

# **Functional importance of the soluble guanylyl cyclase isoforms (sGC $\alpha_1\beta_1$ and sGC $\alpha_2\beta_1$ ) in vascular smooth muscle relaxation**

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If we knew what it was we were doing, it would not be called research, would it

-Albert Einstein-

Science never solves a problem without creating ten more

-George Bernard Shaw-



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## Abbreviations

<b>AC</b>	adenylyl cyclase
<b>ACh</b>	acetylcholine
<b>AGAP-1</b>	the prototype of an <u>A</u> rfGAP protein with a <u>G</u> TPase-like domain, <u>A</u> nkyrin repeats, and a <u>p</u> leckstrin homology domain
<b>5'-AMP</b>	5'-adenosine monophosphate
<b>ArfGAP</b>	ADP ribosylating factor GTPase-activating protein
<b>ATP</b>	adenosine triphosphate
<b>ATR</b>	atropine
<b>BK</b>	bradykinin
<b>BAY 41-2272</b>	5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4- <i>b</i> ] pyridine-3-yl]pyrimidin-4-ylamine
<b>BAY 41-8543</b>	2-[1-[(2-fluorophenyl)methyl]-1H-pyrazolo[3,4- <i>b</i> ]pyridine-3-yl]-5-(4-morpholinyl)4,6-pyrimidinediamine
<b>BAY 58-2667</b>	4-[[[(4-carboxybutyl) {2-[(4-phenethyl-benzyl)oxy] phenethyl} amino)methyl] benzoic acid
<b>Bay k 8644</b>	1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid
<b>BPA</b>	bovine pulmonary arteries
<b>8-Br-cGMP</b>	8-Bromo-guanosine 3', 5'-cyclic monophosphate
<b>BSA</b>	bovine serum albumin
<b>[Ca<sup>2+</sup>]<sub>i</sub></b>	intracellular Ca <sup>2+</sup> concentration
<b>cAMP</b>	adenosine 3', 5'-cyclic monophosphate
<b>CC</b>	corpora cavernosa
<b>CCT</b>	chaperonin containing t-complex polypeptide
<b>CDK</b>	cyclin-dependent kinase
<b>CFM-1571</b>	1-benzyl-3-[3-(dimethylamino) propoxy]-N-(4-methoxyphenyl)-1 H-pyrazole-5-carboxamide
<b>cGMP</b>	guanosine 3', 5'-cyclic monophosphate
<b>CO</b>	carbon monoxide
<b>CO<sub>2</sub></b>	carbon dioxide
<b>COX</b>	cyclooxygenase
<b>CNG</b>	cyclic nucleotide-gated ion channel
<b>CPA</b>	cyclopiazonic acid
<b>CPI-17</b>	PKC-potentiated myosin phosphatase inhibitor, 17 kDa
<b>C-terminal</b>	carboxy-terminal
<b>CYP</b>	cytochrome P450
<b>DOBE</b>	calcium dobesilate

<b>DMSO</b>	dimethylsulfoxide
<b>ED</b>	erectile dysfunction
<b>EDHF</b>	endothelium-derived hyperpolarising factor
<b>EDRF</b>	endothelium-derived relaxing factor
<b>EET</b>	epoxyeicosatrienoic acid
<b>EFS</b>	electrical field stimulation
<b>eNOS</b>	endothelial NOS
<b>ES</b>	embryonic stem
<b>G418</b>	geneticin
<b>GC</b>	guanylyl cyclase
<b>GFP</b>	green fluorescent protein
<b>5'-GMP</b>	5'-guanosine monophosphate
<b>GSH</b>	glutathion
<b>GST</b>	glutathione S-transferase
<b>GTP</b>	guanosine triphosphate
<b>GUA</b>	guanethidine
<b>HA-1077</b>	fasudil
<b>HEK</b>	human embryonic kidney
<b>His</b>	histidine
<b>H<sub>2</sub>O<sub>2</sub></b>	hydrogen peroxide
<b>HPRA</b>	human penile resistance artery
<b>Hsp</b>	heat shock protein
<b>HuR</b>	human R, embryonic lethal abnormal visual [ELAV]-like RNA binding protein
<b>ICP</b>	intracavernosal pressure
<b>IK<sub>Ca</sub></b>	intermediate-conductance Ca <sup>2+</sup> -activated K <sup>+</sup> channels
<b>iNOS</b>	inducible NOS
<b>IP<sub>3</sub></b>	inositol 1,4,5-triphosphate
<b>IRAG</b>	IP <sub>3</sub> receptor-associated PKG I substrate
<b>JNK</b>	c-Jun NH <sub>2</sub> -terminal kinase
<b>K<sub>ATP</sub></b>	ATP sensitive K <sup>+</sup> channel
<b>K<sub>Ca</sub> channel</b>	Ca <sup>2+</sup> sensitive K <sup>+</sup> channel
<b>KRB</b>	Krebs-Ringer bicarbonate
<b>Lev</b>	levcromakalim
<b>LPS</b>	lipopolysaccharide
<b>L-NA</b>	N <sup>G</sup> -nitro-L-arginine
<b>L-NAME</b>	N <sup>G</sup> -nitro-L-arginine methyl ester
<b>L-NMMA</b>	N <sup>G</sup> -monomethyl-L-arginine
<b>LY-83583</b>	6-anilino,5,8-quinolinedione

<b>MAPK</b>	mitogen-activated protein kinase
<b>MB</b>	methylene blue
<b>MLC</b>	myosin light chain
<b>MLC20</b>	20 kDa light chain of myosin
<b>MLCK</b>	myosin light chain kinase
<b>MLCP</b>	myosin light chain phosphatase
<b>MYPT</b>	myosin phosphatase target subunit
<b>NADPH</b>	nicotinamide adenine dinucleotide phosphate
<b>NANC</b>	non-adrenergic non-cholinergic
<b>neo</b>	neomycine
<b>NGF</b>	nerve growth factor
<b>7-NI</b>	7-nitroindazole
<b>NMDA</b>	N-methyl-D-aspartate
<b>nNOS</b>	neuronal NOS
<b>NO</b>	nitric oxide
<b>NOS</b>	NO synthase
<b>N-terminal</b>	amino-terminal
<b>O<sub>2</sub></b>	molecular oxygen
<b>ODQ</b>	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
<b>ONOO<sup>-</sup></b>	peroxynitrite
<b>PC12</b>	pheochromocytoma cells
<b>8-pCPT-cGMP</b>	8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate
<b>PDE</b>	phosphodiesterase
<b>PDZ</b>	post-synaptic density protein-95/Discs large/zona occludens-1
<b>PG</b>	prostaglandin
<b>PGF<sub>2α</sub></b>	prostaglandin F <sub>2α</sub>
<b>PGI<sub>2</sub></b>	prostacyclin
<b>pGC</b>	particulate guanylyl cyclase
<b>Phe</b>	phenylalanine
<b>PI3K<math>\gamma</math></b>	phosphoinositide 3-kinase gamma
<b>PKA</b>	cAMP-dependent protein kinase
<b>PKC</b>	protein kinase C
<b>PKG</b>	cGMP-dependent protein kinase
<b>PLB</b>	phospholamban
<b>PLC</b>	phospholipase C
<b>PP1c</b>	protein phosphatase 1 catalytic subunit
<b>PP<sub>i</sub></b>	pyrophosphate
<b>PSD-95</b>	post synaptic density protein-95

<b>Rho-GDI</b>	Rho-guanine nucleotide dissociation inhibitor
<b>ROS</b>	reactive oxygen species
<b>rPASM</b>	rat pulmonary artery smooth muscle cells
<b>RyR</b>	ryanodine-sensitive Ca <sup>2+</sup> release
<b>SERCA</b>	SR Ca <sup>2+</sup> -pumping ATPase
<b>sGC</b>	soluble guanylyl cyclase
<b>SHR</b>	spontaneously hypertensive rats
<b>SIN-1</b>	3-morpholinopyridone
<b>SK<sub>ca</sub></b>	small-conductance Ca <sup>2+</sup> -activated K <sup>+</sup> channel
<b>SNAP</b>	S-nitroso-N-acetylpenicillamine
<b>SNP</b>	sodium nitroprusside
<b>SOCC</b>	store-operated Ca <sup>2+</sup> channel
<b>SR</b>	sarcoplasmic reticulum
<b>STOC</b>	spontaneous transient outward current
<b>T-1032</b>	(methyl 2-(4-aminophenyl)-1,2-dihydro-1-oxo-7-(2-pyridylmethoxy)-4-(3,4,5-trimethoxy-phenyl)-3-isoquinoline carboxylate sulfate)
<b>TCA</b>	trichloroacetic acid
<b>TEA</b>	tetraethylammonium chloride
<b>TNF-<math>\alpha</math></b>	tumor necrosis factor-alpha
<b>TXA<sub>2</sub></b>	thromboxane A <sub>2</sub>
<b>UV</b>	ultraviolet
<b>VASP</b>	vasodilatory-stimulated phosphoprotein
<b>VIP</b>	vasoactive intestinal polypeptide
<b>WKY</b>	Wistar-Kyoto rats
<b>Y-27632</b>	N-(4-pyridyl)-4-(1-aminoethyl)cyclohexanecarboxamide
<b>YC-1</b>	3-(5'-hydroxymethyl-3'-furyl)-1-benzylindazole

# Chapter I

General introduction





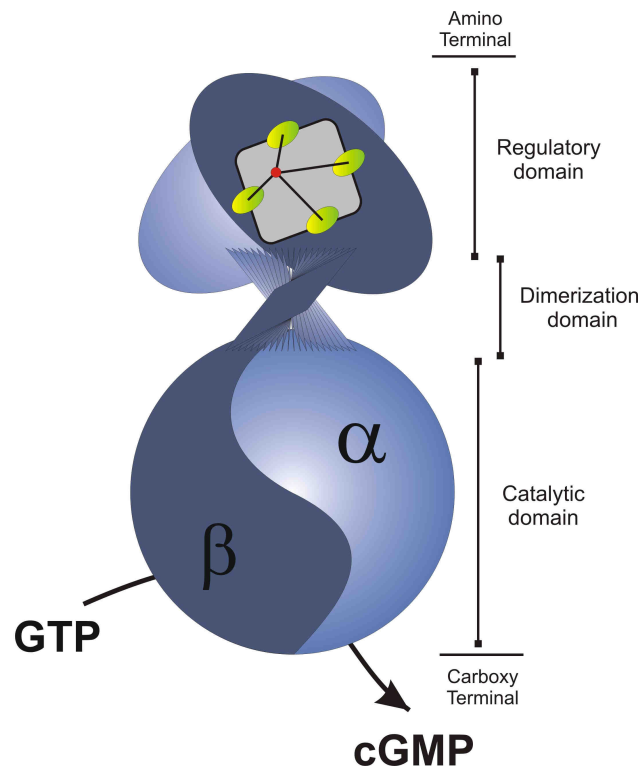
## **I.1. Soluble guanylyl cyclase**

### **I.1.1. Historical perspective**

A few years after the detection of guanosine 3', 5'-cyclic monophosphate (cGMP) in the urine of rats <sup>1</sup>, an enzyme capable of synthesizing cGMP, guanylyl cyclase (GC) was found in several mammalian tissues <sup>2-6</sup>. It took until the mid-1970's to find out that there are two different types of GC <sup>7</sup>, which were subsequently found to differ not only in their cellular localization (cytosolic and membrane-bound) but also in their structure and regulation. In further research, nitric oxide (NO) released from vasodilators such as nitroglycerin and nitroprusside, was shown to activate the cytosolic GC enzyme or soluble guanylyl cyclase (sGC) <sup>8</sup>. As at that point, NO was unknown in biological systems, the NO mediated activation of sGC was considered as a non-physiological phenomenon. However, when in the early 1980's Furchgott and Zawadzki discovered an endothelium-derived relaxing factor (EDRF) <sup>9</sup>, which was later identified as NO <sup>10, 11</sup>, it was realized that sGC mediated many of the beneficial effects of endogenous NO in the cardiovascular system (vasodilatation, inhibition of platelet aggregation) <sup>12</sup>. Later on, the importance of sGC in mammalian physiology further increased, as it became apparent that NO and so also its intracellular receptor sGC were not only involved in vasorelaxation but also in peripheral and central neurotransmission <sup>13</sup>, in phototransduction <sup>14</sup> and in immunomodulation <sup>15</sup>.

### **I.1.2. sGC isoforms**

In 1979 it was first described that the purified sGC enzymes from rat and bovine lung consisted of two subunits of around 72 kDA <sup>16</sup>. Several years later, Kamisaki et al., showed that those two subunits, later designated  $\alpha$  and  $\beta$ , were similar but not identical <sup>17</sup>. Based on the protein sequence of the purified  $\alpha\beta$  heterodimer, the first subunits,  $\alpha_1$  and  $\beta_1$  were cloned <sup>18-21</sup>. By homology screening, two other subunits were identified, known as  $\alpha_2$  and  $\beta_2$  <sup>22, 23</sup>. Subsequently,  $\alpha\beta$  pairs were cloned from Homo sapiens, which were originally termed  $\alpha_3$  and  $\beta_3$  <sup>24</sup>. However, the so-called  $\alpha_3$  and  $\beta_3$  subunits have, based on sequence homology, come to be generally accepted as the human orthologs of the rat  $\alpha_1$  and  $\beta_1$  subunits rather than different isoforms <sup>25</sup>. So, two isoforms of each subunit currently exist and are termed  $\alpha_1$ ,  $\alpha_2$ ,  $\beta_1$ ,  $\beta_2$ , respectively. Despite multiple possible combinations, only the  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  heterodimers have been demonstrated to occur at the protein level and to be catalytically active <sup>26</sup>.



**Figure I.1:** Schematic representation of the sGC heterodimer (adapted from Evgenov et al. <sup>27</sup> and Lucas et al. <sup>28</sup>)

### I.1.3. Structure of the subunits

Each sGC subunit contains three domains (figure I.1) that make up its structure and function: an N-terminal regulatory domain involved in heme-binding, a central domain commonly referred to as dimerization domain and a C-terminal catalytic domain.

Compared to the central and catalytic domains, the N-terminal parts of the sGC subunits are relatively diverse. Only a stretch of about 100 amino acids shows a high degree of conservation among the isoforms of the  $\alpha$  or  $\beta$  subunits, but not between the  $\alpha$  and  $\beta$  subunits. Conceivably, the properties of an  $\alpha$  or a  $\beta$  subunit are defined by these regions <sup>29</sup>. Furthermore, the N-terminal parts bind and coordinate the heme moiety, which mediates the NO sensitivity of the enzyme. The binding of NO to the prosthetic heme group, induces a conformational change in the enzyme, resulting in a several-hundred-fold greater production of cGMP <sup>30</sup>. Although there has been some debate in the past, the heme stoichiometry of sGC has now been agreed to be one mole per mole heterodimer <sup>31</sup>. In contrast to other hemoproteins that use the heme group for the storage and transport of oxygen ( $O_2$ ), the heme group of sGC has remarkably overcome the inherent affinity for  $O_2$  <sup>32</sup>. The discrimination

against O<sub>2</sub> binding, which is based on the lack of a residue capable of forming a strong H-bound, is extremely important for the fidelity of NO-signaling<sup>33</sup>.

By sequential truncation experiments, Foerster et al., demonstrated the need of both ( $\alpha$  and  $\beta$ ) subunits for heme to bind properly to the heterodimeric complex<sup>34</sup>. On the other hand, Koglin and Behrends observed that deletion of the first 259 N-terminal amino acids of the  $\alpha_1$ -subunit, had no effect on heme-binding or NO-sensitivity of the enzyme<sup>35</sup>. Furthermore, the importance of the  $\beta_1$  subunit as primary heme-binding subunit was suggested, as the expression of the N-terminal part of  $\beta_1$  resulted in a domain capable of binding heme<sup>36</sup>. Moreover, a histidine residue (His-105) of the  $\beta_1$  subunit, was identified as the heme-coordinating residue<sup>37, 38</sup>. In addition, two highly conserved cysteines (78 and 214) adjacent to the His-105 on the  $\beta_1$  subunit appear to be important for the heme-binding affinity. Interestingly, mutation of the corresponding cysteines on the  $\alpha_1$ -subunit, did not alter the NO-responsiveness, what provides additional proof for the predominant role of the  $\beta_1$  subunit in heme-binding<sup>39</sup>. So, the function of the N-terminal part of the  $\alpha$ -subunit is not clear, compared to that of the  $\beta_1$  subunit. As the central part of the sGC enzyme, known as the dimerization domain, shows considerable homology to the membrane-bound or particulate GC (pGC), it was suggested in analogy, that this region would be involved in the dimerization of the subunits<sup>40</sup>. Additional evidence was derived from multiple N- and C-terminal deletion experiments, where in both subunits of the  $\alpha_1\beta_1$  isoform, the central region but also the regulatory domain was reported to contribute to heterodimer formation<sup>41</sup>. Besides hetero- and homodimerization, Koglin and Behrends concluded that the central domain of the  $\alpha$ -subunit would be involved in sensitivity to NO and the exogenous activator YC-1<sup>35</sup>.

The C-terminal domains of sGC (responsible for guanosine triphosphate (GTP) binding and conversion to cGMP) are the most conserved regions, not only among the four subunits but also between both pGC and the adenosine 3', 5' -cyclic monophosphate (cAMP)-generating adenylyl cyclases (AC). This evolutionarily conserved catalytic domain defines sGC, pGC and AC as a single family of nucleotide cyclases<sup>42, 43</sup>. Homology modelling of sGC, based on the crystal structure of AC, revealed the existence of a low affinity nucleotide-like binding site, in addition to the putative catalytic site<sup>44</sup>. Despite its low affinity for nucleotide, this pseudosymmetric counterpart of the catalytic site appears to be of significance for the regulation of sGC activity. It has been shown to be responsible for the allosteric pattern of non-competitive inhibition by purine nucleotides such as GTP and adenosine 3', 5'-cyclic triphosphate (ATP)<sup>45</sup>. This notion was further supported by the finding that the

pseudosymmetric site acts as an ATP sensor site of sGC<sup>46</sup>. Besides inhibition of the NO-stimulated activity, the pseudosymmetric site was also found to be a target for sGC-stimulators like YC-1 and BAY 41-2272, that lead to potentiation of the NO-stimulated activity<sup>47</sup>.

Many aspects of the sGC-catalysed conversion of GTP to cGMP are poorly understood. According to Senter et al., the catalysis proceeds by way of a single displacement reaction, with the pyrophosphate-leaving group in the nucleotide being displaced by the 3'-hydroxyl group on the ribose<sup>48</sup>. On activation, under conditions of surplus substrate (GTP) and activator (NO), the enzyme exhibits straightforward Michaelis-Menten-type kinetics: cGMP accumulates at a constant rate with time<sup>49</sup>. Nuclear magnetic resonance spectroscopy demonstrated that only pyrophosphate and cGMP in amounts corresponding to the amount of GTP utilized were produced<sup>50</sup>. As for all nucleotide cyclases, divalent cations ( $Mn^{2+}$  and  $Mg^{2+}$ ) are needed as substrate cofactors and allosteric modulators to express maximum catalytic activity<sup>51</sup>. As the residues required for substrate recognition and catalysis are distributed on the  $\alpha$  and  $\beta$  subunit of the heterodimeric sGC enzyme, cyclase activity is dependent upon the presence of both subunits<sup>52</sup>. Co-expression of the C-terminal regions of the  $\alpha$  and  $\beta$  subunits, which form a heterodimer and that possess dimerization and catalytic regions, is sufficient for basal cGMP production<sup>53</sup>.

Clearly, the above observations demonstrate that the regulatory, catalytic as well as dimerization properties of sGC can be attributed to different domains of each subunit and that both subunits are required to render the sGC enzyme function.

#### I.1.4. Genomic organisation of sGC

In medaka fish<sup>54</sup>, rat<sup>55</sup>, human<sup>56</sup> as well as mouse<sup>57</sup>, the genes encoding the sGC $\alpha_1$  and  $\beta_1$  subunit are found on the same chromosome and are located very close to each other (mouse: 3E3-F1, rat: 2q31, human: 4q32). Moreover, in the medaka fish genome, the sGC $\alpha_1$  and  $\beta_1$  genes are tandemly organized like a single gene, which suggests that the transcriptional regulation of the two subunits is cis-coordinated<sup>54</sup>. However, this has not been duplicated in any mammalian system. The sGC $\alpha_2$ <sup>58</sup> and  $\beta_2$ <sup>59</sup> subunits on the other hand appear to be localized on different chromosomes and separate chromosomes than the  $\alpha_1$  and  $\beta_1$  subunit<sup>60</sup>. The chromosomal localisation of the sGC subunits, gave the opportunity to address and identify whether the  $\alpha$  and  $\beta$  subunits of sGC are possible candidate genes for linkage in NO/cGMP-mediated disease states. Azam et al., investigated such a linkage for the sGC $\alpha$  and

$\beta$  genes with respect to blood pressure in the Dahl salt-sensitive rat <sup>55</sup>. They showed that the genes for the sGC $\alpha_1$  and  $\beta_1$  subunits are closely linked to the GC-A locus, which has been shown to cosegregate with blood pressure, and the Na<sup>+</sup>, K<sup>+</sup>-ATPase  $\alpha_1$  isoform and calmodulin-dependent protein kinase II- $\delta$  genes, which have been shown to flank a quantitative trait locus for blood pressure in the Dahl rat <sup>61-63</sup>. In addition, the  $\beta_2$  locus was also closely linked to the endothelin-2 locus, known to cosegregate strongly with systolic blood pressure. Moreover, the mRNA and protein level for the sGC $\beta_2$  subunit are increased in kidney of Dahl salt-sensitive vs. salt-resistant rats <sup>64, 65</sup>. Together, these results suggest that the sGC subunit loci of  $\alpha_1$ ,  $\beta_1$  and  $\beta_2$  are good candidates for genes controlling salt-sensitive hypertension in the Dahl rat <sup>55</sup>.

### I.1.5. Expression of sGC

The expression of the sGC subunits does not appear to be limited to any specific tissue. The  $\alpha_1\beta_1$  heterodimer, is the most abundant and ubiquitously distributed isoform in mammalian tissues with  $\alpha_1$  and  $\beta_1$  mRNA being expressed at high levels in the lung, brain, heart, kidney, spleen and muscle <sup>21</sup>. The  $\alpha_2$  subunit has a more restricted expression pattern, with the highest level in the brain, where the  $\alpha_1$  subunit was present in comparable amounts. The placenta, pancreas, spleen and uterus contain a lower level of the  $\alpha_2$  subunit. In most tissues sGC $\alpha_1\beta_1$  is the prevailing isoform <sup>22</sup>. The  $\beta_2$  subunit was detected in kidney and liver <sup>66</sup>. However, methods and sensitivity of measuring the transcripts of sGC vary in each report. In the study of Mergia et al., the  $\beta_2$  subunit mRNA levels were almost undetectable in most tissues and relatively most abundantly in the testis and the placenta <sup>67</sup>. No sGC heterodimer containing the  $\beta_2$  subunit has been isolated from tissues or cells and several investigators failed to express a catalytically active  $\alpha_1\beta_2$  or  $\alpha_2\beta_2$  complex. Therefore, the physiological relevance of the  $\beta_2$  subunit is questionable. However, using a chimeric form in which the rat  $\beta_2$  is fused downstream of the green fluorescent protein (GFP), Gupta et al., demonstrate that an  $\alpha_1\beta_2$  heterodimer does exhibit enzyme activity, albeit considerably lower when compared to the  $\alpha_1\beta_1$  enzyme. More importantly, these authors reported that the  $\beta_2$  subunit could act as a negative regulator of NO-stimulated activation of the  $\alpha_1\beta_1$  sGC enzyme by complexing with the  $\alpha_1$  subunit and reducing the NO-sensitivity of sGC <sup>64</sup>. One explanation for this novel finding of catalytic activity is that GFP in the GFP- $\beta_2$  fusion substitutes for the missing N-terminal heme-binding region of  $\beta_2$ . This may also account for the reported ability of the  $\beta_2$ -

GFP fusion construct to form a catalytically active homodimer <sup>68</sup>. On the other hand, a frameshift deletion has been shown in the human  $\beta_2$  gene population that is incompatible with the occurrence of the  $\beta_2$  subunit on protein level. The fact that the human  $\beta_2$  subunit is exclusively expressed in gastric tumors, raises the possibility that this subunit would have a role in tumorigenesis or cell growth <sup>69</sup>. Anyhow, as the actions of NO are so widespread, sGC, no matter which isoform, is likely to be present in all tissues.

For each  $\alpha$  and  $\beta$  subunit, spliced variants have been reported. In several human tissues and cell lines, Behrends et al. discovered a variant of the  $\alpha_2$  subunit ( $\alpha_{2i}$ ) containing 31 additional amino acids in the catalytic domain. The  $\alpha_{2i}$  subunit retained its ability to heterodimerize, but the resulting  $\alpha_{2i}\beta_1$  isoforms exhibited no sGC activity <sup>70</sup>. For the  $\alpha_1$  subunit, three mRNA species were identified, of which only one contained the full expression sequence, necessary for the formation of a functional  $\alpha_1$  subunit. The other two, N-terminal truncated variants have a significantly reduced enzymatic activity, suggesting that they do not contribute to the cGMP formation <sup>71</sup>. Using PCR, Chhajlani et al. detected a splice variant of the  $\beta_1$  subunit that lacks 33 amino acids when compared to the original  $\beta_1$  subunit <sup>72</sup>. Finally, an alternatively spliced variant of the  $\beta_2$  subunit was reported in human corpus cavernosum. It is suggested that this subunit may be NO insensitive because of the absence of 2 exons that are homologous to the N-terminal heme-binding domain <sup>73</sup>.

#### I.1.6. Transcriptional and post-transcriptional regulation of sGC

Several factors are known to regulate sGC expression. The chronic exposure of cultured rat pulmonary artery smooth muscle cells (rPASMC) to NO donor compounds has been shown to decrease sGC subunit mRNA in a cGMP-dependent way <sup>74</sup>. Also other investigators suggested that cGMP regulates sGC subunit gene expression. Ujiie et al., observed that incubation of cultured rat medullary interstitial cells with cGMP-elevating agents, decreased sGC $\alpha_1$  and  $\beta_1$  subunit mRNA levels <sup>75</sup>. Similarly, in aortic smooth muscle cells the sGC subunit gene expression was shown to decrease upon incubation with the phosphodiesterase (PDE) type 5 inhibitor Zaprinast <sup>76</sup>. Increased cGMP levels may alter sGC subunit gene expression via downregulation of the RNA-binding protein HuR (human R, embryonic lethal abnormal visual [ELAV]-like RNA-binding protein), thereby destabilizing sGC mRNA <sup>77</sup>. Similarly, also cAMP has been reported to decrease the expression and RNA binding of HuR <sup>78</sup>. So, alternatively, cGMP may also inhibit the class III cAMP phosphodiesterase activity <sup>79</sup>, leading to increased cAMP levels that in turn may decrease sGC subunit mRNA levels. This

cAMP-mediated reduction of the sGC mRNA and protein levels has not only been described in rat aortic smooth muscle cells<sup>80</sup> but also in other cells like rat fetal lung fibroblasts<sup>81</sup> and pheochromocytoma (PC12) cells<sup>82</sup>. cGMP may also exert its effect through activation of the cGMP-dependent protein kinase, which is known to phosphorylate splicing factor 1 and thereby inhibiting the prespliceosome assembly<sup>83</sup>.

In certain disease states, such as hypertension, atherosclerosis, hypercholesterolemia and Alzheimer's disease<sup>84, 85</sup>, it is demonstrated that disturbances in sGC levels and/or activity may play an important role in the pathophysiology of these disorders. Ruetten et al. found a significant reduction in mRNA and protein expression of both the sGC $\alpha_1$  and  $\beta_1$  subunits in the aortic rings of young and aging spontaneously hypertensive rats (SHR) when compared with normotensive Wistar-Kyoto rats (WKY), which correlated with an attenuated response to YC-1 activation of sGC<sup>86</sup>. Further evidence for a role of sGC in hypertension comes from a study of Kloss et al, that showed a downregulation of the sGC $\beta_1$  subunit expression in senescent SHR compared with age-matched WKY<sup>87</sup>. In addition to hypertension, both research groups demonstrated that also aging lowers the mRNA and protein levels of sGC in aortic smooth muscle cells and so worsen the NO-dependent vasodilator mechanism of the rat aorta. Furthermore it is suggested that the sGC $\alpha$  and  $\beta$  subunits can be independently regulated, as the expression of the  $\beta$  subunit and not of the corresponding  $\alpha$  subunit has been shown to be switched off in aortic smooth muscle cells of old rats compared with newborn and adult rats<sup>88</sup>. An age-induced reduction of the sGC activity has also been reported in nonvascular smooth muscle cells<sup>89</sup>, suggesting that the downregulation of smooth muscle sGC may be a common response to aging. Furthermore, reduced  $\beta_1$  but increased  $\beta_2$  mRNA and protein levels have been reported in kidney of Dahl salt-sensitive rats<sup>64</sup>. Therefore, the reduced renal sGC activity found in the Dahl salt-sensitive rats was suggested to be due to a higher formation of the physiologically non-active  $\alpha_1\beta_2$  heterodimer.

Hypercholesterolemia is reported to induce an overexpression of the sGC enzyme, albeit in a dysfunctional form<sup>90</sup>, which may contribute to the pathogenesis of atherosclerosis<sup>91</sup>. Similarly, chronic myocardial infarction has been shown to increase the sGC expression despite the altered vasodilator responses<sup>92</sup>. As many cardiovascular diseases are known to be associated with an increased vascular oxidative stress, it is not surprising that inhibition of sGC activity under pathological conditions such as hypercholesterolemia and atherosclerosis is redox-controlled<sup>93</sup>. Further evidence is provided by the involvement of reactive oxygen

species (ROS) and cyclooxygenase (COX)-2 in the downexpression of the sGC $\beta_1$  subunit induced by lead in the rat vascular wall <sup>94</sup>.

The effect of hypoxia on sGC expression has been a source of controversy. Crawley and colleagues reported that sGC function was impaired in pulmonary arteries from rats exposed to 10 % oxygen, leading to decreased cGMP accumulation and vasorelaxation <sup>95</sup>. On the other hand, Li and associates reported that exposure of rats to hypoxia significantly upregulates pulmonary sGC expression <sup>96</sup>. The in vitro report of Hassoun et al., is however in line with the in vivo study of Crawley et al., as in rPASMC sGC subunit mRNA levels were significantly reduced in response to hypoxic concentrations of oxygen <sup>97</sup>. Furthermore, also allergic asthma has been documented as a down-regulating condition for sGC <sup>98</sup>.

sGC subunit levels have also been shown to be developmentally regulated. During pregnancy, stimulation of the sGC $\beta_1$  mRNA and protein levels is evident in uterine arterial smooth muscle tissue <sup>99</sup>. Concomitantly, sGC $\alpha_1$  and  $\beta_1$  subunit mRNA levels rise in unborn rat pulmonary artery, beginning at approximately 20 days of gestation, and mRNA and protein remain elevated for at least 8 days following birth <sup>100</sup>. This concurs with the gentle decrease of  $\alpha_1$  mRNA in mouse kidney after birth <sup>101</sup>. Also in the rat brain, sGC activity was found to be high immediately after birth and to decrease during the early postnatal weeks <sup>102</sup>. In the prenatal rat brain, it has also been shown that only  $\alpha_1$  mRNA is expressed, while  $\beta_1$  is absent. In the early postnatal stage, however, the expression pattern was reported to be essentially the same as at adult age, with a more widespread distribution of the  $\beta_1$  subunit compared to its heterodimeric partner sGC $\alpha_1$  <sup>103</sup>. Also in rat heart, sGC expression underlies a dynamic change, as it was shown to shift from endothelial and smooth muscle cells in the neonatal stage to only endothelial cells in adult animals. In addition to this anatomical change, also a higher cardiac sGC activity was found in the perinatal period <sup>104</sup>. The reported changes in expression levels, activity and cell types expressing sGC at different times of development and in different organs, clearly demonstrate a dynamic regulation of the sGC enzyme.

Various additional factors like the nerve growth factor (NGF), estradiol, cytokines and lipopolysaccharide (LPS) also appear to reduce the sGC enzyme expression. The NGF-dependent decrease of the sGC subunit mRNA levels was shown to occur via activation of the Ras-MAPK signaling cascade <sup>82</sup>. Furthermore, it is suggested that the estrogen receptor-dependent pathway for the regulation of the sGC $\alpha_1$  and  $\beta_1$  subunits by estradiol <sup>105, 106</sup>, proceeds via NGF, as an elevated level of the NGF is reported in the rat uterus during pregnancy <sup>107</sup>. Takata and coworkers have demonstrated that inflammatory cytokines, such as



interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) negatively modulate sGC subunit gene expression via NO-dependent and NO-independent mechanisms<sup>108</sup>. Also exposure to the bacterial inflammatory mediator LPS, causes a reduction in sGC mRNA abundance, enzyme mass and enzyme activity<sup>109, 110</sup>. The possibility of NGF<sup>111</sup>, estradiol<sup>112</sup> and cytokines<sup>113</sup> to stimulate the JNK signaling pathway and the blocking effect of an c-Jun NH<sub>2</sub>-terminal kinase (JNK)-II inhibitor on the inhibition of sGC $\alpha_1$  mRNA expression by NFG, TNF- $\alpha$  and interleukin-1 $\beta$ <sup>114</sup>, implicate a role for the c-Jun kinase in the regulation of the sGC expression.

### I.1.7. Post-translational regulation of sGC

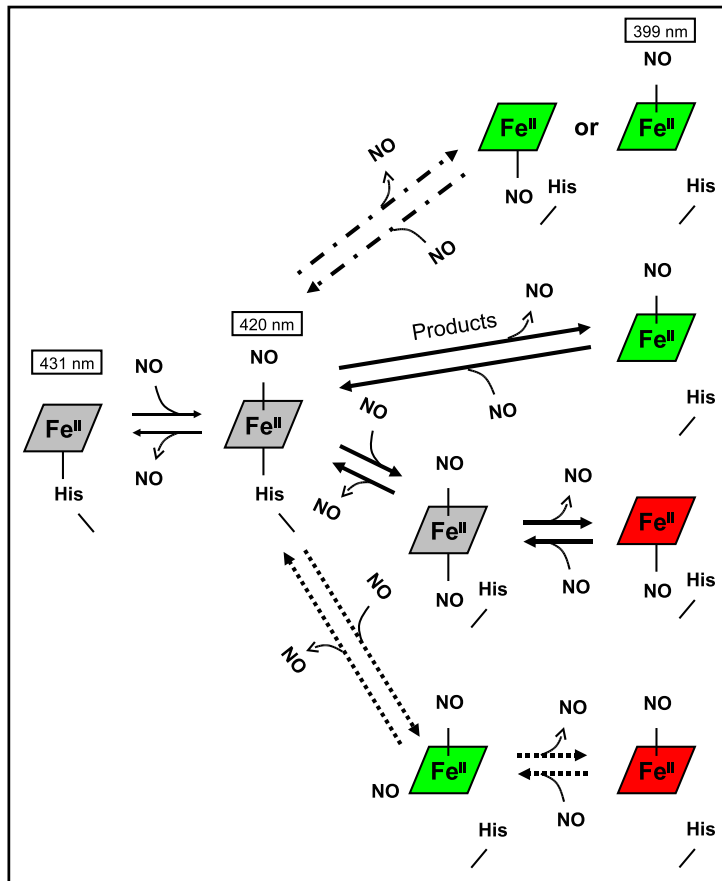
#### *I.1.7.1. sGC activation by ligands*

##### *\*Nitric oxide (NO)*

It is generally accepted that NO binds to the prosthetic heme group associated with the  $\beta$  subunit of sGC, which finally leads to activation of the enzyme and enhanced cGMP production. In the unligated state, the heme group displays an absorbance maximum at 431 nm, the Soret band, indicative of five-coordinated ferrous heme with a histidine as axial ligand. The presence of the heme moiety in the reduced Fe<sup>2+</sup> state is a major prerequisite for the NO-induced activation of sGC, as its removal or oxidation abolishes any NO-induced enzyme activation.

Upon binding of NO, the absorbance maximum shifts to 399 nm<sup>32</sup>. Together with the finding that heme-free sGC could be activated by the iron-free heme precursor, protoporphyrin IX, it was hypothesized that active sGC required a five-coordinate nitrosyl heme complex<sup>115</sup>. However, much speculation has centered on the precise steps involved in the formation of this five-coordinate nitrosyl heme complex (figure I.2). First, sGC was thought to behave as a traditional signal transduction receptor and to follow a simple two-step activation process. In the first step, NO binds to the sixth coordination position of the heme, forming a 6-coordinate ferrous-nitrosyl species with an absorbance maximum at 399 nm. Subsequently, the bond joining the heme to His105 breaks, resulting in the five-coordinate species. This second step is considered to initiate conformational changes in sGC that greatly increase the catalytic activity of the enzyme<sup>116</sup>. It is agreed that the initial rate of association is a fast second-order process, whereas for the second step, different kinetics have been reported. Zhao et al., found that the rate of transition from the six-coordinate to the five-coordinate sGC also depends on the NO-concentration<sup>117</sup>. This suggested a novel mechanism for sGC regulation, whereby NO

not only determines the amount of occupied enzyme but also how quickly the enzyme is activated. It was concluded that various additional reactions of NO with sGC, including the presence of a second NO-binding site, accounted for this finding. The requirement for two NO molecules for full sGC activation, was supported by the work of Russwurm and Koesling<sup>118</sup>. They presented the all-heme site model that proposes two five-coordinated NO-bound states of the enzyme: an active and nonactive state. In the presence of the reaction products or substrate and additional NO, the high catalytic activity or active species is formed, whereas in the absence of the enzyme's products, the low catalytic activity or nonactive species is formed. Based on the crystal structure of cytochrome c', it was suggested that the binding of the second NO molecule occurred to the heme<sup>119</sup>. However, more recently, a second non-heme site model has been proposed, in which the second NO-binding event involves the binding of NO at a non-heme site, or at least at a site that does not influence the heme spectrum<sup>120</sup>. This model, derived from the observation that the rate at which NO dissociates from sGC is much slower than the rate of enzyme deactivation, has significant in vivo implications. Under normal resting conditions, during which basal levels of NO are synthesized, sGC is most likely to have NO stably bound at the heme and to be partially activated (long-lasting tonic activity). Other physiological processes require a rapid rise in cGMP levels, driven by an acute increase in NO. In this case the enzyme becomes fully activated by NO binding to the non-heme site (phasic activity) and deactivation occurs rapidly when levels of NO drop. Both two-site models (all-heme site and non-heme site) account for much of the behaviour of sGC in vitro, especially the presence of a low-activity form that is spectrally indistinguishable from the fully active sGC. According to Roy and Garthwaite, the non-heme site model for the regulation of sGC activity by NO is of doubtful relevance to cells<sup>121</sup>. Their in vivo measurements of cGMP production by platelet and cerebellar cells are entirely consistent with the involvement of only a single ligand binding site and with the concept that activation and deactivation follow binding and unbinding of NO to this site. Earlier on, criticism on the existence of a second NO-binding site was raised by Bellamy et al., who swear by the simple two-state, single-site model<sup>122</sup>. To conclude that there is some fundamental difference between the behaviour of purified sGC compared to the behaviour of sGC in the intracellular milieu, further research has to be done.



**Figure I.2:** Three possible mechanisms for the activation of sGC by NO. In all schemes, NO binds rapidly to the five-coordinate ferrous heme (431 nm), forming a six-coordinate ferrous-nitrosyl intermediate (420 nm). The scheme depicted by the interrupted line (• -•) represents the simple two-step binding model. The full lines (—) show the possible steps of the all-heme site model, whereas the dotted lines (•••) represent the steps of the non-heme site model. The latter two models account for the experimental observation that there is a low-activity form of sGC (depicted in red) that is spectrally indistinguishable from the fully active sGC (depicted in green)

(adapted from Poulos<sup>123</sup>, and Ballou et al.<sup>124</sup>).

#### \*Carbon monoxide (CO)

CO, similarly to NO, binds to the distal side of the prosthetic heme, leading to a 4-6 fold increase in cGMP production<sup>32</sup>. The binding of CO results in formation of a hexacoordinate, rather than pentacoordinate complex without axial ligand displacement. It has been suggested that the dissociation of CO from sGC, proceeds through a pentacoordinate intermediate, which, by its structural similarity to the heme-nitrosyl complex, presumably is responsible for the activation of sGC<sup>125</sup>. By steric hindrance, the tyrosine phenyl ring in the sGC distal heme pocket, creates a heme environment in which CO binding is strongly impeded<sup>126</sup>. Given its rather poor sGC-activating property *in vitro* (30-100 times lower than that of NO), it remains unclear how CO can exert physiological signaling effects. However, multiple studies have demonstrated modulation of cGMP levels by endogenous produced CO. Moreover, there is substantial evidence that CO can regulate sGC, in particular in olfactory neurons in which heme oxygenase produces CO<sup>127</sup>. In the presence of YC-1, a synthesized benzyl indazole derivative, CO has been shown to activate sGC to the same level as that of NO<sup>128</sup>. So, the

modulation of sGC activity by an endogenous substance mimicking YC-1, could provide the molecular basis for CO functioning as a signaling molecule.

#### \*YC-1

YC-1 or 3-(5'-hydroxymethyl-3'-furyl)-1-benzylindazole, was first shown to inhibit platelet aggregation, to disaggregate platelets and to prolong bleedings in mice <sup>129</sup>. Additional findings, all consistent with its ability to activate sGC, showed that YC-1 inhibits proliferation of vascular smooth muscle cells <sup>130</sup> and relaxes smooth muscle cells <sup>131-133</sup>. YC-1 activates sGC directly, without the intermediate production of NO or some other factor capable of activating sGC <sup>30, 128, 133</sup>. However, similar to NO, the activating effect of YC-1 crucially depends on the presence of the reduced prosthetic heme moiety of sGC.

In vitro studies with the purified sGC enzyme demonstrated that incubation with YC-1 leads to a 10-fold increase of purified sGC enzyme activity, an effect that was slightly further potentiated in the presence of NO <sup>128, 132</sup>. In intact cells on the other hand, this synergistic effect of NO and YC-1 on activation of sGC was obvious as YC-1 combined with NO was reported to be up to 100 fold more effective than the individual compounds <sup>132, 134-136</sup>. Moreover, YC-1 has been reported to potentiate the weak sGC activator CO to a level of effectiveness comparable to NO <sup>30, 128, 137</sup>. Those overadditive effects are suggested to be due to the facilitated formation of an active five-coordinate NO- or CO-heme complex <sup>138</sup>. In other studies, the sensitizing action of YC-1 is explained by a reduction of the ligand dissociation rate from the heme group yielding a prolonged half-life of the ligand-heme complex <sup>133, 139</sup>. Besides the data on the mechanism of the YC-1-induced sGC activation, also the data on the location of the YC-1 binding site on sGC are controversial. YC-1 was shown not to alter the ultraviolet-visible absorptionspectrum of basal or stimulated sGC <sup>128</sup> and, in addition, to still bind to a heme-depleted enzyme <sup>133</sup>, suggesting that YC-1 combines with a site different from the heme group. Furthermore, the inability of YC-1 to sensitize a sGC mutant with a changed substrate specificity utilizing ATP instead of GTP, proposed the involvement of a special structure within the catalytic domain <sup>139</sup>. Guided by structure modelling of sGC with AC as template <sup>44, 140</sup> and by the results of mutational studies <sup>45, 47, 141</sup>, it was suggested that YC-1 binds to a pseudosymmetrical site in the catalytic domain, which is equivalent to the allosteric regulatory forskolin-binding pocket in AC. Although these findings do not support a direct interaction of YC-1 with the heme group, some reports indicate that YC-1 binding to CO-sGC results in a shift of the Soret absorption band by 4 nm to a shorter wavelength <sup>142, 143</sup>.

