also $K_{ir}$ channels could be excluded, the latter because the small inhibitory effect of $Ba^{2+}$ on the highest CORM-2 concentration is probably not relevant. More interestingly, we found that 4-AP significantly reduces the vasodilatory properties of CORM-2, indicating for the first time that $K_v$ channels play a significant role in the CORM-2-induced vasorelaxation. A previous study using the patch-clamp technique already illustrated that CO affects $K_v$ channels [20], although up till now no other studies supported a role for $K_v$ channels in CO- or CORM-2-induced vasorelaxation.

A limitation of the present study is that we were not able to identify the mechanism involved in the vasodilatory effect of CORM-2 refractory to the presence of both ODQ and TEA. Furthermore, it is noteworthy that CORM-2 has been shown to possess non-specific actions independent of CO-release [11;12].

## IV.4.6 Conclusion

Interest on the (patho)physiological effects of CO and pharmacological potential of CORMs is just starting. Knowledge on the HO/CO pathway will rapidly grow in the near future. The present study expands the knowledge on the physiological properties and the pharmacological characteristics of CO and CORM-2, a molecule developed in order to mimic CO. Our study clearly shows that one should not presume that the molecular mechanisms underlying CORM-2-induced vasorelaxation are equal to the mechanisms involved in the vasodilatory properties of CO as a gas and shows for the first time the involvement of $K_v$ channels in CORM-2-induced relaxation.

## IV.4.7 Acknowledgements

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# Manuscript 5

# Divergent molecular mechanisms underlay CO and CORM-2 induced corporal relaxation

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**Based on a publication accepted in J. Sex. Med**

## IV.5.1 Abstract

**INTRODUCTION:** Similar to NO, the principal mediator of penile erection, CO possesses vasodilator capacities. However whether CO could be a therapeutic target for treating ED is unexplored. The danger associated with systemic administration of CO has led to the development of CORMs, releasing CO in a local, safe and controlled way. These CORMs have shown positive outcomes in cardiovascular studies. More knowledge on the (patho)physiological functions of CO in erectile function and the potential therapeutic role of CORMs is required.

**AIMS:** The present study aims the assessment of the effect of CO and CO-donor CORM-2 on the corporal tension and the underlying molecular mechanisms.

**METHODS:** Organ bath studies were performed measuring isometric tension on isolated mice CC strips. Responses to CO (10 – 300 µmol/L) and CORM-2 (10 – 100 µmol/L) were measured in the presence/absence of activators/inhibitors of different molecular pathways.

**MAIN OUTCOME MEASURES:** CO and CORM-2 relax corporal strips concentration-dependently, although the molecular mechanism behind the corporal relaxation seems to differ completely.

**RESULTS:** CO induces corporal relaxation by activating sGC, increasing cGMP concentrations. The molecular mechanism involved in CORM-2 induced corporal relaxation is not related to sGC activation and remains obscure. A small contribution of voltage-dependent potassium channel opening could be involved.

**CONCLUSIONS:** Both CO and CORM-2 induce corporal relaxation, although the underlying molecular mechanisms show no resemblance. That CO induces corporal relaxation through a mechanism similar to that of NO could be of importance as it indirectly offers the possibility that endogenous CO might serve as a back-up system for insufficient NO availability in cases of ED. Whether CORM-2 possesses the same capacity remains questionable and requires further research.

## IV.5.2 Introduction

At present ED is mainly treated using PDE-5 inhibitors. However a large percentage of patients (30 – 35%) is or becomes refractory to this treatment. Other therapeutic options are limited and less attractive as they are more invasive. This necessitates research for the development of new therapeutic targets [1].

An evolving potential new therapeutic target is the HO/CO pathway [2]. Hedlund et al. (2000) detected immuno-reactivity to both HO-1 and HO-2 in the endothelium of CC [3]. Research reports on CO in the field of erectile (dys)function are scarce. One study illustrated that HO-1 cDNA-liposome complex transfer augments cGMP concentrations in cavernosal tissue with subsequent sinusoidal relaxation. Moreover, HO-1 gene transfer enhanced corporal relaxation in aged rats, suggesting that activating the HO/CO pathway may ameliorate the erectile function even in elderly [4]. Furthermore, improved erectile function by the antioxidant α-tocopherol in hypertensive rats could be blocked with a HO-inhibitor, implying that HO-inducers may also ameliorate the erectile function in hypertensive rats [5]. Guo et al. (2006) illustrated that enhancing endogenous CO production using a HO-1 inducer had a relaxant effect on the cavernosal smooth muscle cells [6]. This observation further provides evidence that the HO/CO pathway possesses potential as a new therapeutic target for treating ED. This is further confirmed by the observation that high blood pressure and intracavernosal responses in SHRs are normalized after chronic administration of the HO-1 inducer hemin [7]. Finally, Abdel Aziz et al. reported that both the gene expression and enzymatic activity of HO-1 was strongly decreased in cavernosal tissue of diabetic rats, resulting in diminished erectile function. The use of a HO-1 inducer elevated expression and activity of HO-1 and significantly improved the erectile function of these rats [8]. This study suggested that the decline in erectile function in diabetic rats may be attributed to a down-regulation of the HO/CO pathway and may indicate that stimulating this pathway is efficient to treat ED in diabetic patients. Despite these promising results, more research is required to assess the role of the HO/CO pathway in erectile (dys)function before clinical trials can be started targeting the HO/CO pathway.

Based on the increasing knowledge on the physiological importance of the HO/CO pathway [9], molecules have been developed releasing CO with controllable kinetics, the so-called CORMs [10-13]. Development of these molecules has been a crucial step in pharmacological research of CO. One of these CORMs is CORM-2, which is being frequently used to evaluate the (patho)physiological properties of CO and its potential therapeutic applications [14].

## IV.5.3 Aims of the study

The present study aims to evaluate relaxation responses induced by CO and CORM-2 in mice isolated CC and to compare the molecular mechanisms underlying the relaxation effects observed with CO and CORM-2.

## IV.5.4 Materials and Methods

### IV.5.4.1 Animals

Male mice (129SvJ background) (10 - 14 weeks-old) were used. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication no. 85-23, revised 1996). The studies were approved by the local Ethical Committee for Animal Experiments, Faculty of Medicine and Health Sciences, Ghent University, Belgium. The animals were housed on a 12-h light/dark cycle and fed a standard chow diet with water ad libitum. On the day of the experiment, the mice were sacrificed by cervical dislocation.

### IV.5.4.2 Preparation of corporal strips

Penile tissue was dissected free and the CC were separated from each other by cutting the fibrous septum between them and excised at the base. Following, CC were transferred into a Petri-dish containing ice-cold KRB solution. Both corporal strips (1x1x5mm) were then mounted horizontally for isometric tension recordings in 10 mL myograph chambers (made by own technical staff), containing KRB solution at 37°C (pH 7.4) and continuously gassed with a mixture of 95% $O_2$ – 5% $CO_2$. After 30 minutes resting time, the preparations were gradually stretched to a stable resting force of 0.45 g and allowed to equilibrate during 60 minutes. Changes in isometric force were recorded as one end was fixed to a force-displacement transducer and the other to a micrometer. To verify the contractile activity of the preparations, CC were contracted 3 times with NOR (5 µmol/L) at the end of the equilibration period and each time washed and allowed to relax to the basal tension. When reaching a stable resting tension, corporal strips were pre-contracted again with NOR and when the contraction was stabilized, the response to ACh (1 µmol/L) was examined to evaluate the functionality of the endothelium within the corporal strips. Preparations that were not able to relax minimally 10% from the maximum tension were excluded from the study. Thereafter, the CC were once again washed. When reaching a stable resting tension the experimental protocols were started.

### IV.5.4.3 Experimental protocols

Cumulative concentration-response curves to CO (10 – 300 µmol/L) and CORM-2 (10 – 100 µmol/L) were obtained in cavernosal strips contracted with NOR (5 µmol/L). After measuring the corporal responses to CO and CORM-2, we tested their effects in the presence of activators/inhibitors of different molecular pathways. As preliminary experiments showed that the response to CORM-2 is not reproducible in the same strip, experiments testing the influence of different agents on the concentration-response curve elicited by CORM-2 were conducted in parallel corporal strips. The activators/inhibitors used in our experiments act on either the sGC enzyme, the NOS enzymes or as (non)specific $K^+$ channel inhibitors. In addition, responses to the solvents of CO and CORM-2 were also tested.

### IV.5.4.4 Drugs and chemicals

The experiments were performed in a KRB solution of 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 $H_2O$. Dimetylsulfoxide (DMSO), acetylcholine chloride (ACh), 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1), Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), norepinephrine bitartrate (NOR), tricarbonyldichlororuthenium(II)dimer (CORM-2), tetraethyl-ammonium chloride (TEA), glibenclamide, barium chloride ($Ba^{2+}$), 4-aminopyridine (4-AP) and (±)-miconazole nitrate (miconazole) were purchased from Sigma-Aldrich (St.Louis, MO). Iberiotoxin was obtained from Alomone labs (Jerusalem, Israel). ODQ was dissolved in ethanol (stock concentration is 10 mmol/l); YC-1, glibenclamide and CORM-2 were dissolved in DMSO (stock concentration is 10 mmol/l; 30 mmol/l and 1 mmol/l respectively) and ACh in 50 mmol/L $K^+$ hydrogen phthalate buffer (stock concentration is 10 mmol/l), pH 4.0. All other drugs were dissolved in distilled water. The incubation time for the different agents was set at 20 minutes, except for ODQ and YC-1, which were incubated during 10 minutes. A saturated CO solution (1 mmol/L) was prepared from CO gas (Air liquide, Belgium) as described before [15]. CORM-2 was always freshly prepared before administration into the organ baths. All concentrations are expressed as final molar concentrations in the organ bath.

### IV.5.4.5 Statistical analysis

Data are presented as mean values ± SEM; *n* represents the number of cavernosal strips (each obtained from a different mouse). Relaxations are expressed as the percentage decrease of the

precontracted tone developed by the addition of NOR. Statistical significance was evaluated by using Student's *t*-test for paired and unpaired observations (SPSS, version 20) or with two-way ANOVA with Bonferroni post-hoc test (GraphPad Prism, version 4), when appropriate. Additional to the Student's t-test for unpaired observation, the Mann-Whitney U test was performed to evaluate the statistics. $P < 0.05$ was considered as statistically significant.