Additionally, raman resonance studies of the YC-1 interaction with sGC, detected spectroscopic alterations of the CO-heme bound in full-length sGC and in a heme-containing fragment of the  $\beta$ -subunit in the absence of the catalytic domain<sup>144</sup>. Moreover, YC-1 would lack the ability to increase the basal activity of a heme-deficient sGC mutant<sup>128</sup>. Furthermore, a region in the regulatory domain of the  $\alpha_1$  subunit has been reported to be important in the signal transduction of NO and to represent the target for YC-1<sup>35</sup>. These apparently conflicting results postulate two or more binding mechanisms, but definitive conclusions await protein crystallography<sup>27</sup>. Besides the stimulatory effect on sGC, YC-1 has been shown to increase the intracellular cGMP level in different cell types by inhibition of cGMP breakdown through PDE's<sup>129, 145</sup>. In addition to the cGMP-degrading PDE-1 and 5, the activity of cAMP specific PDE- isoenzymes (2-4) was also significantly suppressed by administration of YC-1<sup>134</sup>. Furthermore, it has been suggested that YC-1 has an indirect effect to stimulate sGC. Wohlfart et al. argued that YC-1 stimulates the NO-production by endothelial cells through activation of endothelial NO synthase (eNOS) and that the released NO may stimulate sGC and increase the intracellular cGMP level<sup>146</sup>. However, according to the study of Chun et al., the stimulatory effect of YC-1 on the synthesis of endothelial NO is not due to the activation of eNOS<sup>147</sup>. On the other hand, the results of Schmidt et al., suggest that a heat-labile factor present in the endothelial cells potentiates the effect of YC-1 on NO-activated sGC<sup>135</sup>. Even many other reports have established that YC-1 exhibits some additional effects that do not involve cGMP. For example, in rat neutrophils, YC-1 has been shown to block the  $\text{Ca}^{2+}$  entry through inhibition of the tyrosine kinase activity<sup>148</sup> and to inhibit the generation of superoxide anions<sup>149</sup>. Additionally, it has an inhibitory effect upon the human white blood cell function through a cAMP-dependent pathway<sup>150</sup>. Besides the antiproliferation effect through activation of sGC, YC-1 also demonstrates an inhibitory effect on cell proliferation via a cGMP-independent pathway. In rat mesangial cells, the YC-1-induced cell-cycle arrest, proceeded through suppression of cyclin D1 synthesis and related cyclin-dependent kinase (CDK)4 kinase activity<sup>151</sup>, whereas in human umbilical vein endothelial cells, the increased level of the CDK-inhibitory protein p21 and associated lower CDK2 activity, was the underlying mechanism<sup>152</sup>. A study in endothelial and chromaffin cells from bovine adrenal medulla showed that prolonged exposure to YC-1 at concentrations that activate sGC and cause vasorelaxation, results in apoptotic death in association with increased caspase-3-like activity<sup>153</sup>. An additional mode of action of YC-1 is the enhancement of the gene expression and production of the TNF- $\alpha$  in LPS/interferon- $\gamma$ -activated rat alveolar macrophages<sup>154</sup>. According to Yeo et al., YC-1 has the potential to become an anticancer agent, as it appears to

inhibit hypoxia-inducible factor-1- $\alpha$  and consequently halts tumor growth by blocking tumor angiogenesis and tumor adaptation to hypoxia<sup>147, 155</sup>.

The effects of YC-1 seems to vary dependent on the cell type and perhaps also on the experimental conditions. In isolated rat aortic smooth muscle cells, YC-1 increases the intracellular cGMP concentration, whereas in the ventricular myocardium it does not<sup>131</sup>. On the other hand, in rat cardiomyocytes in vivo, YC-1 was shown to induce apoptosis via cGMP signaling<sup>156</sup>. YC-1 increases cGMP levels in platelets and smooth muscle cells by direct activation of sGC without any effect on the production of NO<sup>157</sup>. Nevertheless, in bovine aortic endothelial cells and human umbilical vein endothelial cells, YC-1 stimulates the production of NO<sup>146</sup>, as discussed above.

#### *\*BAY 41-2272*

From a new series of potent pyrazolopyridine derivatives synthesized using the YC-1 chemical lead structure, BAY 41-2272 was selected as a promising heme-dependent, but NO-independent, sGC-stimulator. In vitro studies showed that BAY 41-2272 activates purified sGC up to 30-fold and that it potentiates sGC activation by NO at ~ 100-fold lower concentrations than its analog YC-1<sup>158</sup>. However, the potency of BAY 41-2272 in vivo, with respect to platelet aggregation, is considerably reduced and certainly much lower than its hypotensive actions<sup>159</sup>. The structural similarity between BAY 41-2272 and YC-1 suggests that both compounds have similar mechanisms of action. However, the effect of BAY 41-2272 on PDE-5 activity is at present controversial. Stasch et al.,<sup>158</sup> and Bischoff and Stasch<sup>160</sup> observed that BAY 41-2272 was devoid of any inhibitory effect on PDE-5 up to a concentration of 10  $\mu\text{mol/L}$ . However, at higher doses, Mullershausen et al. demonstrated PDE-5 inhibitory effects of BAY 41-2272 in platelets. They observed that this inhibitory action critically depends on the substrate concentration, indicating a competitive component in the mechanism of inhibition<sup>161</sup>. Still, as the concentration of BAY 41-2272 used by Mullershausen et al., is several orders of magnitude above those needed for sGC stimulation, the PDE-5 inhibition by BAY 41-2272 is rather considered as irrelevant<sup>27</sup>. In addition, other non-cGMP specific effects have been reported for BAY 41-2272, although much less in comparison to YC-1. In ovine pulmonary artery, BAY 41-2272 seems to stimulate the sarcolemmal sodium pump independent from cGMP<sup>162</sup>. Furthermore, it was demonstrated that besides stimulating sGC, inhibition of the  $\text{Ca}^{2+}$  entry also represents an important mechanism in BAY 41-2272-induced relaxation of rat basilar<sup>163</sup> and mesenteric artery<sup>164</sup>. In

rabbit aorta<sup>165</sup> as well as in human and rabbit corpus cavernosum<sup>166</sup>, the relaxant responses to BAY 41-2272 were only partially attenuated by ODQ, suggesting an additional cGMP-independent mechanism.

As a <sup>3</sup>H-labelled derivative of BAY 41-2272 was bound to the cysteines 238 and 243 of the  $\alpha_1$  subunit of sGC after radiation, it was suggested that these residues or the surrounding region might represent the target site for non-NO sGC-stimulators<sup>158, 167</sup>. However, the observation that this region is poorly conserved in the  $\alpha_2$  subunit of sGC and that the  $\alpha_2\beta_1$  heterodimer is also activated by YC-1 and BAY 41-2272<sup>168</sup>, argue against the binding of those sGC-stimulators to this N-terminal region of the  $\alpha$  subunit. As with YC-1, the postulated allosteric site involved in NO sensitization of sGC will have to be validated by co-crystallization studies.

*\*BAY 41-8543, CFM-1571 and A-350619*

Like BAY 41-2272, BAY 41-8543 and CFM-1571 were synthesized based on YC-1 as lead structure, whereas the chemical structure of A-350619 shares no structural similarity with YC-1. As members of the class of heme-dependent sGC-stimulators, their activity depends on the presence of the reduced heme moiety of sGC and is characterised by strong synergistic enzyme activation when combined with NO. They also increase the maximal catalytic rate of the sGC enzyme, except for CFM-1571<sup>27</sup>. BAY 41-8543 is regarded as the most promising compound with respect to potency, specificity as well as availability after oral administration. Like its close chemical analogue BAY 41-2272, it is about two orders of magnitude more potent than YC-1 in vitro<sup>169</sup>. However, in several in-vivo models a 3-fold higher dose of BAY 41-2272 would be needed to show an effect comparable to BAY 41-8543<sup>170</sup>.

*\*BAY 58-2667*

Through a high-throughput screening using a read-out system consisting of a chinese hamster ovary cell line expressing sGC<sup>171</sup>, a cyclic GMP-sensitive cation channel and aequorin<sup>172</sup>, a class of aminodicarboxylic acids were identified as a new type of sGC activators. Following chemical optimization, BAY 58-2667 was identified as the most potent member. With an EC<sub>50</sub> value in the subnanomolar range, it is about 160 fold more potent than BAY 41-2272. In contrast to heme-dependent sGC stimulators like BAY 41-2272, that show a strong synergism when combined with NO and a loss of effect after the removal or oxidation of the heme moiety, BAY 58-2667 produces an additive, not synergistic effect when combined with NO

and its activity is maintained, even enhanced, in oxidized or heme-free enzyme <sup>173</sup>. This differential activity of BAY 58-2667 on sGC activity together with binding studies and photoaffinity labelling studies, suggested two high affinity binding sites for BAY 58-2667: one that is saturable at nanomolar concentrations and shows no direct interference with the heme moiety and a second one, which changes its affinity for BAY 58-2667 from low to high upon oxidation or removal of the heme moiety of sGC <sup>174</sup>. Subsequently, it was shown that this heme-dependent high affinity site for BAY 58-2667 exhibits a direct competitive inhibition between the negatively charged carboxylic groups in BAY 58-2667 and the propionic acids of the heme for the tyrosine 135 and arginine 139 residues of the  $\beta$  subunit. As those two aminoacids, together with serine 137, form a unique heme-binding motif (**Y-x-S-x-R**) <sup>175</sup>, this competition results in the displacement of the heme moiety and as a consequence activation of sGC due to release of the axial heme ligand. Those actions are facilitated by oxidation of the heme moiety as this has been shown to strongly reduce the binding affinity for the heme-binding motif <sup>176</sup>.

To overcome a pathological state of NO-deficiency, different NO donor compounds have been clinically used for over 100 years, e.g. in angina pectoris and coronary heart disease. However, there are certain disadvantages with NO donor-based therapies, such as the development of tolerance, insufficient biometabolism, unpredictable pharmacokinetics of NO and non-specific interactions of NO with other biological molecules, including the formation of peroxynitrite (ONOO<sup>-</sup>) resulting in tyrosine nitration. Therefore compounds that directly activate sGC in an NO-independent manner might offer considerable efficacy and safety advantages over current therapies in a variety of applications, including cardiovascular and sexual dysfunctions <sup>27, 177</sup>. Indeed, the vasorelaxant and/or anti-platelet actions of non-NO sGC-stimulators like YC-1, BAY 41-2272 and BAY 41-8543 and non-NO sGC-activators like BAY 58-2667 *in vitro* are mirrored *in vivo* <sup>170, 174, 178, 179</sup>. Moreover, the selectivity of BAY 58-2667 to activate oxidation-impaired/heme free sGC has serious implications for its use as diagnostic tool or as highly innovative therapy for vascular disease. After all, the NO-insensitive ferric/heme-free form of sGC is present physiologically and its level increases under pathological conditions associated with oxidative stress <sup>180-182</sup>. Not only do these compounds offer novel therapeutic approaches, they also highlight previously unknown regulatory sites on the enzyme which may be important physiologically, representing target sites for endogenous molecules modulating sGC activity.



### *I.1.7.2. sGC inhibition*

#### *\*Methylene blue*

Methylene blue (MB) has been widely used to probe the involvement of the NO/cGMP signal-transducing pathway in various biological processes. It is known to be an inhibitor of sGC<sup>183-185</sup>, although its action is not specific for this enzyme. This is apparent from the numerous reports in the literature which showed that MB was more potent in inhibiting endothelium-dependent than nitrovasodilator-induced relaxation<sup>186-188</sup>. Subsequent experiments revealed that MB directly inhibits eNOS at concentrations some 100-fold lower than those that inhibit sGC<sup>189</sup>. In addition, it was demonstrated that MB lowers NO concentrations by the production of superoxide anions from its reduced form<sup>187, 190</sup>. Both non-sGC specific effects were also reported for LY-83583, another compound recognized as sGC-inhibitor<sup>191, 192</sup>. Additional studies that have questioned the mechanism of action of MB, have revealed that this compound is also active in inhibiting prostacyclin synthesis<sup>193</sup> and in altering the noradrenaline uptake, release and metabolism<sup>194</sup>. Furthermore, MB appears to modulate the cholinergic system<sup>195, 196</sup> by inhibition of the cholinesterase activity and by muscarinic receptor antagonism<sup>197, 198</sup>. Clearly, those drawbacks may lead to misinterpretations. Therefore, the identification and study of cGMP-dependent and -independent effects, benefit substantially from a more potent and selective sGC-inhibitor, such as the oxadiazoloquinoxaline derivative, ODQ.

#### *\*ODQ*

Garthwaite et al., demonstrated that ODQ inhibited the basal and NO-stimulated sGC-activity without interfering with the steps leading to NO synthesis<sup>199</sup>. Its mechanism of action is believed to be specifically related to the oxidation of Fe<sup>2+</sup> into Fe<sup>3+</sup><sup>200</sup>, which decreases the sensitivity of the heme group for NO and the subsequent cGMP elevation<sup>201</sup>. This is translated into a significant rightward shift of the NO concentration-response curve. In addition, ODQ also attenuates the maximal sGC activity, pointing to a mixed competitive/noncompetitive type of inhibition, instead of a simple competitive antagonism. However, in the study of Feelisch et al., no evidence for a mixed type of inhibition was obtained<sup>202</sup>. In contrast to the irreversible inhibition of purified sGC<sup>200</sup>, ODQ was shown to reversibly inhibit basal and NO-enhanced sGC activity in neuronal tissues *in vivo*<sup>203</sup>. Removal of the oxidized heme and insertion of the ferrous heme into the apoenzyme was suggested as endogenous mechanism to reactivate sGC. This hypothesis is supported by the

finding that the heme may move in and out of its sGC-pocket in the presence of lipophilic compounds<sup>34</sup>, and that NO promotes heme transfer from hemoproteins to apo-sGC<sup>204</sup>.

The previous reports on the ability of ODQ to inhibit the basal sGC activity are contradicted by the study of Zhao et al., showing that ODQ-treated sGC had the same basal activity as untreated sGC. They state that sGC inhibition is based on a selective interaction and oxidation of the heme group, without adversely affecting the catalytic domain. In addition, re-reduction of the heme by dithionite completely restored the NO sensitivity<sup>205</sup>. On the contrary, Kosarikov et al. did not observe a reversal of sGC inhibition when in the presence of ODQ, the heme is re-reduced by dithionite. This suggests that in addition to its role as oxidant of the sGC heme, ODQ may have another mechanism, which involves the binding to a region that is not associated with NO, metal or nucleotide binding. Moreover, since the changes in the far-UV circular dichroism spectrum of NO-activated sGC induced by ODQ were strikingly similar with those induced by YC-1, it was concluded that these compounds bind to sGC at the same site or overlapping sites<sup>206</sup>.

Although ODQ has been shown to be a potent and highly selective inhibitor of sGC, it is not infallible. Besides its action on sGC, it was found to inhibit basal and stimulated NO-production. It should however be mentioned that this effect is related to relative high concentrations of ODQ. Still, preincubation with ODQ, markedly potentiated its NOS inhibitory effect, suggesting that ODQ may be metabolically converted to a more potent NOS inhibitor. Moreover, ODQ would also affect vasorelaxation induced by organic nitrates and NO-donors like sodium nitroprusside by inhibiting their bioactivation via the heme-containing cytochrome P-450 (CYP) system<sup>202</sup>. In addition, also the heme enzymes myoglobin and hemoglobin have been shown to be targets of ODQ. The ODQ scavenger function of myoglobin was suggested to account for the failure of ODQ to inhibit the NO-induced increase in cGMP content of the myoglobin-rich ventricular cardiomyocytes<sup>207</sup>. Indirectly, this also reflects the higher effectiveness of ODQ in aortic tissue, which contains no detectable amounts of myoglobin<sup>208</sup>. It was shown that the inhibitory effect of ODQ is abolished by its reaction with oxyhemoglobin and the resulting formation of methemoglobin. Given the high level of oxyhemoglobin in blood, this reaction is likely to significantly alter the potency of ODQ in vivo and has therefore serious implications for the therapeutic use of ODQ<sup>205, 209</sup>. Furthermore, the inhibitory effect of ODQ on N-acetylcysteine-induced relaxation in LPS-treated aortic rings<sup>210</sup> and on nonadrenergic-noncholinergic (NANC) relaxation in rectum longitudinal muscle strips<sup>211</sup>, has also been related to mechanisms other than inhibition of sGC.

### *1.1.7.3. sGC phosphorylation*

Phosphorylation delineates one of the most important molecular mechanisms by which several signals produce their biological responses. Both  $\alpha_1$  and  $\beta_1$  subunits of sGC contain putative phosphorylation sites for multiple kinases and may represent potential target sites contributing to the regulation of sGC activity. Early studies on sGC have demonstrated that sGC could be phosphorylated ‘in vitro’ by the  $\text{Ca}^{2+}$ -dependent protein kinase C (PKC) and cAMP-dependent protein kinase (PKA), resulting in increased activity<sup>212, 213</sup>. In yet another study, PKC was reported to increase phosphorylation and activity of sGC in PC12 cells<sup>214</sup>. Kostic et al., showed that the stimulatory action of PKA on the production of cGMP proceeds through the phosphorylation of two N-terminal serine residues of the sGC  $\alpha$  subunit and this mechanism accounts for the NO-independent AC-controlled production of cGMP<sup>215</sup>.

The cGMP-dependent protein kinase (PKG) on the other hand, attenuates the catalytic capacity of sGC, most likely through an inhibitory feedback mechanism. The studies of Murthy et al., demonstrate inhibition of sGC activity via PKG-dependent phosphorylation, which is analogous to PKA-mediated inhibition of AC<sup>216, 217</sup>. Those data are not in line with those of Ferrero et al., who demonstrate that a PKG-dependent stimulation of protein phosphatase(s), that reduces the Ser/Thr phosphorylation level of the sGC $\beta_1$ , is the underlying mechanism for the feedback sGC inhibition<sup>218</sup>. In line with the notion that phosphorylation is important for sGC function, 17 $\beta$ -estradiol is reported to inhibit sGC by stimulation of protein tyrosine phosphatase activity and subsequent dephosphorylation<sup>219</sup>. Moreover, it was recently reported that both inhibition of tyrosine phosphatases and the presence of ROS induce tyrosine phosphorylation of the sGC $\beta_1$  subunit accompanied by a reduced NO sensitivity and enhanced basal activity<sup>220</sup>. This latter effect can be due to Src-like kinase-mediated phosphorylation of AGAP-1 (the prototype of an ArfGAP protein with a GTase-like domain, Ankyrin repeats, and a pleckstrin homology domain), which binds to sGC in a phosphorylation-dependent way<sup>221</sup>.

### *1.1.7.4. Protein-protein interactions involving sGC*

Protein-protein interactions can alter both the activity and subcellular localization of the interacting partners. In the case of sGC, the chaperonin containing t-complex polypeptide (CCT) subunit  $\eta$  interacts with the  $\beta_1$  subunit of sGC and, in cooperation with some other factor, inhibits NO-stimulated sGC activity by modifying the binding of NO to the heme group or the subsequent conformational changes<sup>222</sup>. On the other hand, the interaction of sGC

with the heat shock protein (Hsp) 70 has been reported to increase the cGMP-forming ability of sGC<sup>223</sup>. Additionally, the binding of Hsp90 to the  $\beta_1$  subunit of sGC was shown to regulate the pool of active enzymes by affecting the protein levels of the two subunits. The abundantly expressed Hsp90, has also been found to bind to eNOS<sup>224, 225</sup>, to facilitate the calmodulin-dependent disruption of eNOS binding with the inhibitory protein caveolin-1<sup>226</sup> and to mediate the interaction with between eNOS and protein kinase B/Akt<sup>227, 228</sup>. Therefore it was suggested that in cells that express eNOS or nNOS along with sGC, Hsp90, in addition to preserving sGC levels, would facilitate the autocrine actions of NO by bringing together the NO source and its target<sup>229</sup>. This prevents the inactivation of NO by superoxide anion and the formation of peroxynitrite, which is a toxic molecule that has been implicated in the pathology of several vascular diseases. Data by Venema et al. show that in endothelial cells, the majority of the sGC is membrane-associated, whereas in vascular smooth muscle cells most of sGC is found in the cytosol. Therefore it was speculated that eNOS, which is located in specific plasmalemmal microdomains called caveolae<sup>230</sup>, recruits sGC to the plasma membrane through their mutual interaction with Hsp90, whereas in the eNOS-negative smooth muscle cells, sGC remains mostly in the cytosol<sup>231</sup>. Analogously Hsp90 is suggested to play an essential role in the translocation of sGC to the plasma membrane in response to an agonist-induced elevated cytosolic  $\text{Ca}^{2+}$  concentration<sup>232, 233</sup>. The membrane-associated sGC had a higher apparent sensitivity toward NO, demonstrating that the subcellular localization of sGC can modulate its activation properties and is functionally important. Also in the sarcolemmal region of skeletal muscles, sGC was found in colocalization with NOS and the CO-producing enzyme Heme Oxygenase-2<sup>234</sup>. In contrast with Hsp90 and CCT $_{\eta}$  that only bind to the sGC  $\beta_1$  subunit, the multivalent scaffold protein AGAP1 has been reported to bind to the  $\alpha_1$  and  $\beta_1$  subunit of sGC. Thereby, it serves as a bidentate partner accommodating both subunits of sGC, without affecting its enzymatic capacity or NO sensitivity. The finding that AGAP1 is subject to tyrosine phosphorylation, most likely by members of the Src family and the fact that this significantly enhances its interaction with sGC, points to a convergence of signal transduction pathways at the level of AGAP1 involving Arf-type GTPases, Src-like kinases, and the NO/cGMP signaling cascade<sup>221</sup>. Moreover, cross-talk between the NO/cGMP and tyrosine kinase signaling pathways has also been reported at the level of sGC<sup>220</sup>.

Besides the association of the  $\alpha_1\beta_1$  heterodimer with the plasma membrane, the  $\alpha_2$  subunit has been shown to target to synaptosomal membranes through its interaction with the third PDZ

(post-synaptic density protein-95/Discs large/zona occludens-1)-domain of the post-synaptic density protein-95 (PSD-95)<sup>235</sup>. As PSD-95 integrates the NMDA receptor with its first PDZ-domain<sup>236, 237</sup> and nNOS with its second PDZ-domain<sup>238</sup>, a signal transduction unit is formed downstream of the NMDA receptor which is important for synaptic plasticity and learning<sup>239</sup>. So, PSD-95, just like Hsp90 for eNOS and sGC $\alpha_1\beta_1$ , serves as a linker between nNOS and sGC $\alpha_2\beta_1$ . In both cases NO would not need to travel through the cell to reach its cytosolic receptor sGC, but NO/cGMP signaling would be more confined to a protein complex, presumably at or near the cell membrane<sup>233</sup>. Clearly, those findings challenge the designation of sGC as a purely cytosolic enzyme. The term ‘soluble’ or ‘cytosolic’ GC may therefore be misleading and instead some prefer to use the term ‘NO-sensitive GC’.

#### *1.1.7.5. Other*

Before the identification of NO as the physiological activator of sGC, a multitude of redox active substances have been proposed as activators. Several mechanisms of activation have been postulated and subsumed as “redox-regulation” of sGC<sup>240-242</sup>. The importance of thiol groups of sGC has been shown by various reports. The use of several thiol blockers led to inhibition of basal as well as stimulated activity of sGC, a process prevented by an excess of the thiol-reductant dithiothreitol<sup>243, 244</sup>. Furthermore, formation of mixed disulfides occurred as <sup>32</sup>S-labeled cysteines were shown to be incorporated into sGC<sup>245</sup>. Those findings clearly favour an oxidative-reductive mode of sGC modulation which involves sulfhydryl-disulfide interconversions on the sGC enzyme. In contrast to the various reports observing largely inhibitory effects of thiol-oxidizing compounds such as diamide, the study of Wu et al., proposed a concentration-dependency of the diamide-induced regulatory effect on sGC. They demonstrated in platelets that low concentrations of thiol-oxidizing compounds or partial oxidation is associated with increased sGC activity, whereas higher concentrations or massive oxidation results in loss of enzymatic activation<sup>246</sup>. Also a more recent study demonstrated an inhibitory effect on NO-elicited relaxation of endothelium-denuded bovine pulmonary arteries (BPA) and stimulation of sGC activity in BPA homogenates by 1 mM of diamide, however not with lower doses of diamide. Furthermore, this study supports the possibility that besides the direct oxidation of sGC thiols, diamide also inhibits the NO-induced stimulation of sGC through oxidation of glutathione (GSH). The level of cytosolic NADPH controlled by the pentose phosphate pathway, appears to regulate this GSH balance and therefore this system is suggested to be an additional potentially important physiological mechanism of controlling

sGC activity<sup>247</sup>. In addition to modulation of the redox status of sGC thiols, also more indirect effects such as an improved NO-generation from NO-donors<sup>248</sup> and the reconversion of reactive NO-derived metabolites such as ONOO<sup>-</sup> to NO<sup>249</sup>, participate in the thiol-mediated effect on sGC stimulation. Like ONOO<sup>-</sup>, also the reactive oxidant species hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), has been shown to stimulate sGC activity and to promote vascular relaxation<sup>250-252</sup>. Besides a direct effect of H<sub>2</sub>O<sub>2</sub> on sGC activity, also activation of NO-synthase was reported to be partially involved<sup>253-256</sup>. This was not supported by Garcia-Gardena et al, who claim that H<sub>2</sub>O<sub>2</sub> increases eNOS tyrosine phosphorylation, which reduces its activity<sup>257</sup>. However, in several following reports, the relaxation and sGC activation elicited by H<sub>2</sub>O<sub>2</sub>, could be confirmed and the participation of NO in this response excluded<sup>258-260</sup>. Recently, tyrosine phosphorylation as mentioned above, was reported as a potential mechanism for the H<sub>2</sub>O<sub>2</sub>-mediated regulation of sGC<sup>220</sup>.

In human platelets<sup>261</sup> and bovine lung<sup>262</sup>, an endogenous inhibitor of sGC has been isolated. However, the molecular identity of this inhibitor is still unknown. The 149 kDa protein detected in lung tissue is suggested to be an allosteric regulator, whereas the inhibitory effect by the platelet-derived inhibitor, results from the presence of the heme.

Isoliquiritigenin, a chalcone derivative of vegetable origin, was shown to exert a vasorelaxant effect on precontracted rat aortic rings by activating sGC and increasing cGMP<sup>263</sup>. However, later on, this finding was refuted by the lack of the potent and selective sGC inhibitor ODQ to influence the action of isoliquiritigenin. Instead, it was suggested that the effects of isoliquiritigenin are due to direct inhibition of phosphodiesterase activity for which evidence has also been obtained in cell-free preparations<sup>264</sup> and in rat ventricular heart muscle<sup>265</sup>.

Copper sulphate has been reported to inhibit sGC by the reversible high affinity binding of Cu<sup>+</sup>-ions to a site of the protein that is critically involved in enzyme catalysis. This Cu<sup>+</sup>-mediated inhibition of sGC may not play a major role under normal physiological conditions but become significant under pathophysiological conditions<sup>266</sup>.

Also rather likely in pathological states is the inhibition of basal and NO stimulated sGC activity by biliverdin IX, a heme-degradation product of heme oxygenase besides CO. The inhibitory mechanism of biliverdin IX is suggested to be different from ODQ as biliverdin IX was able to inhibit protoporphyrin IX activation whereas ODQ was not<sup>267</sup>.

For many enzymes, product inhibition is a relevant mechanism of regulating catalytic activity. In case of sGC, feedback inhibition by both cGMP and pyrophosphate (PP<sub>i</sub>) has been demonstrated in an adapted Sf9 cells/baculovirus system for overexpression of an enzymatically active human sGC protein. Both reaction products displayed independent

binding and cooperativity with respect to enzyme inhibition. The extent of inhibition by cGMP depended on the activation state of the enzyme, whereas inhibition by  $PP_i$  was not affected by the enzyme state <sup>268</sup>. The sodium nitroprusside (SNP)-stimulated enzyme was reported most susceptible to cGMP inhibition, although even in this case the  $IC_{50}$  was in the 6-10 mM range, concentrations that are unlikely to occur in vivo. Moreover, kinetic studies of sGC catalysis reveal a linear relation between the cGMP concentration and the time, indicating that the enzyme is not subject to feedback, or 'end-product', inhibition <sup>49</sup>.

$Ca^{2+}$  has been reported to be a negative allosteric modulator of sGC, which is in line with the antagonistic functions of  $Ca^{2+}$  and cGMP in several physiological systems <sup>269-271</sup>. According to Parkinson et al.,  $Ca^{2+}$  decreases both  $V_{max}$  and  $K_m$  of sGC, consistent with an uncompetitive mechanism of inhibition, in which  $Ca^{2+}$  directly interacts with the enzyme <sup>269</sup>. The calcium concentrations used to inhibit sGC in the studies (5 to 250  $\mu\text{mol/L}$ ) are, however, much higher than those commonly observed in cells, even when responding to  $Ca^{2+}$ -elevating stimuli. Therefore, a relevant role for  $Ca^{2+}$  in regulating sGC under physiological conditions can not be assumed. Moreover, the study of Parkinson et al. that shows a decrease in the cGMP accumulation in NO-stimulated HEK (human embryonic kidney)-293 cells in response to an influx of  $Ca^{2+}$ , failed to address the possible involvement of a cGMP-hydrolysing  $Ca^{2+}$ -calmodulin sensitive PDE-1 which has a sensitivity to  $Ca^{2+}$  in the appropriate range for physiological  $Ca^{2+}$  signaling <sup>269</sup>. However, as discussed above,  $Ca^{2+}$  appears to regulate the association of sGC with cellular membranes accompanied by increased sensitivity toward NO <sup>232</sup>.

On protein level, also alternative heterodimerization and changes in the extent of homodimerization may play a role in regulating sGC activity. As discussed above, the study of Gupta et al., indicates that the  $\beta_2$  subunit complexes with the  $\alpha_1$  subunit of sGC to form a heterodimer with reduced sensitivity to NO and that the  $\beta_2$  subunit can inhibit the NO-stimulated cGMP accumulation in cells containing the  $\alpha_1\beta_1$  form of sGC by exchanging with the  $\beta_1$  subunit. Therefore, enhanced expression of the  $\beta_2$  subunit has been implicated in the downregulation of renal sGC activity in Dahl salt-sensitive rats <sup>64</sup>. Expression of alternative splice variants exposing modified N-or C-terminal portions but retaining the dimerization region may contribute to the fine tuning of sGC activity through proteasomal degradation of the resultant complexes <sup>41</sup>. Likewise, the variant of the  $\alpha_2$  subunit  $\alpha_{2i}$  can compete with the  $\alpha_1$  subunit and may act as a dominant negative inhibitor <sup>70</sup>. Although naturally occurring homodimers have not been isolated, glutathione S-transferase (GST)-tagged recombinant

human  $\alpha_1$ - and  $\beta_1$ -subunits have been shown to form homodimeric GST- $\alpha_1\alpha_1$  and GST-  $\beta_1\beta_1$  complexes that are devoid of enzymatic activity. Upon coexpression of the respective complementary subunits, heterodimers were preferentially formed, whereas homodimers were still detectable. Those observations suggest the possibility of an physiological equilibrium between homo-and heterodimers that regulates sGC activity in cells<sup>272</sup>.

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## **I.2. Soluble guanylyl cyclase-mediated smooth muscle relaxation**

### **I.2.1 Soluble guanylyl cyclase effectors**

Stimulation of sGC and the resultant accumulation of cGMP regulate complex signaling cascades through immediate downstream effectors, including PKG's, cyclic nucleotide-gated (CNG) ion channels and cGMP-regulated PDE's. The specificity of cellular responses to cGMP is dictated by cGMP-binding motifs in the target proteins. Two evolutionarily distinct allosteric sites for binding of cGMP are present in eukaryotic cells. One occurs with significant sequence homology in PKG's and PKA's and in the CNG channels, while the other occurs in PDE's. In addition, the outcome of an increased intracellular cGMP level is determined by the type and combination of target proteins and substrates, the cGMP-metabolizing enzymes expressed in cells, and their intracellular colocalization and organization into selective compartments and organelles <sup>1</sup>.

The principal intracellular mediator of the cGMP signal is the family of PKG's, that become active as serine/threonine kinase upon binding of cGMP <sup>2</sup>. In mammals, two types of PKG's have been described: the cytosolic 76 kDa homodimer PKG I <sup>3</sup> and the membrane-associated 86 kDa monomer PKG II <sup>4</sup>. The type I form is more widely distributed and has a particularly high expression level in the cerebellum, platelets and all types of smooth muscle cells <sup>5</sup>. Furthermore, it consists of an  $\alpha$  and a  $\beta$  isoform, which arise from alternative splicing <sup>3, 6</sup>. By immunological analysis, PKG I $\beta$  was shown to be mainly expressed in cells that also have been reported to contain high concentrations of sGC, such as smooth muscle cells. It is suggested that the I $\beta$  isoform is predominantly expressed in those tissues to dampen the physiological effects of short-term increases of cGMP <sup>7</sup>, as the I $\beta$  isoform requires a 10-fold-higher concentrations of cGMP for activation than the I $\alpha$  isoform <sup>8</sup>. The type II form of PKG is mainly expressed in intestine, kidney, chondrocytes and specific brain regions, but notably absent from cells of the cardiovascular system <sup>5</sup>. Because of the significant homology between the cyclic nucleotide binding domains, PKA's can also be targets for cGMP, although with a 50-fold lower selectivity than for cAMP <sup>5, 9</sup>. Insight into PKG functions were obtained from analyses of substrates phosphorylated by PKG and from PKG knockout mice. Homozygous deletion of the PKG I gene in mice abolishes relaxation of vascular <sup>10</sup>, visceral <sup>10, 11</sup> and penile smooth muscle cells <sup>12</sup>, resulting in a very low ability to reproduce and severe vascular and intestinal dysfunction with death at early age <sup>10</sup>. In addition, the PKG I knockout

mice also showed an increased platelet adhesion and aggregation<sup>13</sup>. The phenotype of the PKG II knockout mice on the other hand, included a normal lifespan<sup>14</sup>, decreased longitudinal bone growth<sup>14</sup>, decreased intestinal chloride secretion<sup>14, 15</sup> and altered renin secretion<sup>16</sup>. Those data support that PKG I plays an important role in regulating smooth muscle tone, proliferation and differentiation of vascular smooth muscle cells, endothelial cell and platelet function, whereas PKG II has an influence on the hemodynamic parameters via regulation of renin release and ion transport in the kidney<sup>17</sup>.

Another target for cGMP includes the voltage-gated CNG channels. In the principal family of CNG channels, each member consists of 4 subunits that form a voltage-responsive core in the plasmamembrane that directly opens upon binding of cyclic nucleotides<sup>18</sup>. They regulate the influx of Na<sup>+</sup> and Ca<sup>2+</sup> into cells and are critical for the regulation of phototransduction and neurotransmission in the retina<sup>19</sup>.

PDE's are the last group of cGMP activated effectors. Those enzymes which catalyze the conversion of cAMP or cGMP to 5'-adenosine monophosphate (5'-AMP) or 5'-guanosine monophosphate (5'-GMP), respectively, are crucial for the modulation of the intracellular concentration of cyclic nucleotides<sup>20</sup>. Based on their specificity for cGMP and/or cAMP hydrolysis and tissue specific expression, multiple families of PDE's have been characterized<sup>21</sup>. PDE-5, a homodimer of 93 kDa subunits specifically degrades cGMP and is predominantly expressed in smooth muscle cells<sup>22</sup>. Direct binding of cGMP to allosteric sites has been shown to promote phosphorylation of PDE-5 by either PKG or PKA, thereby indirectly stimulating enzyme activity<sup>1</sup>.

### I.2.2. Mechanisms of cGMP-dependent smooth muscle relaxation

The mechanism underlying the contractile activity of the smooth muscle cell is based on synergistic and antagonistic forces regulating the intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>). Increases in the levels of cytosolic [Ca<sup>2+</sup>]<sub>i</sub> initiate smooth muscle contraction by binding to the universal intracellular Ca<sup>2+</sup> receptor calmodulin<sup>23</sup>, which in turn binds to and activates smooth muscle light chain kinase (MLCK)<sup>24</sup>. Activated MLCK catalyzes the phosphorylation of serine 19 on the 20 kDa light chain of myosin (MLC20)<sup>25</sup>, thereby increasing its actin-activated ATPase activity<sup>26</sup> and subsequent cross-bridge cycling. As a means to balance MLCK activity, myosine's actin-activated ATPase activity is attenuated by dephosphorylation of MLC20 by myosin light chain phosphatase (MLCP), a smooth muscle specific

serine/threonine protein phosphatase<sup>27, 28</sup>. Decreases in  $[Ca^{2+}]_i$  and MLC dephosphorylation are considered as 2 salient, independently controlled events in smooth muscle relaxation<sup>29</sup>.

#### *1.2.2.1. Regulation of $[Ca^{2+}]_i$*

As increases in  $Ca^{2+}$  are required for MLC phosphorylation and contraction, reduction of the free intracellular cytosolic  $Ca^{2+}$  concentrations was the first mechanism proposed for cGMP-dependent smooth muscle relaxation<sup>30, 31</sup>. Several sites of action have been reported to account for the cGMP-dependent reduction of  $[Ca^{2+}]_i$ , with PKG I as principal mediator (figure I.3). PKG I inhibits the release of  $Ca^{2+}$  from the sarcoplasmic reticulum (SR), either by phosphorylation of the inositol 1,4,5-triphosphate ( $IP_3$ ) receptor<sup>32, 33</sup> and/or by inhibition of agonist-induced generation of  $IP_3$ <sup>34</sup>. An SR-targeting protein for PKG I termed IRAG ( $IP_3$  receptor associated PKG I substrate) has been characterized and shown to be necessary for the phosphorylation of the  $IP_3$  receptor<sup>35</sup>. Furthermore, PKG I has been shown to increase the  $Ca^{2+}$  sequestration through activation of the smooth endoplasmic reticulum  $Ca^{2+}$ -pumping ATPase (SERCA)<sup>36</sup>. The correlation between vascular smooth muscle relaxation and phosphorylation of the SR membrane protein, phospholamban (PLB), supports the hypothesis that PKG I regulates the  $Ca^{2+}$ -ATPase activity via PLB phosphorylation. Moreover, in rat aortic smooth muscle cells, PLB has been shown to have an inhibitory effect on the  $Ca^{2+}$ -ATPase, which is relieved when PLB is phosphorylated and subsequently dissociates from the ATPase<sup>37, 38</sup>. However, a study on the aorta of mice lacking the PLB gene has demonstrated that PLB plays only a minor role, if any, in cGMP-mediated vasorelaxation<sup>39</sup>. In aortic<sup>40</sup> and airway<sup>41</sup> smooth muscle cells, this SERCA-dependent refilling of the intracellular stores, has also been reported to prevent the  $Ca^{2+}$  influx through store-operated  $Ca^{2+}$  channels (SOCC)<sup>42, 43</sup>, thereby maintaining the decrease in  $Ca^{2+}$ . Another PKG I substrate group in the SR, are the tightly clustered ryanodine-sensitive  $Ca^{2+}$  release (RZR) channels. The combined effect on SR  $Ca^{2+}$  load and RZR channels, results in an increased subplasmalemmal  $Ca^{2+}$  transient or  $Ca^{2+}$  spark frequency, which in turn activates nearby sarcolemmal  $Ca^{2+}$ -sensitive  $K^+$  ( $K_{Ca}$ ) channels<sup>44</sup>. In addition, PKG I has also been reported to directly increase the open probability of the  $K_{Ca}$  channels<sup>45, 46</sup>. Together these actions increase the frequency and amplitude of the “spontaneous transient outward currents” (STOC’s) of  $K^+$  ions<sup>47</sup>. This results in hyperpolarization, which in turn closes voltage-dependent L-type  $Ca^{2+}$  channels leading to reduced global  $[Ca^{2+}]_i$ <sup>48-50</sup>. Besides this indirect hyperpolarization-mediated inhibition of the  $Ca^{2+}$  channels, the  $Ca^{2+}$  influx is also decreased through a direct impairment of the  $Ca^{2+}$

channel activity by PKG I<sup>51</sup>. Furthermore, activation of two different ionic channels, the plasma membrane Ca<sup>2+</sup>-pumping ATPase<sup>52</sup> and the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger<sup>53</sup>, may mediate an increased efflux of Ca<sup>2+</sup> from the smooth muscle cell. The driving force for extrusion of Ca<sup>2+</sup> from the cell through the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger, in turn, is dependent on two other effects mediated by PKG I, depletion of intracellular Na<sup>+</sup> via activation of Na<sup>+</sup>/K<sup>+</sup> ATPase<sup>53</sup> and hyperpolarization of the cell membrane via an enhanced STOC activity.

According to the study of Zolle et al. localized increases in cGMP have distinct regulatory effects on the concentration of [Ca<sup>2+</sup>]<sub>i</sub> within individual cells. They claim that pGC or the particulate pool of cGMP is involved in the inhibition of Ca<sup>2+</sup> efflux via the plasma membrane Ca<sup>2+</sup>-pumping ATPase, whereas sGC or the soluble pool of cGMP modulates [Ca<sup>2+</sup>]<sub>i</sub> via an increased reuptake of Ca<sup>2+</sup> into the intracellular stores without affecting cation influx or Ca<sup>2+</sup> efflux<sup>54</sup>.

#### *1.2.2.2. Regulation of MLC dephosphorylation/Ca<sup>2+</sup> sensitization*

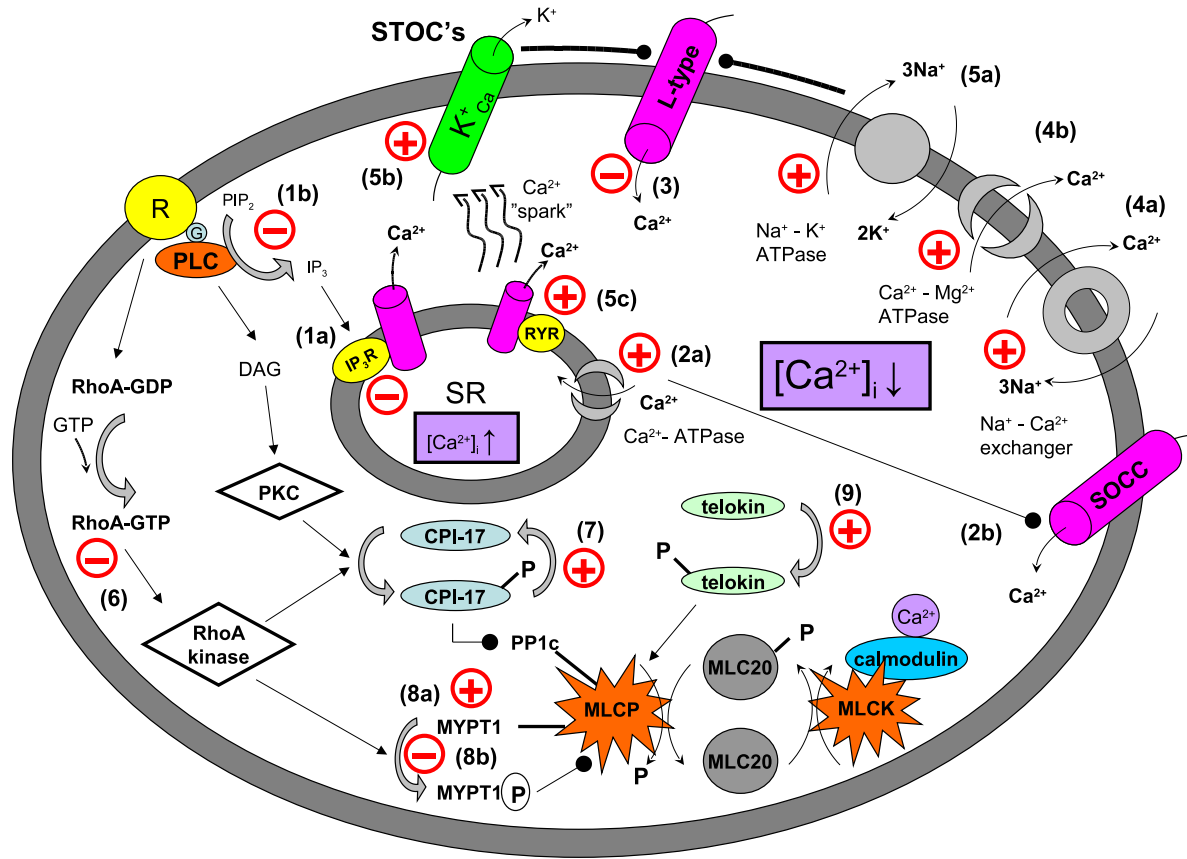
In addition to the multiple Ca<sup>2+</sup> lowering mechanisms, PKG I also mediates cGMP-dependent relaxation of the smooth muscle cell by lowering the Ca<sup>2+</sup> sensitivity of the contractile apparatus or “Ca<sup>2+</sup> desensitization”<sup>24</sup> by stimulation of the MLCP activity (figure I.3). An appreciation for the role of MLCP in the regulation of MLC phosphorylation and smooth muscle contraction began with studies on “calcium sensitization” of contraction<sup>55</sup>. These studies demonstrated that G-protein-coupled agonist-induced MLC phosphorylation and contraction of smooth muscle were apparent at relatively low concentrations of [Ca<sup>2+</sup>]<sub>i</sub> compared with phosphorylation and contraction initiated by membrane depolarization using elevated K<sup>+</sup> solutions<sup>56-58</sup>. Later on, several protein kinase pathways were reported to mediate calcium sensitization including PKC, which is activated on agonist-dependent stimulation of phospholipase C (PLC), and the small molecular weight G-protein, Rho A, which activates Rho kinase<sup>59</sup>. Rho kinase inhibits the MLCP, resulting in an increase in MLC20 phosphorylation and force in the smooth muscle cell<sup>60</sup>. It has been shown that PKG I inhibits the RhoA-mediated Ca<sup>2+</sup> sensitization pathway through phosphorylation of RhoA at Ser 188, which subsequently induces its translocation from the membrane to the cytosol<sup>61</sup>. Thus PKG I inactivates RhoA and consequently activates MLCP to dephosphorylate MLC. The phosphatase inhibitor protein CPI-17 is another potential mediator of Ca<sup>2+</sup> sensitization, which on phosphorylation by a variety of kinases including Rho kinase and PKC<sup>62</sup>, inhibits the MLCP catalytic subunit (PP1c), resulting in Ca<sup>2+</sup> sensitized force independent of the



inhibitory phosphorylation of the regulatory subunit (MYPT1) of MLCP<sup>63</sup>. PKG I has been shown to prevent the inhibition of MLCP in vivo by accelerating dephosphorylation of CPI-17<sup>64</sup>. Additionally, MYPT1 is also reported to be a target of PKG I. The phosphorylation of Ser 695 of MYPT1, prevents phosphorylation of Thr 696 by MYPT1 kinase and RhoA-activated kinases, thereby blocking inactivation of MLCP<sup>65</sup>. Moreover, interaction between the leucine zipper region at the C terminus of some MYPT1 isoforms and the leucine zipper domain of PKG leads to direct stimulation of MLCP<sup>66</sup>. A role for this interaction in Ca<sup>2+</sup> desensitization is supported by in vivo experiments demonstrating that only smooth muscles expressing MYPT1 isoforms containing the leucine zipper motif were sensitive to relaxation by 8-Bromo-guanosine 3', 5' -cyclic monophosphate (8-Br-cGMP), a membrane permeable cGMP analogue. The expression of leucine zipper positive isoforms has been demonstrated to be tissue specific and developmentally regulated, suggesting a correlation with the diversity in NO-mediated smooth muscle relaxation<sup>67</sup>. Although the MYPT1 leucine zipper may serve to target PKG, it is not known whether this is required for phosphorylation of Ser 695 or structurally whether this is feasible<sup>68</sup>. Furthermore, Wu et al. demonstrated that PKG-mediated Ca<sup>2+</sup> desensitization of visceral smooth muscle involves phosphorylation of the myosin-binding protein telokin, which accelerates the dephosphorylation of MLC<sup>69</sup>.

#### *1.2.2.3. Thin filament regulation*

Despite numerous studies performed aiming to unravel the role of the thin filament in actin-myosin cross-bridge cycling or force regulation in smooth muscle cells, this issue remains controversial. Thin filament-associated proteins regulate and contribute to the contractile activity of the cell, but there have been very few reports regarding the role of second messenger regulation of thin filament protein function<sup>70</sup>. The thin filament/actin-binding proteins vasodilatory-stimulated phosphoprotein (VASP) and the 20 kDa Hsp (Hsp20) raised interest in possible regulation of smooth muscle contraction, as they have been shown to be phosphorylated by PKG. This PKG-specific phosphorylation decreases the binding of VASP to actin filaments, possibly through its dissociation from the actin binding protein profilin<sup>71</sup>. In case of Hsp20 this PKG-mediated phosphorylation is reported to be associated with smooth muscle relaxation<sup>72</sup>. Tropomyosin, caldesmon, calponin and SM22 are also purported to bind actin and possibly regulate cross-bridge function<sup>73</sup>, but this is not regulated by PKG.



**Figure I.3:** The  $[Ca^{2+}]_i$  lowering and  $Ca^{2+}$  desensitization mechanisms underlying smooth muscle relaxation mediated by cGMP. cGMP reduces  $[Ca^{2+}]_i$  by decreasing  $Ca^{2+}$  mobilization through (1a) inhibition of the  $IP_3$  receptor in the sarcoplasmic reticulum (SR) or (1b) inhibition of agonist-induced  $IP_3$  formation; (2a) increasing  $Ca^{2+}$  sequestration through activation of the SR  $Ca^{2+}$ -pumping ATPase ((2b) the SERCA-dependent reuptake into the SR will in turn inhibit the SOCC's); (3) inhibiting  $Ca^{2+}$  influx through L-type  $Ca^{2+}$  channels; increasing  $Ca^{2+}$  efflux through activation of (4a)  $Na^+/Ca^{2+}$  exchanger and (4b) the  $Ca^{2+}$ -pumping ATPase; also, cGMP may produce hyperpolarization through activation of (5a) the  $Na^+/K^+$  ATPase and (5b)  $K_{Ca}$  channels (increased STOC of  $K^+$ ). Those  $K_{Ca}$  channels can also be activated by an increased  $Ca^{2+}$  spark frequency, which results from the cGMP-mediated activation of (5c) RYR channels and SERCA. The resulting hyperpolarization will in turn increase  $Ca^{2+}$  extrusion by the  $Na^+/Ca^{2+}$  exchanger. cGMP desensitizes the contractile apparatus to  $Ca^{2+}$  by (6) inhibition of the RhoA-dependent suppression of the myosin light chain phosphatase activity, (7) accelerating the dephosphorylation of CPI-17, activating the myosin light chain phosphatase by (8a) leucine zipper interaction with MYPT1 and by (8b) exclusion of phosphorylation of MYPT1 and (9) phosphorylation of telokin.

R, receptor; G, G protein; PLC, phospholipase C; MLC20, 20 kDa light chain of myosin; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PKC, protein kinase C; DAG, diacylglycerol; CPI-17, 17 kDa PKC-potentiated myosin phosphatase

inhibitor; STOC, spontaneous transient outward current; SOCC, store-operated  $\text{Ca}^{2+}$  channel; RYR, ryanodine receptor;  $\text{IP}_3\text{R}$ ,  $\text{IP}_3$  receptor;  $\text{K}_{\text{Ca}}$ ,  $\text{Ca}^{2+}$  sensitive  $\text{K}^+$  channel; MYPT1, MLCP regulatory subunit; PP1c, MLCP catalytic subunit;  $\ominus$  : inhibitory/negative effect;  $\oplus$  : stimulatory/positive effect (adapted from Porter et al. <sup>47</sup>, Jaggar et al. <sup>50</sup>, Lucas et al. <sup>1</sup> and Jin and Burnett <sup>74</sup>).

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## **I.3. Role of the NO/cGMP pathway**

### **I.3.1. Role of the NO/cGMP pathway in the vascular system**

As illustrated in the previous part, NO plays an important role in vascular tone regulation through the activation of sGC and subsequent production of cGMP. Beyond its vasodilator actions, NO was found to inhibit vascular smooth muscle proliferation and platelet aggregation and to regulate interactions between leucocytes and the blood vessel wall. All these findings established NO as a homeostatic regulator in the vasculature, the absence of which plays a role in a number of conditions and pathological states <sup>1</sup>.

#### *I.3.1.1. Source of NO*

The endothelium, a cell monolayer which constitutes the internal lining of the entire circulatory system, has long been considered as a passive physical barrier whose only function was to provide vessels a smooth surface that prevented clotting and turbulence of the blood flow <sup>2</sup>. This vision changed upon the findings of Furchgott and Zawadzki, who first demonstrated that the relaxation of vascular smooth muscle cells in response to acetylcholine is dependent on the anatomical integrity of the endothelium <sup>3</sup>. They named the factor responsible for this intercellular relationship the EDRF. Some years later, EDRF was recognized as the free radical gas NO <sup>4, 5</sup>. Following the identification of NO, many laboratories jumped into this field of cardiovascular research to study the physiological importance and pathophysiological roles of endothelium-dependent vasodilatation and endogenous NO and cyclic cGMP in the regulation of systemic blood pressure, organ blood flow, hemostasis and cell proliferation.

In mammalian tissues, NO is derived from the amino acid L-arginine, that is accumulated from the extracellular space and is synthesized intracellularly. Under physiological conditions, not the availability of L-arginine <sup>6</sup>, but the activity and expression of the NO-producing enzymes termed NOSs are the rate-limiting factors for the production of NO. This family of enzymes uses NADPH as electron donor and employs five enzyme cofactors (e.g. tetrahydrobiopterin) <sup>7</sup> to catalyse a five electron oxidation of L-arginine to NO with stoichiometric formation of citrulline <sup>8</sup>. The now widely accepted nomenclature identifies the three mammalian enzyme isoforms as neuronal NOS, inducible NOS, and endothelial NOS, reflecting the tissues of origin for the original protein and cDNA isolates. However, their expression patterns are now known to overlap in a complex manner. For example, muscle cells appear to express all three NOS isoforms. Both nNOS and eNOS are expressed in

cardiac myocytes and hippocampus neurons<sup>9</sup>. Vascular endothelial cells express eNOS but can be induced to express iNOS. The nNOS and eNOS isoforms are similar, in that they are generally constitutively expressed and their activity is regulated by  $[Ca^{2+}]_i$ . In contrast, iNOS is only present after induction by immuno-inflammatory stimuli such as tumor necrosis factor or interferon<sup>9,10</sup> and is not regulated by  $Ca^{2+}$ . However, it is now known that the level of gene expression as well as the activity of both eNOS and nNOS can be induced or upregulated under different physiological conditions<sup>11</sup>. Mechanical stimulation, especially fluid shear stress exerted on the endothelium by the streaming blood, represents a major stimulus for the production of NO *in vivo*<sup>12</sup>. The ability of shear stress to enhance the activity of eNOS has been reported to involve the opening of stretch-activated  $Ca^{2+}$  channels, thereby leading to an increase in  $[Ca^{2+}]_i$ <sup>13-15</sup>. Under basal conditions, eNOS interacts directly with caveolin-1, leading to an inhibitory effect on eNOS activity. This inhibitory effect can be completely reversed by  $Ca^{2+}$ /calmodulin, a complex elicited upon the influx of  $Ca^{2+}$ . The activated eNOS synthesizes NO until the  $[Ca^{2+}]_i$  decreases to the point where the calmodulin dissociates and the inhibitory eNOS-caveolin-1 complex reforms<sup>16, 17</sup>. This mechanism accounts for the initial peak of shear stress-induced NO production but not for the following sustained plateau phase which is maintained as long as shear stress is applied. In case of sustained shear stress, NOS has been reported to be activated at basal levels of  $[Ca^{2+}]_i$  via a mechanotransduction cascade that involves tyrosine phosphorylation and that can be modulated by changes in the intracellular pH<sup>18</sup>.

Besides physico-chemical stimuli such as shear stress, pressure and hypoxia, also receptor-mediated mechanisms have been shown to initiate the  $Ca^{2+}$ -dependent activation of eNOS. A number of agonists varying as neurotransmitters (acetylcholine (ACh)), hormones (insulin), autacoids (bradykinin, histamine) and some released by aggregating platelets (serotonin, adenosine) have been reported to act on receptor-operated  $Ca^{2+}$  channels, thereby leading to an increase in  $[Ca^{2+}]_i$ <sup>12</sup>. The initial increase in the endothelial cell  $Ca^{2+}$  is however mediated by the phosphatidylinositol pathway, which mobilizes intracellular  $Ca^{2+}$  from the endoplasmatic reticulum<sup>2</sup>. As the extent of  $Ca^{2+}$  increase is modified by the membrane potential of the endothelial cells, the opening of potassium channels with hyperpolarisation of the endothelium will facilitate  $Ca^{2+}$  entry and thus synthesis of NO<sup>19</sup>. Recently, several reports have shown that activation of eNOS can even occur in the presence of low  $Ca^{2+}$  concentrations by the action of the serine/threonine kinase Akt. Akt phosphorylates the serine 1177-residue and renders NOS activated by increasing the affinity of eNOS for calmodulin<sup>20</sup>.

### *1.3.1.2. Direct vascular effects of NO*

It is now appreciated that NO is continuously generated from vascular endothelial cells in the absence of added endothelium-dependent vasodilators. Despite the fact that the intrinsic stimulus for basal production of NO was not appreciated in the early 1980's, later studies revealed that the shear stress generated by flowing blood against the endothelial cell surface triggered the generation of NO in the endothelial cells<sup>21-23</sup>. The mechanism by which the endothelium is able to sense changes in shear stress on its luminal surface remains obscure, although there have been reports that disruption of the endothelial cytoskeleton may be responsible for initiating some of the changes associated with the exposure to shear stress<sup>24</sup>.<sup>25</sup> The level of shear stress and the release of NO elicited by altering either diameter or flow are positively correlated, suggesting that local changes in tone are as important as changes in flow for the regulation of endothelial NO release in vivo<sup>26, 27</sup>. The main physiological consequence of this relationship is that any decrease in vessel diameter (myogenically- or neurogenically-induced contraction), at constant flow, increases the shear stress to which the endothelial layer is exposed and elicits the release of NO, which in turn feeds back to inhibit the original vasoconstriction<sup>28</sup>. This tone-dependent NO release constitutes a local vascular reflex mechanism to sustain regional blood flow into arterial beds in the presence of vasoconstriction. The basis of the autoregulatory properties of any given vascular bed is the myogenic contraction, which occurs in response to instantaneous increases in transmural pressure. The modulation of myogenic constriction by NO is more pronounced in larger than in smaller (terminal) arterioles. As the metabolic activity of a tissue increases, the local metabolic changes (e.g. decrease in the partial pressure of O<sub>2</sub>, increase in the partial pressure of CO<sub>2</sub>, and decrease in pH) promote the relaxation of precapillary sphincters, causing an increase in capillary blood flow. Despite the fact that the products of energy metabolism are effective dilators, the accumulation of metabolites alone has relatively little effect on vascular resistance. The reason for this is that in order to achieve optimal vascular conductance both small arterioles and the larger arterioles feeding them must dilate in concert. This phenomenon of "conducted dilation" is thought to be determined by local changes in membrane potential as well as by flow-dependent dilation of larger arterioles<sup>29</sup>. The flow-induced dilatation of proximal arterioles, combined with metabolite-induced dilation of distal arterioles, allows a perfect adaptation between tissue O<sub>2</sub> consumption and tissue O<sub>2</sub> supply. The significance of the shear stress-dependent endothelium-derived NO in global

cardiovascular homeostasis is demonstrated by the increase in mean arterial blood pressure and the reduction in blood flow upon the selective inhibition of the NO.

The most often used approach to modulate the actions of NO involves the inhibition of NOS activity. Compounds with chemical modifications of the guanidine group of L-arginine (e.g. N<sup>G</sup>-monomethyl-L-arginine (L-NMMA), N<sup>G</sup>-nitro-L-arginine (L-NA), and N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME))<sup>30</sup> compete with the interaction of L-arginine on the NOS active site or with the uptake of L-arginine, thus reducing the availability of substrate for NOS catalysis<sup>31</sup>. In 1989 Rees et al., observed that L-NMMA by itself produced a dose-dependent and endothelium-dependent contraction of rabbit aorta, suggesting the loss of a continuous NO-dependent relaxing tone<sup>32</sup>. Subsequent studies in the whole animal demonstrated that the intravenous administration of L-NMMA caused an immediate and marked increase in blood pressure that could be reversed by infusion of L-arginine<sup>33</sup>. This finding was very important for the understanding of the mammalian cardiovascular system, as it was concluded that peripheral resistance was the main determinant of blood pressure regulation. Blood pressure increase after inhibition of NO synthesis has been confirmed in a variety of studies in animals and humans. In Wistar rats, chronic blockade of NOS with L-NAME induced a dose-dependent increase in blood pressure and a reciprocal decrease in aortic cGMP, which is mainly dependent on sGC activity<sup>34</sup>. In healthy humans, L-NMMA increased mean arterial blood pressure by 10% and total peripheral resistance by 46%<sup>35</sup>. Those data have led to the conviction that basal NO exerts a continuous dilatory influence on vascular tone, counterbalancing the contractile influences. Later on, those observations were extended to models of genetic manipulation. In these, knocking out the gene encoding eNOS in mice resulted in significant hypertension, although the magnitude of hypertension reported by different laboratories varies. Differences in systolic arterial pressure in conscious eNOS knockout mice compared with wild type mice range from 20<sup>36</sup> to 50<sup>37, 38</sup> mmHg, while differences in mean blood pressure in anesthetized mice range from 14<sup>39</sup> to 37<sup>40</sup> mmHg. The varying magnitude of the hypertension observed in eNOS knockout mice may be due to the use of different methods for measuring blood pressure or the genetic backgrounds of the strains used. However, even with these differences, eNOS knockout mice were found to be hypertensive in all cases. In vitro, endothelium intact aortic rings removed from these animals display no relaxation to acetylcholine and are unaffected by treatment with NOS inhibitors<sup>41</sup>. In concert with those findings, mice with transgenic overexpression of eNOS are reported to be hypotensive<sup>42</sup>. The importance of eNOS in the regulation of blood pressure and vascular

homeostasis is further demonstrated by the observation that a missense variant of the eNOS gene is positively associated with essential hypertension and myocardial infarction <sup>43</sup>.

It is frequently assumed that all hypertension in eNOS knockout mice is caused by the lack of endothelium-derived NO and the resulting increase in arterial tone and peripheral resistance. However, also other NO-related mechanism such as increased cardiac contractility and increased fluid absorption by the kidney may be involved in hypertension <sup>44</sup>. Those mechanisms are known to control the cardiac output, which determines arterial blood pressure together with total peripheral resistance. Finally, genetic deletion of eNOS may disrupt the function of other important regulators of blood pressure by affecting central nervous system activity. Stauss et al. found that in resting eNOS deficient mice, arterial blood pressure fluctuations were more pronounced than in the control mice, indicating that baroreflex responses are blunted in the former <sup>38</sup>. So, hypertension may also be caused by the involvement of eNOS in determining the baroreceptor blood pressure setpoint.

Besides eNOS, also nNOS which is present both in vasomotor centres of the central nervous system and in peripheral nerves as well as certain vascular smooth muscle cells, is likely to play a role in the global regulation of blood pressure. This was concluded from the surprising observation that in eNOS deficient mice, the acute administration of the selective nNOS inhibitor 7-nitroindazole (7-NI) resulted in a decrease in mean arterial blood pressure which was prevented by L-arginine <sup>36</sup>. Although this finding suggests that nNOS may be prohypertensive, in other cases it appears to be antihypertensive. For example, the chronic administration of 7-NI in drinking water significantly increased blood pressure in rats <sup>45</sup>. However, blood pressure in nNOS knockout mice has generally been shown to be similar to that of wild type controls <sup>46-48</sup>. This finding is in line with the observation that the systolic blood pressure in mice deficient in both eNOS and nNOS, is higher than in wild type mice and similar to eNOS knockout mice <sup>49</sup>. So, those data suggest that genetic deletion of nNOS is compensated for, in terms of blood pressure regulation or that the hypotensive actions of nNOS may be counterbalanced by its hypertensive effects. Moreover they suggest that nNOS does not play an important role in the regulation of vascular tone <sup>44</sup>. However, there is evidence that when eNOS-dependent vasodilatation is impaired, nNOS-derived NO mediates flow and agonist-induced dilatations. For example, inhibition of nNOS or sGC attenuated the acetylcholine-induced pial vessel dilatation in eNOS knockout mice, whereas in wild type mice inhibition of nNOS activity was ineffective <sup>50</sup>. Those data suggest that nNOS-cGMP-dependent mechanisms compensate or upregulate after deletion of the eNOS gene. This is supported by the Huang's study, showing that nNOS-derived NO, together with

prostaglandins, maintains flow-induced dilatation in coronary arteries of eNOS knockout mice<sup>51</sup>. In addition, these investigators found an upregulation of nNOS expression in the endothelium and smooth muscle of coronary arteries. Those data suggest that despite the small amount of nNOS in blood vessels, low levels of nNOS-derived NO could compensate for the lack of eNOS. In contrast to eNOS and nNOS, iNOS expression has not been found in the vasculature under physiological conditions and is thus not likely to play a role in the basal regulation of vascular tone<sup>52</sup>. This is in line with the observation that in iNOS knockout mice, basal systolic blood pressure was not elevated<sup>53,54</sup>. Only in pathological states such as septic shock, iNOS-derived NO has been shown to affect vascular tone and to be responsible for severe systemic hypotension<sup>55</sup>.

### *I.3.1.3. Other endothelium-derived relaxing substances*

#### *\*Prostaglandins*

Although the era of endothelium-derived relaxing factors began with the seminal discovery of the EDRF, later identified as NO, prostaglandins were the first endothelium-derived relaxing factor to be discovered. In 1976, Moncada et al. described an anticlotting agent that was also capable of relaxing vascular smooth muscle cells<sup>56</sup>. They called the entity PGX which, soon after, was identified as prostacyclin (PGI<sub>2</sub>)<sup>57</sup>. In common with NO, PGI<sub>2</sub> is lipid soluble and highly unstable in the body and thus it leaves the endothelial cell following its production and acts as a local anticoagulant and vasodilator<sup>58</sup>. It is formed via the cyclooxygenase (COX) pathway, most commonly from arachidonic acid. After chemical (e.g. histamine, bradykinin) or mechanical (shear stress) stimuli, the enzyme phospholipase A<sub>2</sub> in the endothelial cells, mobilizes arachidonic acid from membrane phospholipids. Subsequently, COX converts arachidonic acid to the prostaglandin endoperoxide H<sub>2</sub>, the precursor of all vasoactive prostanoids<sup>59</sup>. Of the COX enzyme, two isozymes have been identified so far: COX-1 and COX-2, which are produced from two different genes. COX-1 is a constitutive enzyme and responsible for many physiological functions; it has been detected in most mammalian tissues under basal conditions, primarily in stomach, platelets and vascular endothelium<sup>60</sup>. COX-2 on the other hand, is an inducible isozyme<sup>61-63</sup> that is thought to be expressed in the cardiovascular system (and immune cells) only during pathogenic episodes. Nevertheless, COX-2 may have some homeostatic functions in some organs because it is constitutively present in the brain<sup>64</sup>, kidney<sup>65</sup>, gastrointestinal system<sup>66</sup> and vascular endothelium<sup>67</sup>. In the last step of the prostaglandin biosynthesis, several PGH<sub>2</sub> isomerases catalyse the

isomerisation of PGH<sub>2</sub> to a variety of biologically active end-products (PGD<sub>2</sub>, PGE<sub>2</sub>, PGI<sub>2</sub>, PGF<sub>2α</sub> or thromboxane (TXA<sub>2</sub>)), depending on the enzymatic machinery present in a particular cell type<sup>59</sup>. PGI<sub>2</sub> is the major prostanoid produced by the endothelial cells, while platelets synthesize mainly TXA<sub>2</sub><sup>58</sup>. PGI<sub>2</sub> elicits smooth muscle relaxation by activating specific cell surface receptors (IP) that are G-protein-coupled to AC and thereby elevate cAMP levels<sup>68, 69</sup>. The formed cAMP activates PKA which phosphorylates selected target proteins, thereby reducing the [Ca<sup>2+</sup>]<sub>i</sub> and subsequently cause relaxation<sup>70</sup>. The effect of PGI<sub>2</sub> is tightly connected with NO effects. Koller et al., demonstrated that increases in perfusate flow elicit endothelium-dependent dilatation of gracilis muscle arterioles, a response that is mediated by the combined release of NO and prostaglandins<sup>71</sup>. Other studies demonstrated that NO stimulates prostaglandin synthesis in endothelial cells and macrophages via a mechanism involving a reaction with the heme-component that binds to the active site of COX enzymes<sup>72, 73</sup>. Furthermore, NO has been shown to potentiate the effect of PGI<sub>2</sub> in the smooth muscle cells through inhibition of cAMP-degrading PDE's<sup>74</sup>. In turn, PGI<sub>2</sub> has been reported to increase eNOS gene expression and the release of NO from endothelial cells via cAMP signaling<sup>75</sup>. On the other hand, there are also studies that indicate an enhanced production of prostaglandins as a consequence of inhibition of NO synthesis and thus support a negative relation between NO and prostaglandin synthesis. Also in eNOS knockout mice, COX-2 derived prostaglandins were reported to be enhanced and responsible for the maintenance of flow-induced dilatation<sup>76, 77</sup>.

In most vascular beds, PGI<sub>2</sub>, PGD<sub>2</sub> and PGE<sub>2</sub> have been shown to induce vasorelaxation, whereas PGF<sub>2α</sub> and TXA<sub>2</sub> have been reported to initiate vascular smooth muscle contraction. The ratio of PGI<sub>2</sub>/TXA<sub>2</sub> appears to provide an important input into determining vascular tone<sup>78</sup>. The ratio of these two vasoactive agents is reduced in a number of vascular diseases, namely pulmonary hypertension<sup>79</sup>, selenium deficiency<sup>80</sup> and pre-eclampsia<sup>81</sup>. In mice lacking the PGI<sub>2</sub> synthase gene, the absence of PGI<sub>2</sub> metabolism is associated with a five fold increase in the production of TXA<sub>2</sub><sup>82</sup>. This increase in TXA<sub>2</sub> may enhance vasoconstriction and attribute to the elevated blood pressure in PGI<sub>2</sub> synthase-deficient mice. The finding that the blood pressure of those mice increases with age, lends support to the contention that polymorphism of the human PGI<sub>2</sub> synthase gene, which decreases the promoter activity, seems to be a risk factor for higher pulse pressure and systolic hypertension in the Japanese population<sup>83</sup>. The PGI<sub>2</sub> receptor-deficient mice, on the other hand, have been reported to be normotensive, suggesting that PGI<sub>2</sub> is not involved in the regulation of blood pressure<sup>84</sup>. Furthermore, those mice did not show renal abnormalities as reported in the PGI<sub>2</sub> synthase-

deficient mice. In the latter study, the high blood pressure could be secondary to the associated gross abnormalities in the kidney, as also suggested for the COX-2-deficient mice<sup>85, 86</sup>. The genetic deficiency of COX-2 appears to disrupt the signaling pathway in the salt sensing region of the kidney, which is important for activation of the renin-angiotensin system<sup>87</sup>. Under conditions where renin expression and activity are induced in wild type mice, renin production was not stimulated in the kidneys of COX-2-deficient mice<sup>88, 89</sup>. Therefore, it is suggested that prostaglandins produced by COX-2 are responsible for maintaining renal homeostatic functions involved in the regulation of salt resorption, fluid volume and blood pressure<sup>90</sup>. This was also confirmed by the study of Muscara et al., in which the chronic suppression of the COX-2 activity by the daily administration of selective COX-2 inhibitor celecoxib, significantly influenced the renal function, leading to severe fluid retention and elevated blood pressure. Therefore, they suggest that the hypertensive effects of celecoxib may have been due to effects of this drug on the kidney and not by virtue of suppression of vascular PGI<sub>2</sub> synthesis<sup>91</sup>. However, in contrast to the COX-2 deficient mice, mice deficient in COX-1 fail to produce an identifiable renal pathology<sup>92</sup>. Still, they have been shown to have an increased mean arterial blood pressure<sup>93</sup>. Besides positive studies regarding the importance of COX in the regulation of blood pressure, there are also negative reports. The blood pressure of both COX-1<sup>94</sup> and COX-2<sup>95</sup> knockout animals has been reported to be not different from the corresponding control animals, suggesting that prostanoid products of COX-activity do not regulate baseline blood pressure or that compensatory mechanisms completely abolish the appearance of any effects. Likewise, baseline sodium and water balance has been shown to be unaffected by COX-2 deficiency<sup>95</sup>. As COX produces vasoconstrictor and vasodilator metabolites, its role in the regulation of blood pressure and hemodynamics is difficult to predict and may explain the positive and negative results regarding the effect of COX inhibition.

*\*Endothelium-derived hyperpolarising factor (EDHF)*

Not all endothelium-dependent relaxations can be fully explained by the release of either NO and/or prostacyclin. In 1984, Bolton and collaborators reported that an additional relaxant pathway associated with smooth muscle hyperpolarisation also exists<sup>96</sup>. This hyperpolarisation was originally attributed to the release of an endothelium-derived hyperpolarizing factor (EDHF) that diffuses to and activates smooth muscle K<sup>+</sup> channels. More recent evidence suggests that endothelial cell receptor activation by these



neurohumoural substances opens endothelial cell  $K^+$  channels<sup>97</sup>. Several mechanisms have been proposed to link this pivotal step to the subsequent smooth muscle hyperpolarization, e.g.  $K^+$ <sup>98</sup>, CYP metabolites<sup>99, 100</sup>, lipoxygenase products<sup>101</sup>, NO itself<sup>102</sup>, ROS ( $H_2O_2$ )<sup>103</sup>, cAMP<sup>104</sup>, C-type natriuretic peptide<sup>105</sup>, and electrical coupling through myoendothelial gap junctions<sup>106</sup>. Up until today, a single molecule or pathway could not be identified which explained all features of EDHF signaling in different vascular beds and species. This led to the assumption that there are several distinct EDHF's acting alone, in parallel, or even together<sup>107</sup>. Although NO is the predominant endothelium-derived vasodilator in conduit arteries, as one descends the vascular tree the role of NO diminishes, whereas the influence of EDHF increases<sup>108</sup>. In this way, the absolute vasodilator capacity of the arterial system is maintained. In mice, EDHF-mediated responses in resistance vessels appear to be at least as important as endothelium derived NO in mediating agonist-induced, endothelium-dependent vasodilatation because neither deletion of the gene encoding eNOS nor inhibition of NOS attenuates agonist-induced vasodilator responses in vivo and in vitro<sup>109, 110</sup>. The large contribution of EDHF to endothelial vasodilatory mechanism in resistance-sized arteries and arterioles, which play a critical role in the control of organ blood flow and peripheral resistance, makes it probable that the EDHF system is an important determinant in regulating blood pressure and vascular homeostasis. There is some evidence that EDHF contributes to regulation of blood flow in vivo<sup>111-113</sup>, however, extensive in vivo characterization of the physiological role of EDHF has been delayed because of the complicating effects of the agents used to explore EDHF responses. As also NO<sup>114</sup> and  $PGI_2$ <sup>115</sup> can elicit dilatation via hyperpolarization of vascular smooth muscle cells, the isolation of EDHF responses necessitates NOS and COX inhibition, which can have profound inherent hemodynamic effects. Recently, Scotland et al. described the vascular phenotype of mice with targeted disruption of both the predominant endothelial isoform of NOS (eNOS) and COX (COX-1) and demonstrated that this "EDHF mouse" is the ideal model to investigate the physiological role of EDHF<sup>116</sup>. Those mice showed that the EDHF system is capable of overcoming the loss of NO and  $PGI_2$ . Interestingly, this occurs more effectively in female "EDHF mice", which are normotensive, whereas male "EDHF mice" are hypertensive. This gender-specific prevalence of EDHF may point to the cardiovascular protective role of the EDHF system in females. Sex-related differences in EDHF activity were also reported in estrogen receptor  $\alpha$  and  $\beta$  knockout mice<sup>117</sup> and soluble epoxide hydrolase deficient mice<sup>118</sup>. Studies using transgenic mice in which the expression of the small-conductance  $Ca^{2+}$ -activated  $K^+$  channels ( $SK_{ca}$ ) is constitutively suppressed by dietary doxycycline<sup>119</sup> and mice deficient in

intermediate-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels ( $\text{IK}_{\text{ca}}$ )<sup>120</sup>, further highlighted the functional role of EDHF in the control of vascular tone. Nowadays, there is growing evidence for the importance of the  $\text{SK}_{\text{ca}}$  and  $\text{IK}_{\text{ca}}$  channels in endothelial hyperpolarisation, which is first required to initiate EDHF-mediated vasodilatation. Both suppression of the  $\text{SK}_{\text{ca}}$  channels and deletion of the  $\text{IK}_{\text{ca}}$  channels increased the mean arterial blood pressure with respectively 20 mm Hg and 14 mmHg<sup>121</sup>. The finding that genetic manipulation of the EDHF signaling has an impact on systemic blood pressure supports the notion that the EDHF system is important in the overall circulatory regulation.

### I.3.2. Role of the NO/cGMP pathway in penile erection

Penile erection is a complicated neurovascular event modulated by psychological factors and hormonal status. Not all details of the mechanism are fully understood but ultimately an erection results from an increased blood flow into the penis and a concomitant decreased outflow through the compressed venous outflow. The engorgement is possible by relaxation of the blood vessels and the specialized smooth muscle cells of the penis, which is mainly mediated by the NO/cGMP signaling pathway.

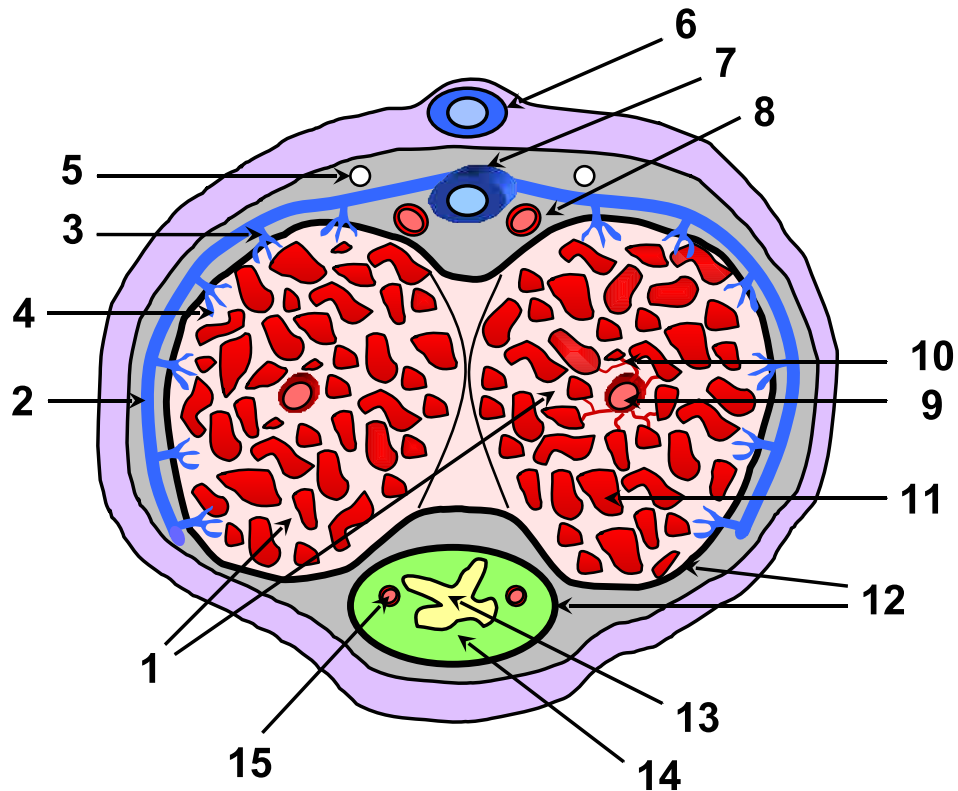
#### *I.3.2.1. Anatomy of the penis*

The penis in man and in most other mammalian species consists of three corpora: dorsally paired corpora cavernosa (CC) which comprise the erectile tissue and the ventral corpus spongiosum that surrounds the urethra and forms the glans penis distally (Figure I.4). Surrounding each cavernosal body is a thick connective tissue sheath, the tunica albuginea, which fuses in the midline to form a perforated septum. The proximal portion of each CC, the crus, is attached to the inferior pubic ramus. Each crus is surrounded by the ischiocavernosus muscle. The proximal part of the corpus spongiosum, the bulb, lies in the midline in the perineum enclosed by the bulbospongiosus muscle. Both the ischiocavernosus and the bulbospongiosus muscles are important in producing a rigid erection and in ejaculation<sup>122</sup>.

The CC resembles a sponge, composed of a meshwork of interconnected hollow cavernous spaces or sinusoids, lined by vascular endothelial cells and separated by trabeculae, containing bundles of smooth muscle cells in a framework of elastic fibers and connective tissue<sup>123</sup>. The main arterial supply to the penis is from the pudendal artery on each side that divides into the cavernosal and dorsal arteries. From the two cavernosal arteries, the resistance-like helicine arteries branch off and flow into the sinusoids, which fill with blood

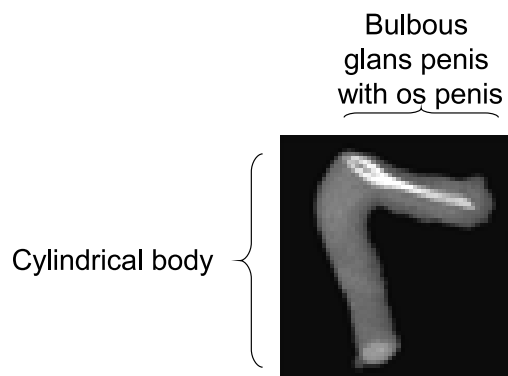
and expand during an erection. The venous drainage of the penis consists of emissary veins that drain the sinusoidal spaces and pierce the tunica to drain into the circumflex veins, and then join the deep dorsal vein of the penis. Two dorsal arteries in the dorsal neurovascular bundle (which consists of two dorsal arteries, the deep dorsal vein, and the dorsal nerves) supply the glans penis. The flow of blood into and out of the penis is regulated by the autonomic erection centre. This provides sympathetic and parasympathetic input to the pelvic nerve plexus including innervation of the cavernosal arteries and trabecular smooth muscle<sup>124</sup>.

In a diverse range of mammals including carnivores, bats, rodents, insectivores, flying lemurs, and some primates, an os penis or baculum has been described. The os penis, designed for ready intromission, often shows great morphological diversity among even closely related species, especially among rodents and carnivores<sup>125</sup>. It is a heterotopic bone that in rats and mice extends from the distal end of the body of the penis to the tip of the glans penis and is responsible for the right angle bend between the body and the glans penis in a non-erect penis (figure I.5)<sup>126</sup>. It consists of a proximal segment and a distal segment. The proximal segment is formed by fusion of a hyaline growth cartilage in its proximal half and a membrane bone in its distal half. The distal segment is formed initially as fibrocartilage, and, in case of rats, becomes gradually ossified<sup>127, 128</sup>. Although there is no os in human glans, a strong equivalent-ligamentous structure, termed the distal ligament is arranged centrally and acts as a supporting trunk for the glans penis. Without this important structure, the glans would be too weak to bear the bulking pressure generated during coitus and too limber to serve as a patent passage for ejaculation, and it could be too difficult to transmit the intracavernosal pressure (ICP) surge along the entire penis during ejaculation. A fixed os penis in the human male would cause much awkward inconvenience in carrying out daily activities; therefore this strong distal ligament is optimal for concealment without sacrifice of tissue strength<sup>129</sup>.



**Figure I.4:** Cross section through the human penis. 1: corpora cavernosa; 2: circumflex vein; 3: emissary vein; 4: subtunical venule; 5: nerve (n. dorsalis penis); 6: superficial vein (v. dorsalis); 7: deep dorsal vein (v. dorsalis profunda); 8: dorsal artery (a. dorsalis penis); 9: cavernosal artery (a. profunda penis); 10: helicine artery; 11: sinusoidal space; 12: tunica albuginea; 13: urethra; 14: corpus spongiosum; 15: urethral artery.

Penile structure in mammals is thought to be generally similar to that in humans (adapted from Morton et al. <sup>130</sup>)



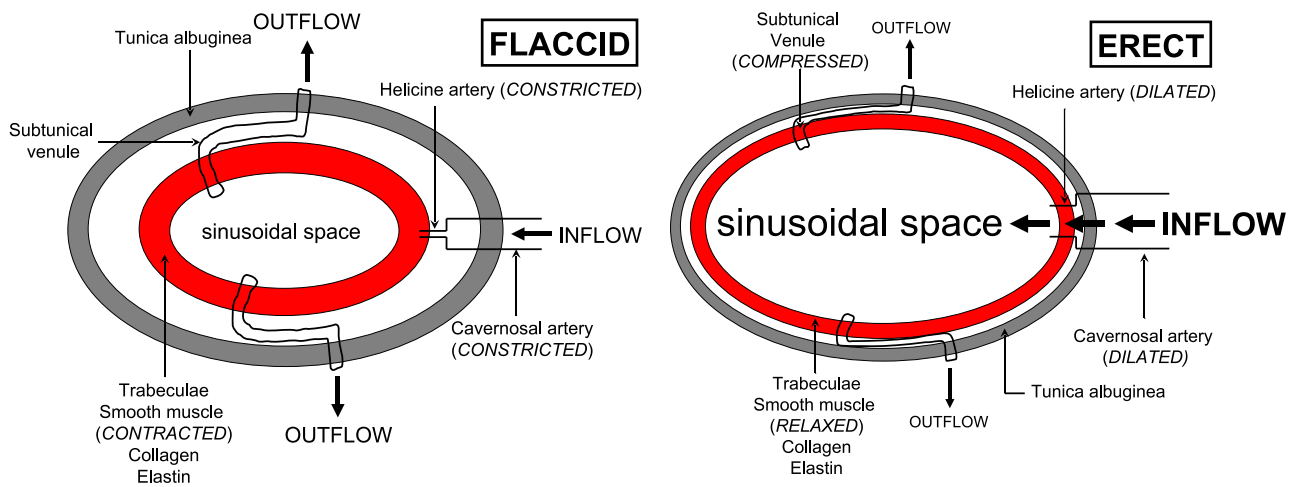
**Figure I.5:** Radiograph of the mice penis (adapted from Goyal et al. <sup>126</sup>)

### *1.3.2.2. Physiology of the penile erection*

Erection is basically a spinal reflex that can be initiated by recruitment of penile afferents but also by visual, olfactory and imaginary stimuli. The generated nervous signals will influence the balance between the contracting and relaxing factors, which control the degree of contraction of the corporal smooth muscle cells and thus the functional state of the penis <sup>131</sup>.

Under basal conditions, cavernosal vasoconstriction narrows the arteriolar lumen and sinusoids, restricting blood flow to maintain low ICP and a flaccid penis (figure I.6). Various studies have suggested the endogenous constriction to be mediated primarily by the sympathetic nerve release of norepinephrine <sup>132-134</sup> and secondarily through the local production of endothelin-1 <sup>135-137</sup>. On sexual stimulation, nerve impulses cause the release of neurotransmitters from the parasympathetic nerve terminals and of relaxing factors from the endothelial cells, resulting in the relaxation of smooth muscle in the arteries and arterioles supplying the erectile tissue (figure I.6). This leads to a several fold increase in the penile blood flow and ICP. When the pressure begins to exceed diastolic pressure, there is a pulsing of blood flow into the penis during systole. At the same time, relaxation of the trabecular smooth muscle increases the compliance of the sinusoids, facilitating rapid filling and expansion of the sinusoidal system <sup>138</sup>. There is an established base of evidence to support the idea that NO released during NANC neurotransmission and from the endothelium is the principal neurotransmitter mediating penile erection <sup>139</sup>. The neuropeptide vasoactive-intestinal polypeptide (VIP) <sup>140</sup> and prostanoids <sup>122</sup> have also been identified and shown to exert potent relaxing actions on penile tissue but the physiological role and involvement in the erectile process remains unclear. The corpora cavernosa will expand until they are restricted by the tunica albuginea. Herewith, the emissary veins and subtunical venules are compressed between the trabeculae and the tunica albuginea, resulting in an almost total occlusion of venous outflow. This process, known as veno-occlusion <sup>141</sup>, temporarily retains the blood within the corpora cavernosa and further increases ICP to approximately 100 mmHg. During sexual intercourse, the ischiocavernosus muscles forcefully compress the base of the blood-filled corpora cavernosa and the tumescence of the penis even increases, with an ICP equal to the mean arterial blood pressure <sup>123</sup>. During this phase, the inflow and outflow of blood are approximately equal, and ICP remains constant <sup>142</sup>. A veno-occlusive mechanism has not been demonstrated in the corpus spongiosum. Thus the corpora cavernosa may be a “closed system”, whereas the corpus spongiosum is a “one-way flow system”, draining freely through

the penile veins<sup>123</sup>. Therefore, the corpus spongiosum does not contribute to the rigidity and may, in fact, serve only to make the urethra an efficient conduit for the ejaculate<sup>138</sup>.



**Figure I.6:** Schematic illustration (only one sinusoidal space is depicted) of the mechanisms of erection and flaccidity. During erection, relaxation of the trabecular smooth muscle and vasodilatation of the cavernosal and helicine arteries, results in a several fold increase in blood flow, which expands the sinusoidal spaces to lengthen and enlarge the penis. The expansion of the sinusoids compresses the subtunica venules against the tunica albuginea, resulting in a physically obstructive reduction in outflow of blood. This simultaneous increase in outflow resistance, along with an increased inflow and relaxed sinusoidal smooth muscles produces an erection. In the flaccid state, inflow through the constricted and tortuous helicine arteries is minimal, and there is free outflow via the subtunica venular plexus.

### 1.3.2.3. Source of NO

Among several organs of the body in which it was early found to operate, also the penis as well as female genital tissue was shown to be a site of action of NO<sup>143</sup>. Several functional studies involving isolated corporal tissues obtained from different specimens showed that NO causes relaxation of the corporal smooth muscle cells and thereby induces penile erection. The fact that those relaxant responses were susceptible for tetrodotoxin or inhibition of axonal conduction and resistant to cholinergic and adrenergic receptor blockade, affirmed the NANC neurogenic basis of the responses<sup>144-146</sup>. The biological role for NO as mediator of cavernosal smooth muscle cell relaxation was strengthened by several in vivo studies in which the electrical stimulation of cavernous nerves and subsequent increase in the intracavernous pressure could be inhibited by NO synthase inhibitors<sup>145, 147, 148</sup>. Moreover,

immunohistochemical tracing methods localized NO's synthetic enzyme, nNOS in the autonomic innervation of the penis and the pelvic ganglion <sup>149, 150</sup>.