## IV.5.5. Main outcome measures

The main findings of this study are that both CO and CORM-2 relax isolated CC strips in a concentration-dependent manner and that CO acts by activating sGC, while the molecular mechanism underlying CORM-2-induced relaxation is not related to sGC and remains obscure.

## IV.5.6 Results

### IV.5.6.1 CO-induced responses

CO relaxes mice cavernosal strips in a concentration-dependent manner (Figure IV.5.1A). Maximal relaxation at the highest concentration was 29.09 % ± 3.93 The solvent of the CO solution also induces a small response at the highest concentration.

Incubation of the preparation with the sGC inhibitor ODQ (10 µmol/L) effectively inhibits the relaxation of CO (Figure IV.5.1B), suggesting that CO relaxes mainly through the activation of sGC. The involvement of sGC was further confirmed in experiments using YC-1 (1 µmol/L). Incubation with this sGC activator enhanced the CO-induced relaxation (Figure IV.5.1C).

To elucidate whether the sGC activation results from stimulation of the endogenous NOS/NO pathway, we compared the effect of CO in the absence and presence of the NOS inhibitor L-NAME (100 µmol/L) (Figure IV.5.1D). No significant inhibition was found, suggesting that CO does not activate sGC through stimulation of the endogenous NO pathway.

Furthermore, the non-specific $K_{Ca}$ channel blocker TEA (3 mmol/L) did not influence the relaxation responses to CO, illustrating that $K_{Ca}$ channels are not involved (Figure IV.5.1E).

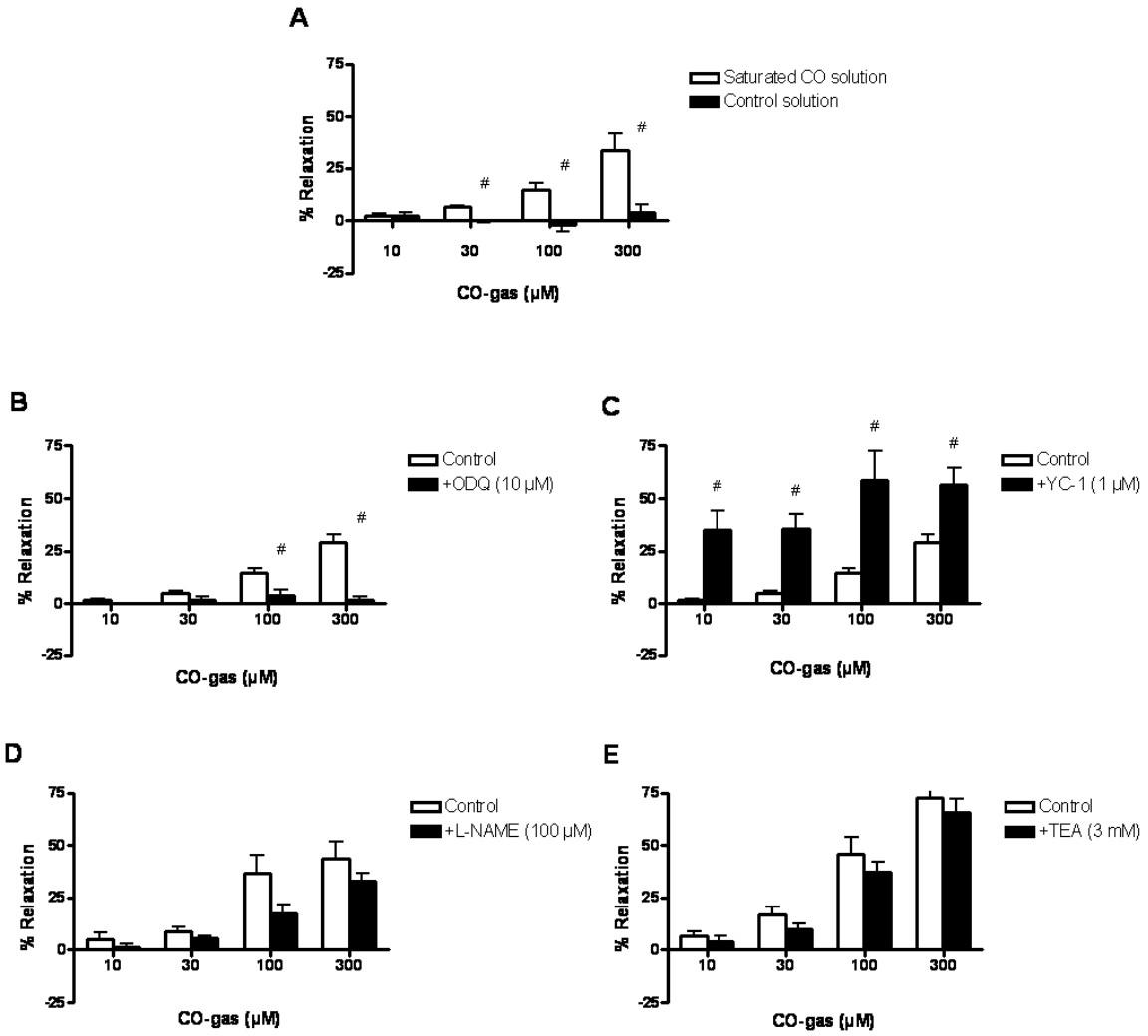


**Figure IV.5.1** (A) shows that CO (10 – 300 µmol/L) elicits a concentration-dependent relaxation of the CC after precontraction with NOR (5 µmol/L), while the solvent solution only induces a very small relaxation when used at the highest concentration. (B – C) indicate that CO induced corporal relaxation is (B) attenuated in the presence of ODQ (10 µmol/L, 10 minutes incubation) (C) potentiated in the presence of YC-1 (1 µmol/L, 10 minutes incubation). (D – E) indicate that CO induced corporal relaxation is unaltered after incubation during 20 minutes with L-NAME (100 µmol/L) or TEA (3 mmol/L). Data are expressed as % relaxation of the NOR-induced tone; Student's t-test for paired observations; # $P < 0.001$.

That large-conductance $K_{Ca}$ channels are of no importance in CO-induced corporal relaxation is further supported by the unaffected CO-induced relaxation in the presence of iberiotoxin (100 nM) (Figure IV.5.2A). Moreover, no evidence is found for a contribution of the $K_V$ channels, the $K_{ATP}$ channels and $K_{IR}$ channels in CO-induced corporal relaxation, as incubation with the $K_V$ channel blocker 4-AP (3 mmol/L), the $K_{ATP}$ channel blocker glibenclamide (3 µmol/L) and the $K_{IR}$ channel blocker $Ba^{2+}$ (10 µmol/L) had no influence on the effect of CO (Figure IV.5.2B, IV.5.C and IV.5.D).

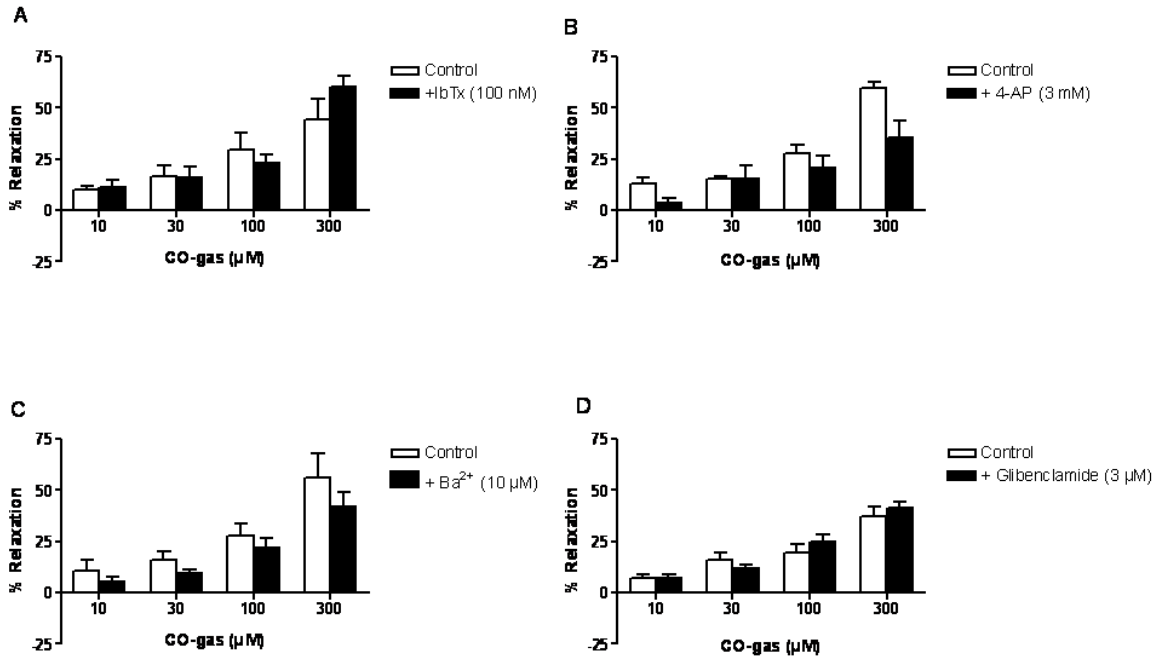


**Figure IV.5.2** illustrates that CO (10 – 300 µmol/L) elicits a concentration-dependent relaxation of precontracted (NOR 5 µmol/l) penile tissue that is similar in the absence or presence of (A) iberiotoxin (100 nmol/L), (C) $Ba^{2+}$ (10 µmol/L) and (D) glibenclamide (3 µmol/L). However, CO-induced corporal relaxations were significantly impaired in the presence of (B) 4-AP (3 mmol/L). Data are expressed as % relaxation of the NOR-induced tone; Student's t-test for paired observations.

### IV.5.6.2 CORM-2-induced responses

CORM-2 relaxes mice cavernosal strips in a concentration-dependent manner. The maximum relaxation was 97.15 % ± 2.21 (Figure IV.5.3A). It should be noted that the solvent DMSO also induce relaxation although less than CORM-2, maximum 46.92 % ± 4.90.