The ultimate proof for the neurotransmitter role of NO in the NANC mediation of penile erection would be lent by mice lacking the nNOS gene (nNOS<sup>-/-</sup> mice). Surprisingly, although the absence of nNOS in those mutant mice, NO-dependent erectile function was preserved <sup>151</sup>. Further exploration of this discrepancy, revealed the alternative expression of splice variants of the nNOS gene. Residual NO produced by the shorter nNOS variant, nNOS $\beta$  is shown to be sufficient for normal physiological functioning and explains why the nNOS<sup>-/-</sup> mice maintain erection <sup>152</sup>. Another important variant of the nNOS cDNA, penile nNOS also survives in the nNOS<sup>-/-</sup> mice and thus has a functional significance for penile erection in those mice <sup>153</sup>. The other NO-producing enzymes eNOS and iNOS were also proposed as candidates for the NO-dependent penile erection in the nNOS<sup>-/-</sup> mice. As it was not possible to demonstrate the presence of iNOS in the penile tissue of nNOS<sup>-/-</sup> mice, it does not appear to be the source of NO in those mice <sup>151</sup>. There is however evidence for a modulating role of iNOS in the erectile response, since mice deficient in iNOS (iNOS<sup>-/-</sup>), showed an altered function in the corporal tissue. However, further study is needed to affirm a potential role of iNOS in erectile function in vivo <sup>154</sup>.

By contrast, the eNOS gene expression is significantly upregulated in nNOS<sup>-/-</sup> corporal tissue, indicating a role for endothelial derived NO in mediating corporal smooth muscle relaxation. In many instances, compensatory mechanisms preserve the function in transgenic mice engineered with disruption of specific genes. Therefore the augmented eNOS activity in nNOS<sup>-/-</sup> mice presumably compensatory overcame the nNOS deficiency, which might suggest that nNOS derived NO is the primary source involved in penile erection <sup>151, 155</sup>. However, the contribution of eNOS to the regulation of penile erection should not be underestimated, since the administration of the eNOS agonist, carbachol elicited penile erection in wild type mice but not in eNOS<sup>-/-</sup> mice. This suggests that eNOS physiologically would mediate erection via cholinergic stimulation <sup>156</sup>. Moreover, it has been shown that upon nerve stimulation, acetylcholine can be released in the presence of endothelial cells, since neurons staining for choline acetyltransferase extend branches throughout the corpora cavernosa including the area of the endothelium <sup>157</sup>. Despite several arguments in favour of the involvement of an endothelium dependent mechanism in penile erection, there are also arguments that do not support this hypothesis e.g. in isolated monkey and dog penile tissues, atropine and endothelium damage could not reduce the relaxant response to electrical field stimulation

(EFS)<sup>158, 159</sup> and a lack of eNOS immunoreactivity has been shown in the endothelium of cavernous sinusoids in rats<sup>160</sup>.

New concepts however, support the idea that besides the liberation of an ancillary source of NO from the penile endothelium by cholinergic stimulation, eNOS performs an even bigger assignment in penile erection. eNOS would facilitate maximal erection, whereas nNOS would initiate the penile process<sup>161</sup>. The augmented blood flow, induced by the rapid, brief activation of nNOS at the onset of penile erection, generates a shear force on the endothelium, which activates the phosphatidyl-inositol 3-kinase / Akt pathway. The phosphorylated serine/threonine kinase Akt causes in turn direct phosphorylation of eNOS<sup>20</sup>. Classified as a Ca<sup>2+</sup>/calmodulin-dependent enzyme, eNOS is normally activated by agonists after an increase in the intracellular Ca<sup>2+</sup> concentration<sup>162</sup>. Phosphorylation however, reduces its Ca<sup>2+</sup> requirement and makes a maintained (over hours) production of NO and thus more sustained relaxation possible<sup>163</sup>.

Without any doubts, we can conclude that NO, produced by nNOS, acts as a neurotransmitter of NANC nerves innervating the penile tissue and plays a major role in penile erection. The role of eNOS derived NO is not established. However, irrespective of the fact that its relative importance is species-dependent<sup>164</sup>, it can be accepted that in response to chemical and possibly mechanical stimuli, NO liberated from the endothelium also participates in penile erection.

#### *1.3.2.4. Other peripheral neurotransmitters*

##### *\*Vasoactive-intestinal polypeptide (VIP)*

The possible role of VIP as a transmitter mediating the relaxation necessary for penile erection has been discussed since the first reports were published on the occurrence of this peptide in the penis and on its effects on isolated penile tissue<sup>165</sup>. Moreover, VIP immunoreactive vesicles were localized in the nerve fibers of the CC of several species<sup>166</sup> and an increased concentration of VIP was detected in blood drawn from the CC during penile erection<sup>166, 167</sup>. However, the latter finding could not be confirmed in the studies of Dixson et al.<sup>168</sup> and Kiely et al.<sup>169</sup>. The expression of VIP in rat CC appears to be androgen-independent and to be unaffected by age of the animals<sup>170, 171</sup>. However, an impairment of VIP-ergic innervation in penile tissue of diabetic rats<sup>172</sup> as well as a decreased level of VIP has been observed in the penis of impotent men<sup>173, 174</sup>. Despite all efforts, no definitive consensus has been reached as to the exact role of VIP in the physiology of penile erection.



Many *in vitro* studies of strips of human CC tissue and cavernous vessels have shown that VIP has an inhibitory and relaxation-producing effect<sup>175, 176</sup>, but it has been difficult to show convincingly that VIP released from nerves is responsible for relaxation of penile smooth muscle. Whilst the relaxant activity of exogenously administered VIP was blocked by VIP antiserum or  $\alpha$ -chymotrypsin, it had no effect on the relaxation induced by EFS of the nerves, suggesting that VIP is unlikely to be the NANC transmitter under these conditions<sup>177</sup>. Further evidence that VIP is not the key NANC transmitter mediating erection is that intracavernosal administration of the peptide did not produce full erections in normal<sup>178</sup> or impotent men<sup>169, 179</sup>. However, the use of higher doses of VIP in combination with phentolamine has been reported to induce erections sufficient for intercourse<sup>180</sup>. Moreover, Kim et al. reported that in rabbit CC a VIP antagonist inhibited electrically induced relaxations, suggesting that the peptide was released from nerves during stimulation<sup>181</sup>. They concluded that VIP appeared to contribute to NANC mediated cavernosal relaxation, and that its mechanisms of relaxation are dependent on prostanoids and the generation of NO. This suggestion is supported by reports on the colocalization of VIP and NOS in the parasympathetic nerves innervating the CC and penile vasculature<sup>182-184</sup>. It seems that most of these NO- and VIP-containing neurons are cholinergic, since they all contain the vesicular acetylcholine transporter<sup>185</sup>, which has been suggested to be a unique marker for investigation of the distribution of cholinergic nerves in the peripheral and central nervous systems<sup>186</sup>. Also in the submandibular gland<sup>187, 188</sup>, VIP has been shown to contribute to the parasympathetic control of blood flow via NO. However, Hempelmann et al., who studied the effects of NO and the NO donor SIN-1 on human isolated cavernous artery and CC, found nonsynergistic, independent relaxant effects in both types of preparation<sup>189</sup>. This finding does not favour the view of cooperative actions in neurotransmission or the simultaneous use of these agents in the treatment of erectile dysfunction. Not only NOS but also other peptides seem to be colocalized with VIP. Peptide histidine methionine<sup>190, 191</sup>, pituitary adenylate cyclase-activating peptide<sup>192</sup> and helospectin<sup>193</sup> have been shown to be localized to nerves in close proximity of bundles of smooth muscle, around arteries in the human CC and in circumflex veins<sup>194</sup>. Like VIP, those peptides seem to act through one of the VIP receptors<sup>195</sup>, leading to activation of AC and increase in cAMP. They were found to be as effective as VIP for producing relaxation of human corporal tissue and for inhibiting contractions evoked by electrical stimulation of nerves<sup>193</sup>. However, a role for those peptides as neurotransmitters and/or neuromodulators in the nervous control of penile erection has not been established.

*I.3.2.5. Other endothelium derived modulators of penile erection*

In the periphery, NO appears to play a key, if not pivotal role in the erectile process. Still, as with other vascular beds, the endothelium in the penis (both arterial and trabecular) produces a number of other endothelium-derived vasoactive factors, which may also be important in the control of corporal smooth muscle tone.

*\*Prostaglandins*

The widespread use of intracavernosally injected PGE<sub>1</sub> for the treatment of erectile dysfunction<sup>196, 197</sup> focused interest on the physiological functions of prostanoids in the mechanisms of penile erection. The ability of penile tissue to generate and metabolise prostaglandins (PGE<sub>2</sub>, PGI<sub>2</sub>, PGE<sub>1</sub> and PGF<sub>2α</sub>) has amply been demonstrated in vitro<sup>198-201</sup>. In strips of rabbit CC, there is a basal or tonic release of prostanoids including PGF<sub>2α</sub>, TXA<sub>2</sub>, PGI<sub>2</sub>, PGE<sub>2</sub> and PGD<sub>2</sub>. Incubation of cavernosal strips with acetylcholine increases the level of all these prostaglandins, as determined by radioimmunoassay, implying that endothelium-dependent stimulation by acetylcholine results not only in the production of NO, but also in stable COX products<sup>202</sup>.

PGI<sub>2</sub> is known to be a potent vasodilator in a number of vascular beds<sup>58, 203</sup>. By contrast, in human CC tissue, PGI<sub>2</sub> released by muscarinic receptor stimulation was reported to cause contraction<sup>198</sup>. Also when added to isolated corporal preparations pre-contracted with noradrenaline or PGF<sub>2α</sub>, PGI<sub>2</sub> had no relaxant effect. Isolated pre-contracted preparations of the cavernosal artery, on the other hand, were effectively relaxed<sup>204</sup>, which suggests that PGI<sub>2</sub> may serve as a vasodilator in the initial phase of penile erection<sup>205</sup>. Direct intracavernous injection of PGI<sub>2</sub> into the penis of pigtailed monkeys resulted in a large reduction of the cavernosal compliance, owing to smooth muscle contraction, but there was no increase in cavernosal arterial blood flow<sup>206</sup>. This argues against the fact that PGI<sub>2</sub> directly contributes to regulation of corporal smooth muscle tone. It is more likely that during penile enlargement and blood stasis this prostanoid, synthesised from the vascular endothelium and corporal trabecular tissue, counteracts local thrombosis formation<sup>123</sup>.

By contrast, PGE<sub>1</sub>, seems to be a better candidate for the modulation of the corporal smooth muscle tone. PGE<sub>1</sub> effectively relaxed human trabecular tissue, and pre-contracted segments of the cavernous artery<sup>204</sup>. Furthermore, radioligand binding studies have demonstrated the presence of PGE<sub>1</sub> receptors in penile tissues from humans and monkeys<sup>207, 208</sup>. Correspondingly, intracorporal injection of PGE<sub>1</sub> in those species resulted in erection. Besides

through the activation of AC and the subsequent increase in the production of cAMP<sup>209</sup>, PGE<sub>1</sub> has been shown to decrease the [Ca<sup>2+</sup>]<sub>i</sub> through inhibition of the voltage-dependent L-type Ca<sup>2+</sup> channels<sup>210</sup>. Moreover, PGE<sub>1</sub> appears to inhibit the electrical release of noradrenaline from human penile adrenergic nerves, which may contribute to its relaxant action. In contrast, PGD<sub>2</sub> increases its release, suggesting the possibility that prostanoid receptors may exist presynaptically, thereby modulating tonic sympathetic tone<sup>211</sup>. Moreover, constrictor prostanoids have been shown to negatively modulate the antagonizing effect of NO on the adrenergic tone. In rabbit CC, the contractile response to noradrenaline, following treatment with the sGC inhibitor LY-83583 (thereby blocking the effect of NO) was significantly increased but reversed by COX blockade<sup>212</sup>. This indicates that under physiological conditions, cGMP may not only be a second messenger involved in smooth muscle relaxation, but it may also directly inhibit the contractile effects of noradrenaline. This alteration in vascular reactivity secondary to altered NO production has also been shown in a number of other vascular beds<sup>213</sup>. For example, in rat caudal artery<sup>214</sup>, endothelium derived NO/cGMP was shown to directly attenuate contractile responses to exogenous noradrenaline and sympathetic EFS induced contractions. This process appears to be modulated by prostaglandins, which sensitize the smooth muscle cell to  $\alpha$ -agonist stimulation. Besides corporal smooth muscle, the sensitizing effect has also been reported in other vascular models, i.e. human chorionic artery<sup>215</sup> and pig testicular artery<sup>216</sup>. In those ring segments treated with L-NA, there was an increase in the resting tension and enhanced contractile responses to potassium and serotonin respectively. Both these effects were reduced in the presence of indomethacin. These findings indicate that there is an important physiological interaction between contractile prostanoids and endothelium-derived NO. Under pathological conditions of impaired NO production, the unopposed actions of the contractile prostanoids may result in an increase in corporal smooth muscle tone, thus contributing to the development of erectile dysfunction<sup>217</sup>.

#### *\*EDHF*

In addition to NO, recent reports indicate that EDHF may be critical to successful erection. Angulo et al. demonstrated that in human penile resistance arteries (HPRA) a consistent relaxation to acetylcholine remains after NOS and COX inhibition that is abolished by a high extracellular K<sup>+</sup> concentration that prevents hyperpolarisation of smooth muscle and endothelium<sup>218</sup>. This observation is consistent with the existence of a hyperpolarizing factor

contributing to endothelium-dependent dilatation in horse penile resistance arteries <sup>219</sup> and with the relevant role of relaxations mediated by EDHF in other human resistance vessels <sup>112, 220</sup>. Additionally, calcium dobesilate, an angioprotective agent that is proposed to potentiate the vasodilator effect of EDHF in human arteries, significantly improved endothelium-dependent EDHF-like relaxation of HPRA and potentiated erectile responses *in vivo* <sup>218</sup>. Further evidence for the involvement of EDHF in the endothelium-dependent relaxation is provided by the other study of Angulo et al., showing that sildenafil, which potentiates the NO/cGMP pathway through inhibition of PDE-5, was able to completely reverse endothelial dysfunction in human CC from diabetic patients but not in penile resistance arteries from those patients. Moreover, calcium dobesilate completely recovered endothelial function in penile arteries from diabetic patients <sup>221</sup>. However, administration of DOBE also failed to significantly improve erectile function in diabetic rats while it had significant potentiating effects in nondiabetic rats <sup>222</sup>. Those findings demonstrate that potentiation of EDHF alone is insufficient to reverse endothelial dysfunction or that normal function of both CC and penile arteries is essential for the hemodynamic events that initiate and maintain penile erection. The observations with calcium dobesilate are in line with a study showing that chronic treatment with evening primrose oil did not improve endothelium-dependent relaxation of CC from diabetic rats while this treatment recovered endothelial function in mesenteric resistance vessels from these animals by enhancing EDHF-mediated responses, thus in a similar way as DOBE differentially affects endothelial function in CC and penile arteries <sup>223</sup>. Several candidates for EDHF have been proposed, and CYP epoxygenase metabolites, epoxyeicosatrienoic acids (EET's) are one of the several putative mediators of endothelium-dependent relaxation. Recently, Jin et al. proposed that EET's function as endothelium-derived relaxing factors in penile tissue and are essential to achieve full erection <sup>224</sup>. In human penile arteries, the nonselective inhibition of CYP activities by miconazole, significantly attenuated the effect of DOBE on EDHF-mediated relaxations, suggesting that this agent is related to the enhancement of the production or the action of CYP derivatives <sup>218</sup>. Additionally, the pharmacological inhibition of CYP by sulfaphenazole, attenuates cavernosal nerve mediated erectile responses in the rat, suggesting that a CYP metabolite may mediate an EDHF-dependent smooth muscle effect in the penis that may contribute to the erectile response <sup>225</sup>. As this area of penile vascular biology is not fully elucidated, further research must be undertaken to evaluate the potential importance of this endothelium-derived relaxing factor in the regulatory control of penile erection.

### *I.3.2.6. RhoA/Rho kinase*

The RhoA/Rho kinase signaling pathway (see also caption I.2.2.2. Regulation of MLC dephosphorylation/ $\text{Ca}^{2+}$  sensitization) is suggested to be the major  $\text{Ca}^{2+}$  sensitization mechanisms in the smooth muscle.  $\text{Ca}^{2+}$  sensitization is brought about by agonist activation of heterotrimeric G-protein-coupled receptors, the exchange of GTP for GDP on the small GTPase RhoA, its activation and dissociation from its partner Rho-GDI (guanine nucleotide dissociation inhibitor). GTP-RhoA activates Rho-kinase, which inhibits MLCP by phosphorylating its regulatory subunit; this leads to an increase in MLC20 phosphorylation and tension at constant  $[\text{Ca}^{2+}]_i$ <sup>226</sup>. This mechanism has been shown to make a significant contribution to agonist-induced contraction under physiological conditions in peripheral vasculature<sup>227, 228</sup>. Using an in vivo rat model, the functional role of RhoA/Rho kinase was also demonstrated in penile corpus cavernosum. Injection of the specific Rho kinase antagonist Y-27632 into the CC sinuses, without nerve stimulation, resulted in a dose-dependent increase in ICP<sup>229</sup>. This suggests that the RhoA/Rho kinase is constantly active and plays an important role in maintaining the penis in the flaccid state. Inhibition of Rho kinase also potentiates EFS-induced erections<sup>229</sup>. Electrical stimulation of the major pelvic ganglion has been demonstrated to result in a voltage-dependent increase in ICP, a process that is inhibited by NOS blockade (and is thus attributed to NO release from NANC nerves)<sup>230</sup>. Additional experiments have demonstrated a similar potentiation of the effect of Rho kinase inhibition on ICP upon prior injection with a NO donor drug<sup>231</sup>. These data suggest that NO potentiates the effect of Y-27632 and provide support for the hypothesis that NO-induced penile erection is through the inhibition of endogenous Rho kinase constrictor activity. However, pre-injection of L-NA and MB or ODQ did not significantly alter the effect of Rho kinase antagonism, indicating that the Y-27632-induced rise in ICP is independent of a NO-mediated pathway<sup>229, 232</sup>. Thus, antagonism of Rho kinase may offer a potential alternate avenue for the treatment of erectile dysfunction. Supporting such a suggestion, Wilkes et al. investigated the effects of Y-27632 on erectile responses in male SHR, and found a significant increase in neurogenic response after intracavernosal injection of Y-27632. They suggested that an increased hypertension-induced vasoconstrictor tone and impaired erectile response may be due to up-regulation of Rho kinase activity<sup>233</sup>. Additionally, diabetic rabbit CC have been shown to have an increased sensitivity to stimulation by the endothelin-1 agonist<sup>234</sup>, an increased expression of Rho kinase and

decreased relaxation of Y-27632<sup>235</sup>. Also in aging male Brown-Norway rats, Rho kinase inhibition with Y-27632 was reported to improve erectile function<sup>236</sup>.

In vitro, Rho kinase inhibition has been shown to inhibit CC smooth muscle tone including human and rabbit<sup>237</sup>. Furthermore, Y-27632 was found to relax isolated rat CC tissue that had been precontracted with KCl or phenylephrine to a similar extent<sup>229</sup>. As in conduit vessels pre-constricted with KCl the effect of Y-27632 on vasorelaxation was attenuated as opposed to phenylephrine<sup>238, 239</sup>, Rho kinase activity is suggested to be elevated in penile vasculature, as compared to conduit arteries. In addition, RhoA protein concentration was reported to be 17 times greater in rabbit CC than in ileum, bladder, femoral artery and portal vein<sup>240</sup> suggesting that RhoA/Rho kinase  $Ca^{2+}$  sensitization may play a substantial role in CC smooth muscle contraction. Furthermore, the detection of the MLCP inhibitory protein CPI-17 in human and rabbit CC, supports the possibility of CPI-17-mediated  $Ca^{2+}$  sensitization in the penis<sup>240</sup>. Finally, the adeno-associated viral gene transfer of a dominant-negative RhoA mutant into the rat CC, confirmed the significance of the RhoA/Rho kinase signaling pathway in penile erection, as the mutant markedly increases basal ICP as well as cavernous nerve stimulation-induced ICP in vivo<sup>241</sup>.

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## **I.4. Summarising conclusions**

sGC is the only conclusively proven receptor for NO and so intimately involved in a wide range of physiological functions, most notably in the cardiovascular system (platelet reactivity, vascular tone) and peripheral and central nervous system (neurotransmission). In its molecular makeup, sGC exists as an obligatory  $\alpha\beta$ -heterodimer, of which 2 two isoforms for each subunit have been found ( $\alpha_1/\alpha_2$  and  $\beta_1/\beta_2$ ). However, only two isoforms (sGC $\alpha_1\beta_1$  and sGC $\alpha_2\beta_1$ ) so far have been shown to be physiologically active in which  $\beta_1$  acts as the dimerizing partner for both types of  $\alpha$  subunits. While sGC $\alpha_1\beta_1$  is ubiquitously expressed in mammalian tissue, sGC $\alpha_1\beta_2$  is less widely distributed. The low basal activity of sGC increases several hundred-fold when NO binds to the heme prosthetic group, which is associated with the N-terminal region of the  $\beta_1$  subunit through a weak histidine 105-iron bond. The histidine-residue is essential for the stimulation by NO as substitution by phenylalanine leads to a heme-deficient enzyme that retains basal cyclase activity but fails to respond to NO. In addition to endogenous NO, pharmacological NO-donors (e.g. nitroglycerin, isosorbide dinitrate and SNP) and agents such as BAY 41-2272 and YC-1 stimulate sGC, the latter two also in an NO-independent way. Upon activation of sGC, GTP is converted to cGMP. Acting as a second messenger, cGMP regulates the activity of several enzymes including PKG, CNG ion channels and cGMP-regulated phosphodiesterases (e.g. PDE-5) which culminates in a reduction of intracellular  $\text{Ca}^{2+}$  concentration and a decrease in the sensitivity of the contractile system to  $\text{Ca}^{2+}$ . Both mechanisms ultimately lead to smooth muscle relaxation, which is the key event in penile erection and a very important determinant of blood flow and blood pressure.



# Chapter II

Aims of the work



## **II.1. General aim**

The physiological importance of NO/cGMP signaling is now widely appreciated. As the predominant intracellular receptor for NO and as cGMP-producing enzyme, sGC plays a pivotal role in regulating smooth muscle tone, thereby controlling, among other things, blood pressure <sup>1</sup> and penile erection <sup>2</sup>. For a long time, the scientific attention for sGC remained significantly lower than the focus on NO itself. However, the finding that different sGC isoforms (the main sGC $\alpha_1\beta_1$  and minor sGC $\alpha_2\beta_1$ ) and splice variants exist and that other substances besides NO <sup>3</sup> can activate sGC, has led to a renewed interest in the sGC enzyme. Nonetheless, the involvement and the specific role of the different sGC isoforms remain elusive. This is mainly due to the unavailability of sGC isoform-specific inhibitors. Therefore, targeted disruption of the genes encoding the various sGC subunits is a valuable approach to study the function of each isoform. In the present study we made use of the advantages of sGC subunit knockout/knockin mice to explore the **functional importance of the sGC subunits in smooth muscle relaxation**. Knowledge hereof could allow the development of more selective sGC agonists with potentially less-side effects.

## **II.2. Specific aims**

### **II.2.1. Relaxation of vascular smooth muscle cells in sGC $\alpha_1$ knockout mice**

#### **(sGC $\alpha_1$ <sup>-/-</sup> mice)**

Known the significance of endothelial NO-signaling, many cardiovascular disorders are related with an impairment of the NO/cGMP signaling cascade. Current therapies that involve the use of organic nitrates and other NO donors have severe limitations, including unpredictable pharmacokinetics of NO, lack of response and the development of tolerance following prolonged administration <sup>4</sup> and peroxynitrite formation, which can lead to protein S-nitrosylation <sup>5</sup>, as well as tyrosine nitration <sup>6</sup>. Understanding of the functional importance of the different sGC isoforms, is of interest in developing improved NO-independent therapeutic strategies for the treatment of NO/cGMP signaling pathway-related disease. Therefore, by the use of aortic and femoral artery segments isolated from mice deficient for the sGC $\alpha_1$  gene (sGC $\alpha_1$ <sup>-/-</sup> mice), we explored **the physiological contribution of the sGC $\alpha_1\beta_1$  isoform in sGC-regulated vascular smooth muscle relaxation (chapter IV)**.

### II.2.2. Relaxation of corpus cavernosum smooth muscle cells in sGC $\alpha_1$ <sup>-/-</sup> mice

Although there are many neurohumoral factors involved in penile erection, NO is generally accepted as the most important to initiate and maintain erection. This is also illustrated by the extensive use of therapeutic agents targeting the NO/cGMP pathway for the treatment of erectile dysfunction. Today's most successful therapy, sildenafil citrate (better known as Viagra<sup>®</sup>), is based on the decreased breakdown of cGMP by PDE-5, resulting in increased levels of cGMP<sup>7</sup>. Because PDE-5 is also present in the systemic vasculature, sildenafil can cause a synergistic and potentially life threatening hypotensive response when combined with organic nitrates. The co-administration of a nitrate within the first 24 h after a dose of Viagra<sup>®</sup> is therefore absolutely contraindicated<sup>8</sup>. Moreover, the efficacy of the treatment with PDE-5 inhibitors is significantly lower in specific populations such as patients with diabetes<sup>9, 10</sup>. Therefore, there is a call for alternative therapeutic targets. Especially since ED is worldwide estimated to affect more than 150 million men, and that number is expected to exceed 300 million men by the year 2025<sup>11</sup>. In order to determine the possible value of the sGC $\alpha_1\beta_1$  isoform as pharmacological target, we investigated the **sGC-regulated responsiveness of corpora cavernosa isolated from sGC $\alpha_1$ <sup>-/-</sup> mice (chapter V)**.

### II.2.3. Relaxation of coporal and vascular smooth muscle cells in sGC $\beta_1$ knockin mice (sGC $\beta_1$ <sup>ki/ki</sup> mice)

In the vessel and corpus cavernosum preparations isolated from the sGC $\alpha_1$  knockout mice, we observed a significantly reduced response to exogenously applied NO and NO-independent sGC activators. However, those responses were not completely abolished; there still remained a substantial relaxant effect, suggesting that besides the predominant sGC $\alpha_1\beta_1$  isoform, also the less abundantly expressed sGC $\alpha_2\beta_1$  isoform and/or an (an) sGC-independent mechanism(s) are involved. In order to characterise the mechanism responsible for the remaining response in the sGC $\alpha_1$  knockout preparations, **the sGC-regulated relaxant properties of vessel and coporal tissues isolated from sGC $\beta_1$ <sup>ki/ki</sup> mice were investigated (chapter VI)**. In those sGC $\beta_1$  knockin mice, the heme-dependent activation of sGC is abolished by the substitution of the histidine 105 residue (axial ligand) of the  $\beta_1$  subunit by phenylalanine<sup>12</sup>.

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# Chapter III

## Materials and methods



### **III.1. Introduction**

Our knowledge on basic pharmacodynamics is mainly based on studies of drugs acting on preparations of isolated tissues or blood vessels mounted in organ chambers. Such *in vitro* experiments allow to analyse the influence of local mechanisms, without the interference of other competing or compensatory mechanisms being active in *in vivo* conditions, which complicate the correct interpretation of *in vivo* data.

To study the vasoreactivity of ring segments of isolated large and small arteries, we used a wire myograph. In this method, the vessel segments are mounted in an organ bath onto two thin wires that are fixed on two holders. One of the holders is connected to a force transducer. The length of the ring segment is maintained constant while the force is recorded during contraction or relaxation. The mechanical properties of isolated corpora cavernosa were investigated using a myograph in which the tissues are clasped on two holders, based on the same principles of the wire myograph for vessel segments. The strength of this technique is that the tension measurements are very sensitive. Those functional studies were supplemented with measurements of cGMP concentrations in vascular tissue.

### **III.2. Mice models**

The sGC $\alpha_1^{-/-}$  mice, sGC $\beta_1^{ki/ki}$  mice and the corresponding control mice, used throughout the experiments, 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. The Mice 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).

#### **III.2.1. sGC $\alpha_1^{-/-}$ mice**

In the sGC $\alpha_1$  knockout mice, exon 6 of the sGC $\alpha_1$  gene, which encodes an essential part of the conserved catalytic domain, is deleted. The targeting vector was constructed using 4.3 kb 5' and 2.5 kb 3' sGC $\alpha_1$  homology fragments, isolated from a 129/SvJ 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 (ES) 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) and/or the floxed exon 6 (full knockout), targeted ES cell clones were transiently transfected with a Cre-expressing plasmid. Chimeras were generated by aggregating ES cell clones, carrying the desired deletion with Swiss morula stage embryos, and identified by agouti contribution of the ES cells to coat color. Male chimeric mice were mated to Swiss females to check whether the ES cells, containing the mutant  $sGC\alpha_1$  allele are represented in the gametes. The heterozygous offspring, identified by dominant agouti coat coloration and Southern blot analysis, were then mated to one another to produce homozygous recombinants.

### III.2.2. $sGC\beta_1^{ki/ki}$ mice

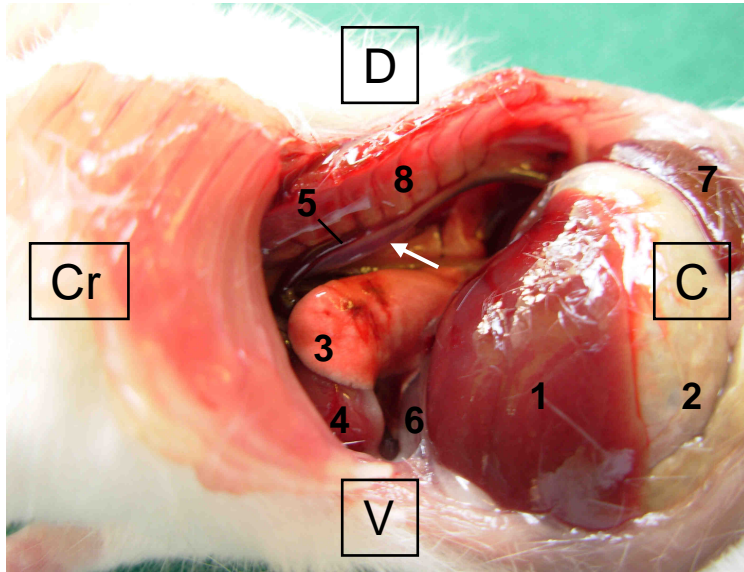
In the  $sGC\beta_1$  knockin mice, residue 105, the codon of which is located in exon 5, is converted from histidine (His) to phenylalanine (Phe). The targeting construct contains 4.1 kb 5' and 3 kb 3' flanking regions of homology cloned from a 129/SvJ lambda FIXII mouse genomic library (Stratagene), a positive and negative selection marker (a floxed neo cassette and a thymidine kinase cassette respectively). In addition to the three mutations which replace 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 region, 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  $sGC\beta_1$  knockin mice were generated following the same procedure as the  $sGC\alpha_1$  knockout mice. But, the recipient blastocyst was derived from a C57BL/6J 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.3. Arteries**

### III.3.1. Dissection

Experiments were performed on arteries isolated from adult male and female mice (age: 10-15 weeks). After cervical dislocation, the mouse is placed on its side and the skin is removed in

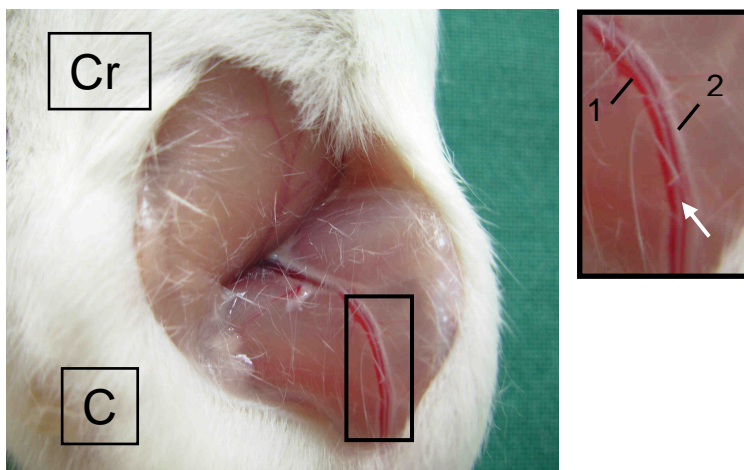
the area from the diaphragm to the foreleg. Next, the diaphragm is cut free from the ribs and the thorax is opened. The flap of the thoracic wall is turned over, exposing the heart and the lungs. Next the lungs are moved aside so that the aorta can be dissected out from the aortic arch to the diaphragm (figure III.1). The isolated aorta is then transferred to fresh Krebs-Ringer-Bicarbonate (KRB) solution and cleansed from surrounding tissue without traumatising the vessel wall. Finally, an arterial segment of about 2.5 mm in length is cut and ready for mounting in the wire myograph.



**Figure III.1:** Thorax and upper part of the abdomen of the mouse. The arrow indicates the thoracic aorta used in the study

1: liver, 2: stomach, 3: lung, 4: heart, 5: vena cava, 6: diaphragm, 7: spleen, 8: ribs, D: dorsal, V: ventral, Cr: cranial, C: caudal

For the isolation of the femoral artery, the mouse is placed on its back with the legs extended. Next, the skin of the groin area of the rear leg is removed and the femoral artery is carefully dissected free from the femoral vein (figure III.2). The dissected vessel is then transferred to fresh KRB solution and cut into segments of about 2.5 mm in length. After the ring segment is slid over the first wire (see mounting of a ring segment), the surrounding tissue is being removed

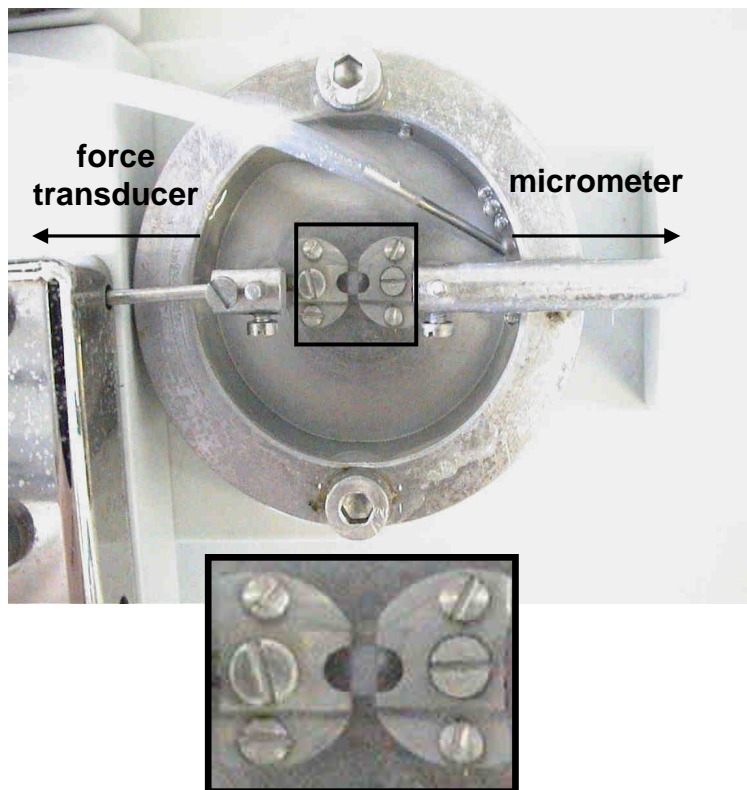


**Figure III.2:** Groin area of the mouse. The arrow indicates the femoral artery used in the study. 1: femoral vein (v. femoralis), 2: femoral nerve, Cr: cranial, C: caudal

### III.3.2. Tension measurements

#### III.3.2.1. The apparatus

We have used a manual wire myograph (figure III.3) (based on the automated dual channel vessel myograph, model 500A, JP trading, Aarhus, Denmark) constructed by the technical staff of our laboratory (Dirk De Gruytere and Cyriel Mabilde). It consists of a 10 ml organ bath with two holders in it. One holder is connected to a micrometer which is used to change the distance between the two holders and which allows precise measurement of the distance between the wires during the normalisation procedure. The other one is connected to a force-displacement transducer that measures the isometric tension changes. Each ring segment is mounted under a dissecting microscope onto two thin stainless steel wires (40  $\mu\text{m}$  diameter) that are fixed at those holders.



**Figure III.3:** A photograph of the organ bath of the wire myograph. Vessel segments are mounted on two thin wires, fixed on the two holders in the organ bath. One holder is connected to a micrometer which is used to change the distance between the wires. The other holder is connected to a force transducer which allows measurement of isometric tension changes in the ring segment.

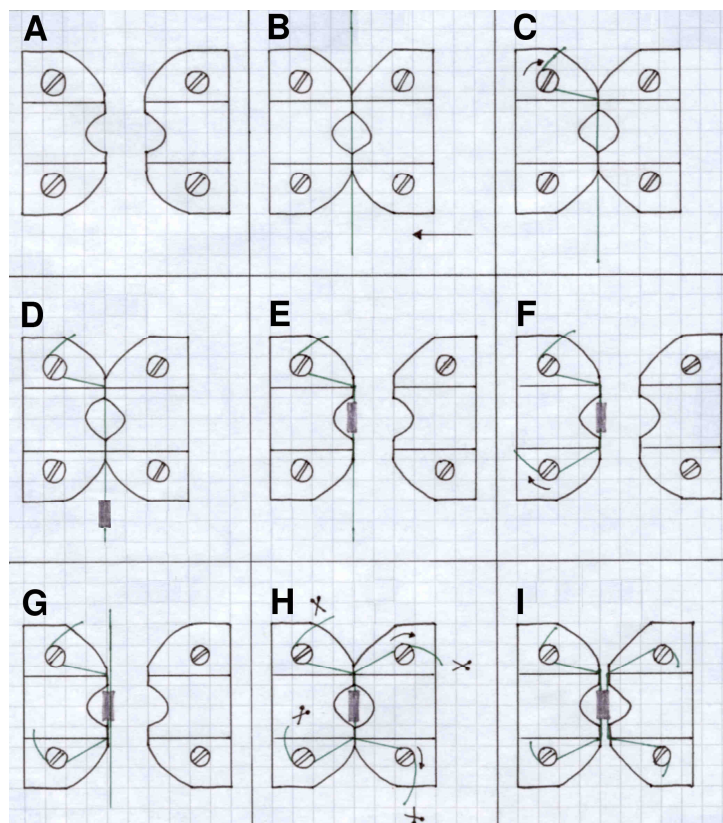
#### III.3.2.2. Mounting of a ring segment

First, a thin wire (40  $\mu\text{m}$ ) is cut to length and clamped between the two holders in the organ bath (figure III.4B). Then, the far end of the wire is fixed on the left holder with a screw, whereas the near end is kept free, pointing towards the operator (figure III.4C).

Using a pair of tweezers, the ring segment is slid over the free end of the wire (figure III.4D). Herewith, care is taken not to damage the endothelium. Next, the holders are set apart, and the vessel segment is slid further over the wire until the segment is situated just between the holders (figure III.4E). Subsequently, the free end of the wire is fixed with a screw to the other side of the left holder (figure III.4F).

Now, the second wire is guided through the lumen of the artery (figure III.4G) and the holders are brought together again. Furthermore the ends of the second wire are fixed on the right holder (figure III.4H). The wires must be levelled so that both wires are in a single horizontal plane (figure III.4I). Finally, the length of the segment (l) is measured with a micrometer eyepiece (Zeiss, Germany).

After mounting, the preparations are allowed to equilibrate in a KRB solution at 37°C, pH 7.4 and oxygenated with 95% O<sub>2</sub>-5% CO<sub>2</sub> for 30 minutes.



**Figure III.4:** Schematic representation of the different steps involved in the mounting of an arterial segment on two stainless steel wires, fixed on the holders.

*III.3.2.3. Preparation of the femoral artery segments before the experiment*

Active force development of a muscle cell depends on its length<sup>1, 2</sup>. In small arteries, the length of the circumferentially oriented smooth muscle cells can be influenced by stretching the vessel. It is commonly accepted that the active force development in vascular tissue peaks at a certain internal circumference or diameter, which corresponds to a specific length of the smooth muscle cell aiming optimal experimental conditions. The vessels should therefore be set to their optimal internal diameter at the beginning of each experiment.

It has been found that the active force production is maximal when the internal circumference is 0.9 times the internal diameter a vessel would have in situ when relaxed and under a transmural pressure of 100 mm Hg ( $IC_{100}$ )<sup>1, 3-5</sup>. To calculate the  $IC_{100}$  for a vessel mounted in the vessel myograph, we use the normalisation procedure. This allows us to determine the position of the holders corresponding to the vessels ideal internal circumference. Also, the size of the vessel, normally defined as its size when fully relaxed and under a transmural pressure of 100 mm Hg, can be calculated through the normalisation procedure.

*\*The normalisation procedure*

First of all, the vessel is brought into a resting position corresponding to a force of 0 mN. Hence, the holders in the organ bath are brought together until the mounting wires touch and the force transducer registers a negative force. Then, the holders are partly pulled back until the registered force returns to baseline. At this point, the force registered by the force transducer is 0 mN ( $Y_0$ ) and the distance between the two wires is 0  $\mu$ M (corresponding micrometer setting =  $X_0$ ).

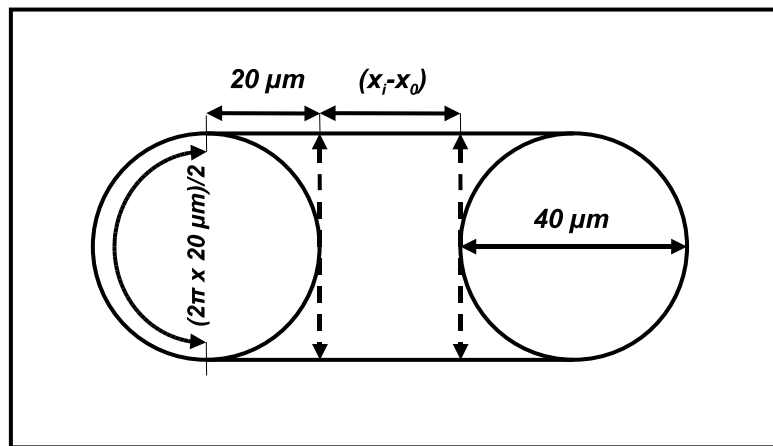
During the normalisation procedure, the micrometer is gradually moved to enlarge the distance between the wires and subsequently stretch the vessel step by step (figure III.5). One minute after each step (i), the corresponding micrometer setting ( $x_i$ ) and force ( $y_i$ ) are registered. Finally, the internal circumference ( $IC_i$ ), the wall tension ( $T_i$ ) and the corresponding transmural pressure ( $P_i$ ), can be calculated from those recorded values.

- Internal circumference ( $IC_i$ )

The  $IC_i$  is calculated from the distance between the wires ( $x_i - x_0$ ) and the diameter of the mounting wires (40  $\mu$ m)



$$IC_i = 2 \times ((2\pi \times 20 \mu\text{m}) / 2) + 4 \times 20 \mu\text{m} + 2 \times (x_i - x_0) = 205.66 \mu\text{m} + 2 \times (x_i - x_0)$$



**Figure III.5:** a schematic representation of the blood vessel after distention i.

- Wall tension ( $T_i$ )

The measured force ( $y_i$ ) divided by the wall length equals  $T_i$ . The wall length corresponds to 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 after mounting.

$$T_i = y_i / (2 \times l)$$

- Transmural pressure ( $P_i$ )

The calculation of  $P_i$  is based on the law of Laplace, which states that in a cylinder, wall tension ( $T_i$ ) is proportional to the pressure times the radius of the cylinder. Since the radius ( $r$ ) can be computed from the  $IC_i$  ( $IC_i = 2\pi r$ ), the  $P_i$  can be determined with the following formula:

$$P_i = T_i / (IC_i / 2\pi)$$

It should be noted that the calculated pressure is only an approximation for the intraluminal pressure that would be necessary to expand the vessel to the measured  $IC_i$ .

For each pair of  $x_i$  and corresponding  $y_i$ , the matching pressure is calculated. The stepwise stretching of the vessel segments is stopped when the pressure exceeds 100 mm Hg (= 13.3 kPa). Then, an exponential curve is constructed, showing the relation between the internal circumference and the equivalent pressure. On this graph, the point corresponding with a pressure of 100 mmHg denotes  $IC_{100}$ . With this value, the ideal position of the holders to obtain an internal circumference of 90 % of the  $IC_{100}$ , can be calculated by inserting this value in the formula for  $IC_i$ . Finally, the holders are set in this position and the experiment can be started. Additionally, the diameter the ring segment would have when exposed to a transmural pressure of 100 mm Hg can be deduced from the  $IC_{100}$  value ( $IC = 2\pi r$ ).

*\*A detailed example of a normalisation procedure*

This example demonstrates the successive steps in the normalisation procedure for a mouse femoral artery with a length of 2.40 mm.

Step (i)	$x_i$ ( $\mu\text{m}$ )	$y_i$ (mN)
0	4245	0.00
1	4654	2.10
2	4773	6.48
3	4845	9.98
4	4919	16.10

1 minute after the first distension:

$$IC_1 = 205.66 \mu\text{m} + (2 \times (x_1 - x_0)) \mu\text{m} = 205.66 \mu\text{m} + (2 \times (4654 - 4245)) \mu\text{m} = 1023.66 \mu\text{m}$$

$$T_1 = y_1 / (2 \times l) = 2.1 \text{ mN} / (2 \times 2.40 \text{ mm}) = 0.438 \text{ mN/mm}$$

$$P_1 = T_1 / (IC_1 / 2\pi) = 0.438 \text{ mN/mm} / (1.02366 \text{ mm} / 2\pi) = 2.69 \text{ kPa}$$

After the same calculations were made for the following distensions, the internal circumference is expressed in function of the pressure. On this curve (figure III.6), the point corresponding with a pressure of 13.3 kPa corresponds with an  $IC_{100}$  of 1521.66  $\mu\text{m}$ . With this value, the ideal position of the holders and the size of the artery can be calculated:

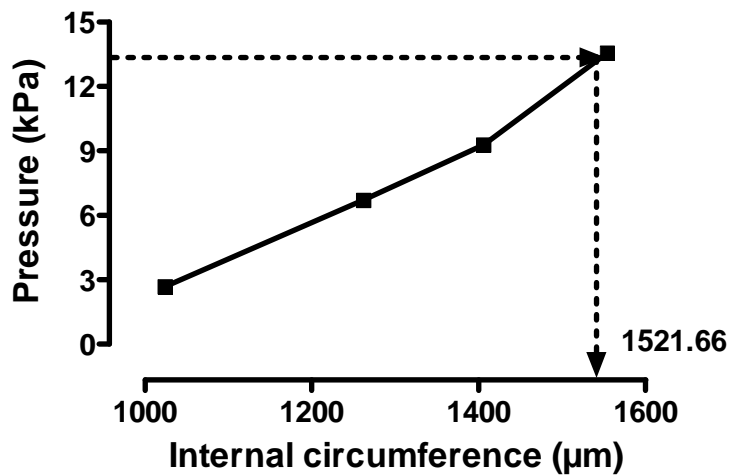
$$\text{The ideal internal circumference } IC_{90} = 0.9 \times IC_{100} = 1369.50 \mu\text{m}$$

The ideal micrometer setting ( $x_1'$ ) yielding the  $IC_{90}$  can be computed with the following formula:  $IC_{90} = 205.66 \mu\text{m} + (2 \times (x_1' - x_0)) \mu\text{m}$

$$\rightarrow x_1' = ((IC_{90} - 205.66 \mu\text{m}) / 2) + x_0 \mu\text{m} = ((1369.50 \mu\text{m} - 205.66 \mu\text{m}) / 2) + 4245 \mu\text{m} = 4826.92 \mu\text{m}$$

The diameter of the ring segment, when it would be exposed to a transmural pressure of 13.3 kPa ( $I_{100}$ ) equals  $IC_{100} / \pi = 484.36 \mu\text{m}$

Before the start of the experiment, the micrometer is set at 4827  $\mu\text{m}$ .



**Figure III.6:** Relation between the internal circumference of the ring segment (X-as) and the intraluminal pressure that would be necessary to extend the vessel to this internal circumference (Y-axis). From this exponential curve, the internal circumference that the vessel would have under a transmural pressure of 100 mm Hg (13.3 kPa) can be deduced.

#### III.3.2.4. Preparation of the aortic segments before the experiment

When studying ring segments of larger arteries such as the mouse aorta, the ring segments are gradually stretched until a fixed stable preload is obtained. In mouse aorta a preload of 0.5 g is used, as it has been shown that this resulted in a maximum response.

#### III.3.3. cGMP measurements

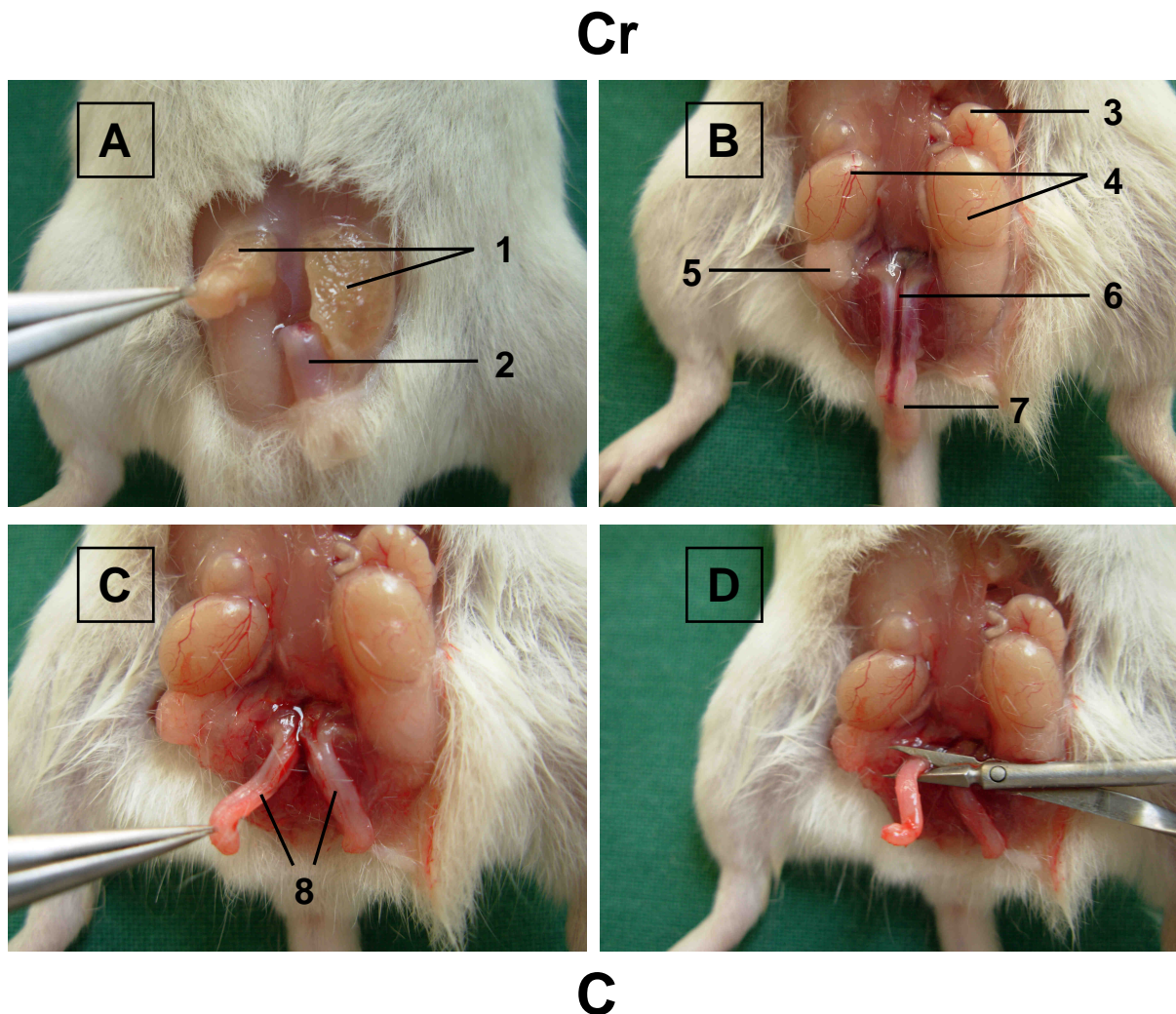
After cervical dislocation, the thoracic aorta was rapidly dissected and transferred to cooled KRB solution. Subsequently two aortic ring segments of equal length were prepared, which were incubated for 30 minutes in 10 ml KRB solution at 37°C, bubbled with 95%  $O_2$ -5%  $CO_2$  (pH 7.4). Next, the aortic rings were treated with 30  $\mu\text{mol/L}$   $PGF_{2\alpha}$  for 10 minutes. Then, the segments were treated with SNP (10  $\mu\text{mol/L}$ ) or vehicle for 1 minute, which was terminated

by a quick freeze with liquid nitrogen. In the other set of experiments, the segments were treated for 20 minutes with ODQ (1  $\mu\text{mol/l}$ ) or vehicle together with  $\text{PGF}_{2\alpha}$ . Subsequently, the relaxation reaction was initiated by SNP (10  $\mu\text{mol/L}$ ) and 1 minute later terminated by snap freezing in liquid nitrogen. Frozen tissues were stored at  $-80^{\circ}\text{C}$  until further processing. Different homogenizing strategies were performed in order to optimize the measurement of cGMP in the mouse aorta. A few samples were homogenized in ice cold trichloroacetic acid (TCA) (6% w v<sup>-1</sup>) with a glass-Teflon homogenizer at  $4^{\circ}\text{C}$ . In another set of samples, the Teflon pestle was replaced by a glass one. Besides the potter homogenizers, also a polytron homogenizer at 3000 r.p.m. was used to grind the ring segments. All those methods were not able to fully scatter the aorta, which is a very tough tissue due to the large amount of elastic fibres. A more homogenous solution was obtained by pulverizing the frozen segments by a Mikrodismembrator U (B-braun Biotech International, Germany) and subsequently dissolving the tissue debris in ice-cold trichloroacetic acid. However, this process is very labour-consuming and not very practical. Considering the degree of homogenization and the practical feasibility, the best results were obtained by crushing the frozen segments by hand prior to the addition of TCA. Immediately after the tissue was transferred to a cooled 15 ml Falcon tube, a few tight thrusts were applied on the tissue. For that purpose, we used a pestle made of Teflon which was designed to perfectly fit the Falcon tube. Subsequently, the tissue homogenates were centrifuged at  $1500 \times g$  for 10 minutes at  $4^{\circ}\text{C}$ . The resulting pellets were used for a protein assay according to the Bradford method <sup>6</sup> with bovine serum albumin (BSA) as standard. The supernatant fractions were extracted 4 times with 5 volumes of water-saturated diethyl ether and dried in a Speed-Vac centrifuge. Further processing of the samples for acetylation and subsequent determination of cGMP was carried out according to the manufacturer's instructions using a commercially available non-radioactive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). This assay, performed in duplicate, is based on the competition between unlabelled cGMP and a fixed quantity of peroxidase-labeled cGMP for a limited number of binding sites on a cGMP-specific antibody. With fixed amounts of antibody and peroxidase-labeled cGMP, the amount of peroxidase-labeled ligand bound by the antibody was inversely proportional to the concentration of added unlabeled ligand. The absorbance was measured with a 96-well plate reader at 405 nm and the arterial cGMP content was expressed as femtomoles per microgram protein.

### **III.4. Corpora cavernosa**

#### **III.4.1. Dissection**

Experiments were performed on corpora cavernosa isolated from sexually mature male mice (age: 10-15 weeks). After cervical dislocation, the mouse is placed on its back with the legs extended. Then the skin in the uro-genital area and the underlying adipose tissue is cut away (figure III.7A). Next the connective and adventitial tissue along the shaft of the penis is removed, followed by the dorsal arteries, dorsal vein and the corpus spongiosum with the urethra (figure III.7B). After, the glans penis is cut off, the corpora cavernosa are separated by cutting the fibrous septum between them (figure III.7C). Finally, the corpora cavernosa are excised at the base (figure III.7D) and transferred to fresh KRB solution.

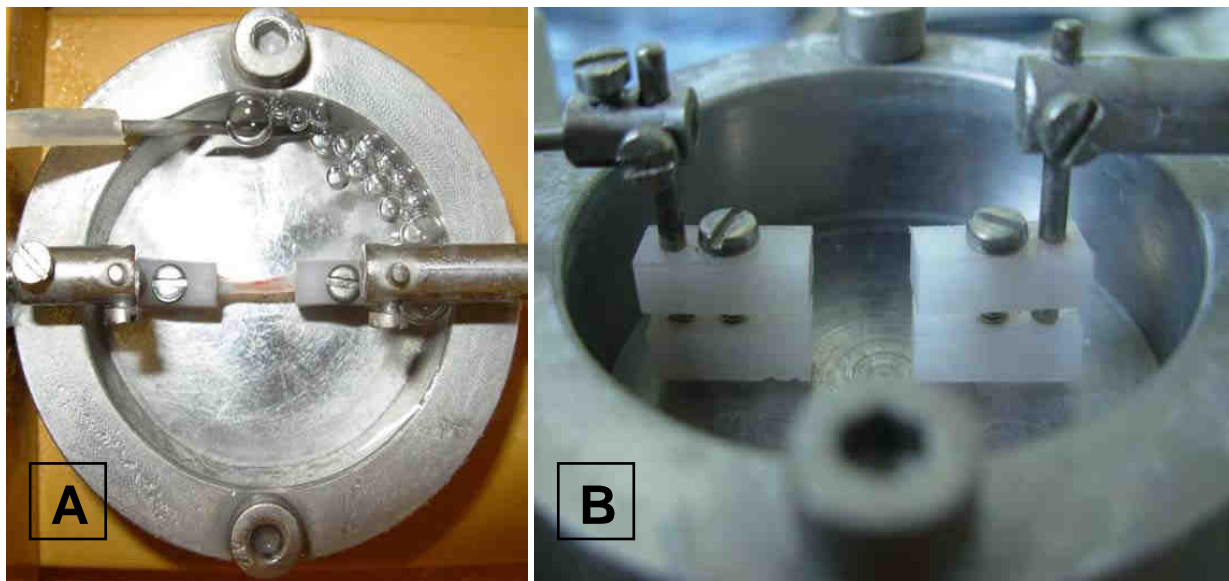


**Figure III.7:** Different steps involved in the isolation of the mice corpora cavernosa.

1: fat, 2: penis, 3: seminal vesicle, 4: testis, 5: cauda epididymis, 6: superficial vein, 7: glans penis, 8: corpora cavernosa, Cr: cranial, C: caudal

### III.4.2. The apparatus

We have used a manual myograph (figure III.8) constructed by the technical staff of our laboratory (Mr. Dirk De Gruytere and Mr. Cyriel Mabilde). It contains two holders which are placed in an organ bath (10 ml capacity). One holder is connected to a micrometer that is used to change the distance between the two holders. The other one is connected to the lever of a force-displacement transducer that measures the isometric tension changes. Between those holders, each corpus cavernosum is fixed horizontally (figure III.8A).



**Figure III.8:** A photograph of the organ bath of the adapted myograph: **A.** top view; **B.** lateral view. Corpora cavernosa are horizontally fixed on the two holders in the organ bath of the myograph. One holder is connected to a micrometer which is used to change the distance between the holders. The other holder is connected to a force transducer which allows measurement of isometric tension changes in the corporal tissues.

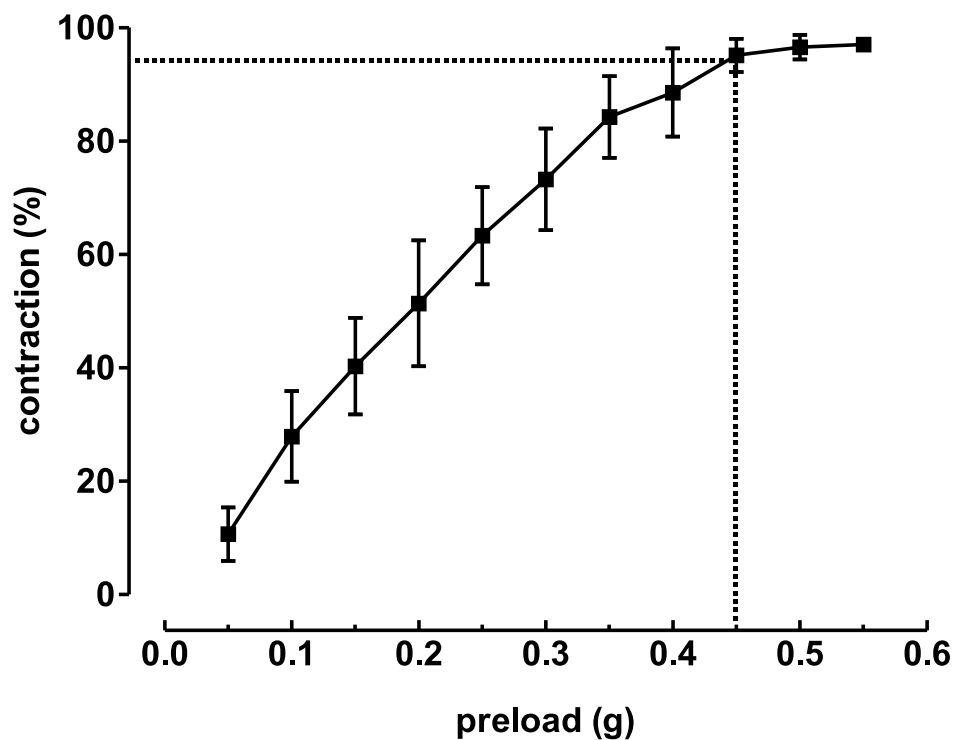
### III.4.3. Mounting of the penile tissue

First, the screw of the left holder is loosened so that one end of the tissue can be placed between the clasp parts of the holder. Then by tightening the screw, the tip of the corpus cavernosum is fixed on the left holder. Next the holders are brought together so that in a similar way, the other end of the penile tissue can be fixed on the right holder.

After mounting, the preparations are allowed to equilibrate in a KRB solution at 37°C, pH 7.4 and oxygenated with 95% O<sub>2</sub>-5% CO<sub>2</sub> for 30 minutes.

#### III.4.4. Preparation of the tissue before the experiment.

During an equilibration of 60 min, tension is adjusted until a fixed stable tension was obtained. To investigate the optimal preload for this tissue a preliminary study was performed, in which the preload was augmented in several steps (beginning with 0.05 g and increasing by 0.05 g). After each increase in preload, the contractile response to 5  $\mu\text{mol/l}$  norepinephrine was registered. Subsequently the CC was washed and allowed to relax to the imposed basal tone. This stepwise procedure was stopped when maximum contractile effect was obtained with norepinephrine. From those measurements, a curve that represents the relationship between the preload and the subsequent norepinephrine-induced contraction was constructed (figure III.9). The point on the curve corresponding with the maximal response to norepinephrine (95.10 mN) then denotes the optimal preload (0.45 g), applied in all further experiments on penile tissue.



**Figure III.9:** Relation between the preload and subsequent contraction elicited by 5  $\mu\text{molL}$  norepinephrine (n=4). From this curve, the optimal preload which results in a maximal contraction was deduced (0.45 g).

### **III.5. References**

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# Chapter IV

## Functional role of the soluble guanylyl cyclase $\alpha_1$ subunit in vascular smooth muscle relaxation.

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## **IV.1. Abstract and keywords**

**OBJECTIVE:** Soluble guanylyl cyclase (sGC), the predominant receptor for nitric oxide (NO), exists in 2 active isoforms ( $\alpha_2\beta_1$  and  $\alpha_1\beta_1$ ). In vascular tissue sGC $\alpha_1\beta_1$  is believed to be the most important. The aim of our study was to investigate the functional importance of the sGC $\alpha_1$ -subunit in vasorelaxation.

**METHODS:** Aortic and femoral artery segments from male and/or female sGC $\alpha_1^{-/-}$  mice and wild type mice were mounted in a small vessel myograph for isometric tension recording. This was supplemented with biochemical measurements of the cGMP concentration and sGC enzyme activity.

**RESULTS:** The functional importance of sGC $\alpha_1\beta_1$  was demonstrated by the significantly decreased relaxing effects of acetylcholine (ACh), sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP), NO-gas, YC-1, BAY 41-2272 and T-1032 in the sGC $\alpha_1^{-/-}$  mice of both genders. Moreover, the basal and SNP-stimulated cGMP levels and basal sGC activity were significantly lower in the sGC $\alpha_1^{-/-}$  mice. However, the relaxing effects of NO, BAY 41-2272 and YC-1 seen in blood vessels from sGC $\alpha_1^{-/-}$  mice indicate a role for an sGC $\alpha_1\beta_1$ -independent mechanism. The increase in sGC activity after addition of BAY 41-2272 and the inhibition of the ACh-, SNP-, SNAP and NO-gas-induced response by the sGC-inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) in the sGC $\alpha_1^{-/-}$  mice, are observations suggesting that also the sGC $\alpha_2\beta_1$  isoform is functionally active. However, the non-significant increase in cGMP in response to SNP and the non-upregulated sGC $\alpha_2$  expression level in the sGC $\alpha_1^{-/-}$  mice, rather suggest the involvement of (an) sGC-independent mechanism(s).

**CONCLUSIONS:** We conclude that sGC $\alpha_1\beta_1$  is involved in the vasorelaxations induced by NO-dependent and NO-independent sGC activators in both genders. However the remaining relaxation seen in the sGC $\alpha_1^{-/-}$  mice, suggests that besides sGC $\alpha_1\beta_1$  also the minor isoform sGC $\alpha_2\beta_1$  and/or (an) sGC-independent mechanism(s) play(s) a substantial role.

*Key words:* arteries, nitric oxide, endothelial function, second messengers, vasoconstriction/dilatation

Preliminary reports of these findings have been presented at the International Conference on cGMP, Potsdam<sup>1</sup>.

## **IV.2. Introduction**

Soluble guanylyl cyclase (sGC) is considered to be the predominant intracellular receptor for nitric oxide (NO) and hence a very important enzyme for the physiological regulation of vascular tone and blood pressure. The heterodimeric hemoprotein is composed of a larger  $\alpha$  and a smaller  $\beta$  subunit, both necessary for catalytic activity<sup>2</sup>. Two isoforms for each subunit ( $\alpha_1/\alpha_2$  and  $\beta_1/\beta_2$ ) have been identified in various species<sup>3</sup>. Theoretically, the association of  $\alpha$  and  $\beta$  subunits could give rise to at least four different isoforms, but only the  $\alpha_2\beta_1$  and  $\alpha_1\beta_1$  isoforms are reported to be active<sup>4</sup>. In the brain, the levels of both isoforms are comparable but in all other tissues, including vascular tissue, the  $\alpha_1\beta_1$  isoform is predominant<sup>5</sup>.

The most important endogenous activator of sGC is NO. In addition to endogenous NO, pharmacological NO-donors (e.g. nitroglycerin, isosorbide dinitrate and SNP) and agents such as BAY 41-2272 and YC-1 stimulate sGC, the latter two also in an NO-independent way<sup>6</sup>. The resulting rise in the intracellular cGMP concentration induces vascular smooth muscle relaxation by lowering the intracellular  $\text{Ca}^{2+}$  concentration and by desensitization of the contractile apparatus to  $\text{Ca}^{2+}$ <sup>7</sup>.

Dysfunction of the endothelial NO/cGMP signaling pathway contributes to the pathophysiology of a variety of cardiovascular disorders including hypertension, thrombosis, atherosclerosis, myocardial infarction and angina pectoris<sup>8</sup>. This makes the different isoforms of sGC, as effector molecule for NO, attractive therapeutic targets for the treatment of the above mentioned conditions and drugs aiming to target sGC isoforms are currently in development<sup>9</sup>. However, due to the lack of sGC isoform-specific inhibitors, little is known about the specific role and relative importance of the sGC isoforms on vascular tissue response to endogenous and exogenous sGC stimulators. The recently developed knockout mice for the sGC $\alpha_1$  gene<sup>10</sup> allow to unravel this. In the present study the functional importance of the sGC $\alpha_1$ -subunit in the vascular system was analysed using aortic and femoral artery segments isolated from soluble guanylyl cyclase alpha 1 knockout (sGC $\alpha_1^{-/-}$ ) mice. Experiments were performed on preparations from both genders as it was found that male but not female animals develop hypertension<sup>10</sup>.

## **IV.3. Materials and methods**

### **IV.3.1. Animals and tissue collection**

All experiments were performed on male and/or female homozygous soluble guanylyl cyclase alpha 1 knockout (sGC $\alpha_1^{-/-}$ ) mice, bred in the Department of Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology, Ghent, Belgium (age: 10-15 weeks; genetic background: mixed Swiss-129), using sGC $\alpha_1^{+/+}$  mice as control<sup>10</sup>. 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). The sGC $\alpha_1^{-/-}$  mice lack exon 6 of the sGC $\alpha_1$  gene, which codes for an essential part in the catalytic domain. After cervical dislocation, the thoracic aorta and femoral artery were carefully removed from the animals and transferred to cooled Krebs-Ringer bicarbonate (KRB) solution.

### **IV.3.2. Tension measurements**

*Artery preparations and general experimental protocol* Ring segments of the collected arteries (internal diameter: aorta:  $978.0 \pm 39.5 \mu\text{m}$  (n=9); femoral artery:  $424.7 \pm 11.8 \mu\text{m}$  (n=15)) were mounted in a small-vessel myograph with a tissue chamber filled with 10 ml of KRB solution and were cleansed from adhering tissue. Two stainless steel wires ( $40 \mu\text{m}$  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<sub>2</sub>-5% CO<sub>2</sub> (pH 7.4) at 37 °C. The aortic rings were gradually stretched until a stable preload of 0.5 g was obtained, whereas the femoral arteries were set to their normalized internal diameter<sup>11</sup>. In short, the arteries were stretched in progressive steps. From the passive wall-tension-internal circumferences 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 mm Hg.

After applying the optimal resting tension, the preparations were contracted 3 times with a KRB solution containing 120 mmol/L K<sup>+</sup> and 5  $\mu\text{mol/L}$  norepinephrine (NOR), washed, and allowed to relax to basal tension before starting the protocol. Precontraction was elicited with 30  $\mu\text{mol/L}$  prostaglandin (PGF<sub>2 $\alpha$</sub> ) or 5  $\mu\text{mol/L}$  NOR. When a stable contraction plateau was obtained, relaxation responses were examined in a cumulative manner by increasing the

concentration in log increments, once the response to the previous concentration had stabilized. Segments of  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_1^{+/+}$  mice were always tested in parallel.

*Specific experimental protocols* First, the relaxation responses to acetylcholine (ACh) (1 nmol/L–10  $\mu$ mol/L), sodium nitroprusside (SNP) (1 nmol/L–10  $\mu$ mol/L), S-nitroso-N-acetylpenicillamine (SNAP) (1 nmol/L–10  $\mu$ mol/L) and NO-gas (1  $\mu$ mol/L–100  $\mu$ mol/L) were measured in varying order. These relaxing substances were also tested in the presence of the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (1  $\mu$ mol/L for ACh, SNP and SNAP or 10  $\mu$ mol/L for NO-gas; 20 minutes preincubation) or the NO-synthase (NOS) inhibitor *N*<sup>o</sup>-nitro-L-arginine (L-NA) (0.1 mmol/L; 10 minutes preincubation). Besides vasodilators with NO as active metabolite, also NO-independent sGC stimulators such as BAY 41-2272 (1 nmol/L–10  $\mu$ mol/L) and YC-1 (10 nmol/L–10  $\mu$ mol/L) were tested. Also the influence of 1  $\mu$ mol/L ODQ on the BAY 41-2272-induced response was measured. The influence of accumulation of cGMP formed under basal conditions was investigated by addition of the PDE-5 inhibitor, T-1032 (1 nmol/L–10  $\mu$ mol/L). sGC-independent relaxation was assessed using the  $K_{ATP}$ -channel opener levcromakalim (Lev) (1 nmol/L–10  $\mu$ mol/L) and the cGMP-analogue 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate (8-pCPT-cGMP) (100 nmol/L–100  $\mu$ mol/L).

### IV.3.3. Measurement of cGMP levels in thoracic aortic rings

After 30 minutes of equilibration in a KRB solution at 37°C, bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub> (pH 7.4), the thoracic aorta segments were precontracted with 30  $\mu$ mol/L PGF<sub>2 $\alpha$</sub> . In the first series of experiments, a single concentration of either SNP (10  $\mu$ mol/L) or vehicle was added to the preparations 10 minutes after the addition of PGF<sub>2 $\alpha$</sub>  1 minute later, the reaction was stopped by snap freezing in liquid nitrogen. In the second series of experiments, 1  $\mu$ mol/L ODQ or vehicle was added together with PGF<sub>2 $\alpha$</sub> . After 20 minutes, the ring segments were exposed to a single concentration of SNP (10  $\mu$ mol/L) for 1 minute, before being rapidly frozen in liquid nitrogen.

The collected segments were kept at -80°C until further processing. The frozen aortic rings were pulverized, homogenized in 6 % w v<sup>-1</sup> trichloroacetic acid, followed by centrifugation at 1,500 x g for 10 minutes at 4°C. The resulting pellets were used for total protein determination according to the method of Bradford<sup>12</sup>. The supernatants were extracted 4 times with 5 volumes of water-saturated diethyl ether before being dried in a Speed-Vac

centrifuge. The redissolved samples were acetylated and cyclic GMP concentration was determined using a non-radioactive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). Assays were performed in duplicate and the amount of cGMP in each blood vessel ring was expressed as fmol  $\mu\text{g}^{-1}$  protein.

#### IV.3.4. Measurement of sGC activity in femoral artery preparations

sGC enzyme activity was measured as described by Bloch et al.<sup>13</sup>. After collecting, the femoral artery tissues were homogenized in buffer containing 50 mmol/L tris(hydroxymethyl)aminomethane (Tris).HCl (pH 7.6), 1 mmol/L EDTA, 1 mmol/L dithiothreitol, and 2 mmol/L phenylmethylsulfonyl fluoride. After centrifugation at 20,000  $\times g$  for 1h at 4°C, the supernatants were incubated for 10 min at 37°C in a reaction mixture containing 50 mmol/L Tris.HCl (pH 7.5), 4 mmol/L MgCl<sub>2</sub>, 0.5 mmol/L 1-methyl-3-isobutylxanthine, 7.5 mmol/L creatine phosphate, 0.2 mg/ml creatine phosphokinase, 1 mmol/L L-NAME and 1 mmol/L GTP with or without 100  $\mu\text{mol/L}$  BAY 41-2272. The reaction was terminated by the addition of 0.9 ml of 0.05 mol/L HCl and the cGMP content in the reaction mixture was measured using a commercial radioimmunoassay (Biomedical Technologies, Stoughton, MA). sGC enzyme activity is expressed as pmol of cGMP produced per minute per milligram of protein in femoral artery extract supernatant.

#### IV.3.5. Drugs

The experiments were performed in a KRB solution of the following composition (mmol/L): NaCl, 135; KCl, 5; NaHCO<sub>3</sub>, 20; glucose, 10; CaCl<sub>2</sub>, 2.5; MgSO<sub>4</sub>, 1.3; KH<sub>2</sub>PO<sub>4</sub>, 1.2 and EDTA, 0.026 in H<sub>2</sub>O. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), acetylcholine chloride, T-1032, YC-1, 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate (8-pCPT-cGMP), trichloroacetic acid, norepinephrine bitartrate, dithiothreitol, phenylmethylsulfonyl fluoride, tris(hydroxymethyl)aminomethane (Tris).HCl, 1-methyl-3-isobutylxanthine, creatine phosphate, creatine phosphokinase, *N*<sup>0</sup>-nitro-L-arginine (L-NA), *N*<sup>0</sup> nitro-L-arginine methyl ester (L-NAME) and GTP were obtained from Sigma-Aldrich (St.Louis, MO); sodium nitroprusside from Merck (Darmstadt, Germany); BAY 41-2272 and S-nitroso-N-acetylpenicillamine from Alexis (San Diego, USA) and prostaglandin F<sub>2 $\alpha$</sub>  (Dinolytic) from Upjohn (Puurs, Belgium). ODQ, T-1032, SNAP, YC-1 and BAY 41-2272 were dissolved in dimethylsulfoxide and acetylcholine in 50 mmol/L potassium hydrogen phthalate 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<sup>14</sup>. 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.3.6. Calculations and statistics

Data are presented as mean values  $\pm$  SEM;  $n$  represents the number of arteries (each obtained from a different mouse). 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.  $P < 0.05$  was considered as significant.

### **IV.4. Results**

#### IV.4.1. NO-dependent sGC-induced relaxations

##### *IV.4.1.1. Effect of ACh*

Responses to stimulated release of endothelium-derived NO were determined by recording concentration-relaxation curves to ACh in the aorta (figure IV.1A) and femoral artery (figure IV.1B) from male and female  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_1^{+/+}$  mice. The results were essentially similar in both genders. ACh-induced concentration-dependent relaxation was nearly abolished in the aortic rings of the  $sGC\alpha_1^{-/-}$  mice (10  $\mu\text{mol/L}$  ACh: female:  $67.8\% \pm 3.5$  vs.  $16.4\% \pm 5.2$  ( $n=6$ ),  $P < 0.05$ ). Also in the femoral artery segments of the  $sGC\alpha_1^{-/-}$  mice, the ACh-induced response was significantly reduced compared to the  $sGC\alpha_1^{+/+}$  mice (10  $\mu\text{mol/L}$  ACh: female:  $89.0\% \pm 2.9$  vs.  $43.1\% \pm 10.7$  ( $n=6$ ,  $P < 0.05$ )).

A second relaxation curve to ACh was established in the presence of the sGC inhibitor ODQ. ODQ inhibited the ACh-induced response in the aorta (figure IV.1A) (10  $\mu\text{mol/L}$  ACh: female:  $67.8\% \pm 3.5$  vs.  $28.8\% \pm 3.0$  ( $n=6$ ,  $P < 0.05$ )) and femoral artery (figure IV.1B) (10  $\mu\text{mol/L}$  ACh: female:  $89.0\% \pm 2.9$  vs.  $50.3\% \pm 10.9$  ( $n=6$ ,  $P < 0.05$ )) from control mice. As the response to ACh in the  $sGC\alpha_1^{-/-}$  aortic rings was very small, the reducing effect of ODQ was also confined (10  $\mu\text{mol/L}$  ACh: female:  $16.4\% \pm 5.2$  vs.  $2.6\% \pm 1.3$  ( $n=6$ ,  $P > 0.05$ )). After treatment with ODQ, the maximal relaxation in the femoral arteries of  $sGC\alpha_1^{-/-}$  mice, was approximately reduced by 74% and 52% ( $43.1\% \pm 10.7$  vs.  $20.9\% \pm 9.9$  ( $n=6$ ,  $P < 0.05$ )) respectively in male and female  $sGC\alpha_1^{-/-}$  mice.

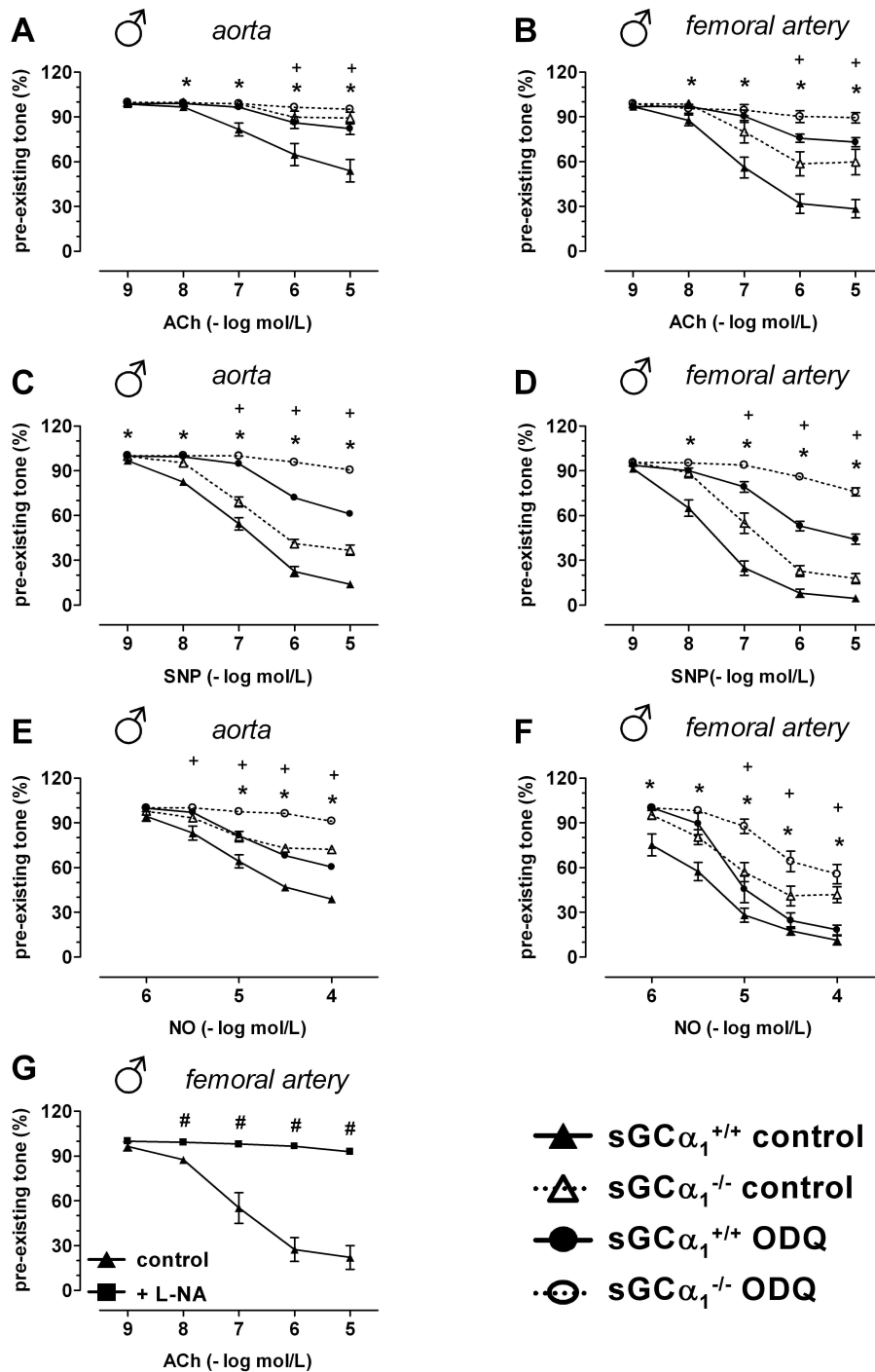


In order to evaluate the involvement of non-nitric oxide vasodilating substances such as the endothelium-derived hyperpolarising factor (EDHF) in the ACh-induced response, the effect of the NOS inhibitor L-NA was investigated in femoral arteries isolated from male sGC $\alpha_1^{+/+}$  mice (figure IV.1G). In those arteries, the vasorelaxing influence of ACh was almost completely abolished by L-NA (10  $\mu$ mol/L ACh: female: 77.9% $\pm$ 8.0 vs. 7.0% $\pm$ 1.9 (n=4, P<0.05)).

#### IV.4.1.2. Effect of SNP

In this series of experiments, the relaxant effects of increasing concentrations of the NO-donor SNP were compared on precontracted aortas (figure IV.1C) and femoral arteries (figure IV.1D; figure IV.2A,B) from male and female sGC $\alpha_1^{-/-}$  and sGC $\alpha_1^{+/+}$  mice. The results were essentially the same in both genders. The cumulative addition of SNP resulted in a concentration-dependent response in the ring segments of both sGC $\alpha_1^{-/-}$  and sGC $\alpha_1^{+/+}$  mice. However, the relaxing effect of SNP was significantly reduced in the preparations of the sGC $\alpha_1^{-/-}$  mice (10  $\mu$ mol/L SNP: female: aorta: 77.9% $\pm$ 4.0 vs. 48.6% $\pm$ 3.6 (n=15, P<0.05); femoral artery: 89.9% $\pm$ 1.8 vs. 70.3% $\pm$ 5.1 (n=14, P<0.05)).

Following preincubation with ODQ, the relaxing effect of SNP was significantly reduced in the aorta and femoral artery of both sGC $\alpha_1^{-/-}$  and sGC $\alpha_1^{+/+}$  mice (10  $\mu$ mol/L SNP: female: aorta: 77.9% $\pm$ 4.0 vs. 36.8% $\pm$ 2.6 (n=15, P<0.05); femoral artery: 89.9% $\pm$ 1.8 vs. 49.5% $\pm$ 3.9 (n=14, P<0.05)). The relaxing effect of 10  $\mu$ mol/L SNP was reduced by approximately 85% in male aortic rings (figure IV.1C) of sGC $\alpha_1^{-/-}$  mice and 80% (48.6% $\pm$ 3.6 vs. 9.8% $\pm$ 1.9 (n=15, P<0.05)) in the corresponding female segments. Also in the femoral artery preparations (figure IV.1D) of the sGC $\alpha_1^{-/-}$  mice, approximately 70% (female: 70.3% $\pm$ 5.1 vs. 20.5% $\pm$ 1.9 (n= 14, P<0.05)) of the response to SNP was eliminated by ODQ.



**Figure IV.1:** Relaxation effect of ACh (A, B), SNP (C, D) and NO-gas (E, F) on precontracted (30  $\mu\text{mol/L}$   $\text{PGF}_{2\alpha}$  (ACh, NO-gas,) and 5  $\mu\text{mol/L}$  NOR (SNP)) aortas (A, C, E) and femoral arteries (B, D, F) from male *sGC $\alpha_1$ <sup>+/+</sup>* and *sGC $\alpha_1$ <sup>-/-</sup>* mice in control conditions ( $\blacktriangle$  and  $\triangle$ ) and in the presence of ODQ ( $\bullet$  and  $\circ$ ) (1  $\mu\text{mol/L}$  (ACh, SNP) and 10  $\mu\text{mol/L}$  (NO-gas)). G, effect of L-NA ( $\blacksquare$ ) (0.1 mmol/L) on the ACh-induced response in the femoral artery of male *sGC $\alpha_1$ <sup>+/+</sup>* mice. \*(*sGC $\alpha_1$ <sup>+/+</sup>* vs. *sGC $\alpha_1$ <sup>-/-</sup>*), +(*sGC $\alpha_1$ <sup>+/+</sup>* ODQ vs. *sGC $\alpha_1$ <sup>-/-</sup>* ODQ):  $P < 0.05$ , (n=7-15); # (control conditions vs. presence of L-NA):  $P < 0.05$ , (n=4).

*IV.4.1.3. Effect of SNAP*

In these experiments, we investigated the relaxant effect of the NO-donor SNAP on aortic rings of female female sGC $\alpha_1^{-/-}$  and sGC $\alpha_1^{+/+}$  mice. The concentration-dependent relaxant effect of SNAP was nearly abolished in the ring segments of the sGC $\alpha_1^{-/-}$  mice compared to the sGC $\alpha_1^{+/+}$  mice (10  $\mu\text{mol/L}$  SNAP: 64.4% $\pm$ 4.5 vs. 7.0% $\pm$ 3.6 (n=6, P<0.05)).

Treatment of the aortic rings with ODQ, resulted in a large, significant reduction of the SNAP-induced response in the sGC $\alpha_1^{+/+}$  aortic rings. In those preparations, the 10  $\mu\text{mol/L}$  SNAP-induced relaxation was reduced by approximately 94% (64.4% $\pm$ 4.5 vs. 3.8% $\pm$ 2.1 (n=6, P<0.05)). Since SNAP had a very small effect in the aortic rings of the sGC $\alpha_1^{-/-}$  mice, the influence of ODQ was rather negligible (7.0% $\pm$ 3.6 vs. 0.7% $\pm$ 0.7 (n=6, P>0.05)).