Pretreatment with ODQ (10 µmol/L) did not attenuate the response of CORM-2. Moreover, incubation with YC-1 (1 µmol/L) had no influence on the CORM-2-induced relaxation (Figure IV.5.3B and IV.5.C).

After incubation of the CC with the NOS inhibitor L-NAME (100 µmol/L) no statistically significant inhibitory response was observed, excluding contribution of the NOS/NO pathway in the CORM-2-induced corporal vasodilation (Figure IV.5.3D). Relaxation of CORM-2 is not altered after incubation with TEA (3 mmol/L) (Figure IV.5.3E).

Data obtained with iberiotoxin (100 nmol/L) confirmed the data obtained with TEA (Figure IV.5.4A). Surprisingly we found that incubation of the corporal strips with 4-AP (3 mmol/L)

potentiated the effects of CORM-2, although this potentiating effect was only statistically significant at a concentration of 25 µM CORM-2 (Figure IV.5.4B). $K_{ATP}$ and $K_{IR}$ channels or of no significance in the relaxation induced by CORM-2, as incubation with glibenclamide (3 µmol/L) or $Ba^{2+}$ (10 µmol/L) had no influence (Figure IV.5.4C and IV.5.4D).

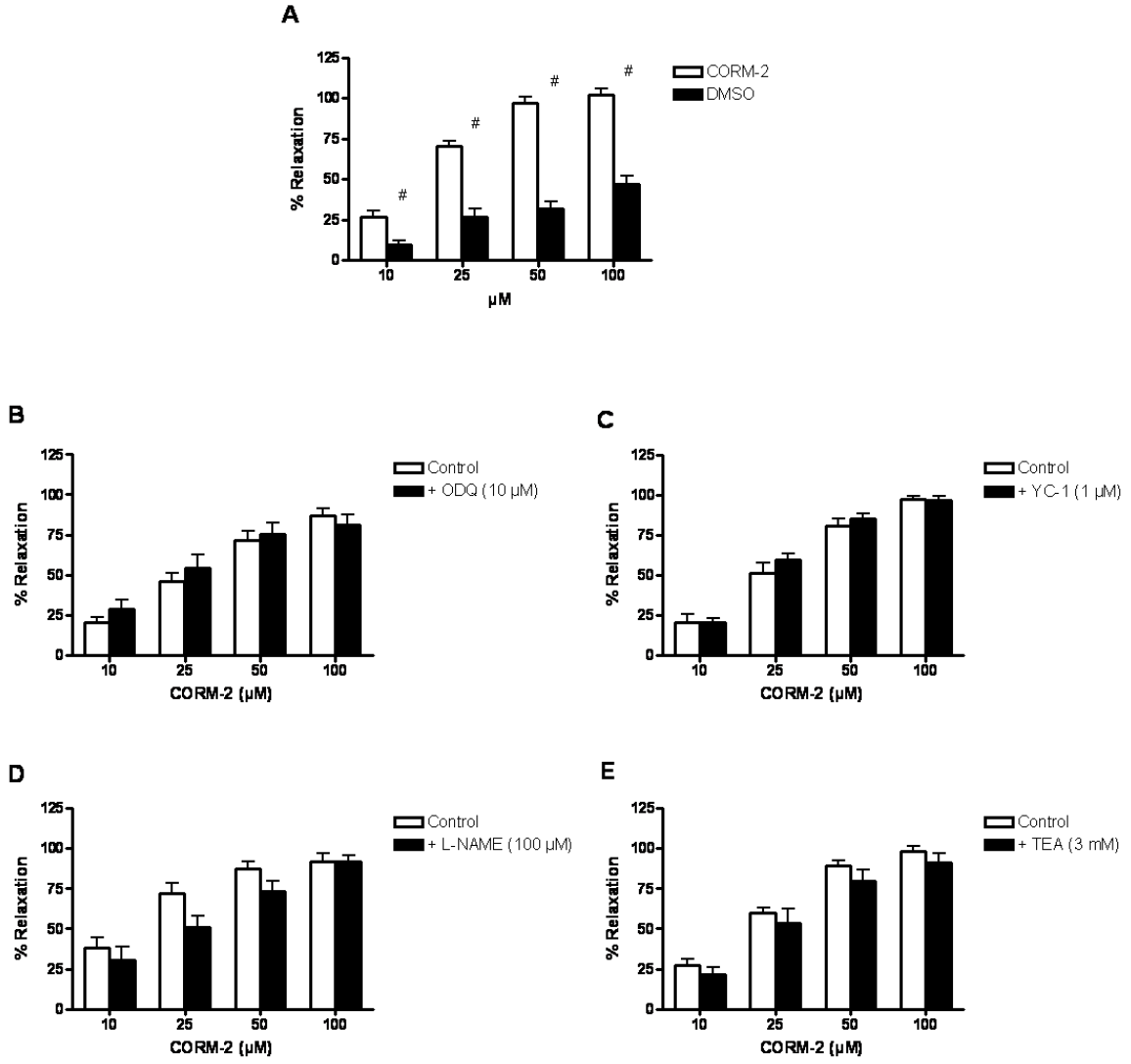


**Figure IV.5.3** (A) shows that CORM-2 (10 – 100 µmol/L) and its solvent DMSO elicit a concentration-dependent relaxation of the CC after precontraction with NOR (5 µmol/L). (B – C) CORM-2 induced corporal relaxation is similar in the absence and presence of (B) ODQ (10 µmol/L) and (C) YC-1 (1 µmol/L). (D - E) illustrate that 20 minutes incubation with (D) L-NAME (100 µmol/L) and (E) TEA (3 mmol/L) does not influence CORM-2 responses. Data are expressed as % relaxation of the NOR-induced tone; Student's t-test for unpaired observations plus Mann-Whitney U test; # $P < 0.001$.

It is of interest to note that none of the activators/inhibitors used had an effect on the precontraction levels elicited by NOR.

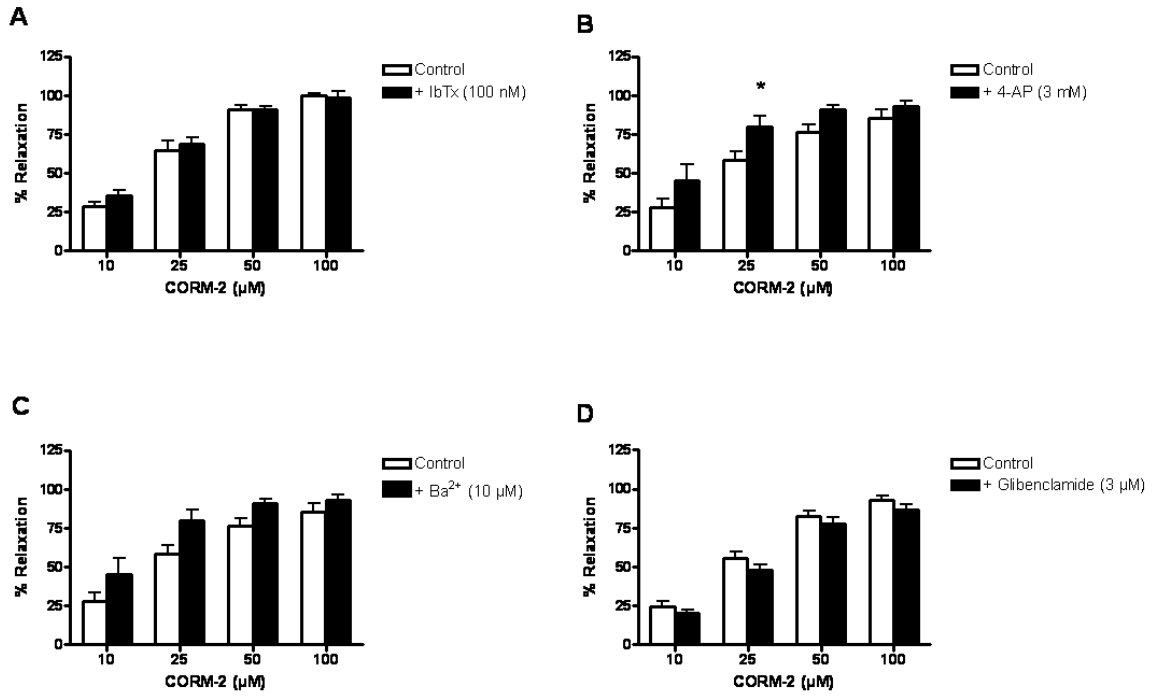


**Figure IV.5.4** illustrates that (A) CORM-2 (10 – 100 µmol/L) elicits a concentration-dependent relaxation of precontracted (NOR 5 µmol/l) CC that is similar in the absence or presence of iberiotoxin (100 nmol/L, 20 minutes incubation). (B) the CORM-2 induced corporal relaxation is slightly but significantly potentiated by the $K_V$ channel inhibitor 4-AP (3 mmol/L). (C – D) Similar responses to CORM-2 in the absence and presence of 20 minutes incubation of (C) $Ba^{2+}$ (10 µmol/L) or (D) glibenclamide (3 µmol/L). Data are expressed as % relaxation of the NOR-induced tone; Student's t-test for unpaired observations plus Mann-Whitney U test ; * $P < 0.05$.

Additional experiments were performed evaluating the contribution of the cytochrome P450 pathway on CORM-2 induced responses. The hypothesis that CORM-2 partially acts through the activation of the cytochrome P450 pathway however could be refuted as the non-specific cytochrome inhibitor miconazole (10 µM) had no influence on CORM-2-induced corporal relaxation.

## IV.5.7 Discussion

The main and original finding of the present study is that both CO and CORM-2 relax CC in a concentration-dependent manner albeit through different molecular mechanisms. Plenty of reports show convincing data that exogenous CO exerts vasoactive properties and indirect evidence suggests that also endogenous CO induces vasodilation in many vascular beds [16-18]. However, CO-induced vasorelaxation is certainly not a universal phenomenon [19;20]. While we previously found that mice isolated blood vessels are unresponsive to CO, a pronounced relaxation on mice CC is observed in the present study [20]. An explanation for

the discrepancy remains to be elucidated, although a hypothesis could be that mice CC, in contrast to mice aorta and femoral artery, possess a specific molecular target for CO or that this molecular target shows a higher activity/sensitivity in penile tissue. This surprising selectivity demands further research.