*IV.4.1.4. Effect of NO-gas*

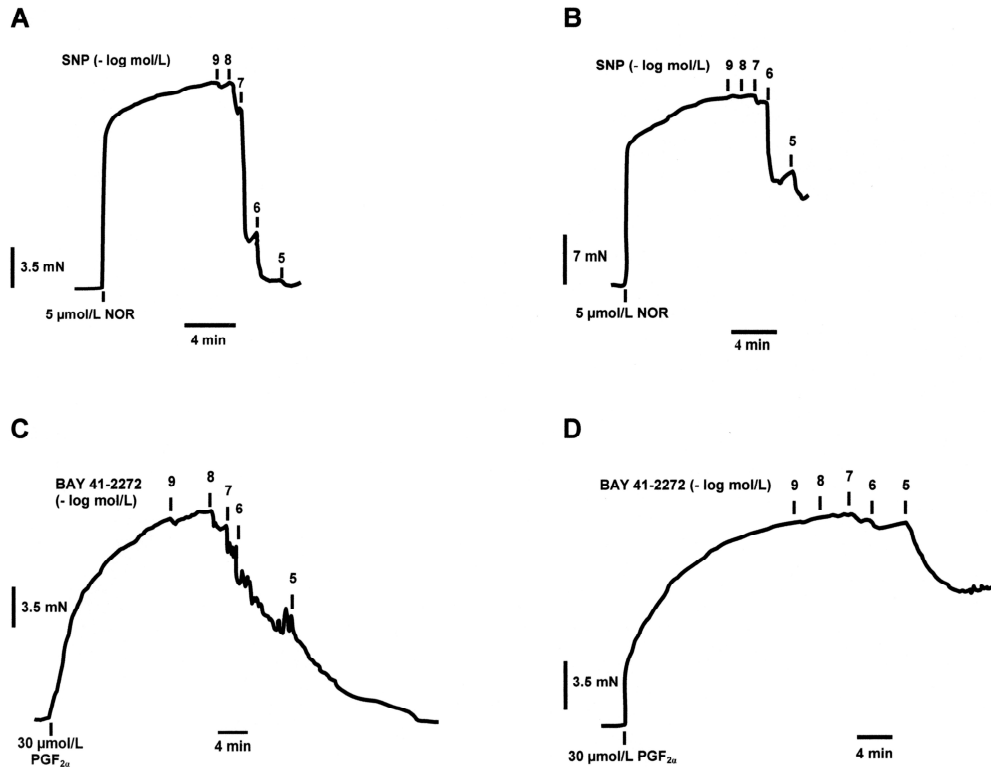
The relaxing effect of exogenous NO delivered as gas was also examined on the aorta (figure IV.1E) and femoral artery (figure IV.1F) from male and female sGC $\alpha_1^{-/-}$  and sGC $\alpha_1^{+/+}$  mice. The results were essentially similar in both genders. In the ring segments of both sGC $\alpha_1^{+/+}$  and sGC $\alpha_1^{-/-}$  mice, NO-gas showed a concentration-dependent relaxing effect. The response to NO-gas was significantly reduced in the aorta (100  $\mu\text{mol/L}$  NO-gas: female: 58.0% $\pm$ 3.5 vs. 33.0% $\pm$ 3.0 (n=7, P<0.05)) and femoral artery (100  $\mu\text{mol/L}$  NO-gas: female: 86.4% $\pm$ 2.7 vs. 56.35% $\pm$ 6.3 (n=7, P<0.05)) of the sGC $\alpha_1^{-/-}$  mice.

Preincubation with ODQ before adding NO-gas caused a rightward shift of the concentration-response curve in the aorta (figure IV.1E) and femoral artery (figure IV.1F) of both sGC $\alpha_1^{-/-}$  and sGC $\alpha_1^{+/+}$  mice (100  $\mu\text{mol/L}$ : female: aorta: 58.0% $\pm$ 3.5 vs. 36.9% $\pm$ 3.1; femoral artery: 86.4% $\pm$ 2.7 vs. 75.8% $\pm$ 4.7 (n=7, P<0.05)). The relaxation induced by 100  $\mu\text{mol/L}$  NO-gas on male and female sGC $\alpha_1^{-/-}$  aortas was reduced by respectively 68% and 56% (33.0% $\pm$ 3.0 vs. 14.6% $\pm$ 3.4 (n=7, P<0.05)), while 24% and 32% (56.4% $\pm$ 6.3 vs. 38.1% $\pm$ 7.5 (n=7, P<0.05)) for respectively male and female sGC $\alpha_1^{-/-}$  femoral artery segments.

*IV.4.1.5. Effect of basal NO*

We also analysed the effect of ODQ on the contraction elicited by 5  $\mu\text{mol/L}$  NOR in the aorta (figure IV.3F) and femoral artery (data not shown) of male and female sGC $\alpha_1^{-/-}$  and sGC $\alpha_1^{+/+}$  mice. The results were similar for both genders. Addition of ODQ elicited a small rise or even no rise in the precontraction level of the femoral arteries, while in the aortic rings the

contraction to norepinephrine was substantially increased by ODQ. This ODQ-induced increase in vascular tone was significantly smaller in the aortas of the  $sGC\alpha_1^{-/-}$  mice.

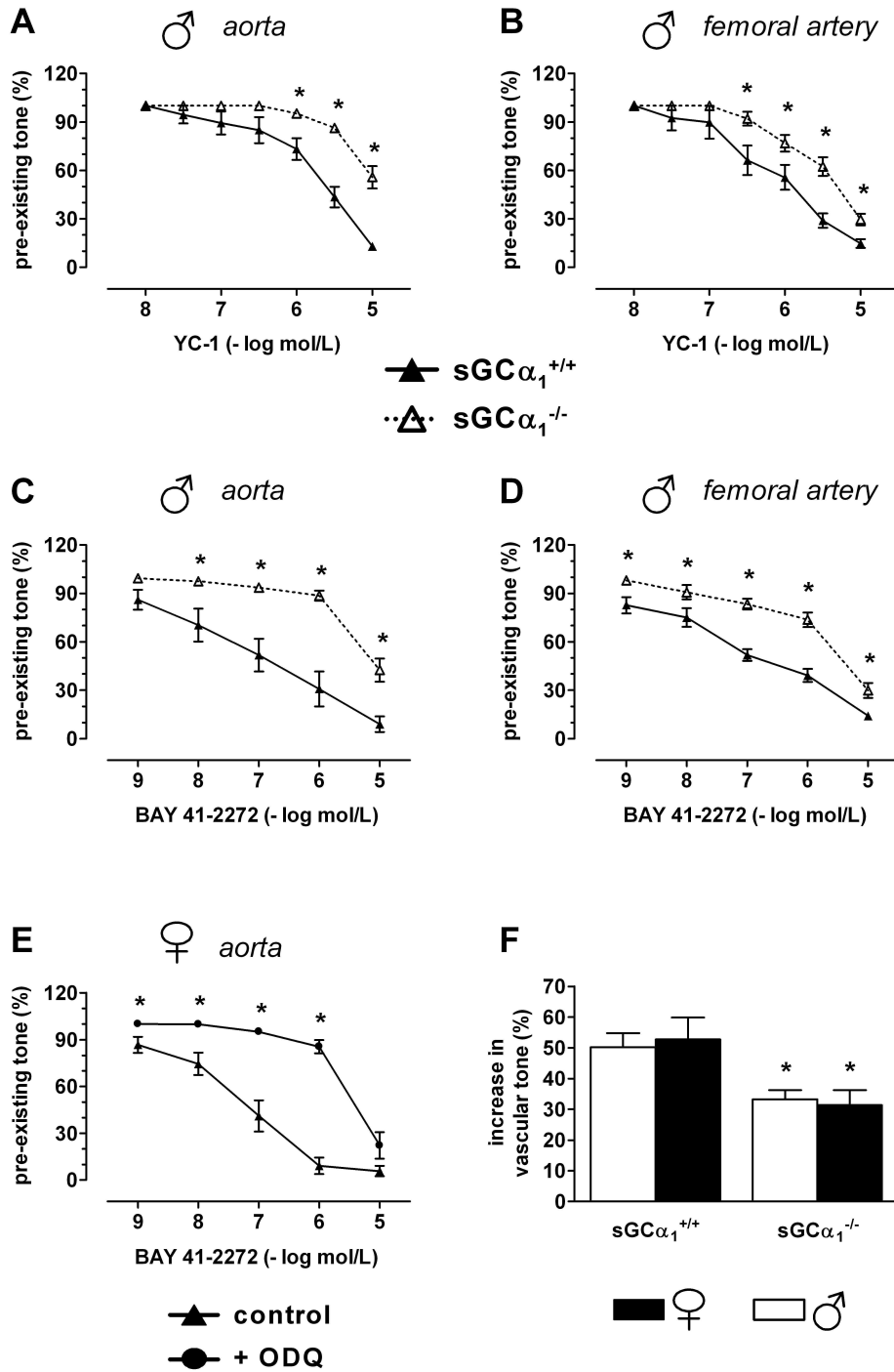


**Figure IV.2:** Original tracings showing a concentration-response curve to SNP in the femoral artery of a male  $sGC\alpha_1^{+/+}$  (A) and  $sGC\alpha_1^{-/-}$  mouse (B) and to BAY 41-2272 in the aorta of a male  $sGC\alpha_1^{+/+}$  (C) and  $sGC\alpha_1^{-/-}$  mouse (D).

#### IV.4.2. NO-independent sGC-induced relaxations

##### *IV.4.2.1. Effect of YC-1*

YC-1 stimulates sGC in an NO-independent way and sensitizes it to NO<sup>6</sup>. To find out which sGC isoform is involved in the vasodilating effect of YC-1, cumulative concentration-response curves to YC-1 were obtained in the aorta (figure IV.3A) and femoral artery (figure IV.3B) from male  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_1^{+/+}$  mice. YC-1 induces a concentration-dependent relaxing effect in the ring segments of both  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_1^{+/+}$  mice. However, the YC-1-induced response was significantly reduced in the preparations of the  $sGC\alpha_1^{-/-}$  mice.



**Figure IV.3:** Relaxation effect of YC-1 (A,B) and BAY 41-2272 (C,D) on precontracted (30  $\mu$ mol/L PGF $_{2\alpha}$ ) aortas (A,C) and femoral arteries (B,D) from male sGC $\alpha_1^{+/+}$  ( $\blacktriangle$ ) and sGC $\alpha_1^{-/-}$  ( $\triangle$ ) (n=7-9). E, influence of ODQ (1  $\mu$ mol/L) on the BAY 41-2272-induced response in the aorta of female sGC $\alpha_1^{+/+}$  mice (n=4). F, effect of ODQ (1  $\mu$ mol/L) on the contraction elicited by 5  $\mu$ mol/L NOR in the aorta of male and female sGC $\alpha_1^{+/+}$  and sGC $\alpha_1^{-/-}$  mice (n=13-15). \* P<0.05

#### *IV.4.2.2. Effect of BAY 41-2272*

BAY 41-2272, another NO-independent type of sGC stimulator<sup>9</sup> was added to aortic (figure IV.2C,D; figure IV.3C) and femoral artery (figure IV.3D) ring segments of male and female  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_1^{+/+}$  mice. This resulted in a relaxing response that was concentration-dependent in the femoral arteries of  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_1^{+/+}$  mice. In the aorta, however, the BAY 41-2272-induced response was only clearly concentration-dependent in the  $sGC\alpha_1^{+/+}$  preparations. In the aortic rings from  $sGC\alpha_1^{-/-}$  mice, a substantial relaxation was only obtained with a concentration of 10  $\mu\text{mol/L}$  (female:  $96.3\% \pm 1.4$  vs.  $66.5\% \pm 5.6$  ( $n=8$ ,  $P<0.05$ )). Also in the femoral arteries of  $sGC\alpha_1^{-/-}$  mice, the relaxing effect of BAY 41-2272 was significantly impaired (female:  $90.2\% \pm 3.6$  vs.  $61.5\% \pm 4.4$  ( $n=8$ ,  $P<0.05$ )). The results were similar for both genders.

The treatment of  $sGC\alpha_1^{+/+}$  aortic rings with ODQ (figure IV.3E) shows that ODQ had a strong inhibitory influence on the BAY 41-2272-induced response, except on the highest BAY 41-2272 concentration (10  $\mu\text{mol/L}$ ).

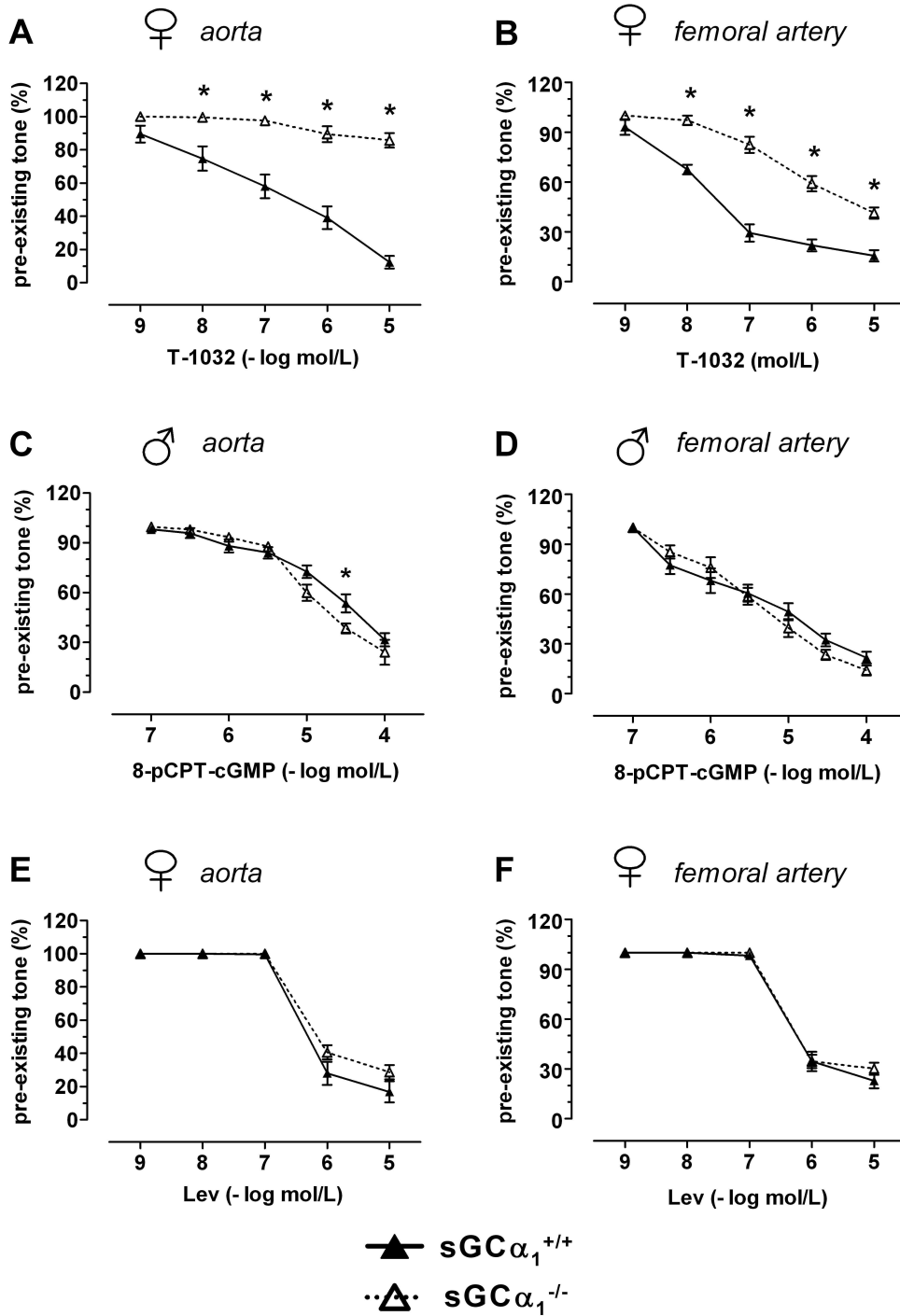
#### IV.4.3. Relaxation induced by PDE-5 inhibition

The influence of the PDE-5 inhibitor T-1032 was explored in the aorta (figure IV.4A) and femoral artery (figure IV.4B) of female  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_1^{+/+}$  mice. Increasing concentrations of T-1032 induced a concentration-dependent relaxation which was almost completely abolished in the aortas isolated from  $sGC\alpha_1$  knockout mice. Also in the femoral artery segments of the  $sGC\alpha_1^{-/-}$  mice, the T-1032-induced response was significantly reduced compared to the control mice.

#### IV.4.4. sGC-independent relaxations

##### *IV.4.4.1. Effect of 8-pCPT-cGMP*

Concentration-response curves to 8-pCPT-cGMP, a cell membrane permeable cGMP-analogue, were recorded in the aorta (figure IV.4C) and femoral artery (figure IV.4D) from male  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_1^{+/+}$  mice. The concentration-dependent responses to 8-pCPT-cGMP were not significantly altered in the aorta and femoral artery of  $sGC\alpha_1^{-/-}$  mice compared to  $sGC\alpha_1^{+/+}$  preparations.



**Figure IV.4:** Relaxation effect of T-1032 on precontracted (30  $\mu$ mol/L PGF $_{2\alpha}$ ) aortas (A) and femoral arteries (B) from female sGC $\alpha_1^{+/+}$  (▲) and sGC $\alpha_1^{-/-}$  mice (△) (n=6) in control conditions. Relaxation effect of 8-pCPT-cGMP on precontracted (30  $\mu$ mol/L PGF $_{2\alpha}$ ) aortas (C) and femoral arteries (D) from male sGC $\alpha_1^{+/+}$  (▲) and sGC $\alpha_1^{-/-}$  mice (△) (n=5-8) in control conditions. Relaxation effect of Lev on precontracted (30  $\mu$ mol/L PGF $_{2\alpha}$ ) aortas (E) and femoral arteries (F) from female sGC $\alpha_1^{+/+}$  (▲) and sGC $\alpha_1^{-/-}$  mice (△) (n=6-9) in control conditions. \* P<0.05.

#### *IV.4.4.2. Effect of levchromakalim*

In these experiments, relaxation curves were obtained by addition of cumulative concentrations of the  $K_{ATP}$  channel opener Lev to the aorta (figure IV.4E) and femoral artery (figure IV.4F) of female  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_1^{+/+}$  mice. There was no significant difference in the concentration-dependent response to Lev between the preparations of  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_1^{+/+}$  mice.

#### IV.4.5. cGMP-measurements in thoracic aorta rings

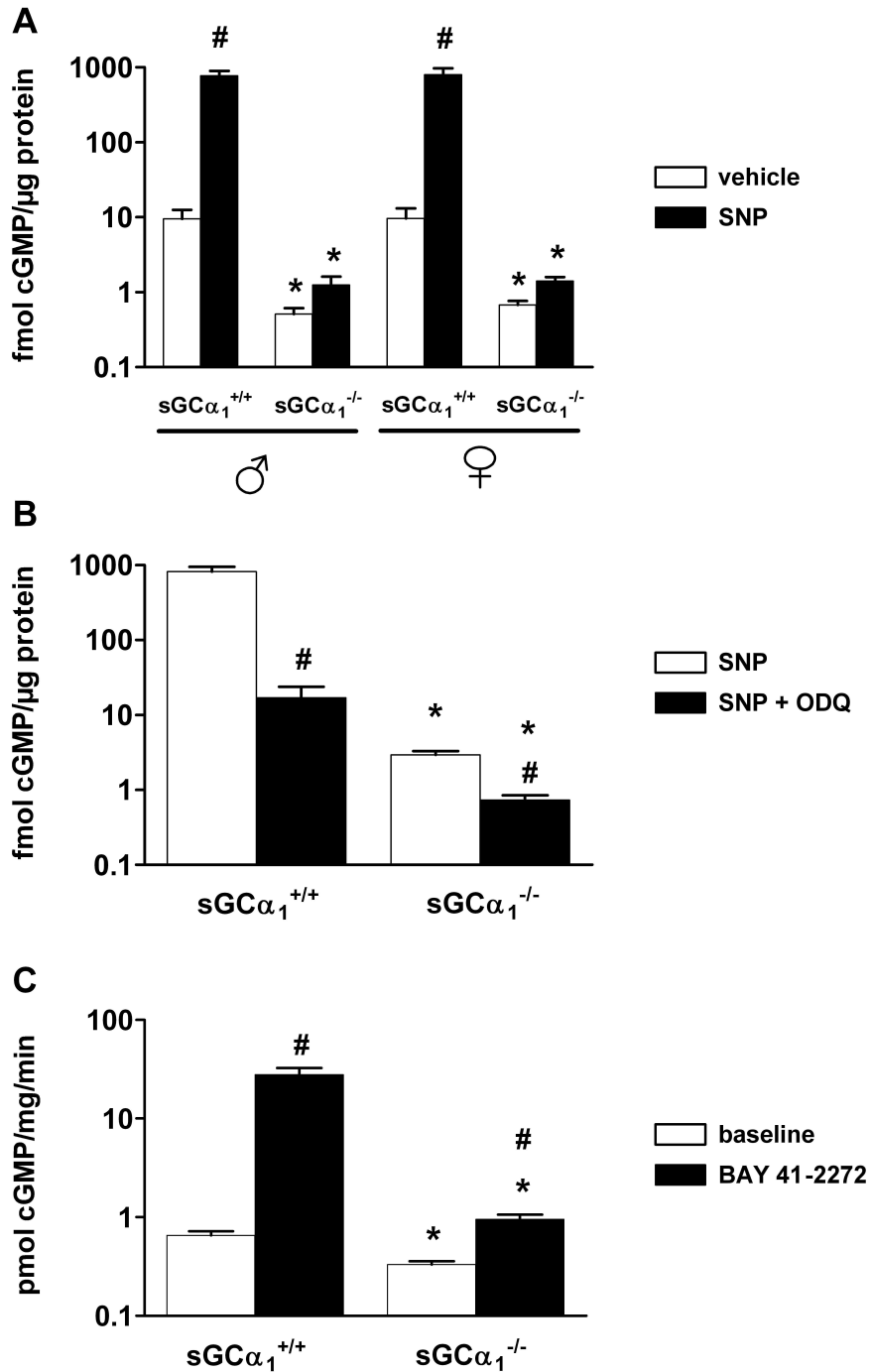
Figure IV.5A shows that the basal cGMP content in aortic rings isolated from  $sGC\alpha_1^{-/-}$  mice was significantly smaller than in rings from  $sGC\alpha_1^{+/+}$  mice. In the  $sGC\alpha_1^{+/+}$  ring segments stimulated with SNP (10  $\mu\text{mol/L}$ ), the cGMP levels increased 100-fold above basal values. Those isolated from  $sGC\alpha_1^{-/-}$  mice showed only a non-significant two-fold increase upon stimulation with SNP.

In another series of experiments, the effect of ODQ on the SNP-induced cGMP increase was assessed in aortic segments of female  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_1^{+/+}$  mice. The results (figure IV.5B) demonstrate that ODQ significantly reduced the cGMP content of the SNP-treated aortic rings in both  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_1^{+/+}$  mice.

#### IV.4.6. sGC enzyme activity levels in femoral artery rings

In the femoral arteries isolated from male and female  $sGC\alpha_1^{-/-}$  mice, the basal sGC activity was significantly smaller compared to  $sGC\alpha_1^{+/+}$  mice. Upon stimulation with 100  $\mu\text{mol/L}$  BAY 41-2272, the sGC activity level increased approximately 50-fold above basal values in the ring segments of the  $sGC\alpha_1^{+/+}$  mice. The corresponding  $sGC\alpha_1^{-/-}$  preparations showed only a 3-fold increase (figure IV.5C).





**Figure IV.5:** A, Increase of cGMP elicited by SNP (10  $\mu$ mol/L) in aortic rings of male and female sGC $\alpha_1^{+/+}$  and sGC $\alpha_1^{-/-}$  mice (n=6-9). # (vehicle vs. SNP), \*(wild type vs. knockout): P<0.001. B, Influence of ODQ (1  $\mu$ mol/L) on the increase in cGMP elicited by SNP (10  $\mu$ mol/L) in aortic rings of female sGC $\alpha_1^{+/+}$  and sGC $\alpha_1^{-/-}$  mice (n=9). # (SNP vs. SNP+ODQ), \*(sGC $\alpha_1^{+/+}$  vs. sGC $\alpha_1^{-/-}$ ): P<0.001. C, Baseline and BAY 41-2272-stimulated sGC enzyme activity in femoral rings from male and female sGC $\alpha_1^{+/+}$  and sGC $\alpha_1^{-/-}$  mice (n=11-14). # (baseline vs. BAY 41-2272), \*(sGC $\alpha_1^{+/+}$  vs. sGC $\alpha_1^{-/-}$ ): P<0.001. Data from male and female animals were pooled.

## **IV.5. Discussion**

The physiological relevance of the sGC isoforms is of great importance to validate sGC subunits as potential pharmacological targets for the treatment of various diseases. In vascular tissue the sGC $\alpha_1$  subunit is predominantly found<sup>5</sup>. However, our results demonstrate that sGC $\alpha_1\beta_1$  is not the only target of both NO-dependent and NO-independent sGC stimulators.

The importance of the sGC $\alpha_1$  subunit in vasorelaxations induced by endogenous and exogenous NO is illustrated by the significantly reduced responses to ACh, SNP, SNAP and NO-gas in the arteries from sGC $\alpha_1^{-/-}$  mice. The relaxant response to ACh (release of endogenous NO from the endothelium) and SNAP (release of exogenous NO upon nonreductive decomposition) was nearly abolished in the aortic rings of sGC $\alpha_1^{-/-}$  mice, whereas SNP (release of exogenous NO upon biotransformation) and NO-gas (represents exogenous NO as such) still elicited relaxation. Femoral arteries of sGC $\alpha_1^{-/-}$  mice showed a substantial response to all three NO-delivering substances (SNAP was not tested).

Besides NO, the endothelium may release other relaxing substances including prostacyclin (PGI<sub>2</sub>) and EDHF depending on the vascular bed studied. In mouse aorta, Chataigneau et al. demonstrated that the ACh-induced response is completely blocked by N<sup>o</sup>-nitro-L-arginine (L-NA), indicating that NO is the sole endothelium-derived vasodilator<sup>15</sup>. The same conclusion can be made from analogous experiments we performed in the femoral arteries of sGC $\alpha_1^{+/+}$  mice.

The continuous release of basal NO keeps the cardiovascular system in a state of constant active vasodilation and plays a substantial role in regulating blood flow and blood pressure<sup>16</sup>. The increase in vascular tone elicited by the sGC-inhibitor ODQ on precontracted preparations was significantly smaller in the aorta from sGC $\alpha_1^{-/-}$  mice. This suggests that basally released NO acts predominantly through activation of sGC $\alpha_1\beta_1$ .

T-1032 causes an accumulation of the basally produced cGMP by inhibiting phosphodiesterase type 5. In comparison to the femoral arteries of the sGC $\alpha_1^{+/+}$  mice, the corresponding arteries of the sGC $\alpha_1^{-/-}$  mice showed significantly less relaxation in response to T-1032. This is in line with the significantly lower basal sGC activity we found in the sGC $\alpha_1^{-/-}$  femoral artery preparations, using a biochemical technique. In the aorta of sGC $\alpha_1^{-/-}$  mice, the relaxant effect of T-1032 is almost abolished and a significantly lower basal cGMP content is found. All these data suggest a diminished basal influence of sGC in blood vessels of sGC $\alpha_1^{-/-}$  mice.

Because of their sGC-stimulating effect, there is a great interest in molecules such as YC-1 and BAY 41-2272<sup>6</sup> as potential new drugs for the treatment of cardiovascular pathologies<sup>9</sup>. Our results confirm that BAY 41-2272 is approximately 30-fold more potent as a vasodilator than YC-1<sup>9</sup>. The finding that the relaxations in response to YC-1 and BAY 41-2272 were significantly diminished in the arteries from male sGC $\alpha_1$ <sup>-/-</sup> mice, suggests that YC-1 and BAY 41-2272 mainly act through activation of sGC $\alpha_1\beta_1$ . However YC-1 and BAY 41-2272 still induce a response in the sGC $\alpha_1$ <sup>-/-</sup> mice.

The finding that the arteries from both sGC $\alpha_1$ <sup>-/-</sup> and sGC $\alpha_1$ <sup>+/+</sup> mice responded in a similar way to the cGMP-analogue, 8 pCPT-cGMP, and the K<sub>ATP</sub> channel opener Lev, excludes that mechanisms downstream of the cGMP formation are affected by knocking out sGC $\alpha_1$ .

All observations demonstrate the functional importance of the sGC $\alpha_1$  subunit as was expected from its predominant presence in vascular tissue. However, the surprising observation that NO, BAY 41-2272 and YC-1 still elicit a relaxing effect in sGC $\alpha_1$ <sup>-/-</sup> mice, indicates that activation of sGC $\alpha_1\beta_1$  is not the sole mechanism responsible for these relaxations. It should however be mentioned that besides their stimulatory effect on sGC, at higher concentrations BAY 41-2272 and YC-1 also increase intravascular cyclic GMP by inhibition of cGMP breakdown through phosphodiesterase type 5, the major cGMP-degrading enzyme in vascular smooth muscle cells<sup>17</sup>. Despite the fact that the NO-donor drugs, SNP and SNAP release the same NO species (NO $\cdot$ , NO<sup>+</sup> and NO<sup>-</sup>)<sup>18</sup>, the SNP-induced response in the aortic rings of the sGC $\alpha_1$ <sup>-/-</sup> mice is far more pronounced. This could be due to the fact that SNP also decomposes to other bioactive compounds, such as cyanide and iron ions<sup>19</sup>, leading to an enhanced oxidative stress and vasorelaxation<sup>20</sup>.

Recently Mergia et al.<sup>21</sup> also showed the substantial role of an sGC $\alpha_1$ -dependent mechanism in NO-related vasorelaxations. However, possible gender and regional differences were not explored in that study. The potential gender difference could be relevant considering that male but not female sGC $\alpha_1$ <sup>-/-</sup> mice develop hypertension from the age of 14 weeks due to an increase in peripheral resistance<sup>10</sup>. Because of this remarkable gender dependency, we performed experiments on both female and male sGC $\alpha_1$ <sup>-/-</sup> and sGC $\alpha_1$ <sup>+/+</sup> mice. No gender differences of sGC $\alpha_1$  deletion were observed in the response to ACh, SNP, NO-gas and BAY 41-2272 indicating that this is not the underlying cause for the development of hypertension in male sGC $\alpha_1$ <sup>-/-</sup> mice. Potential regional variations were also addressed by studying two different types of arteries: the aorta as an elastic artery and the femoral artery as a muscular artery. Overall, similar results were obtained in both vessel types.

The fact that there is still a substantial relaxation in response to several sGC-stimulators in the sGC $\alpha_1^{-/-}$  mice, indicates that besides sGC $\alpha_1\beta_1$  another mechanism is involved, either activation of the sGC $\alpha_2\beta_1$  isoform or activation of (an) sGC-independent mechanism(s) or a combination of these. Arguments in favour of both hypotheses are obtained in the present study.

Our finding that there is still an increase in sGC activity of sGC $\alpha_1^{-/-}$  femoral arteries after addition of BAY 41-2272 is in line with the contribution of the sGC $\alpha_2\beta_1$  isoform in the BAY 41-2272-induced response. The possible contribution of the sGC $\alpha_2\beta_1$  isoform in the NO-induced relaxations seen in sGC $\alpha_1^{-/-}$  mice is also suggested by the observations that ODQ, which inhibits both sGC isoforms, had a strong inhibitory influence on NO-induced relaxations in both the aorta and femoral artery preparations of the sGC $\alpha_1^{-/-}$  mice and that the cGMP production by SNP was significantly reduced in the sGC $\alpha_1^{-/-}$  aortic rings in the presence of ODQ. From their observations Mergia et al. suggest that sGC $\alpha_1$ -independent relaxation is mediated by sGC $\alpha_2$  and that the limited activity of sGC $\alpha_2$  is enough to elicit a response in sGC $\alpha_1^{-/-}$  mice <sup>21</sup>. It should however be mentioned that there is an important difference between the transgenic mouse model developed by Mergia et al. and the one used in the present study. We isolated arteries from sGC $\alpha_1^{-/-}$  mice expressing a mutant sGC $\alpha_1$  protein that is catalytically inactive <sup>10</sup>. This avoids alterations in phenotype due to potential enzyme structural functions as has recently been demonstrated for PI3K $\gamma$  knockout mice <sup>22</sup>. Conversely, Mergia et al. generated sGC $\alpha_1$ -deficient mice with complete abrogation of sGC $\alpha_1$  expression <sup>21</sup>. Therefore, the possible influence on the phenotype of non-catalytic sGC $\alpha_1$  effects, such as complex formation with e.g. AGAP1 <sup>23</sup>, can not be ruled out in that model.

Several observations suggest that also (an)other sGC-independent mechanism(s) might be involved in the remaining relaxation response in sGC $\alpha_1^{-/-}$ . If sGC $\alpha_2\beta_1$  is the sole isoform responsible for the vasorelaxation seen in sGC $\alpha_1^{-/-}$  mice, one would expect a significant increase in cGMP in the sGC $\alpha_1^{-/-}$  mice upon stimulation with NO. However, the sGC $\alpha_1^{-/-}$  aortic ring segments showed only a two-fold, non-significant increase in cGMP in response to SNP. It is questionable that this small increase in cGMP is sufficient to elicit a substantial relaxation (SNP 10  $\mu$ mol/L: 63.23% $\pm$ 3.38 and 48.59% $\pm$ 3.55 in respectively male and female sGC $\alpha_1^{-/-}$  aortas). Under the assumption that the cGMP-relaxation relation in rat aorta <sup>24</sup> is similar to that of mouse aorta, the level of cGMP we observed in the aorta from sGC $\alpha_1^{-/-}$  mice upon stimulation by SNP, would be able to elicit a relaxation of only 10%. This would imply that the rise of cGMP induced by SNP in the sGC $\alpha_1^{-/-}$  aortas is too small to explain a 50% relaxation. This questions a substantial role of the sGC $\alpha_2\beta_1$  isoform in NO-induced relaxations. It should however be mentioned that this reasoning is purely hypothetical. More

convincing are the QPCR-measurements showing no higher expression of the sGC $\alpha_2$  subunit in the ring segments of sGC $\alpha_1^{-/-}$  mice<sup>10</sup>.

In addition to the sGC $\alpha_2\beta_1$  isoform, perhaps (an) sGC-independent mechanism(s) contribute(s) to the substantial relaxation seen in the sGC $\alpha_1^{-/-}$  mice. Various sGC/cGMP-independent actions of NO have been described, including the activation of (i) calcium- and voltage-dependent potassium channels in vascular smooth muscle cells<sup>25, 26</sup>, (ii) sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase<sup>27</sup>, and (iii) vascular Na<sup>+</sup>/K<sup>+</sup>-ATPase<sup>28</sup>. They have not only been reported for NO-donors but also for authentic NO<sup>29</sup> and endogenous NO synthesized from inducible NO synthase<sup>30</sup>. Also the observation that ODQ failed to inhibit the relaxant effect of 10  $\mu$ mol/L BAY 41-2272 in the sGC $\alpha_1^{+/+}$  aortic ring, suggests the involvement of (a) cGMP-independent mechanism(s) rather than sGC $\alpha_2$  activation. There are reports on cGMP-independent mechanisms underlying BAY 41-2272 and YC-1 induced vasorelaxation, including inhibition of Ca<sup>2+</sup> entry<sup>31</sup> and activation of K<sup>+</sup> channels<sup>32</sup> and Na<sup>+</sup>-K<sup>+</sup>-ATPase<sup>33</sup>.

## **IV.6. Conclusions**

From the present study we can conclude that the predominant sGC isoform in the aorta and femoral artery, sGC $\alpha_1\beta_1$ , is indeed involved in the vasorelaxations induced by NO-dependent and NO-independent sGC activators in both genders. However the remaining relaxation seen in the sGC $\alpha_1^{-/-}$  mice, may indicate that besides sGC $\alpha_1\beta_1$  also the less abundantly expressed isoform sGC $\alpha_2\beta_1$  and/or (an) sGC-independent mechanism(s) play(s) a very important role.

## **IV.7. Acknowledgments**

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# Chapter V

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

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## **V.1. Abstract and keywords**

Soluble guanylyl cyclase (sGC) is the major effector molecule for NO, and as such an interesting therapeutic target for the treatment of erectile dysfunction. To assess the functional importance of the sGC $\alpha_1\beta_1$  isoform in corpus cavernosum (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 acetylcholine, bradykinin and electrical field stimulation) was nearly abolished in the sGC $\alpha_1^{-/-}$  CC. In the sGC $\alpha_1^{-/-}$  mice, the relaxing influence of exogenous NO (from sodium nitroprusside and NO-gas), BAY 41-2272 (NO-independent sGC-stimulator) and T-1032 (phosphodiesterase type 5 inhibitor) were also significantly decreased. The remaining exogenous NO-induced relaxation seen in the sGC $\alpha_1^{-/-}$  mice was 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 (adenylyl cyclase 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 and NO-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).

*key words:* penile erection, nitric oxide, soluble guanylyl cyclase, vasodilatation, impotence

## **V.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<sup>1</sup>. Nitric oxide (NO) is widely accepted as the principal mediator of the erectile response. It is produced by neuronal NO synthase (nNOS) in non-adrenergic, non-cholinergic (NANC) nerves, innervating the penis<sup>2</sup>. Although also sinusoidal and vascular endothelial cells release NO in response to mechanical<sup>3</sup> and chemical stimuli<sup>1,4</sup>, 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<sup>5</sup>. Regardless of the source, NO binds to the heme component of soluble guanylyl cyclase (sGC), leading to a 300-fold increase in the catalytic conversion of guanosine-5'-triphosphate (GTP) to cyclic guanosine-3', 5'-monophosphate (cGMP) and pyrophosphate<sup>6</sup>. This high amount of cGMP conveys signals through activation of cGMP-dependent protein kinase I,

eventually leading to smooth muscle relaxation<sup>7, 8</sup>. sGC is a heterodimer composed of two subunits,  $\alpha$  and  $\beta$ <sup>9</sup>, both essential for catalytic activity<sup>10</sup>. Two isoforms for each subunit ( $\alpha_1/\alpha_2$  and  $\beta_1/\beta_2$ ) have been described<sup>11-13</sup>, but only the  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  heterodimers are found active<sup>14</sup>. 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<sup>15</sup>. Various diseases, including hypertension<sup>16, 17</sup>, hypercholesterolemia<sup>18</sup>, diabetes mellitus<sup>19</sup> and renal failure<sup>20</sup>, that cause erectile dysfunction (ED) are highly associated with impairments of the NO/cGMP signaling pathway. The central role of this pathway is demonstrated by the phosphodiesterase type-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<sup>21, 22</sup>, 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 analyse the functional importance of the sGC $\alpha_1\beta_1$  isoform in penile smooth muscle relaxation using soluble guanylyl cyclase alpha 1 knockout (sGC $\alpha_1^{-/-}$ ) mice.

### **V.3. Materials and methods**

#### **V.3.1. Animals**

All experiments were performed on male homozygous soluble guanylyl cyclase alpha 1 knockout (sGC $\alpha_1^{-/-}$ ; n= 6-9) mice and sGC $\alpha_1^{+/+}$  (n= 6-11) mice (genetic background: mixed Swiss-129)<sup>23</sup>, 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.

#### **V.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 corpora cavernosa (CC) were separated by cutting the fibrous septum between them and were excised at the base. They were kept in cooled Krebs-Ringer bicarbonate (KRB) solution until mounting.

### V.3.3. Tension measurements

Of each mouse, one corpus cavernosum 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<sub>2</sub>-5% CO<sub>2</sub>. 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  $\mu$ mol/L norepinephrine (NOR), washed, and allowed to relax to resting tension before starting the protocol. When the pre-contraction response reached a stable level, electrical field stimulation (EFS: train duration 20s or 40s; 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 analyse the contractile response. EFS was repeated after incubation with atropine (1  $\mu$ mol/L) and guanethidine (4  $\mu$ mol/L) for 30 minutes to eliminate responses mediated by cholinergic and noradrenergic nerves, respectively. The influence of the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (1  $\mu$ mol/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.

### V.3.4. Drugs

The experiments were performed in a KRB solution of the following composition (mmol/L): NaCl, 135; KCl, 5; NaHCO<sub>3</sub>, 20; glucose, 10; CaCl<sub>2</sub>, 2.5; MgSO<sub>4</sub>, 1.3; KH<sub>2</sub>PO<sub>4</sub>, 1.2 and EDTA, 0.026 in H<sub>2</sub>O. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), acetylcholine chloride (ACh), bradykinin acetate (BK), N<sup>w</sup>-nitro-L-arginine, forskolin, 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate (8-pCPT-cGMP), atropine, guanethidine and norepinephrine bitartrate 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 acetylcholine in 50 mmol/L potassium hydrogen phthalate 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<sup>24</sup>. 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%.

### V.3.5. Calculations and statistics

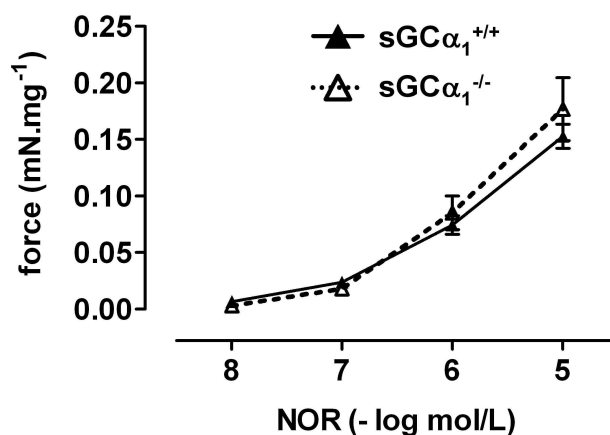
Data are presented as mean values  $\pm$  SEM;  $n$  represents the number of corpora cavernosa (each obtained from a different mouse). Relaxations are expressed as a percentage of the tone developed by the addition of NOR. Contractions are expressed in mN.

Statistical significance was evaluated using Student's  $t$ -test for paired and unpaired observations (SPSS, version 12).  $P < 0.05$  was considered as significant.

## V.4. Results

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

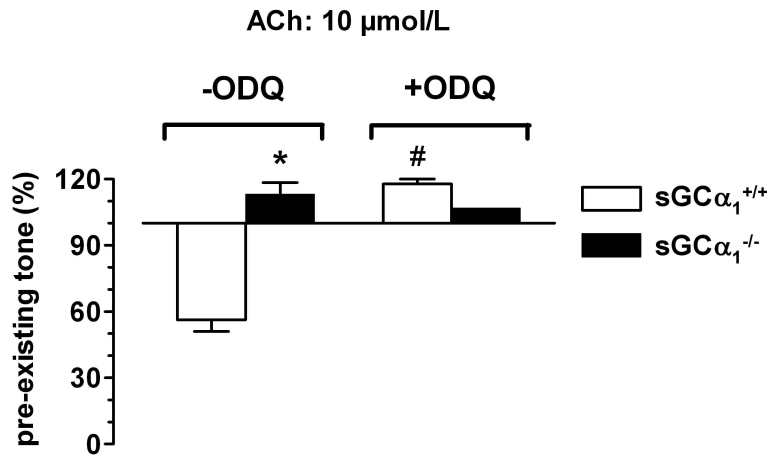
In response to increasing concentrations of NOR (10 nmol/L–10  $\mu$ mol/L), the penile tissue isolated from  $sGC\alpha_1^{-/-}$  mice, developed an equal force per mg tissue compared with the  $sGC\alpha_1^{+/+}$  preparations (figure V.1).



**Figure V.1:** Cumulative concentration-contraction curve to NOR in CC from  $sGC\alpha_1^{+/+}$  ( $\blacktriangle$ ;  $n=11$ ) and  $sGC\alpha_1^{-/-}$  ( $\Delta$ ;  $n=9$ ) mice.

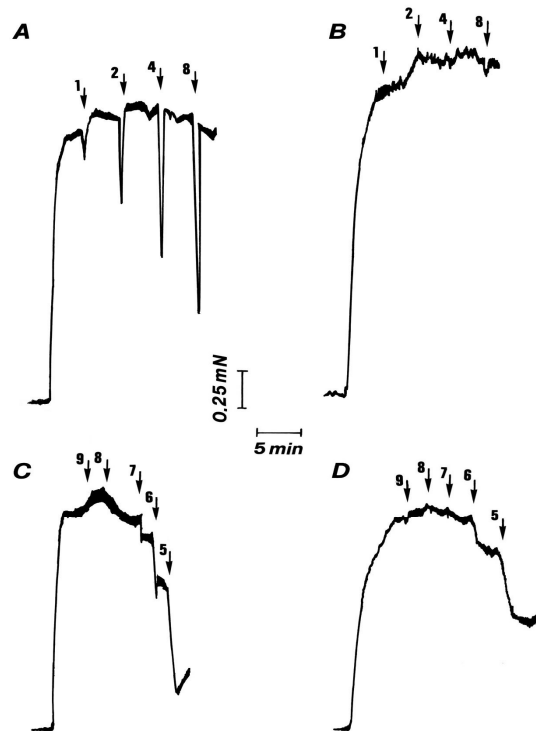
The ability to relax NOR-contracted CC preparations through release of endothelial NO was tested by addition of ACh (10  $\mu$ mol/L) and BK (50  $\mu$ mol/L). ACh relaxed the  $sGC\alpha_1^{+/+}$  preparations, whereas it contracted the tissues of  $sGC\alpha_1^{-/-}$  mice (figure V.2). Inhibition of sGC

by ODQ, resulted in a contractile effect of ACh in both sGC $\alpha_1^{-/-}$  and sGC $\alpha_1^{+/+}$  CC tissues (figure V.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 ( $4.55\% \pm 2.80$  in sGC $\alpha_1^{-/-}$  vs.  $26.82\% \pm 2.86$  in sGC $\alpha_1^{+/+}$  (n=6 each, P<0.05)).