From pharmacological point of view, the finding that CO relaxes CC without effect on other vascular vessels is very interesting. The latter could indicate that an appropriate CO-donor, would lead to less adverse effects compared to current drugs which struggle with smooth muscle relaxation in other vascular beds and in the gastro-intestinal system [1]. Similar studies should be performed on human tissues to evaluate whether human CC are also more sensitive than other vascular tissues. Whatsoever, our observation strengthens the concept that the HO/CO pathway is a valid target to treat ED.

The mechanisms underlying CO- and CORM-2-induced corporal relaxation still remain obscure. Many reports suggest that CO induces vasorelaxation through the activation of sGC although others dispute this finding and suggest that CO-induced vascular relaxation is due to the opening of $K^+$ channels, in particular $K_{Ca}$ channels [21;22]. Still others believe that activation of the P450 pathway underlies CO-induced vasorelaxation [17]. Moreover, there is as yet no indication that CO and CORM-2 induce vasorelaxation through a similar manner. It is often reported that CORMs do not always behave as authentic CO, although no data are available on the CC [10;15;20].

The present study shows that the molecular mechanism underlying CO- and CORM-2-induced corporal relaxation differ completely. CO acts through the activation of sGC, indicated by the inhibiting effect of ODQ and potentiating effect of YC-1 on the CO-induced responses. CO-induced sGC activation has already been shown in different vascular smooth muscle tissues [10;15;17;23]. Furthermore, it was illustrated that water-soluble long-acting curcumin, a HO-1 inducer, significantly potentiated the ICP via up-regulation of cavernous tissue cGMP [24]. This observation might indicate that also endogenous CO acts through sGC activation. As CO only possesses a very low potency to activate sGC, one could suggest that CO interferes with the NOS/NO pathway, inducing corporal relaxation through stimulation of NO production [17;20]. Although a inhibitory tendency could be seen, the inhibitory influence of the NOS inhibitor L-NAME on the CO-induced response was not statistically significant and thus most likely illustrates that CO-induced corporal relaxation acts

independent of the NOS/NO pathway. These results directly suggest that CO activates sGC, a conclusion that is not evident considering the low affinity of CO for the enzyme compared to NO [25]. Interesting to mention is that the HO/CO pathway can be up-regulated when the NOS/NO pathway is deranged in pathophysiological conditions such as oxidative stress and hypertension [26-29]. The HO/CO up-regulation serves as a protective back-up mechanism compensating for the diminished NO responses and keeping the vessels dilated [30]. It would be interesting to find out whether the HO/CO pathway is up-regulated when NO availability is decreased in ED, for instance by using triple knockout NOS mice.

Despite the observation that CO is produced in the CC [3], the physiological role of CO in erectile function was refuted by Burnett et al. who revealed that HO-2 knockout mice have the same mount latency and erection following ES of the cavernosal nerve as wild-type mice [31]. On the other hand, gene therapy with HO-1 in aged rats resulted in higher cGMP concentrations and inhibition of the NOS/NO pathway had no effect on the up-regulation of HO-1 and increased cGMP concentrations. Moreover, the CC of aged rats treated with HO-1 gene transfer demonstrated a significant dilation of the helicinal arteries compared to the control rats [4]. A clarification for the fact that Burnett et al. observed no significant effects in HO-2 deleted mice could be that HO-1 becomes more important, producing higher intracellular CO concentrations and that HO-1 is only important when up-regulated by for example oxidative stress, a contributor to ED in aging.

Besides sGC, $K_{Ca}$ channels have been suggested as a target of CO [22;32]. Moreover, CO might also influence Kv channel activity [20;33;34]. The present study however excludes their contribution and provides evidence that activation of $K^+$ channels is of no importance in CO-induced corporal relaxation (Figure IV.5.1E and IV.5.2B).

As mentioned before, the present study proves that CORM-2 elicits corporal relaxation through another molecular mechanism than CO. First of all, it does not depend on sGC activation as neither ODQ nor YC-1 had an influence on the CORM-2-induced corporal relaxation responses. The reason for the discrepancy between CO and CORM-2 remains to be elucidated but it has been illustrated before on other vascular tissues [20]. A hypothesis is that CO diffuses through the membrane and directly stimulates sGC, while CO released from CORM-2 is still bound somehow to the transition metal not being able to diffuse through the

membrane and thus stimulates membrane-bound receptors ion channels inducing vasorelaxation. However this is pure speculation which requires further investigation.

Consistent with CO, CORM-2-induced relaxation was not affected by a high TEA concentration. Surprisingly 4-AP slightly potentiated the relaxation induced by CORM-2. This observation might suggest that CORM-2 acts at best in the closed state of Kv channels, although as the enhanced response could only be observed at 25 µM CORM-2, further research will need to be performed in order to elucidate the role of $K_V$ channels in the CORM-2-induced responses. A previous study however already illustrated that CORM-2 is an allosteric inhibitor of Kv2.1 channels and this inhibitory action was shown to be independent of CO derived from CORM-2 [35].

Additional experiments were performed to evaluate whether CORM-2 exhibits its vasorelaxing effect through the activation of the cytochrome P450 pathway. It has previously been shown that 11,12- epoxyeicosatrienoic acid (EET) is produced and potentially required for normal erection [36]. Moreover, CO might act through the activation of the cytochrome P450 pathway [17]. However, the effect of 11,12-EET has been show to be significantly reduced by both glibenclamide and iberiotoxin [36]. As these inhibitors had no effect on CO- and CORM-2-induced corporal relaxation, it is unlikely that 11,12-EET contributes to their dilatory effects in CC. Moreover, we found that incubation of CC with miconazole had no influence on CORM-2-induced corporal relaxation, further excluding the involvement of cytochrome P450 metabolites.

The molecular mechanism involved in CORM-2-induced relaxation of CC thus remains as yet unknown. Currently water-soluble CORMs are developed such as CORM-3 and CORM-A1, exhibiting better pharmacological handling and potentially more similarity with authentic CO [37]. To our knowledge, only one report is published showing slightly enhanced cGMP levels by CORM-3 in rat penile tissue. Whether this observation can be translated to corporal relaxation was not investigated [38].

## IV.5.8 Conclusion

CO, previously regarded as a waste product of heme metabolism, is now appreciated to exert many physiological effects that resemble those of another gaseous messenger molecule NO.

The present study indicates that CO and CORM-2 elicit corporal vasorelaxation in a concentration-dependent manner. This observation may suggest that interfering with the HO/CO pathway is a valuable option for treating ED. While CORMs can be considered useful drugs with potential in clinical use, much more research is required to examine their effects and the underlying mechanisms.

## IV.5.9 Acknowledgements

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# Chapter V:

# Discussion and future perspectives

Due to the established relevance of the NO/cGMP signaling in the cardiovascular system, the mechanisms responsible for regulation and transduction of NO/cGMP signaling are of major physiological and pharmacological interest. Dysfunction of the vascular NO/cGMP signaling pathway has been illustrated to occur at different levels and is believed to contribute essentially to various cardiovascular disorders, including ED [1]. Many research groups already presented convincing evidence that under pathological conditions NO production may significantly be diminished due to down-regulation of NOS expression/activity and/or reduced NOS substrate/cofactor availability [2]. In addition, NO bio-availability may also be decreased through oxidative inactivation by ROS [3]. ROS react with NO to form peroxynitrite and other RNS. As a result NO levels are reduced and as peroxynitrite is toxic, heightened levels of this agent lead to increased apoptosis of both endothelial and vascular smooth muscle cells. Importantly, decreased NO sensitivity due to reduced expression and/or activity of the NO sensor sGC has also been illustrated in some pathophysiological conditions [4-11]. Moreover, elevated ROS/RNS can lead to oxidation of the ferrous heme group of sGC, rendering the enzyme insensitive towards its natural activators. Furthermore, downstream targets of cGMP might also be affected in different vascular diseases. For instance, PDE-5 activity may be up-regulated [12], resulting in a higher breakdown of cGMP and PKG activity was found to be down-regulated in CC of diabetic rabbits [13].

Currently, PDE-5 inhibitors are the first choice of drugs for treating ED and have been implemented for other cardiovascular diseases as well. However, in conditions where the cGMP production is strongly reduced, PDE-5 inhibitors might possess a low efficiency whereas direct stimulation of sGC may offer benefits. sGC stimulators such as NO-donors and agents which sensitize the enzyme towards its heme ligands, have been shown to exert beneficial effects in both arteries and CC of diseased animals or humans, suggesting that modulation of sGC activity has important pharmacological implications [1]. However, sGC activation has been shown to induce a life-threatening hypotension, an observation that is not surprising knowing that the enzyme is present in both large and small arteries as well as in heart smooth muscle cells [14;15]. Moreover, like PDE-5, sGC is also present in cerebral vessels, the gastrointestinal system, the retina and others, leading to the assumption that sGC stimulators offer no benefit over PDE-5 inhibitors regarding side-effect reduction. However the existence of two different sGC isoforms offers new therapeutic perspectives. Despite strong differences in primary structure of the N-terminal heme binding regions of the $\alpha_1$ and $\alpha_2$ subunits, no differences in heme-coordination, substrate affinity, NO-sensitivity and

catalytic activity were detected during biochemical and kinetic characterization of the $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$ isoforms [16]. Since the regulatory features of the two isoforms are indistinguishable, the physiological relevance of the two isoforms is unclear. The question remains whether the 2 isoforms of sGC serve diverse biological functions or whether one isoform can substitute for the other. Expression under transcriptional control of cell-type specific or developmental promoters may be a plausible explanation for the existence of the two functionally similar isoforms [17].

As molecular studies revealed the existence of both isoforms of sGC in human CC [18], isoform-specific stimulation could be of potential beneficial therapeutic value for ED. Our first studies therefore aimed to increase our knowledge on the functional role of sGC and its isoforms in the mechanism of penile erection. A mouse model was chosen to accomplish our goals. The mouse model affords a unique approach to scientific research when compared with other animal models. Economic feasibility and practicability of this species are optimal. More importantly, application of transgenic technology and availability of molecular biological approaches to investigate genetic determinants make this species superior for scientific research [19]. In our studies, $sGC\alpha_1^{-/-}$ mice helped to elucidate the particular role of the predominantly expressed $sGC\alpha_1\beta_1$ isoform in CC, whereas $sGC\beta_1^{ki/ki}$ mice permitted the investigation on the function of sGC in penile erection and possible cGMP-independent signaling pathways in erectile tissue.