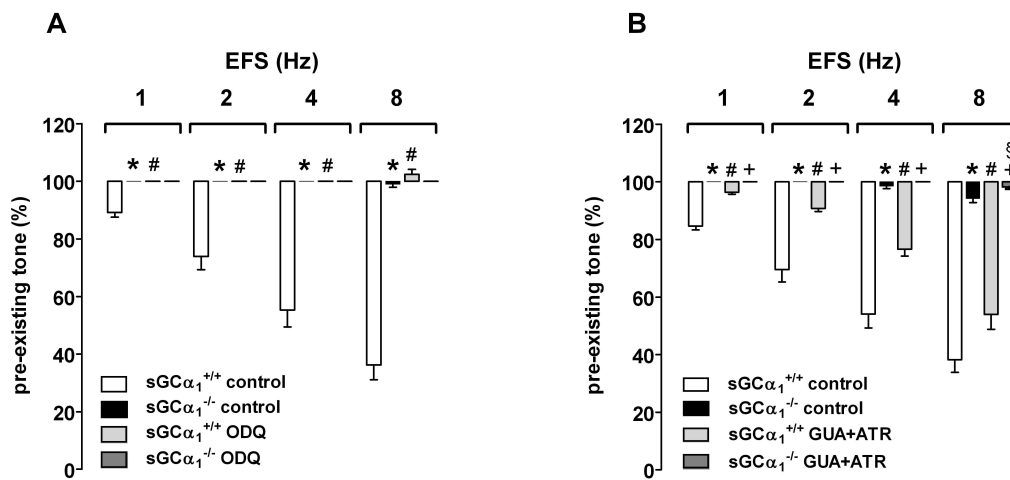


**Figure V.2:** Relaxation effect of ACh on precontracted (5  $\mu\text{mol/L}$  NOR) CC from sGC $\alpha_1^{+/+}$  (n=7) and sGC $\alpha_1^{-/-}$  (n=7) mice in control conditions (-ODQ) and in the presence of ODQ (+ODQ). \*(sGC $\alpha_1^{+/+}$  vs. sGC $\alpha_1^{-/-}$ ), #(-ODQ vs. +ODQ): P<0.05.

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 V.3A), whereas the response in the sGC $\alpha_1^{-/-}$  preparations was nearly abolished (figures V.3B and V.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 V.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 V.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 20s to 40s (data not shown).



**Figure V.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.

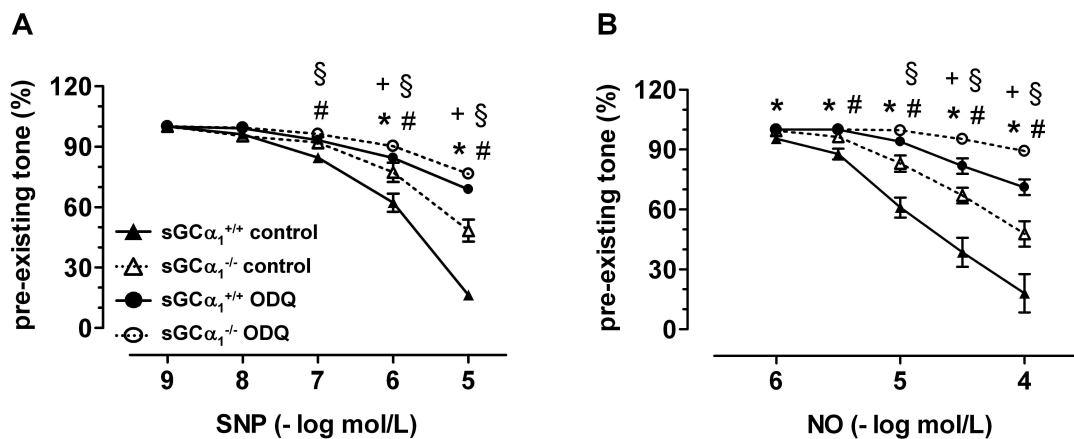


**Figure V.4:** Effect of EFS on precontracted (5  $\mu$ mol/L NOR) CC from  $sGC\alpha_1^{+/+}$  (n=6) and  $sGC\alpha_1^{-/-}$  (n=6) mice in control conditions and in the presence of ODQ (A) or guanethidine (GUA) and atropine (ATR) (B). \*( $sGC\alpha_1^{+/+}$  vs.  $sGC\alpha_1^{-/-}$ ), +( $sGC\alpha_1^{+/+}$  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):  $P < 0.05$ .



Administration of increasing concentrations of the endothelium-independent NO-donor compound SNP (1 nmol/L–10  $\mu$ mol/L) resulted in a concentration-dependent relaxation of the sGC $\alpha_1^{+/+}$  (figure V.3C) and sGC $\alpha_1^{-/-}$  (figure V.3D) CC preparations that was significantly reduced in the sGC $\alpha_1^{-/-}$  mice as compared to sGC $\alpha_1^{+/+}$  mice (figure V.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 V.5A).

Exogenous NO delivered as gas (1  $\mu$ mol/L–100  $\mu$ mol/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 V.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 V.5B).



**Figure V.5:** Relaxation effect of SNP (A) and NO-gas (B) on precontracted (5  $\mu$ mol/L NOR) CC from sGC $\alpha_1^{+/+}$  and sGC $\alpha_1^{-/-}$  mice in control conditions (▲ and Δ) and in the presence of ODQ (● and ○).

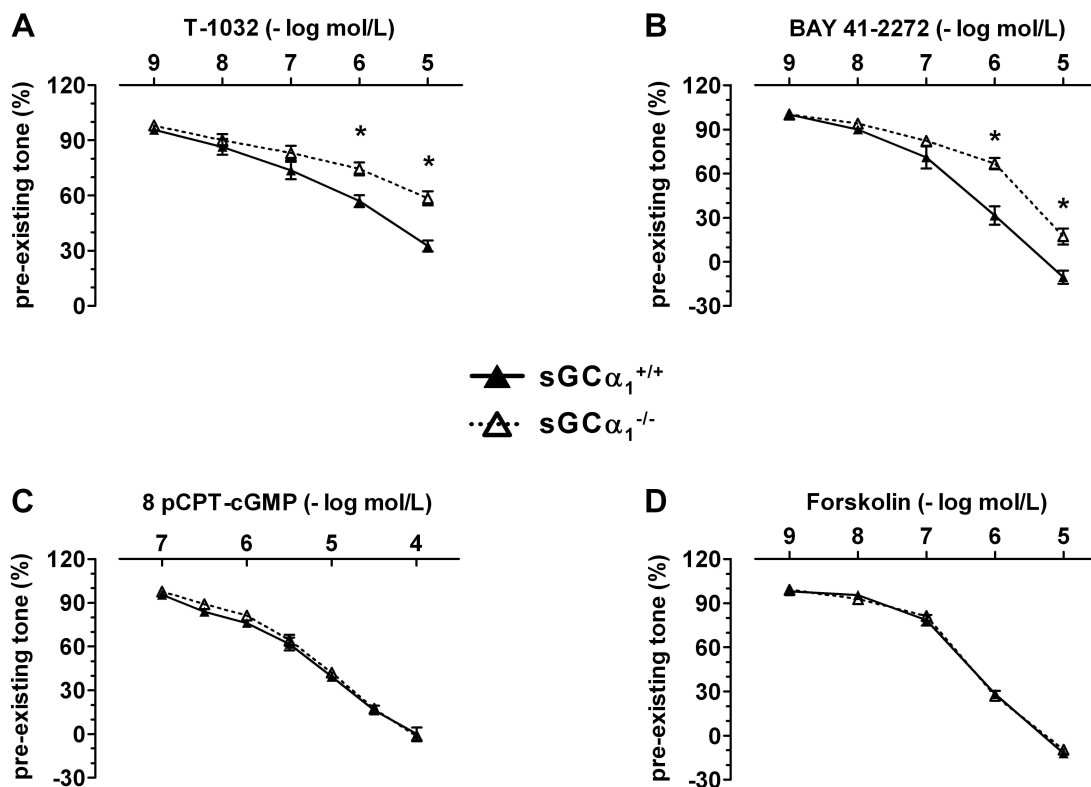
\*(sGC $\alpha_1^{+/+}$  vs. sGC $\alpha_1^{-/-}$ ), +(sGC $\alpha_1^{+/+}$  ODQ vs. sGC $\alpha_1^{-/-}$  ODQ):  $P < 0.05$ , (n=6-7).

The inhibition of phosphodiesterase type 5 by T-1032<sup>25</sup> (1 nmol/L–10  $\mu$ mol/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 V.6A).

Addition of BAY-41-2272, an NO-independent sGC-stimulator<sup>26</sup> (1 nmol/L–10  $\mu$ mol/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 V.6B).

There was no difference in the concentration-dependent response to the cell membrane permeable cGMP-analogue, 8-pCPT-cGMP (100 nmol/L–10  $\mu$ mol/L) between the sGC $\alpha_1^{-/-}$  and sGC $\alpha_1^{+/+}$  preparations (figure V.6C).

Also forskolin (1 nmol/L–10  $\mu$ mol/L), an adenylyl cyclase-stimulator, induced an identical concentration-dependent response in the sGC $\alpha_1^{-/-}$  and sGC $\alpha_1^{+/+}$  preparations (figure V.6D).



**Figure V.6:** Relaxation effect of T-1032 (A), BAY 41-2272 (B), 8-pCPT-cGMP (C) and Forskolin (D) on precontracted (5  $\mu$ mol/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.

## **V.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 corpora cavernosa is sGC $\alpha_1\beta_1$ <sup>27</sup>. 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 $\alpha_1$ <sup>-/-</sup> mice compared to the sGC $\alpha_1$ <sup>+/+</sup> CC tissues. Cavernosum from sGC $\alpha_1$ <sup>-/-</sup> 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<sup>28</sup>. 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<sup>5</sup>. Moreover, our data suggest the involvement of endothelium-derived NO induced by ACh released from parasympathetic nerve fibers<sup>29</sup>, as atropine inhibited the EFS-induced relaxation in the CC of sGC $\alpha_1$ <sup>+/+</sup> 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 $\alpha_1$ <sup>-/-</sup> mice, the response to EFS was completely abolished, even after prolonged stimulation (40 seconds), 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 the vasoactive intestinal peptide (VIP) as inhibitory neurotransmitter in penile erection<sup>30</sup>. VIP, which is present in the nerves of murine corpora cavernosa, as well as other species, stimulates adenylyl cyclase (AC) and subsequently elevates the cAMP-dependent protein kinase<sup>31</sup>. As it has been shown that there are cross-modulatory functions between the sGC/cGMP- and AC/cAMP-signaling pathways<sup>32</sup>, 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 $\alpha_1$ <sup>-/-</sup> and sGC $\alpha_1$ <sup>+/+</sup> 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 $\alpha_1$ <sup>-/-</sup> mice. Furthermore, this unaltered forskolin-induced response in the sGC $\alpha_1$ <sup>-/-</sup> 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<sup>+</sup>/K<sup>+</sup>-ATPase activity, NO (derived from SNP) relaxes human corpus cavernosum smooth muscle cells independently of its ability to increase the intracellular cGMP concentration<sup>33</sup>. The ability of NO to directly activate calcium-dependent potassium channels or sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase, as described for vascular smooth muscle cells<sup>34</sup>, might be involved. The administration of T-1032, which blocks the hydrolysis of cGMP by phosphodiesterase type 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<sup>26</sup>, are of particular interest for ED-patients who respond less well to phosphodiesterase type 5 inhibitors because of severe endothelial and/or nerve dysfunction<sup>35</sup>. 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<sup>36</sup> and also to exert some cGMP-independent actions<sup>37</sup>. 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.

## **V.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.

## **V.7. Acknowledgements**

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# Chapter VI

## Vascular and corporal smooth muscle responsiveness in soluble guanylyl cyclase $\beta_1$ His 105 Phe mutant mice.

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In preparation



## **VI.1. Abstract and keywords**

The binding of nitric oxide (NO) to the heme group of soluble guanylyl cyclase (sGC), results in a burst of cGMP, which in turn mediates vascular and corporal smooth muscle relaxation. The sGC $\alpha_1\beta_1$  and sGC $\alpha_2\beta_1$  heterodimer are reported to be physiologically active, in which the  $\beta_1$  subunit acts as dimerizing partner for both  $\alpha$  subunits. As the histidine (His) residue at position 105 of the  $\beta_1$  subunit functions as axial ligand for the heme prosthetic group, substitution of His by phenylalanine (Phe) will abolish the heme-dependent activation of sGC. This is the case in the sGC $\beta_1$ <sup>ki/ki</sup> mice from which artery segments (aorta and femoral artery) and corpora cavernosa (CC) were isolated and mounted on a myograph for isometric tension recording. In comparison with the preparations isolated from the wild type mice, the response to endogenous NO (released from the endothelium in response to acetylcholine (ACh)) and exogenous NO (from the NO-donor sodium nitroprusside (SNP)) were completely abolished in the aortic rings from the sGC $\beta_1$ <sup>ki/ki</sup> mice. In the sGC $\beta_1$ <sup>ki/ki</sup> femoral arteries, on the other hand, both NO-releasing substances still had a relaxing effect. In the CC from the sGC $\beta_1$ <sup>ki/ki</sup> mice, the response to endogenous NO, which is induced by ACh and electrical field stimulation (EFS) (neuronal derived NO), was completely abolished, whereas SNP could still induce a response. The relaxing influence of the NO-independent sGC stimulator BAY 41-2272 was significantly reduced in the arteries and CC from the sGC $\beta_1$ <sup>ki/ki</sup> mice, though it was not completely abolished. The arteries from the sGC $\beta_1$ <sup>ki/ki</sup> mice showed a higher response to NOR. Moreover, the responses to methoxamine (specific  $\alpha_1$ -adrenoreceptor agonist), prostaglandin (prostanoid receptor agonist) and potassium ( $K^+$ ) were significantly higher in the aortic rings from the sGC $\beta_1$ <sup>ki/ki</sup> mice, but not in the corresponding femoral arteries. In the presence of the Rho kinase inhibitor HA-1077, the response to NOR was still significantly higher in the aortic rings from the sGC $\beta_1$ <sup>ki/ki</sup> mice, whereas in the femoral arteries, the difference in the NOR-induced contraction between the sGC $\beta_1$ <sup>ki/ki</sup> and sGC $\beta_1$ <sup>+/+</sup> mice was abolished. The influx of  $Ca^{2+}$  induced by tetraethylammonium chloride (a non-selective  $K^+$ -channel blocker) and BAY k 8644 (L-type  $Ca^{2+}$ -channel agonist) resulted in a significantly higher force development in the aortic rings, but not in the femoral arteries from the sGC $\beta_1$ <sup>ki/ki</sup> mice. All those data indicate that in the sGC $\beta_1$ <sup>ki/ki</sup> aortic rings, the contractile response is rather unselective, whereas in the corresponding femoral arteries it is less marked and rather specific for NOR. The administration of the sGC-inhibitor ODQ resulted in a significant, comparable increase of the NOR-induced tone in the aorta and femoral artery from C57BL/6J mice. This suggests that the enhanced contractile activity of the sGC $\beta_1$ <sup>ki/ki</sup> preparations is at

least in part due to the abolished vasorelaxing influence of basal endothelial NO. In previous studies <sup>1, 2</sup>, where we characterised mice which are deficient for the sGC $\alpha_1$  gene, we found that the responses to SNP and BAY 41-2272 were significantly reduced but not completely abolished in the preparations of the sGC $\alpha_1^{-/-}$  mice. Apart from the CC, also ACh still had a relaxing effect in the sGC $\alpha_1^{-/-}$  mice. From those data, together with the present data, we can conclude that in the aortic rings, sGC is the sole target for NO, whereas in the femoral arteries and CC, also (an) sGC-independent mechanism(s) play(s) a substantial role. In the CC, the predominant sGC $\alpha_1\beta_1$  isoform is the main target for NO, as well as for BAY 41-2272. This is also the case for the response to endogenous NO and BAY 41-2272 in the arteries, whereas exogenously applied NO mainly activates the less abundantly expressed sGC $\alpha_2\beta_1$  isoform.

*Key words:* arteries, corpora cavernosa, soluble guanylyl cyclase, nitric oxide, vasoconstriction/dilatation

## **VI.2. Introduction**

Soluble guanylyl cyclase (sGC) plays a pivotal role in the transduction of cellular signals conveyed by the messenger molecule nitric oxide (NO). By the formation of cGMP, this enzyme mediates NO-elicited actions, particularly smooth muscle relaxation, thereby controlling blood flow, blood pressure <sup>3</sup>, and erectile function <sup>4</sup>. The cloning of sGC from various species has revealed that the protein is a heterodimer composed of a larger  $\alpha$  and a smaller  $\beta$  subunit <sup>5</sup>, required for catalysis <sup>6</sup>. Although two  $\alpha$  ( $\alpha_1$  and  $\alpha_2$ ) and two  $\beta$  ( $\beta_1$  and  $\beta_2$ ) subunits have been identified so far <sup>7-9</sup>, only the  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  heterodimers have been shown to occur in vivo <sup>10</sup>. The  $\alpha_1\beta_1$  isoform predominates in most tissues except the brain, in which relatively high levels of the  $\alpha_2\beta_1$  isoform are expressed <sup>11</sup>.

The low basal activity of sGC increases several hundred-fold upon the binding of NO to the heme prosthetic group, which is associated with the N-terminal region of the  $\beta_1$  subunit through a weak histidine (His) 105-iron bond. Wedel et al. showed that histidine 105 of the  $\beta_1$  subunit is essential for the stimulation by NO since substitution by phenylalanine yielded an enzyme that was catalytically active but insensitive to NO <sup>12</sup>. Upon activation of sGC, the accumulating intracellular cGMP targets principally cGMP-dependent protein kinase I (PKG I) to bring about smooth muscle relaxation <sup>13</sup>. Due to its ubiquitous nature, the pathogenesis of various disease states has been linked to inappropriate activation of sGC <sup>14</sup>. The different isoforms of sGC are therefore very attractive as potential new therapeutic targets for the

treatment of among others cardiovascular diseases and erectile dysfunction. In previous studies we used sGC $\alpha_1$ <sup>-/-</sup> mice to analyse the functional importance of the sGC $\alpha_1$  subunit in vascular<sup>1</sup> and penile<sup>2</sup> smooth muscle relaxation. We found that besides the predominant sGC $\alpha_1\beta_1$  isoform, also the less abundantly expressed sGC $\alpha_2\beta_1$  and/or (an) sGC-independent mechanism(s) are involved in the NO- and NO-independent sGC mediated smooth muscle relaxation. To assess the relative contribution of sGC $\alpha_2\beta_1$  and (an) sGC-independent mechanism(s), we examined the responsiveness of vessel segments and corpora cavernosa isolated from mice, that express NO-insensitive sGC (sGC $\beta_1$ <sup>ki/ki</sup> mice), to NO and an NO-independent sGC activators.

## **VI.3. Materials and methods**

### **VI.3.1. Animals**

Almost all experiments were performed on male and/or female homozygous soluble guanylyl cyclase beta 1 knockin (sGC $\beta_1$ <sup>ki/ki</sup>) mice and sGC $\beta_1$ <sup>+/+</sup> mice (genetic background: mixed 129/SvJ-C57BL/6J), developed and bred in the Department of Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology, Ghent, Belgium. The Sixth series of experiments were performed on female C57BL/6J mice (Janvier). 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). The mice were euthanized by cervical dislocation.

### **VI.3.2. Blood vessel study**

#### *VI.3.2.1. Tissue collection*

The thoracic aorta and femoral artery were carefully removed from the animals and transferred to cooled Krebs-Ringer bicarbonate (KRB) solution.

#### *VI.3.2.2. Tension measurements*

Ring segments of the collected arteries were mounted in a small-vessel myograph with a tissue chamber filled with 10 ml of KRB solution and were cleansed from adhering tissue. Two stainless steel wires (40  $\mu$ m 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<sub>2</sub>-5% CO<sub>2</sub> (pH 7.4) at 37 °C. The aortic rings were gradually stretched until a stable preload of 0.5 g was obtained, whereas the femoral arteries were set to their normalized internal diameter<sup>15</sup>. In short, the arteries were stretched 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 mm Hg.

After applying the optimal resting tension, the preparations were contracted (1 till 3 times) with a KRB solution containing 120 mmol/L K<sup>+</sup> and 5 μmol/L norepinephrine (NOR), washed, and allowed to relax to basal tension before starting the protocol. Precontraction was elicited with prostaglandin (30 μmol/L PGF<sub>2α</sub>, unless mentioned otherwise). When a stable contraction plateau was obtained, cumulative concentration-response curves were obtained by increasing the concentration in log increments, once the response to the previous concentration had stabilized. Relaxation responses to Acetylcholine (ACh), Sodium nitroprusside (SNP), BAY 41-2272, 8-pCPT-cGMP and forskolin were examined in varying order. The concentration-response curves to ACh and SNP were repeated on vessel segments in which a comparable level of precontraction was obtained in preparations from the sGCβ<sub>1</sub><sup>+/+</sup> and sGCβ<sub>1</sub><sup>ki/ki</sup> mice. Therefore, a concentration-response curve to PGF<sub>2α</sub> was obtained on the sGCβ<sub>1</sub><sup>ki/ki</sup> and sGCβ<sub>1</sub><sup>+/+</sup> vessel segments from which the adequate concentration was deduced. To analyse the differences in contractile responses between preparations from sGCβ<sub>1</sub><sup>+/+</sup> and sGCβ<sub>1</sub><sup>ki/ki</sup> mice, a single dose (tetraethylammonium chloride (TEA)) or increasing concentrations (NOR, PGF<sub>2α</sub>, methoxamine and Bay k 8644) of the vasoconstrictor were added to the preparations at a stable resting tension. The cumulative concentration-response curves to NOR, PGF<sub>2α</sub>, methoxamine and Bay k 8644 were determined by a stepwise increase in the concentration of the contractile substance as soon as a steady response to the preceding concentration had been obtained. The concentration-response curve to NOR was repeated in the presence of HA-1077 (10 μmol/L, 15 minutes preincubation) or ODQ (1 μmol/L, 20 minutes preincubation). The cumulative concentration-response curve to K<sup>+</sup> was made by replacing the KRB solution by a modified KRB solution containing 30, 60 or 120 mmol/l K<sup>+</sup> (changes in the KCl concentration of the KRB solution were compensated by equimolar adjustment of the NaCl concentration). Segments of sGCβ<sub>1</sub><sup>ki/ki</sup> and sGCβ<sub>1</sub><sup>+/+</sup> mice were always tested in parallel.

### VI.3.3. Corpora cavernosa study

#### *VI.3.3.1. 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 corpora cavernosa (CC) were separated by cutting the fibrous septum between them and were excised at the base. They were kept in cooled Krebs-Ringer bicarbonate (KRB) solution until mounting.

#### *VI.3.3.2. Tension measurements*

Of each mouse, one corpus cavernosum 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<sub>2</sub>-5% CO<sub>2</sub>. 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  $\mu$ mol/L norepinephrine (NOR), washed, and allowed to relax to resting tension before starting the protocol. When the pre-contraction response (5  $\mu$ mol/L NOR) reached a stable level, electrical field stimulation (EFS: train duration 20s or 40s; 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. Additionally, the relaxant response to a single concentration of ACh and cumulative concentration-response curves to SNP, NO gas, HA-1077 and 8-pCPT-cGMP were investigated after NOR-induced precontraction. The contractile effect of NOR was assessed by adding increasing concentrations of NOR at a stable resting tension. In between the concentration-response curves, the CC were washed and allowed to recover for 20-30 min. Corporal tissues of sGC $\beta_1$ <sup>ki/ki</sup> and sGC $\beta_1$ <sup>+/+</sup> mice were always tested in parallel. At the end of the experiments, tissues were lightly patted dry and weighed.

### VI.3.4. Drugs

The experiments were performed in a KRB solution of the following composition (mmol/L): NaCl, 135; KCl, 5; NaHCO<sub>3</sub>, 20; glucose, 10; CaCl<sub>2</sub>, 2.5; MgSO<sub>4</sub>, 1.3; KH<sub>2</sub>PO<sub>4</sub>, 1.2 and EDTA, 0.026 in H<sub>2</sub>O. Acetylcholine chloride (ACh), forskolin, 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate (8-pCPT-cGMP), methoxamine hydrochloride, HA-

1077 dihydrochloride, tetraethylammonium chloride (TEA), Bay k 8644, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) 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). BAY 41-2272 and ODQ were dissolved in dimethylsulfoxide, Bay k 8644 in ethanol and ACh in 50 mmol/L potassium hydrogen phthalate 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<sup>16</sup>. All concentrations are expressed as final molar concentrations in the organ bath. The final concentration of dimethylsulfoxide or ethanol in the organ bath never surpassed 0.1%.

### VI.3.5. Calculations and statistics

Data are presented as mean values  $\pm$  SEM;  $n$  represents the number of preparations (each obtained from a different mouse). In the figures, relaxations are expressed as a percentage of the pre-contraction tone developed by the addition of the precontractile agent, whereas in the results section, relaxations are expressed in terms of percentage decrease of the pre-contraction tone. Contractions are expressed in mN. The reduction or the remaining part of a response in the preparations of the transgenic mice is expressed in term of percentage relatively compared to the response in the preparations of the corresponding control mice. Statistical significance was evaluated using Student's  $t$ -test for paired and unpaired observations (SPSS, version 12).  $P < 0.05$  was considered as significant. The estimated maximum relaxation ( $E_{max}$ ) and the negative logarithm of the concentration producing 50% of the maximal effect ( $pEC_{50}$ ) were calculated using nonlinear regression curve fit (GraphPad Prism, version 4).

## VI.4. Results

### VI.4.1. Blood vessel study

#### *VI.4.1.1. First set of experiments*

In a first series of experiments, the relaxing effect of **ACh**, **SNP** en **BAY 41-2272** were investigated on precontracted (30  $\mu$ mol/L  $PGF_{2\alpha}$ ) aortic (ACh: Fig. VI.1A; SNP: Fig. VI.1C; BAY 41-2272: Fig. VI.1E) and femoral artery rings (ACh: Fig. VI.1B; SNP: Fig. VI.1D; BAY 41-2272: Fig. VI.1F) isolated from male  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice.



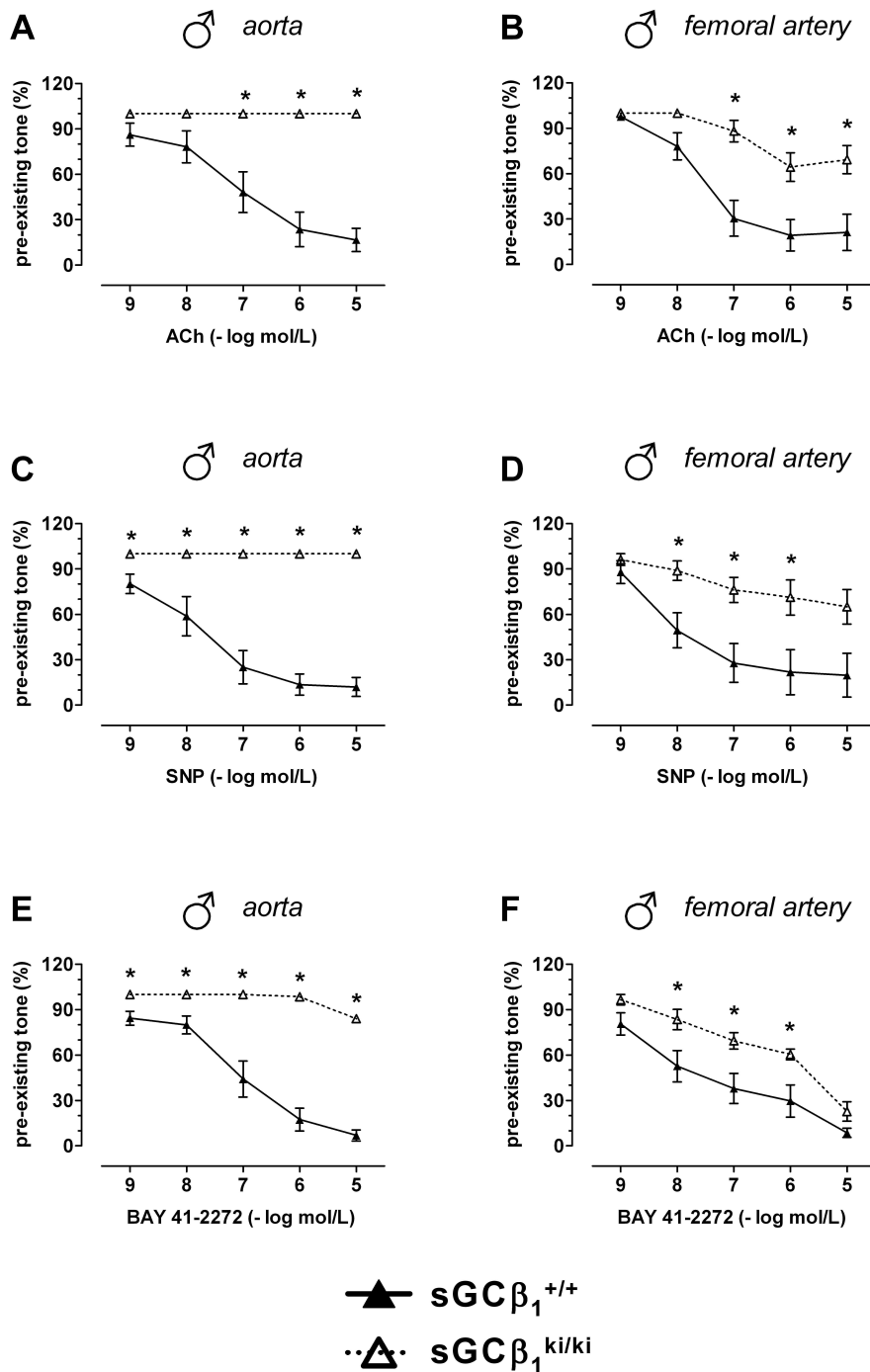
**The endothelium-dependent vasodilator ACh** (1 nmol/L–10  $\mu$ mol/L) elicited a concentration-dependent response in the sGC $\beta_1$ <sup>+/+</sup> vessel segments, whereas in the aortic rings of the sGC $\beta_1$ <sup>ki/ki</sup> mice, this response was completely abolished. Also in the sGC $\beta_1$ <sup>ki/ki</sup> femoral artery segments, the ACh-evoked response was markedly impaired (the response induced by 10  $\mu$ mol/L ACh was reduced by 61.05 %) compared to the femoral artery rings from the sGC $\beta_1$ <sup>+/+</sup> mice.

When added cumulatively to the bathing medium, **the NO-donor SNP** (1 nmol/L–10  $\mu$ mol/L) elicited a concentration-dependent relaxation in the aortic and femoral artery rings of the sGC $\beta_1$ <sup>+/+</sup> mice. In the aorta from sGC $\beta_1$ <sup>ki/ki</sup> mice, the relaxant effect of SNP was completely eliminated, while in the corresponding femoral artery rings a significantly reduced response to SNP was observed (the response induced by 10  $\mu$ mol/L SNP was reduced by 56.37%).

The cumulative addition of **BAY 41-2272** (1 nmol/L–10  $\mu$ mol/L), **an NO-independent sGC-stimulator**, resulted in a concentration-dependent response in the aortic and femoral artery rings of the sGC $\beta_1$ <sup>+/+</sup> mice. In the sGC $\beta_1$ <sup>ki/ki</sup> aortic rings, only a concentration of 10  $\mu$ mol/L BAY 41-2272 had a minor relaxing influence. The femoral artery from the sGC $\beta_1$ <sup>ki/ki</sup> mice, showed a reduced sensitivity as compared to sGC $\beta_1$ <sup>+/+</sup> preparations (pEC<sub>50</sub>: 7.57 $\pm$ 0.48 for sGC $\beta_1$ <sup>+/+</sup> vs. 6.06 $\pm$ 0.24 for sGC $\beta_1$ <sup>ki/ki</sup> (n=4, P<0.05)). The estimated maximal response to BAY 41-2272 in both sGC $\beta_1$ <sup>+/+</sup> and sGC $\beta_1$ <sup>ki/ki</sup> femoral arteries was comparable (E<sub>max</sub>: 94.8% $\pm$ 3.17 for sGC $\beta_1$ <sup>+/+</sup> vs. 97.18% $\pm$ 2.82 for sGC $\beta_1$ <sup>ki/ki</sup> (n=4, P>0.05)).

Additionally, we examined the **NOR**-induced constriction in the aortic and femoral artery rings of male sGC $\beta_1$ <sup>+/+</sup> and sGC $\beta_1$ <sup>ki/ki</sup> mice (data not shown). The concentration-response curve to NOR (10 nmol/L–10  $\mu$ mol/L) showed a remarkable upward shift (indicated by an increase in estimated maximal contractile response: 1.57 mN $\pm$ 0.7 for sGC $\beta_1$ <sup>+/+</sup> vs. 23.52 mN $\pm$ 1.47 for sGC $\beta_1$ <sup>ki/ki</sup> (n=5, P<0.05)) in the aortic rings from the sGC $\beta_1$ <sup>ki/ki</sup> mice in comparison to the corresponding sGC $\beta_1$ <sup>+/+</sup> rings. Also the femoral artery rings of the sGC $\beta_1$ <sup>ki/ki</sup> mice showed an increased contractile response to NOR (10  $\mu$ mol/L NOR: 2.94 mN $\pm$ 0.19 vs. 8.27 mN $\pm$ 1.17 (n=3, p<0.05)). This increased contractile responsiveness of the sGC $\beta_1$ <sup>ki/ki</sup> mice was also observed in the contractions that are elicited by the combination of a **KRB solution containing 120 mmol/L K<sup>+</sup> and 5  $\mu$ mol/L NOR** applied repeatedly on the vessel segments before starting the protocol. The first as well as the third time, the contractile response was significantly higher in the aorta and femoral artery from the sGC $\beta_1$ <sup>ki/ki</sup> mice compared to the matching sGC $\beta_1$ <sup>+/+</sup> rings. The difference in contraction level between sGC $\beta_1$ <sup>+/+</sup> and sGC $\beta_1$ <sup>ki/ki</sup> mice was significantly more pronounced in the aortas than in the

femoral arteries (first response to K120 and 5  $\mu\text{mol/L}$  NOR: 2.03  $\text{mN}\pm 1.13$  for the femoral artery vs. 12.89  $\text{mN}\pm 1.25$  for the aorta; third response to K120 and 5  $\mu\text{mol/L}$  NOR: 2.3  $\text{mN}\pm 0.96$  for the femoral artery vs. 17.67  $\text{mN}\pm 2.31$  for the aorta ( $n=4-6$ ,  $P<0.05$ )).

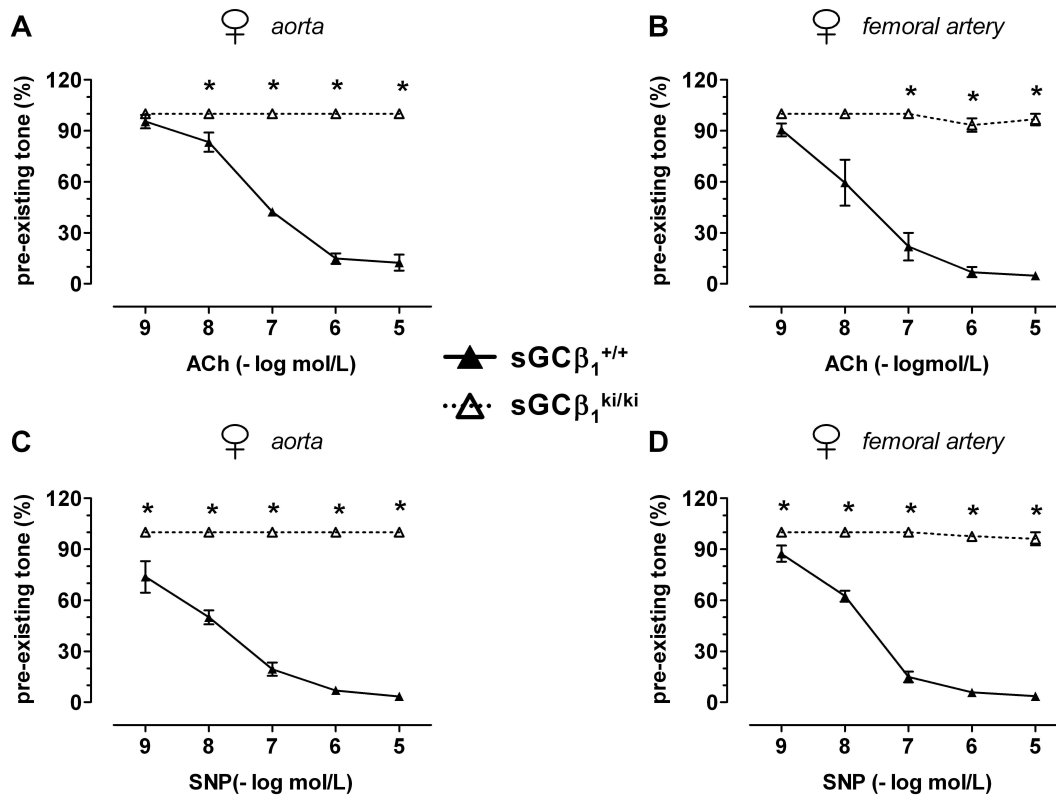


**Figure VI.1:** Relaxation effect of ACh (A, B), SNP (C, D) and BAY 41-2272 (E, F) on precontracted (30  $\mu\text{mol/L}$   $\text{PGF}_{2\alpha}$ ) aortas (A, C, E) ( $n=6$ ) and femoral arteries (B, D, F) ( $n=4$ ) from male  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice. \*( $sGC\beta_1^{+/+}$  vs.  $sGC\beta_1^{ki/ki}$ ):  $P<0.05$ ).

#### VI.4.1.2. Second set of experiments

In a separate series of experiments, the relaxing influence of **ACh** (1 nmol/L–10 μmol/L), **SNP** (1 nmol/L–10 μmol/L) and the **cell permeable cGMP analogue 8-pCPT-cGMP** (100 nmol/L–0.1 mmol/L) (were examined on aortic (ACh: Fig. VI.2A; SNP: Fig. VI.2C; 8-pCPT-cGMP: Fig. VI.7A) and femoral artery (ACh: Fig. VI.2B; SNP: Fig. VI.2D; 8-pCPT-cGMP: Fig. VI.7B) segments in which the concentration of PGF<sub>2α</sub> was adjusted to obtain a similar precontraction level in female sGCβ<sub>1</sub><sup>ki/ki</sup> and sGCβ<sub>1</sub><sup>+/+</sup> vessel segments (precontraction level of the concentration-response curve to ACh : aortic rings: 19.64 mN±0.57 for sGCβ<sub>1</sub><sup>+/+</sup> vs. 19.43 mN±1.68 for sGCβ<sub>1</sub><sup>ki/ki</sup> (n=4, P>0.05), femoral artery: 14.74 mN±0.93 for sGCβ<sub>1</sub><sup>+/+</sup> vs. 13.48 mN±0.87 for sGCβ<sub>1</sub><sup>ki/ki</sup> (n=4, P>0.05); precontraction level of the concentration-response curve to SNP : aortic rings: 18.59 mN±0.56 for sGCβ<sub>1</sub><sup>+/+</sup> vs. 20.34 mN±1.66 for sGCβ<sub>1</sub><sup>ki/ki</sup> (n=4), P>0.05), femoral artery: 13.65 mN±1.1 for sGCβ<sub>1</sub><sup>+/+</sup> vs. 13.30 mN±1.01 for sGCβ<sub>1</sub><sup>ki/ki</sup> (n=4), P>0.05). The **ACh and SNP**-induced concentration-dependent relaxation was completely abolished in the aortic rings of the sGCβ<sub>1</sub><sup>ki/ki</sup> mice and nearly completely abolished in the femoral artery rings of the sGCβ<sub>1</sub><sup>ki/ki</sup> mice.

**8-pCPT-cGMP** induced a concentration-dependent relaxation of the femoral arteries and aorta from female sGCβ<sub>1</sub><sup>+/+</sup> and sGCβ<sub>1</sub><sup>ki/ki</sup> mice. The sGCβ<sub>1</sub><sup>+/+</sup> and sGCβ<sub>1</sub><sup>ki/ki</sup> femoral arteries responded to 8-pCPT-cGMP in a similar way (pEC<sub>50</sub>: 5.81±0.47 for sGCβ<sub>1</sub><sup>+/+</sup> vs. 5.03±0.12 for sGCβ<sub>1</sub><sup>ki/ki</sup> (n=4, P>0.05); E<sub>max</sub>: 89.82%±6.10 for sGCβ<sub>1</sub><sup>+/+</sup> vs. 94.63%±4.22 for sGCβ<sub>1</sub><sup>ki/ki</sup> (n=4, P>0.05)), whereas in the aortic rings, the 8-pCPT-cGMP-induced response was somewhat reduced in the sGCβ<sub>1</sub><sup>ki/ki</sup> mice (pEC<sub>50</sub>: 5.27±0.59 for sGCβ<sub>1</sub><sup>+/+</sup> vs. 4.30±0.17 for sGCβ<sub>1</sub><sup>ki/ki</sup> (n=4, P>0.05); E<sub>max</sub>: 84.28%±10.39 for sGCβ<sub>1</sub><sup>+/+</sup> vs. 64.54%±13.89 for sGCβ<sub>1</sub><sup>ki/ki</sup> (n=4, P>0.05)).



**Figure VI.2:** Relaxation effect of ACh (A, B), SNP (C, D) on precontracted (adjusted concentration of  $PGF_{2\alpha}$ ) aortas (A, C) ( $n=4$ ) and femoral arteries (B, D) ( $n=4$ ) from female  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice (similar precontraction level between  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  arteries). \*( $sGC\beta_1^{+/+}$  vs.  $sGC\beta_1^{ki/ki}$ ):  $P < 0.05$ .

#### VI.4.1.3. Third set of experiments

In another subset of experiments the contractile effect of **NOR**,  **$PGF_{2\alpha}$** ,  **$K^+$**  and **methoxamine** were investigated on aortic (NOR: Fig. VI.3A; methoxamine: Fig. VI.3B;  $PGF_{2\alpha}$ : Fig. VI.3C;  $K^+$ : Fig. VI.3D) and femoral artery rings (NOR: Fig. VI.4A; methoxamine: Fig. VI.4B;  $PGF_{2\alpha}$ : Fig. VI.4C;  $K^+$ : Fig. VI.4D) from male (data not shown) and female  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice. Additionally, the response to 10  $\mu\text{mol/L}$  ACh was assessed in these preparations.

Rings from both  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice responded to **NOR** (aorta: 1 nmol/L–1  $\mu\text{mol/L}$ ; femoral artery: 1 nmol/L–10  $\mu\text{mol/L}$ ) in a concentration-dependent way. The NOR-induced concentration-response curve in the  $sGC\beta_1^{ki/ki}$  aortic rings was shifted to the left as compared with the aortic segments of the  $sGC\beta_1^{+/+}$  mice. Consequently,  $pEC_{50}$  was significantly higher in the aortic segments of the  $sGC\beta_1^{ki/ki}$  mice (Table VI.1). In the aortic rings of the female

$sGC\beta_1^{ki/ki}$  mice also the estimated maximum response to NOR was significantly higher (Table VI.1). Also in the femoral artery rings of  $sGC\beta_1^{ki/ki}$  mice, the contractile effect of NOR was significantly enhanced (response induced by 10  $\mu\text{mol/L}$  NOR was augmented with 34.72% and 44.67% in males and females respectively). The results were essentially similar in both genders.

The  **$\alpha_1$ -receptor agonist methoxamine** (10 nmol/L–0.1 mmol/L) increased the tone of the  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  preparations in a concentration-dependent way. The methoxamine-induced concentration-response curve of the  $sGC\beta_1^{ki/ki}$  aortic rings was shifted to the left with a significant higher  $pEC_{50}$  (Table VI.1). Also the estimated maximal response to methoxamine was significantly higher in the  $sGC\beta_1^{ki/ki}$  aortic rings (Table VI.1). Contractions in response to methoxamine were comparable in the femoral arteries from female  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice. The results were essentially similar in both genders.