In the first study it was illustrated that the $sGC\alpha_1\beta_1$ isoform is of great importance in NO-induced corporal smooth muscle relaxation in *in vitro* condition. Despite the strong attenuation of the NO-induced responses, a substantial response to these agents could still be observed, suggesting that also (an) alternative mechanism(s) is are involved [20]. Activation of the less expressed $sGC\alpha_2\beta_1$ enzyme by NO as well as sGC-independent NO targets may explain the substantial response. However, incubation of CC with ODQ completely blunted the corporal relaxations induced by EFS, indicating that the remaining responses observed in $sGC\alpha_1^{-/-}$ mice might be explained by activation of the $sGC\alpha_2\beta_1$ isoform by NO [20]. However, there are reports on the influence of ODQ on the redox state of other heme-containing proteins such as the cytochrome P450 pathway [21;22]. Moreover, as targets for ODQ, myoglobin and hemoglobin can positively or negatively influence the effectiveness of ODQ [23;24]. The non-specificity of ODQ may account for the complete inhibition of EFS-induced corporal responses.

In order to evaluate the correlation between the functional alteration in the CC of $sGC\alpha_1^{-/-}$ and ED, erectile hemodynamics of $sGC\alpha_1^{-/-}$ mice were determined during electrophysiological and pharmacological induction of penile erection by ICP monitoring. This technique has been thought to be a significant advance because it provides an objective and accurate quantitative index to evaluate penile erections [25]. Results of these *in vivo* studies also illustrated that the erectile hemodynamics in response to ES and NO-donors were significantly diminished in $sGC\alpha_1^{-/-}$ mice. A role for agents other than NO to be released by NANC nerves after applying ES and to induce erectile responses was excluded as the NOS inhibitor L-NAME completely blocked the responses to ES. This observation was initially in contrast to the observation that mice lacking either eNOS or nNOS preserved their erectile capacity [26-29]. However, this discrepancy was later explained by the action of a nNOS splice variant, nNOSβ, mediating a major portion of penile erection [30].

Data obtained with 8-pCPT-cGMP demonstrate undisturbed signal transduction by PKG and further downstream in the $sGC\alpha_1^{-/-}$ mice. Furthermore, unaltered responses to forskolin indicate that the AC/cAMP pathway is not up-regulated in the erectile tissues of $sGC\alpha_1^{-/-}$ mice to compensate for the strongly reduced sGC/cGMP pathway. The observation of remaining responses in $sGC\alpha_1^{-/-}$ mice to ES and NO-donors, however suggests that besides the $sGC\alpha_1\beta_1$ also the $sGC\alpha_2\beta_1$ and/or sGC-independent mechanisms contribute to NO-induced penile erection. Although no up-regulation for $sGC\alpha_2\beta_1$ has been found in the aortic and gastrointestinal tissues of $sGC\alpha_1^{-/-}$ mice as compensation for the loss of $sGC\alpha_1\beta_1$, it could still be of interest to study the expression level of the $sGC\alpha_2\beta_1$ isoform in corporal tissues. In addition, it could be of value to supplement the obtained results with cGMP concentration measurements.

Strain-dependent differences were investigated since it was observed that 129SvJ $sGC\alpha_1^{-/-}$ mice developed hypertension while $sGC\alpha_1^{-/-}$ mice with a C57BL6/J background did not [31]. Our study also indicated strain-dependent differences in penile hemodynamics. Several studies already reported that variations in the genetic make-up of laboratory mouse strains may affect the cardiovascular phenotype of those strains. Recently, Buys et al. demonstrated that the activity of the renin-angiotensin aldosterone system (RAAS) modulates the susceptibility to hypertension in a setting of impaired NO/cGMP signaling [32]. As it has already been demonstrated several times that RAAS also contributes the regulating of corporal tone [33], a genetic difference in RAAS between the strains investigated might

underlay the observed differences. Based on the extensive differences in genomic and amino acid sequence between the murine and human renin 1 genes, one needs to be careful in extrapolating results obtained from murine cardiovascular research to humans.

Data obtained from $sGC\beta_1^{ki/ki}$ mice clearly reveal the unique role of sGC as a receptor for NO in penile tissue. Despite the abrogated responses to EFS and ES of the cavernosal nerve in penile tissue of $sGC\beta_1^{ki/ki}$ mice, the NO-donors still induced a substantial response on isolated CC of $sGC\beta_1^{ki/ki}$ mice. A likely explanation for this discrepancy is that the use of large NO concentrations may lead to increased ROS and RNS, resulting in enhanced oxidative stress and consequently vasorelaxation [34]. Moreover, SNP not only decomposes into NO but also other compounds that may have adverse effects on vascular smooth muscle cells. One of such substances is cyanide which is known to affect oxidative metabolism in smooth muscle and further impair cell function [35]. However, the lack of response to NO-donors in the in vivo experiments confirms that the relaxing effect of NO in the CC is solely conveyed by sGC. These experiments thus indirectly indicate that the observed responses in $sGC\alpha_1^{-/-}$ mice induced by NO are due to the activation of the less expressed $sGC\alpha_2\beta_1$ isoform. Despite a previous observation that NO can activate $Na^+/K^+$-ATPase independent of an increase in cGMP concentration in corporal smooth muscle cells [36], no evidence for the contribution of cGMP-independent mechanisms was found. These findings are in line with a previous report illustrating that the erectile response is completely abrogated in mice lacking PKG [37]. The remaining fertility observed in $sGC\alpha_1^{-/-}$ mice, in contrast to $sGC\beta_1^{ki/ki}$ mice which very rarely produce offspring, may thus be attributed to the residual effect of NO on the $sGC\alpha_2\beta_1$ isoform. However the existence of a penile bone found in most rodents most probably aids penetration without the need for full rigidity of the penis.

The potency of NO donors may vary considerably, an observation that was also made in our studies. NO donors differ in the chemistry of the scaffold substance, the rate and spontaneity of NO release and the NO species they release. Among the three redox forms of NO ($NO^\bullet$ ; $NO^-$ and $NO^+$) only the uncharged NO radical has been shown to significantly activate sGC [38;39].

The corporal responsiveness of $sGC\alpha_1^{-/-}$ and $sGC\beta_1^{ki/ki}$ mice was also tested after the application of the sGC activator BAY 41-2272. The response to this compound was significantly reduced in CC isolated from $sGC\alpha_1^{-/-}$ mice [20]. However, the substantial

corporal relaxation observed with BAY 41-2272 led to the assumption that this sGC activator also exerted its effects on the $sGC\alpha_2\beta_1$ isoform. YC-1 and BAY 41-2272 were already found to exhibit effects on both the $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$ isoform [40]. Surprisingly, elimination of the heme-dependent activation using $sGC\beta_1^{ki/ki}$ mice, did not result in a stronger reduction of the BAY 41-2272-induced corporal responses. In line with our data, Buys et al. illustrated that the $sGC\alpha_2\beta_1$ isoform is not able to mediate BAY 41-2272-induced decreases in blood pressure in $sGC\alpha_1^{-/-}$ mice, implying that BAY 41-2272 is an isoform-specific $sGC\alpha_1\beta_1$ stimulator *in vivo* in mice [31]. In addition, these data indicate that BAY 41-2272 also exerts sGC-independent effects, at least if this sGC activator is completely heme-dependent. It has already been reported that BAY 41-2272 decreases superoxide formation and inhibits NADPH oxidase expression [41].

Most sGC stimulators require the integrity of the reduced heme moiety of the prosthetic group within the sGC, whereas in most diseases ROS generation is increased to such an extent that the heme moiety is oxidized, rendering most sGC stimulators unable to offer benefit. Recently heme-independent sGC stimulators have been developed to overcome this problem [42]. One example is cinaciguat or BAY 58-2667. While cinaciguat is only a moderate activator of regular sGC, it stimulates heme-oxidized or heme-depleted purified enzyme almost two hundred fold [42]. This indicates that BAY 58-2667 may be able to substitute for the heme group or stabilize the heme-free conformation. Interestingly, cinaciguat relaxes CC of both wild-type and $sGC\beta_1^{ki/ki}$ mice, although the relaxing effect was more pronounced in CC isolated from $sGC\beta_1^{ki/ki}$ mice. Our data also illustrate that cinaciguat strongly up-regulates the sGC activity in CC of wild-type mice, which could indicate that even in physiological circumstances a substantial amount of sGC is heme-free or oxidized. Surprisingly, no significant increase was observed in sGC activity of CC isolated from $sGC\beta_1^{ki/ki}$ mice after administration of BAY 58-2667. As yet no explanation for this controversy is available. Investigation of the protein level of the different sGC subunits could provide an explanation. The occurrence of the heme-free form of sGC under physiological conditions remains to be elucidated. Moreover, the corporal responses to BAY 58-2667 as well as its effect on the influence of sGC activity should be investigated in the presence of the sGC oxidant ODQ. Whatsoever, our functional data suggest that cinaciguat might be useful in ED associated with a strong increased ROS formation.

Future perspectives are to evaluate the corporal responses of the different sGC stimulators and activators in a variety of mouse models of ED. For instance, it has been illustrated that using

STZ one can induce a mouse model of diabetes-induced ED [43]. Moreover, cutting the cavernosal nerves will result in a mouse model of neurogenic-related ED [44]. In addition, different types of mouse models for ED-associated hypertension have been developed so far [45].

Investigation of corporal responsiveness in $sGC\alpha_2^{-/-}$ mice could reveal the exact role of the $sGC\alpha_2\beta_1$ isoform in corporal relaxation. Deletion of the $sGC\alpha_2$ subunit has been shown to have no major influence on the murine cardiovascular system, although no functional data are available on CC [29]. Furthermore, global deletion of sGC in mice does not allow identification of the cell/tissue type responsible for the strongly reduced corporal responsiveness. The development of $sGC\beta_1^{ki/ki}$ mice with specific deletion in smooth muscle cells/neuronal cells may provide an answer. The inherent problem of a constitutive KO mouse is the lack of the respective protein during the animal's ontogeny [46]. During the development of the embryo, compensatory mechanisms may veil or counteract the impact of the deletion. The use of an inducible system allows deleting sGC at stages of full development and would be of interest.

Besides NO, the vasoactive responses of CO and the contribution of sGC in CO-induced vasodilation and corporal relaxation were also examined. Originally, CO was regarded only as a toxic waste product [47]. Tissue hypoxia following hemoglobin saturation represents a principle cause of CO-induced mortality in higher organisms. Partial occupation of CO at the binding sites inhibits the release of $O_2$ from the remaining hemoglobin heme groups, shifting the $O_2$ dissociations curve to the left. This property of CO diminishes the capacity of the blood to deliver $O_2$ leading to tissue hypoxia [48]. Later on, it was found that CO was formed endogenously [49]. It is now widely accepted that cells and tissues produce significant amounts of endogenous CO as an elimination product of cellular metabolism, largely from heme degradation catalyzed by microsomal HO [50;51]. For every mole of CO formed by this mechanism, one mole of ferrous iron is released and one mole of the linear tetrapyrrole biliverdin-Ixα is produced [52]. The latter undergoes further metabolism to bilirubin-Ixα by NADPH biliverdin reductase [53]. The remaining fraction of CO arises from other sources that may include lipid peroxidation and xenobiotic metabolism [54;55]. That HO enzymes are of great clinical importance was illustrated by a fascinating case report of a 6 year old boy with severe HO-1 deficiency. He exhibited hematuria, proteinuria, a microcytic hemolytic

anemia, increased iron binding capacity, ferritin and iron depositions alongside raised von Willebrand factor marking endothelial cell damage [56].

Indications of the cardiovascular effects of CO emerged in the late 1970s as it was found to evoke dilation of the pulmonary vasculature under normoxic conditions.[57] It was not until the early 1990s, when the scientific community recognized that NO was EDRF that it became appreciated that these noxious gases possess important physiological vasoactive roles [58;59]. Since then, its vasorelaxant effects in physiologically relevant concentrations have been demonstrated in numerous cases [60]. CO affects vascular function by influencing the regulation of vessel tone, platelet aggregation and smooth muscle proliferation [61-65]. In vivo, CO decreases total peripheral resistance and reduces blood pressure [66]. Exogenously applied CO has been shown to induce a concentration-dependent relaxation of rat tail artery precontracted with PE. CO also relaxes ductus arteriosus, femoral arterial rings as well as porcine and rat thoracic aorta [67;68]. In addition, altered metabolism and functions of CO have been linked to the pathogenesis and maintenance of hypertension. Increased vascular contractility, unbalanced cellular apoptosis, proliferation of the vascular wall, increased oxidative stress and the altered interaction of CO and NO are among the consequences of HO/CO system dysfunction in hypertension [60].

In order to evaluate CO-induced responses in the transgenic $sGC\alpha_1^{-/-}$ and $sGC\beta_1^{ki/ki}$ mice, we performed preliminary studies using wild-type controls. No previous reports had been published on the vasoactive effect of exogenous CO on murine isolated vessels. Surprisingly, these experiments indicated that CO was unable to relax mice isolated blood vessels. To exclude the possibility of the occurrence of technical defaults in our settings, we further examined CO-induced vasorelaxation on rat thoracic aorta. In line with other publications, CO elicited a concentration-dependent relaxation in the rat isolated blood vessels [61;69]. These findings strongly indicate that CO-induced responses are subjected to species-related differences, an observation that has been illustrated by other research groups as well [70].

To our surprise, the gaseous molecule relaxes mice isolated CC in a concentration-dependent manner. The finding that addition of CO results in corporal relaxation although has no vasorelaxing effect in arteries of mice is surprising and could be of great importance. As relaxation of smooth muscle cells other than the corporal smooth muscle cells is the underlying mechanism of the side-effects observed with PDE-5 inhibitor treatment, the HO/CO pathway, which only affects corporal smooth muscle cells, makes an attractive new

therapeutic target for the treatment of ED. However, the HO/CO pathway is subjected to species- as well as tissue-related differences, so it remains to be elucidated whether or not human arteries are also less sensitive to CO in comparison to the human CC.

Regardless of the route of administration, access of exogenous CO to target tissue may be limited by binding to hemoglobin forming carboxyhemoglobin [66;71]. As the clinical use of CO is thus not without danger, CORMs have been developed to release CO in a more controlled way [72]. These compounds include metal carbonyl complexes which release CO in a concentration-dependent manner. The amount of CO released from metal carbonyl complexes has been assessed spectrophotometrically by measuring the conversion of deoxymyoglobin to carbonmonoxymyoglobin. It has been illustrated that like CO, CORMs evoke sustained relaxation of rat PE-precontracted aortic rings and attenuate coronary vasoconstriction in ex-vivo hearts [72;73]. Although CORMs are presumed to mimic CO, little effort has been made to establish the molecular mechanisms underlying CORM-induced vasorelaxation.

In our studies, we used CORM-2, a compound often used in pharmacological studies evaluating CO-specific responses. We could observe that CORM-2, in contrast to CO, relaxes all vascular tissues isolated from mice. This was the first indication that CORM-2 might induce vasorelaxation through (a) molecular mechanism(s) that differ(s) from those of CO-induced vasorelaxation.

The sGC/cGMP pathway has been implicated in mediating the effects of CO on neurotransmission, vascular relaxation, smooth muscle relaxation, bronchodilation, inhibition of platelet aggregation, coagulation as well as smooth muscle proliferation [63;65;74-79]. Thus besides NO, CO is another physiological molecule able to activate sGC. Although both gaseous molecules bind the heme moiety of sGC, CO binds to the sGC heme group with a lower affinity and binding only results in four till six fold activation of the enzyme [80]. The main reason for this difference is that unlike NO, CO binding to the heme of sGC leaves the iron-histidine bond intact, forming a six-coordinate complex rather than penta-coordinate complex, that only weakly increases cyclase activity [81;82]. Furthermore, an excess NO produces a high activity form of sGC by binding to a non-heme site involving cysteine residues, a mechanism unavailable to CO [83].

Despite its low potential to activate sGC, evidence is accumulating suggesting a direct role for CO in the regulation of cGMP levels. In vascular smooth muscle cells an elevation of cGMP

occurred following treatment with exogenous CO [63]. Treatment of these cells with hypoxia also increased the levels of cGMP, which required HO activity and HO-derived CO but excluded the involvement of NO [65]. This hypoxia-induced effect was blocked by the HO-inhibitor Sn-PPIX. Furthermore, chemical inhibitors of sGC inhibited the anti-proliferative effect of CO on hypoxic vascular smooth muscle cells [63]. It was illustrated that CO released from vascular smooth muscle cells stimulated the production of cGMP in co-cultured endothelial cells in a paracrine fashion and suppressed the expression of endothelial-derived mitogens such as platelet-derived growth factor and endothelin-1 [64]. In addition, exogenously applied CO relaxes the aortic rings in a cGMP-dependent way and overexpression of HO-1 inhibited PE-induced vasoconstriction in isolated aortic rings by inducing cGMP production [76].

Comparable to these data, our studies confirmed that CO induces vascular and corporal relaxation through the activation of sGC. Future perspectives are to unravel whether CO acts sGC isoform-specific in corporal smooth muscle relaxation using the $sGC\alpha_1^{-/-}$ and $sGC\beta_1^{ki/ki}$ transgenic mice.

When examining the molecular mechanism(s) underlying CORM-2-induced smooth muscle relaxation, it could be concluded that this compound acts sGC-independent, except for the aorta were CORM-2 induces relaxation partially via sGC activation. These data suggest that either CORM-2 strongly exerts additional effects or that the mechanism(s) for CORM-2-induced smooth muscle relaxation differs (almost) completely from CO-induced smooth muscle relaxation. As different water-soluble CORMs are currently available [73;84], it could be of interest to explore whether these CORMs act in a CO-dependent manner. Moreover, experiments using water-soluble CORMs offer the benefit that they can be applied *in vivo*.

The proposal of CO as a physiological sGC activator is opposed by the rather poor sGC stimulatory properties of CO. Thus it remains controversial whether CO is a physiological activator of sGC *in vivo*. One possible explanation could be the existence of an endogenous molecule that sensitizes sGC to CO [82;83]. The discovery of a naturally occurring YC-1-like molecule would clearly reinforce the role of CO as modulator of biological function via its ability to stimulate sGC. On the other hand, CO signaling may become relevant under oxidative stress or pathophysiological conditions when HO-1 is dramatically induced and/or when the bioavailability of NO is reduced [50]. Many have speculated that CO potentially

serves as a substitute for NO, during NO-deficient states. Under certain pathophysiological conditions, such as hypoxia, down-regulation of constitutive eNOS is occurs in parallel with transient increases in HO-1 protein, indicating a potential compensatory regulation between the two systems [63].

It is becoming increasingly clear that CO and NO do not always act independently but rather modulate each other's activity [85]. Although much is known about the HO/CO and NOS/NO pathways, how these two important systems interact is less well understood. The two diatomic gases share similar molecular mass, solubility in water and basal rates of production [86]. In addition, also the enzymes responsible for generating CO and NO have surprising similarities in their isoforms, requirements for activity and regulation. Both have constitutive and inducible isoforms and both enzymes require molecular oxygen plus the reducing agent NADPH for activity [85]. Despite the similarities, there are important differences as well. While NO is a free radical, CO is a stable gas and is therefore not prone to oxidative and reductive reactions [62]. Moreover, NOS requires additional cofactors and the constitutive isoforms of NOS are $Ca^{2+}$/calmodulin-dependent [59].

Marked similarities have been reported in the localization of HO and NOS in endothelial cells and adventitial nerves of blood vessels, suggesting a possible coordinated function for CO and NO in the control of vascular smooth muscle tone [67]. Interestingly, NO donors can activate HO-1 gene expression and activity in a variety of tissues [87-90]. Conversely, HO could decrease NO production by NOS [85]. NOS requires heme and increased HO activity could directly degrade the heme located at the active site of NOS or could reduce the amount of available heme [49]. Furthermore, iron released during the degradation of heme further inhibits production of NOS by inhibiting its nuclear transcription [85]. Moreover, both HO and NOS require NADPH as cofactor and the subsequent reduction of biliverdin to bilirubin by biliverdin reductase also utilizes NADPH, which could shift competitions for electrons in favor of the HO pathway [49]. In addition, NOS activity has further been shown to be susceptible to direct inhibition by CO [85]. These effects may however be largely dependent on the amount of CO present in the system as Thorup et al. reported that, whereas high levels of CO inhibit NOS activity and NO generation, lower concentrations induce release of NO from intracellular pools [91;92]. Furthermore, at relatively greater concentrations, the binding of CO to heme proteins has a longer lifespan than the binding of NO, notwithstanding the greater affinity of NO for heme proteins such as sGC. Thus the CO-heme protein complex

may competitively reduce the formation of NO-heme complexes at greater CO concentrations.

Our data do not indicate a role for the NOS/NO pathway in CO- or CORM-2-induced vascular and corporal relaxation. However, it could be of value to evaluate the interplay of the HO/CO and NOS/NO pathway in both mice isolated blood vessels and CC. The lack of effect of the NOS inhibitors used in our experiments on CO/CORM-2-induced relaxation, does not indicate whether NO-donors are also potential HO-1 inducers in mice isolated vascular beds. Neither does it indicate whether the HO/CO pathway has an influence on the NOS/NO signaling transduction pathway.

It has often been suggested that CO can also induce relaxation through direct activation or opening of the $K_{Ca}$ channels [93;94]. When evaluating $K^+$ channel involvement in CO- and CORM-2-induced responses, no evidence was found for the $K_{Ca}$ channels, only a small role for the $K_V$ channels could be suggested. The involvement of the $K_V$ channels is not straightforward as the CORM-2-induced responses were decreased in the arteries in the presence of the $K_V$ channel inhibitor 4-AP, while it was increased in the CC. A reason for these discrepancies is unknown and should be subjected to further research.

A limitation of the studies is the lack of data on the functional effect of the transition metal compound, ruthenium. It has been published that ruthenium is responsible for the inhibiting effect of CORM-2 on the exogenous NO-induced responses [95]. Moreover, ruthenium has been shown to exert an intrinsic effect on $K_V$ channels [96]. These data might suggest that the ruthenium compound by itself may exert additional functional effects.

Besides working with CO and CO-donors, it could be of value to examine whether induction of endogenous CO formation also relaxes CC as well as to evaluate the effect of CO induction on arteries. If endogenous-derived CO relaxes CC, further experiments should be done using the transgenic animals in order to explore whether it is possible to regain corporal relaxation in these animals by elevating CO concentrations. Normalization of CO metabolism could restore the homeostatic control of blood pressure by reversing hypertension related to abnormal vascular tone and remodeling [60]. This might suggest that normalization of CO metabolism could also offer benefit to restore erectile function. Similar experiments could be performed using mice suffering from hypertension, diabetes mellitus as well as mice with

cavernous neuronal damage. In addition, it has been illustrated that in hypertensive rats HO-1 gene expression is altered. As male $sGC\alpha_1^{-/-}$ mice are hypertensive, the question remains whether HO-1 expression is also changed within these animals.

In conclusion, our results demonstrate that NO induces vascular and corporal relaxations solely through the activation of sGC. Furthermore, the data illustrate that both the $sGC\alpha_1\beta_1$ and the $sGC\alpha_2\beta_1$ isoform are involved in the vasoactive responses to NO and NO-donors. Selectively targeting one of these isoforms may therefore offer a therapeutic approach to compensate the dysfunctional NO/cGMP pathway present in many cardiovascular diseases. Moreover, our findings show that besides NO, CO also induces vascular and corporal relaxations via sGC activation. However the vasoactive effects of CO are not universal and are subjected to species- as well as tissue-dependent differences. As CO induces corporal relaxations while having no vasorelaxing effect in arteries of mice, this might lead to the assumption that the HO/CO pathway makes an attractive new therapeutic target for the treatment of ED. However, in order to be able to extrapolate our findings to humans, research should be performed exploring whether human CC also have increased sensitivity towards CO in comparison to other vascular tissues. In addition, we have presented data illustrating that the CO-donor CORM-2 is not a simple mimic of CO and exerts vasorelaxing effects through molecular mechanisms other than that of CO.

## V.1 Reference List


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# Chapter VI:

# Summary

Penile erection depends on the delicate balance between smooth muscle contraction and relaxation. Nitric oxide (NO), through the activation of soluble guanylyl cyclase (sGC) and thus the intracellular accumulation of the second messenger cGMP, is generally accepted to be the most important activator of both arterial and corporal smooth muscle relaxation. The importance of the NO/cGMP pathway for the induction and maintenance of penile erection is illustrated by the efficacy of phosphodiesterase-5 (PDE-5) inhibitors for the treatment of erectile dysfunction (ED). However, some limitations and drawbacks in the use of PDE-5 inhibitors, necessitates research on other potential targets. As the intrinsic receptor for NO, sGC represents an attractive new target for the treatment of ED. Different sGC stimulators such as NO-donors, YC-1 and BAY 41-2272 have been illustrated to relax CC *in vitro* and to induce penile erection *in vivo*. Due to the ubiquitous expression of sGC, sGC stimulators develop many side-effects. The finding that sGC exists in two active isoforms opens the possibility of a more selective therapeutic approach. Therefore, one of the aims of this thesis was to gain more knowledge into the physiological role of the two different isoforms of sGC in the mechanism of penile erection.

To evaluate the function of the $sGC\alpha_1\beta_1$ and the $sGC\alpha_2\beta_1$ isoforms, transgenic mice were examined. A targeted deletion of exon 6 of the $sGC\alpha_1$ gene resulted in mice lacking a functional $sGC\alpha_1\beta_1$ isoform. Insertion of a mutation in the $sGC\beta_1$ gene resulted in mice expressing a sGC enzyme which still possesses basal catalytic activity but is no longer activated by its physiological activators. Both *in vitro* and *in vivo* studies were performed using these transgenic mice. Chapter III encloses a detailed description of the techniques applied for the different experiments.

In Chapter IV.1 and IV.2 a correlation is described between the functional alteration in the CC of the $sGC\alpha_1^{-/-}$ mice and ED. We demonstrated that the corporal responses *in vitro* and the penile haemodynamics *in vivo* towards NO from both exogenous and endogenous origin were strongly attenuated in $sGC\alpha_1^{-/-}$ mice compared to their wild-type controls. These data indicate that the $sGC\alpha_1\beta_1$ isoform is of major importance in the NO-induced erectile function. However, the observation that the corporal responsiveness in $sGC\alpha_1^{-/-}$ mice is strongly reduced although not completely abolished, suggests that besides $sGC\alpha_1\beta_1$ also the less abundantly expressed isoform $sGC\alpha_2\beta_1$ and/or sGC-independent mechanism(s) are involved in NO-induced penile erection.

To gain further insight into the functional role of the $sGC\alpha_2\beta_1$ isoform in erectile function, corporal responses and penile haemodynamics of $sGC\beta_1^{ki/ki}$ mice were examined (chapter IV.3). Using these transgenic mice, we were able to explore the potential sGC-independent mechanisms contributing to NO-induced penile erection. Moreover, by comparing the responses in $sGC\beta_1^{ki/ki}$ mice with those in $sGC\alpha_1^{-/-}$ mice, provided indirect evidence on the importance of the $sGC\alpha_2\beta_1$ isoform in erectile function. As NO-induced erectile responses were completely abolished in $sGC\beta_1^{ki/ki}$ mice, it was suggested that NO induces penile erection solely through the activation of sGC. In addition, these data illustrate that the cGMP producing capacity of the $sGC\alpha_2\beta_1$ isoform is sufficient to partially compensate for the loss of the $sGC\alpha_1\beta_1$ isoform. Interestingly, the relaxing effect of the NO-independent sGC activator BAY 58-2667 was significantly enhanced in CC isolated from $sGC\beta_1^{ki/ki}$ mice compared to their wild-type littermates. These functional data support the use of this sGC activator in ED associated with pathological conditions in which the heme group of sGC is oxidized due to increased production of ROS.

The vasodilatory properties of CO have been illustrated in different vascular beds of different species. However, in chapter IV.4 it is illustrated that CO-induced vasodilation is strongly subjected to species differences. While inducing a sustained and concentration-dependent relaxation of rat isolated thoracic aortas, CO did not influence the vascular tone of mice isolated thoracic aortas and femoral arteries. An explanation for this discrepancy is as yet not available. Interestingly, as indicated in chapter IV.5, CO is a potent inducer of mice corporal smooth muscle relaxation. The tissue-dependent differences of CO-induced responses observed in mice might be of great interest as this selectivity makes the HO/CO pathway an attractive therapeutic target for the treatment of ED. Further studies will need to elucidate whether or not human arteries are also less sensitive to CO in comparison to human CC. CO was found to induce corporal relaxation independent of the NOS/NO pathway and activation of potassium channels but through the activation of sGC. This finding suggests that research for the therapeutic use of CO-donors as sGC stimulators in the field of ED might be promising.

Recently, CO-releasing molecules (CORMs) have been developed in order to overcome the obstacles related to delivery of CO in the gaseous form to an organism. CORMs are presumed to mimic CO and have already been shown to induce vasorelaxation. We evaluated the response to one of these CORMs, namely CORM-2, on both vascular and corporal tissues. As described in chapter IV.4, CORM-2 relaxed mice isolated thoracic aorta and femoral arteries,

which is in contrast to the data obtained with CO. When further examining the molecular mechanism underlying CORM-2-induced vasorelaxation, it was found that CORM-2-induced responses were only partially dependent or completely independent upon sGC activation. In line with these results, CORM-2 relaxed mice isolated CC to a much greater extent than CO did (chapter IV.5). While CO-induced corporal relaxation was dependent on sGC activation, CORM-2-induced corporal relaxation in an sGC-independent manner. Taken together, these data suggest that CORM-2 induces vasodilatation and corporal relaxation through (a) molecular mechanism(s) that differ(s) from that of CO, illustrating that CORMs should be used with caution when examining CO-related effects. Further studies will be performed exploring whether the CO-independent effects elicited by CORM-2 may be attributed to the transition metal compound ruthenium. In addition, it would be of value to examine the vascular responses to water-soluble CORMs as well as HO-inducers, which upregulate endogenous CO formation.

In sum, our studies demonstrate that NO induces vasorelaxation solely through the activation of sGC, whereby both the $sGC\alpha_1\beta_1$ and the $sGC\alpha_2\beta_1$ isoforms are involved. Selectively targeting one of these isoforms may have therapeutic potential for the treatment of a series of cardiovascular disorders, including ED. In addition, our studies further indicated that also the HO/CO pathway is an interesting new therapeutic target for the treatment of ED, with the potential of having less side-effects in other vascular tissues.

# Chapter VI:

# Samenvatting

Erectiele functie is afhankelijk van een fijn gereguleerde balans tussen gladde spiercel contractie en relaxatie. Via stimulatie van oplosbaar guanylaat cyclase (sGC) en de uiteindelijke verhoging in intracellulaire cyclisch guanosine monofosfaat (cGMP), wordt NO algemeen aangenomen als de belangrijkste mediator van zowel arteriële als corporale gladde spiercel relaxatie. Het belang van de NO/cGMP signaaltransductie cascade voor zowel de inductie als het behoud van een erectie wordt geïllustreerd door de doeltreffendheid van de behandeling van erectiele dysfunctie (ED) of impotentie met fosfodiësterase-5 (PDE-5) inhibitoren. Toch is een behandeling met PDE-5 inhibitoren niet altijd aangewezen wat verder wetenschappelijk onderzoek naar nieuwe potentiële therapieën noodzaakt. Als intrinsieke receptor voor NO blijkt sGC een aantrekkelijke nieuwe doelwitmolecule voor de behandeling van ED. Verschillende sGC stimulatoren zoals NO-donoren, YC-1 en BAY 41-2772 veroorzaken relaxatie van de corpora cavernosa (CC) *in vitro* en induceren een erectie wanneer ze *in vivo* worden aangewend. Het gebruik van deze sGC stimulatoren is echter gelimiteerd door de ontwikkeling van vele ongewenste neveneffecten als gevolg van de wijdverspreide expressie van sGC in de verschillende orgaanstelsels. Het bestaan van twee verschillende isovormen van het sGC enzyme bestaan biedt echter wel de mogelijkheid tot de ontwikkeling van isovorm-specifieke therapieën. Hiervoor is verdere kennis over de fysiologische relevantie van de verschillende isovormen bij erectie noodzakelijk.

Om de rol van zowel $sGC\alpha_1\beta_1$ en $sGC\alpha_2\beta_1$ te evalueren, hebben we gebruik gemaakt van transgene muizen. Een deletie in het $sGC\alpha_1$ gen resulteerde in het ontstaan van knock-out muizen ($sGC\alpha_1^{-/-}$) met een gebrek aan een functionele $sGC\alpha_1\beta_1$ isovorm. Daarentegen resulteerde een mutatie in het gen coderend voor de $sGC\beta_1$ subeenheid in knock-in muizen ($sGC\beta_1^{ki/ki}$) die een sGC enzym tot expressie brengen met een normale basale activiteit, maar ongevoelig voor de fysiologische stimulatoren. Zowel *in vitro* als *in vivo* studies werden uitgevoerd, gebruik makend van deze transgene muizen. In hoofdstuk III worden de aangewende technieken voor de verschillende experimenten uitvoerig besproken.

Hoofdstuk IV.1 en IV.2 beschrijven een duidelijk verband tussen de functionele veranderingen in de CC van $sGC\alpha_1^{-/-}$ muizen en ED. *In vitro* en *in vivo* studies tonen aan dat NO van zowel endogene als exogene afkomst een sterk verminderde relaxatie van corporale gladde spiercellen teweegbrengt in $sGC\alpha_1^{-/-}$ muizen in vergelijking tot de wild-type muizen. Deze data suggereren dat de $sGC\alpha_1\beta_1$ isovorm een belangrijke rol speelt in de NO-geïnduceerde erectie. De bevinding dat het antwoord van CC afkomstig van $sGC\alpha_1^{-/-}$ muizen

slechts deels is gereduceerd, leidt tot de veronderstelling dat naast de $sGC\alpha_1\beta_1$ isovorm ook de $sGC\alpha_2\beta_1$ isovorm en/of sGC-onafhankelijk(e) mechanism(en) betrokken zijn.

Om verder inzicht te verkrijgen in de functionele rol van de $sGC\alpha_2\beta_1$ isovorm in het erectiele mechanisme, werd de relaxatie van de corporale gladde spiercellen alsook de hemodynamica van het erectiele weefsel bestudeerd in $sGC\beta_1^{ki/ki}$ muizen (hoofdstuk IV.3). Gebruik makend van deze transgene muizen, zijn we in staat potentiële sGC-onafhankelijke mechanismen bij NO-geïnduceerde erecties te achterhalen. Bovendien hebben we, via de vergelijking van resultaten bekomen in $sGC\beta_1^{ki/ki}$ muizen met deze bekomen in $sGC\alpha_1^{-/-}$ muizen, indirect bewijs omtrent het belang van de $sGC\alpha_2\beta_1$ isovorm. NO-geïnduceerde erectiele veranderingen zijn totaal afwezig in $sGC\beta_1^{ki/ki}$ muizen, hetgeen suggereert dat NO enkel en alleen via activatie van sGC een erectie veroorzaakt. Bovendien illustreren deze data dat cGMP geproduceerd door de $sGC\alpha_2\beta_1$ isovorm voldoende is om de afwezigheid van de functionele $sGC\alpha_1\beta_1$ isovorm gedeeltelijk te compenseren. Interessant is dat het relaxerende effect van de NO-onafhankelijke sGC activator BAY 58-2667 significant versterkt is bij CC afkomstig van $sGC\beta_1^{ki/ki}$ muizen, vergeleken met wild-type muizen. Deze functionele data steunen het gebruik van deze sGC activator bij ED geassocieerd met pathologieën waarbij de heem component van het sGC geoxideerd is door de productie van vrije radicalen.

Vasodilaterende eigenschappen van koolstofmonoxide (CO) zijn reeds beschreven ter hoogte van verschillende types bloedvaten in verschillende species. Toch wordt in hoofdstuk IV.4 geïllustreerd dat CO-geïnduceerde vasodilatatie sterk onderworpen is aan species-afhankelijke verschillen. Terwijl CO een blijvende, concentratie-afhankelijke relaxatie veroorzaakt in aorta's afkomstig van ratten, oefende CO geen invloed uit op de vasculaire tonus van muis geïsoleerde aorta's en femorale arteriën geïsoleerd uit de muis. Een verklaring voor deze tegenstrijdigheid is er voorlopig niet. Van belang is dat in hoofdstuk IV.5 wordt aangetoond dat CO een duidelijke relaxatie uitlokt in CC afkomstig van muizen. Deze weefsel-afhankelijke verschillen bij CO-geïnduceerde vasorelaxatie in muizen kunnen een belangrijke meerwaarde hebben. Ze zouden mogelijk suggereren dat de heem oxygenase (HO)/CO signaaltransductie cascade een aantrekkelijk therapeutisch alternatief biedt voor de behandeling van ED. Toch zullen verdere studies eerst moeten uitwijzen of menselijke arteriën ook minder gevoelig zijn voor CO in vergelijking met humane CC, vooraleer deze resultaten kunnen geëxtrapoleerd worden naar de mens. Bovendien werd aangetoond dat CO CC relaxeert onafhankelijk van de NOS/NO cascade en verschillende kaliumkanalen, maar wel via de activatie van sGC. Deze bevinding geeft aan dat wetenschappelijke onderzoek naar

het therapeutisch potentieel van CO-donoren als sGC stimulatoren in de behandeling van ED interessant zou kunnen zijn.

CO vrijstellende moleculen (CORMs) werden recent ontwikkeld om de moeilijkheden die gepaard gaan met het toedienen van CO in gasvorm te overbruggen. CORMs worden verondersteld om de effecten van CO na te bootsen en het is reeds beschreven dat ook deze moleculen vasorelaxatie teweegbrengen. Wij onderzochten de invloed van één van deze CORMs, namelijk CORM-2, op zowel bloedvaten als CC. In hoofdstuk IV.4 wordt geïllustreerd dat CORM-2, in tegenstelling tot CO, aorta's en femorale arteriën afkomstig van muizen relaxeert. Wanneer het moleculaire mechanisme waarmee CORM-2 deze vasorelaxatie veroorzaakt nader wordt bekeken, wordt het duidelijk dat het effect van CORM-2 slechts deels of volledig onafhankelijk is van sGC. CORM-2 relaxeerde ook CC geïsoleerd uit muizen en dit antwoord was eveneens onafhankelijk van sGC (hoofdstuk IV.5). Deze data suggereren dat CORM-2 vasodilatatie en corporale relaxatie induceert via (een) moleculair(e) mechanisme(n) die totaal verschillen van die van CO. De studie illustreert dat CORMs met alle voorzichtigheid dienen aangewend te worden wanneer men CO-gerelateerde effecten wil bestuderen. In de toekomst zullen experimenten uitgevoerd worden om na te gaan of the CO-onafhankelijke effecten van CORM-2 te wijten zijn aan het transitiemetaal ruthenium. Bovendien zou het interessant zijn om de vasculaire effecten na te gaan van water oplosbare CORMs alsook van agentia die de activiteit van HO-1 doen toenemen om zo de endogene CO vorming te verhogen.

Samenvattend tonen deze studies aan dat NO vasorelaxatie veroorzaakt enkel en alleen via de activatie van sGC. Zowel de $sGC\alpha_1\beta_1$ als de $sGC\alpha_2\beta_1$ isovorm is bij deze respons betrokken. Het selectief inwerken op een van deze isovormen zou dus op therapeutisch vlak potentieel kunnen bieden bij de behandeling van een aantal cardiovasculaire aandoeningen, alsook ED. Bovendien geven de studies aan dat ook de HO/CO signaalcascade een interessant therapeutisch doelwit vormt voor de behandeling van ED.

# Curriculum Vitae

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## IV. Scientific publications

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## V. Abstracts and presentations at national/international meetings

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