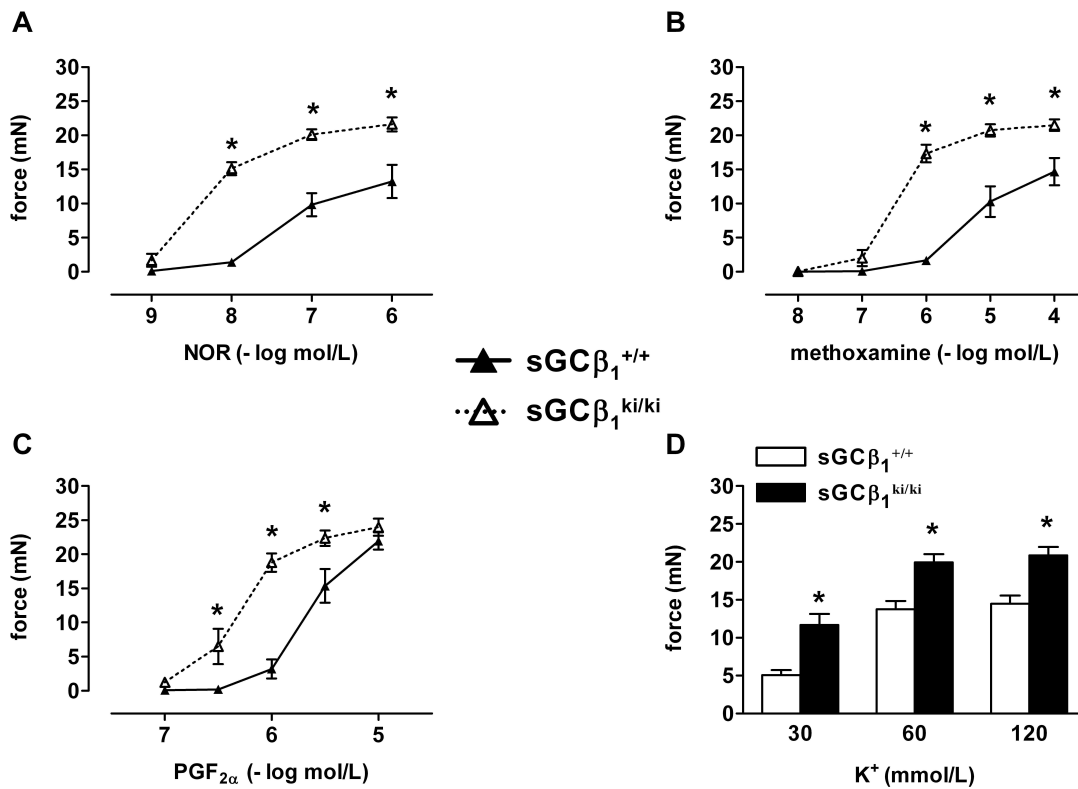
**PGF<sub>2 $\alpha$</sub>**  (aorta: 100 nmol/L–10  $\mu\text{mol/L}$ ; femoral artery: 100 nmol/L–30  $\mu\text{mol/L}$ ) had a concentration-dependent contractile effect in the vessel segments of  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice. But, the aortic rings of  $sGC\beta_1^{ki/ki}$  mice exhibited a greater sensitivity to PGF<sub>2 $\alpha$</sub>  compared with  $sGC\beta_1^{+/+}$  mice, with a significant higher  $pEC_{50}$  (Table VI.1). The estimated maximum response to PGF<sub>2 $\alpha$</sub>  was however comparable between the aortas of  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice (Table VI.1). In the femoral arteries of  $sGC\beta_1^{ki/ki}$  mice, the concentration-response curve to PGF<sub>2 $\alpha$</sub>  was not significantly altered as compared to the  $sGC\beta_1^{+/+}$  preparations. No differences in the response to PGF<sub>2 $\alpha$</sub>  were obtained between male and female preparations.

Administration of increasing concentrations of **K<sup>+</sup>** (30, 60 and 120 mmol/L) resulted in a concentration-dependent contraction of the  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  preparations. Yet, in the  $sGC\beta_1^{ki/ki}$  aortic rings, the contractile effect of 30 mmol/L K<sup>+</sup> was significantly enhanced with 46.11% and 56.61% in male and female mice respectively. Moreover, in the aortic segments of the female  $sGC\beta_1^{ki/ki}$  mice, the responses to 60 and 120 mmol/L were also significantly increased. The femoral artery rings of both  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice responded to K<sup>+</sup> in a similar way. The contractile activity of K<sup>+</sup> was essentially similar in both genders.

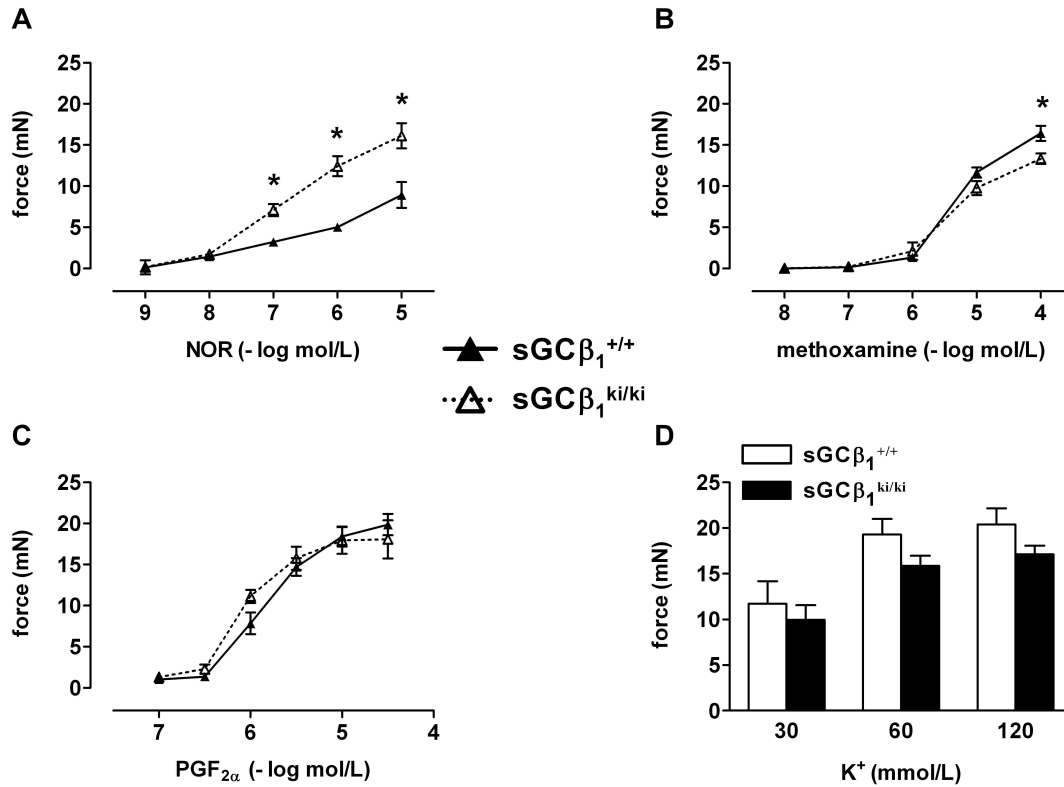
The addition of a single concentration of ACh (10  $\mu\text{mol/L}$ ) resulted in a minor relaxation of the  $sGC\beta_1^{ki/ki}$  femoral arteries as compared to the  $sGC\beta_1^{+/+}$  preparations (response to 10  $\mu\text{mol/L}$  ACh: male: 60.54%  $\pm$ 3.13 for  $sGC\beta_1^{+/+}$  vs. 18.67%  $\pm$ 14.44 for  $sGC\beta_1^{ki/ki}$  (n=5, p<0.05); female: 88.60% $\pm$ 3.59 for  $sGC\beta_1^{+/+}$  vs. 15.09%  $\pm$ 6.63 for  $sGC\beta_1^{ki/ki}$  (n=6, p<0.05)), whereas in the aortic rings of the  $sGC\beta_1^{ki/ki}$  mice, no response was obtained. No differences in the ACh-induced response were observed between the male and female preparations.

		pEC <sub>50</sub>		E <sub>max</sub> (mN)	
		sGCβ <sub>1</sub> <sup>+/+</sup>	sGCβ <sub>1</sub> <sup>ki/ki</sup>	sGCβ <sub>1</sub> <sup>+/+</sup>	sGCβ <sub>1</sub> <sup>ki/ki</sup>
♀	NOR	7.35 ± 0.05 (n=6)	8.27 ± 0.09 *	13.53 ± 2.48 (n=6)	21.12 ± 1.02 *
	PGF <sub>2α</sub>	5.64 ± 0.09 (n=6)	6.31 ± 0.1 *	23.05 ± 1.01 (n=6)	23.43 ± 1.26 (n=5)
	Methoxamine	5.09 ± 0.23 (n=6)	6.38 ± 0.09 *	15.76 ± 1.28 (n=6)	21.14 ± 0.87 *
♂	NOR	7.33 ± 0.17 (n=5)	8.13 ± 0.07 *	13.51 ± 2.98 (n=5)	19.68 ± 0.75 (n=6)
	PGF <sub>2α</sub>	5.7 ± 0.07 (n=6)	6.22 ± 0.03 *	21.73 ± 1.37 (n=6)	21.6 ± 1.02 (n=6)
	Methoxamine	5.32 ± 0.18 (n=6)	6.16 ± 0.06 *	15.81 ± 1.98 (n=6)	19.56 ± 1.01 (n=5)

**Table VI.1:** pEC<sub>50</sub> and E<sub>max</sub> for NOR, PGF<sub>2α</sub> and methoxamine in the aorta of sGCβ<sub>1</sub><sup>+/+</sup> and sGCβ<sub>1</sub><sup>ki/ki</sup> mice.



**Figure VI.3:** Cumulative concentration-response curve to NOR (A), methoxamine (B), PGF<sub>2α</sub> (C) and K<sup>+</sup> (D) in aortas from female sGCβ<sub>1</sub><sup>+/+</sup> and sGCβ<sub>1</sub><sup>ki/ki</sup> mice \*(sGCβ<sub>1</sub><sup>+/+</sup> vs. sGCβ<sub>1</sub><sup>ki/ki</sup>): P<0.05, (n=5-6).



**Figure VI.4:** Cumulative concentration-response curve to NOR (A), methoxamine (B), PGF<sub>2α</sub> (C) and K<sup>+</sup> (D) in femoral arteries from female sGCβ<sub>1</sub><sup>+/+</sup> and sGCβ<sub>1</sub><sup>ki/ki</sup> mice \*(sGCβ<sub>1</sub><sup>+/+</sup> vs. sGCβ<sub>1</sub><sup>ki/ki</sup>): P<0.05, (n=6).

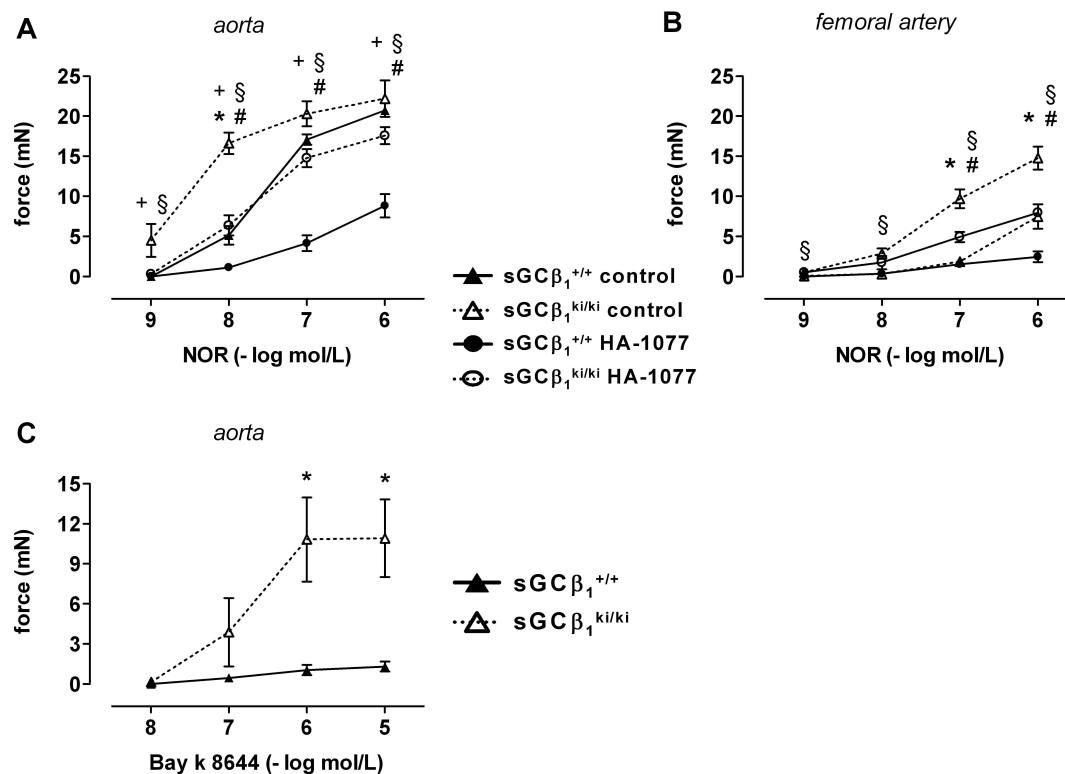
#### VI. 4.1.4. Fourth set of experiments

In this series of experiments, we explored the influence of the **Rho kinase inhibitor HA-1077** on the contractile effect of NOR in the aortic (Fig. VI.5A) and/or femoral artery rings (Fig. VI.5B) from male and/or female sGCβ<sub>1</sub><sup>+/+</sup> and sGCβ<sub>1</sub><sup>ki/ki</sup> mice (male and female mice were pooled). In addition, increasing concentrations of the **L-type Ca<sup>2+</sup>-channel agonist Bay k 8644** (Fig. VI.5C) and a single dose of **the K<sup>+</sup>-channel blocker TEA** were added to the aortic (TEA: Fig. VI.6C) and/or femoral artery rings (TEA: Fig. VI.6D) from male and/or female sGCβ<sub>1</sub><sup>+/+</sup> and sGCβ<sub>1</sub><sup>ki/ki</sup> mice (male and female mice were pooled).

Preincubation with **HA-1077**, resulted in a significant reduction of the NOR-induced response in the aortic and femoral artery preparations from both sGCβ<sub>1</sub><sup>+/+</sup> and sGCβ<sub>1</sub><sup>ki/ki</sup> mice. Moreover, in the sGCβ<sub>1</sub><sup>ki/ki</sup> femoral arteries, the significantly higher contractile effect of NOR observed in control conditions, is abolished in the presence of HA-1077.

**Bay k 8644**, an agonist of L-type  $\text{Ca}^{2+}$ -channels, almost failed to induce a contraction in the aortic rings from the  $\text{sGC}\beta_1^{+/+}$  mice, whereas in the  $\text{sGC}\beta_1^{\text{ki/ki}}$  aortic rings, Bay k 8644 elicited a strong and concentration-dependent contractile effect.

The addition of 1 mmol/L **TEA** to the aortic rings from  $\text{sGC}\beta_1^{+/+}$  and  $\text{sGC}\beta_1^{\text{ki/ki}}$  mice (male and female mice were pooled) at a stable resting tension, resulted in a sustained contraction of both  $\text{sGC}\beta_1^{+/+}$  and  $\text{sGC}\beta_1^{\text{ki/ki}}$  aortic rings. However, the TEA-induced response was significantly higher in the aortic segments of the  $\text{sGC}\beta_1^{\text{ki/ki}}$  mice compared to the  $\text{sGC}\beta_1^{+/+}$  aortas (response to 1 mmol/L TEA:  $1.4 \text{ mN} \pm 0.49$  for  $\text{sGC}\beta_1^{+/+}$  vs.  $14.61 \text{ mN} \pm 0.55$  for  $\text{sGC}\beta_1^{\text{ki/ki}}$  ( $n=6$ ,  $P<0.05$ )). In the femoral arteries, TEA induced a transient augmentation of the basal tone, with a similar peak contraction in both  $\text{sGC}\beta_1^{+/+}$  and  $\text{sGC}\beta_1^{\text{ki/ki}}$  mice.

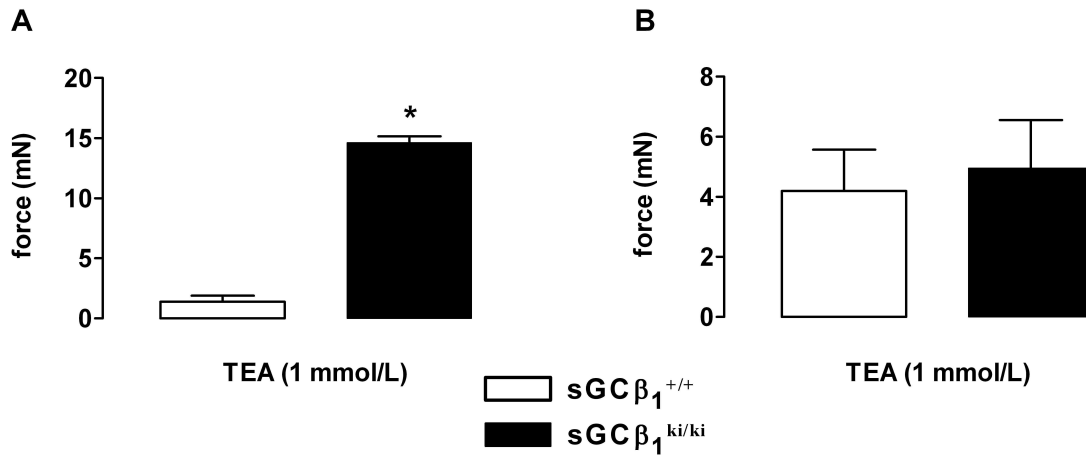


**Figure VI.5:** Cumulative concentration-response curve to NOR in the aorta (A) ( $n=5$ ) and femoral artery (B) ( $\text{sGC}\beta_1^{+/+}$ :  $n=3$  and  $\text{sGC}\beta_1^{\text{ki/ki}}$ :  $n=5$ ) from male and female  $\text{sGC}\beta_1^{+/+}$  and  $\text{sGC}\beta_1^{\text{ki/ki}}$  mice in control conditions (▲ and △) and in the presence of HA-1077 (● and ○). Cumulative concentration-response curve to Bay k 8644 in aortas (C) ( $n=6$ ) from male and female  $\text{sGC}\beta_1^{+/+}$  and  $\text{sGC}\beta_1^{\text{ki/ki}}$  mice.

\*( $\text{sGC}\beta_1^{+/+}$  vs.  $\text{sGC}\beta_1^{\text{ki/ki}}$ ), +( $\text{sGC}\beta_1^{+/+}$  vs.  $\text{sGC}\beta_1^{\text{ki/ki}}$  both in the presence of HA-1077), #(-HA 1077 vs. +HA-1077 for  $\text{sGC}\beta_1^{+/+}$  mice), §(-HA-1077 vs. +HA-1077 for  $\text{sGC}\beta_1^{\text{ki/ki}}$  mice):  $P<0.05$ .

Data from male and female animals were pooled.



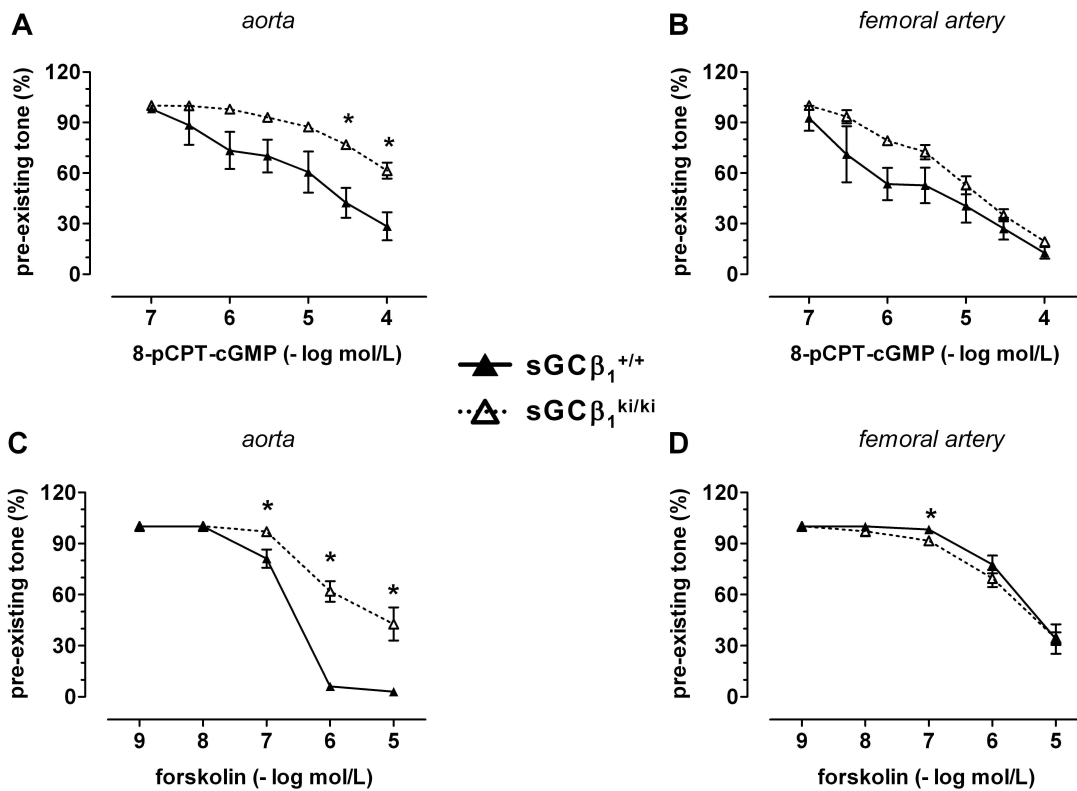


**Figure VI.6:** Contractile effect of TEA (1 mmol/L) on aortic rings (A, C) and femoral arteries (B, D) from male and female  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice. \*( $sGC\beta_1^{+/+}$  vs.  $sGC\beta_1^{ki/ki}$ ):  $P < 0.05$ , (n=5-6). Data from male and female animals were pooled.

#### VI.4.1.5. Fifth set of experiments

$sGC$ -independent vasorelaxation was explored with a concentration-response curve to **8-pCPT-cGMP** (see second set of experiments and Fig. VI.7A, B). Additionally, the relaxing influence of **forskolin**, which stimulates **adenylyl cyclase** and increases the concentration of cAMP, was examined on aortic rings (Fig. VI.7C) and femoral arteries (Fig. VI.7D) from male and/or female  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice (male and female mice were pooled).

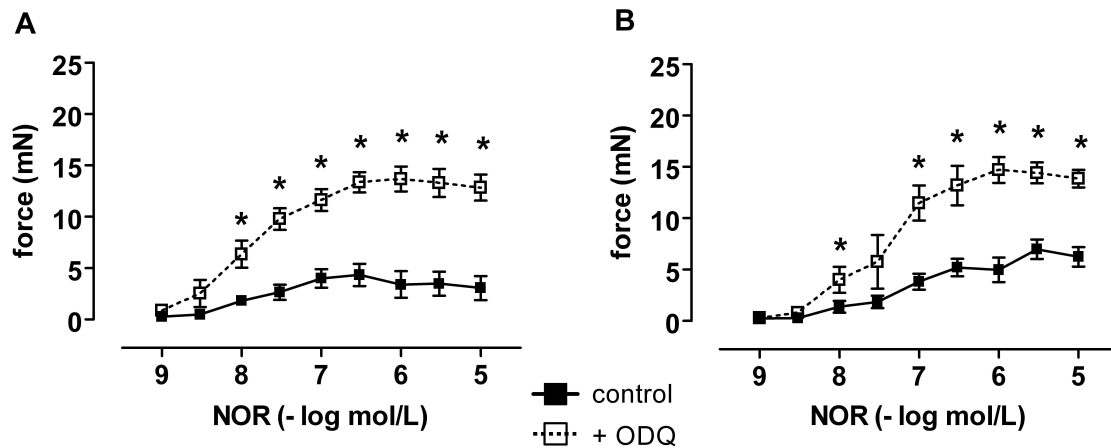
**Forskolin** produced concentration-dependent relaxations in the aortic and femoral artery preparations from  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice. In the aortic rings from the  $sGC\beta_1^{ki/ki}$  mice, 10  $\mu\text{mol/L}$  forskolin had a biphasic vasorelaxing effect: an initial fast phase of relaxation with an average duration of  $8.4 \pm 0.52$  minutes (n=4), followed by a slow phase. For the  $sGC\beta_1^{ki/ki}$  aortic rings, the concentration-response curve to forskolin is in case of the highest concentration, drawn up with the data from the first, fast phase. The concentration-response curve to forskolin is significantly shifted to the right in the aortic rings from the  $sGC\beta_1^{ki/ki}$  mice compared to the  $sGC\beta_1^{+/+}$  aortas, as indicated by the significantly reduced  $pEC_{50}$  and estimated maximum response in the former ( $pEC_{50}$ :  $6.7 \pm 0.07$  vs.  $6.22 \pm 0.11$  (n=5,  $P < 0.05$ );  $E_{\text{max}}$ :  $96.91\% \pm 1.0$  vs.  $58.53\% \pm 10.16$  (n=5,  $P < 0.05$ )). In the femoral artery segments, the forskolin-induced response is not significantly reduced in the  $sGC\beta_1^{ki/ki}$  mice. On the contrary, the concentration-response curve to forskolin is slightly shifted to the left in the femoral artery rings from the  $sGC\beta_1^{ki/ki}$  mice, with a significant higher response in the  $sGC\beta_1^{ki/ki}$  femoral arteries at a concentration of 100 nmol/l forskolin.



**Figure VI.7:** Relaxation effect of 8-pCPT-cGMP (A, B) (n=4) and forskolin (C, D) (n=5) on precontracted 30  $\mu\text{mol/L}$   $\text{PGF}_{2\alpha}$  aortas (A, C) and femoral arteries (B, D) from female (8-pCPT-cGMP) (data from male and female animals were pooled in case of forskolin)  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice. \*( $sGC\beta_1^{+/+}$  vs.  $sGC\beta_1^{ki/ki}$ ):  $P < 0.05$ , (n=7-15).

#### VI.4.1.6. Last set of experiments

We explored the influence of the sGC inhibitor ODQ on the concentration-response curve to NOR (1 nmol/L–10  $\mu\text{mol/L}$ ) in the aorta (Fig. VI.8A) and femoral artery (Fig. VI.8B) isolated from female mice with a C57BL/6J background. The preincubation with ODQ resulted in a significant increase in tone in the aorta (10  $\mu\text{mol/L}$  NOR: 3.06mN $\pm$ 1.16 for -ODQ vs. 12.83mN $\pm$ 1.28 for +ODQ (n=6,  $P < 0.05$ ) and femoral artery (10  $\mu\text{mol/L}$  NOR: 6.24mN $\pm$ 0.95 for -ODQ vs. 13.85mN $\pm$ 0.86 for +ODQ (n=6,  $P < 0.05$ )). This increase in contraction level was comparable in both vessels ( $\Delta$  (-ODQ vs. +ODQ) 10  $\mu\text{mol/L}$  NOR: 9.78mN $\pm$ 0.74 for aorta vs. 7.61mN $\pm$ 0.86 for femoral artery (n=6,  $P > 0.05$ )).



**Figure VI.8:** Effect of ODQ (1  $\mu\text{mol/L}$ ) on the concentration-response curve to NOR in the aorta (A) and femoral artery (B) of female C57BL/6J mice. \*(control conditions vs. the presence of ODQ):  $P < 0.05$ , (n=6).

#### VI.4.2. Corpora cavernosa study

The vasorelaxing influence of **endothelium-derived NO** was examined by the addition of 10  $\mu\text{mol/L}$  **ACh** (Fig. VI.9A) to the NOR-contracted CC preparations of  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice. ACh substantially relaxed CC tissues from the  $sGC\beta_1^{+/+}$  mice, but contracted the CC tissues from the  $sGC\beta_1^{ki/ki}$  mice.

**Neuronal NO** was released by stimulation of the intrinsic nerves by **EFS** (Fig. VI.9B) (1, 2, 4 and 8 Hz). In the  $sGC\beta_1^{+/+}$  CC preparations, EFS generated frequency-dependent relaxant responses. No relaxation in response to EFS was observed in the NOR-contracted CC preparations from  $sGC\beta_1^{ki/ki}$  mice. In contrast, contractions were observed, starting from a frequency of 4 Hz.

Exogenously derived NO from the **NO-donor SNP** (1 nmol/L–10  $\mu\text{mol/L}$ ) (Fig. VI.9C), relaxed the CC tissues from both  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice in a concentration-dependent way. The SNP-induced response was significantly reduced in the CC preparations of the  $sGC\beta_1^{ki/ki}$  mice.

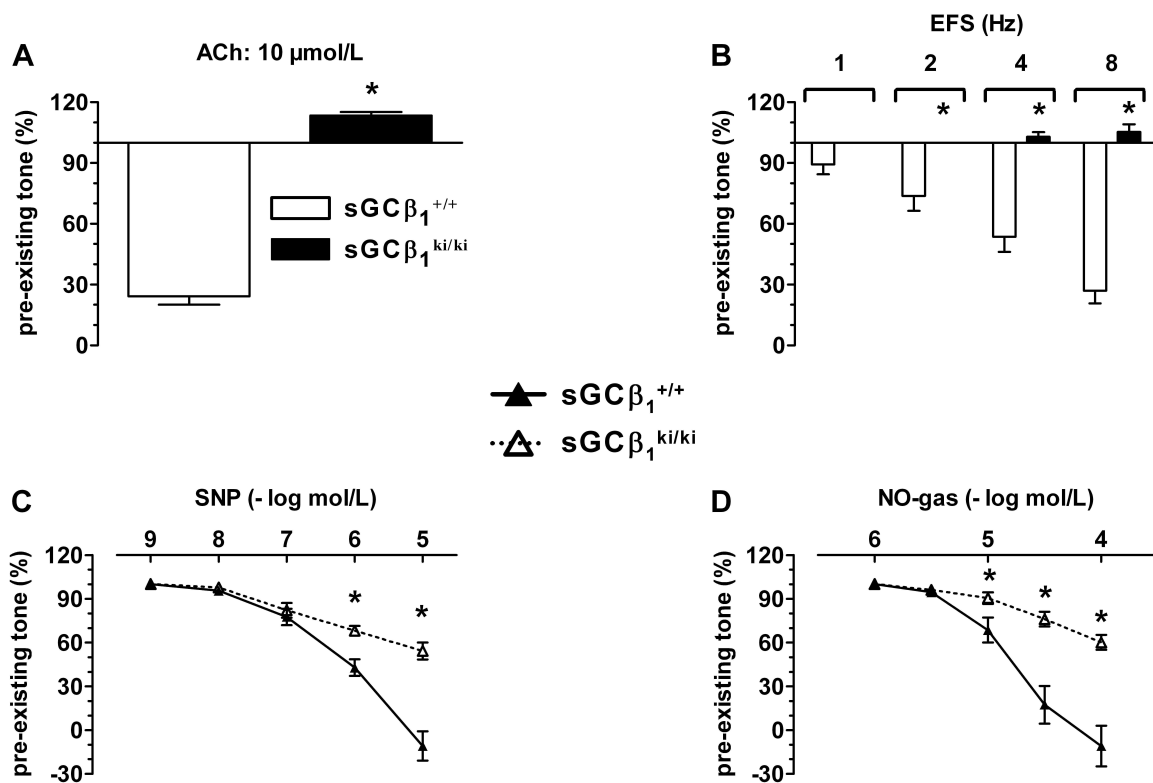
Addition of **NO-gas** (1  $\mu\text{mol/L}$ –0.1 mmol/L) (Fig. VI.9D) induced concentration-dependent and rapid relaxations in NOR-contracted preparations from both  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice. But, in the  $sGC\beta_1^{ki/ki}$  CC preparations, the relaxation evoked by NO-gas was significantly impaired.

**BAY 41-2272** (1 nmol/L–10  $\mu$ mol/L) (Fig. VI.10A), stimulating sGC in a NO-independent way, produced concentration-dependent relaxations in the sGC $\beta_1^{+/+}$  and sGC $\beta_1^{ki/ki}$  CC tissues. In the sGC $\beta_1^{ki/ki}$  CC, the relaxant effect of BAY 41-2272 is slightly but significantly attenuated.

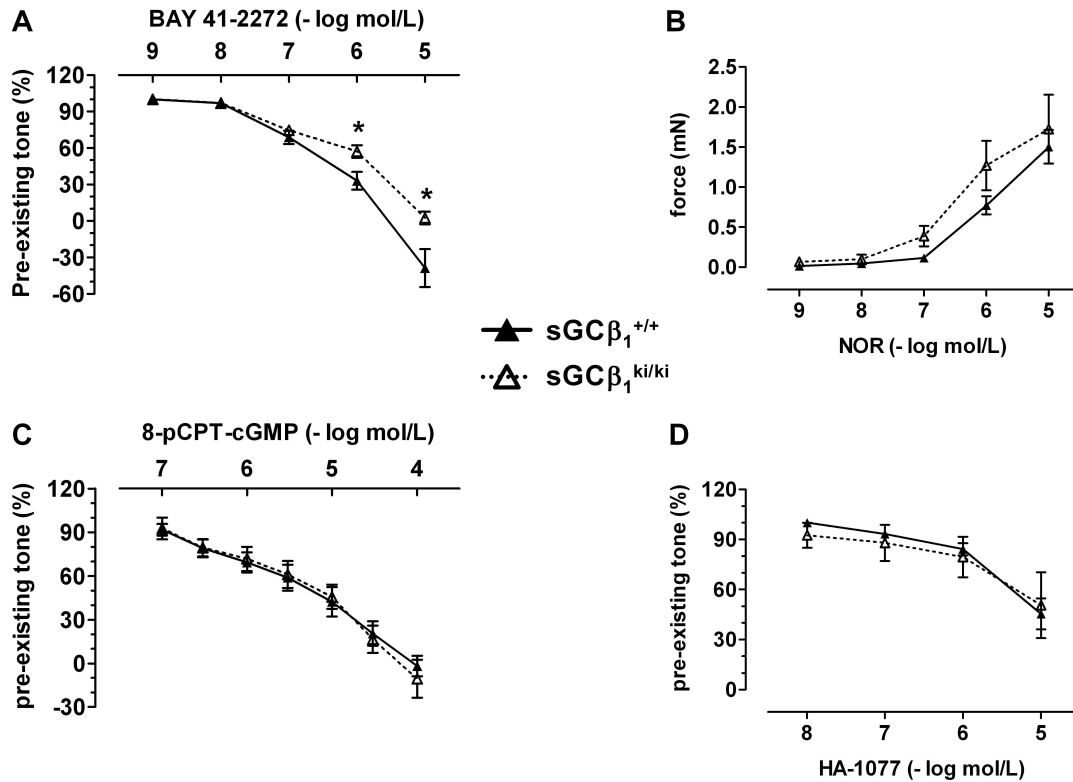
The concentration-dependent relaxation induced by the cGMP analogue, **8 pCPT-cGMP** (Fig. 10C) was not different in CC from sGC $\beta_1^{+/+}$  and sGC $\beta_1^{ki/ki}$  mice.

**HA-1077, a Rho-kinase inhibitor** (Fig. VI.10D), produced concentration-dependent relaxations with similar potency in NOR-contracted CC preparations from sGC $\beta_1^{+/+}$  and sGC $\beta_1^{ki/ki}$  mice.

The concentration-response curve to **NOR** (Fig. VI.10B) was not significantly altered in the CC from the sGC $\beta_1^{ki/ki}$  mice ( $pEC_{50}$ :  $6.83 \pm 0.13$  for sGC $\beta_1^{+/+}$  vs.  $5.82 \pm 0.21$  for sGC $\beta_1^{ki/ki}$  ( $n=5$ ,  $P>0.05$ );  $E_{max}$ :  $1.89mN \pm 0.43$  for sGC $\beta_1^{+/+}$  vs.  $1.79mN \pm 0.46$  for sGC $\beta_1^{ki/ki}$  ( $n=5$ ,  $P>0.05$ )).



**Figure VI.9:** Relaxation effect of ACh (A), EFS (B), SNP (C) and NO-gas (D) on precontracted (50  $\mu$ mol/L NOR) CC from sGC $\beta_1^{+/+}$  and sGC $\beta_1^{ki/ki}$  mice. \*(sGC $\beta_1^{+/+}$  vs. sGC $\beta_1^{ki/ki}$ ):  $P<0.05$ , ( $n=8$ ).



**Figure VI.10:** Relaxation effect of BAY 41-2272 (A), 8-pCPT-cGMP (C) and HA-1077 (D) on precontracted (50  $\mu\text{mol/L}$  NOR) CC from  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice. Cumulative concentration-response curve to NOR (B) in CC from  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice. \*( $sGC\beta_1^{+/+}$  vs.  $sGC\beta_1^{ki/ki}$ ):  $P < 0.05$ , (n=5-8).

## VI.5. Discussion

As NO receptor, sGC plays an important role in numerous physiological processes, thus representing a very attractive pharmacological target. Moreover, the presence of two physiologically active isoforms of sGC, offers a potentially more selective therapeutical approach. Therefore, a quantification of the physiological importance of the different sGC isoforms is of substantial interest. In previous studies, using  $sGC\alpha_1^{-/-}$  mice<sup>1,2</sup>, we established that in vascular and penile smooth muscle relaxation the predominant  $sGC\alpha_1\beta_1$  isoform is a very important, though not the only target of NO-dependent and NO-independent sGC activators. The involvement of the less abundantly expressed  $sGC\alpha_2\beta_1$  isoform as well as (an) sGC-independent mechanisms could be argued. In the present study, in which we used NO-insensitive sGC mutant mice, the issue whether  $sGC\alpha_2\beta_1$  and/or (an) sGC-independent

mechanisms participate in vascular and penile smooth muscle relaxation, was further unravelled.

In the aortic rings from the  $sGC\beta_1^{ki/ki}$  mice, the response to endogenous (induced by ACh) and exogenous NO (induced by SNP) was completely abolished, indicating that in the aorta sGC is the sole receptor for NO. This observation, allows us to conclude that the remaining effect of ACh (remaining effect of 10  $\mu\text{mol/L}$  ACh: 23.24%<sup>1</sup>) and of SNP (remaining effect of 10  $\mu\text{mol/L}$  SNP: 73.45%<sup>1</sup>) in the  $sGC\alpha_1^{-/-}$  aortic rings, is due to the activation of  $sGC\alpha_2\beta_1$ . In contrast with the aortic rings, the femoral arteries from the  $sGC\beta_1^{ki/ki}$  mice showed a significantly reduced but not completely blocked response to endogenous and exogenous NO, suggesting that NO in this preparation also signals via an alternative, sGC-independent target. The response to ACh is slightly more impaired in the femoral arteries from the  $sGC\beta_1^{ki/ki}$  mice compared to the  $sGC\alpha_1^{-/-}$  mice (impairment of the response to 10  $\mu\text{mol/L}$  ACh: 43.75% for  $sGC\alpha_1^{-/-}$  vs. 61.05% for  $sGC\beta_1^{ki/ki}$ ), whereas the relaxant effect of SNP is considerably more reduced in the femoral arteries from the  $sGC\beta_1^{ki/ki}$  mice compared to the  $sGC\alpha_1^{-/-}$  mice (impairment of the response to 10  $\mu\text{mol/L}$  SNP: 13.89% for  $sGC\alpha_1^{-/-}$  vs. 56.37% for  $sGC\beta_1^{ki/ki}$ ). This suggests that activation of  $sGC\alpha_2\beta_1$  is more significant in the response to exogenous NO than in the response to endogenous NO.

The involvement of (an) sGC-independent mechanism(s) is not only suggested by the observations on femoral arteries but also by the response to exogenous NO in the CC. This suggestion is based on the fact that a substantial effect of SNP and NO-gas remains in the CC from the  $sGC\beta_1^{ki/ki}$  mice compared to the CC in  $sGC\beta_1^{+/+}$  mice. The responses to SNP and NO-gas are more impaired in the  $sGC\beta_1^{ki/ki}$  CC than in the CC from the  $sGC\alpha_1^{-/-}$  mice (impairment of the response to 10  $\mu\text{mol/L}$  SNP: 38.3% for  $sGC\alpha_1^{-/-}$  vs. 58.67% for  $sGC\beta_1^{ki/ki}$ ; 100  $\mu\text{mol/L}$  NO-gas: 36.29% for  $sGC\alpha_1^{-/-}$  vs. 64.01% for  $sGC\beta_1^{ki/ki}$ ), suggesting that the response to exogenous NO is partly mediated by the  $sGC\alpha_2\beta_1$  isoform. In contrast to the response induced by exogenous NO, CC smooth muscle relaxation induced by endogenous NO is completely mediated through activation of sGC, as shown by the total loss of response to ACh and EFS in the  $sGC\beta_1^{ki/ki}$  CC. This finding is in line with the suggestion of our study on  $sGC\alpha_1^{-/-}$  mice where we found that in the CC,  $sGC\alpha_1\beta_1$  is the sole receptor for endogenously produced NO<sup>2</sup>.

During our experiments it became apparent that the  $sGC\beta_1^{ki/ki}$  arteries exhibited a significant stronger response to NOR compared to the  $sGC\beta_1^{+/+}$  preparations. In order to exclude a possible influence of the higher precontraction tone of the  $sGC\beta_1^{ki/ki}$  arteries on the NO-mediated vasorelaxation, concentration-response curves to ACh and SNP were obtained in

which the precontraction of preparations from sGCβ<sub>1</sub><sup>+/+</sup> and sGCβ<sub>1</sub><sup>ki/ki</sup> mice was set to a similar supramaximal level. Under these conditions, the vasorelaxing influence of ACh and SNP was completely abolished in both the aortic and femoral artery rings from the sGCβ<sub>1</sub><sup>ki/ki</sup> mice. These data on the femoral arteries did not correspond with the observations in our first series of experiments and reject the conclusion of sGC-independent effect of NO in those vessels. It should however be mentioned that besides the precontraction level, there were also other differences between these series of experiments. The first NO-induced responses were examined on vessel segments from male sGCβ<sub>1</sub><sup>+/+</sup> and sGCβ<sub>1</sub><sup>ki/ki</sup> mice of 7 to 10 weeks old, whereas for the following concentration-response curves only older mice (12 to 13 weeks) of the female sex were used. Moreover, the younger animals had a weaker state of health and related to this a higher mortality at early age. Because of the age and gender variations between the mice used to explore the vasorelaxing effect of NO, the response to 10 μmol/L ACh was reassessed on aortic and femoral artery rings from sGCβ<sub>1</sub><sup>+/+</sup> and sGCβ<sub>1</sub><sup>ki/ki</sup> mice of both genders and with a broader range of age (9 to 12 weeks). ACh was not able to relax the aortic rings from those sGCβ<sub>1</sub><sup>ki/ki</sup> mice, whereas the sGCβ<sub>1</sub><sup>ki/ki</sup> femoral arteries still showed a minor response to ACh. Those data confirm our first suggestion that is that in the aorta, sGC is the only target of NO regulating vasorelaxation, whereas in the femoral artery also (an) sGC-independent mechanism(s) participate in the NO-induced smooth muscle relaxation. Activation of Ca<sup>2+</sup>- and voltage-dependent potassium channels<sup>17, 18</sup>, sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase<sup>19</sup> and Na<sup>+</sup>/K<sup>+</sup>-ATPase<sup>20</sup> have been described as sGC-independent vasorelaxing actions of NO. Still, the remaining response to ACh in the femoral arteries could also point to the participation of the endothelium-derived hyperpolarising factor (EDHF), particularly since this non-prostanoid, non-nitric oxide factor has been reported to be play an important role in the endothelium-dependent relaxation of femoral arteries isolated from mice with a C57BL/6J background<sup>21</sup>. No gender differences in the ACh-induced response were obtained, indicating that this is not the underlying cause for the variations in the responsiveness of the sGCβ<sub>1</sub><sup>ki/ki</sup> femoral arteries towards NO.

NO-independent sGC stimulators like BAY 41-2272 are emerging as valuable tools for the treatment of numerous pathologies caused by the reduced bioavailability and/or responsiveness to endogenously produced NO<sup>22</sup>. Despite the fact that the sGC-activating effect of BAY 41-2272 is dependent on the presence of the reduced prosthetic heme moiety of sGC<sup>23</sup>, BAY 41-2272 could still elicit a substantial response in the femoral arteries and CC isolated from the sGCβ<sub>1</sub><sup>ki/ki</sup> mice. This suggests that in those tissues, BAY 41-2272 partly exerts its relaxant effect through (an) sGC-independent mechanism(s). BAY 41-2272 has

been reported to induce vasorelaxation independently from cGMP-production through activation of the Na<sup>+</sup>-K<sup>+</sup>-ATPase<sup>24</sup> and inhibition of the Ca<sup>2+</sup>-entry<sup>25</sup>. As abolition of the sGC $\alpha_1$  subunit or of the NO-stimulated sGC activity decreased the response to BAY 41-2272 in a similar way (response to 10  $\mu$ mol/l BAY 41-2272: 18.28%<sup>1</sup> vs. 15.45%), only the sGC $\alpha_1\beta_1$  isoform is suggested to be responsible for the sGC-dependent vasorelaxing effect of BAY 41-2272. This is also suggested by the observations with BAY 41-2272 on the CC, where similar results were obtained in the preparations from sGC $\beta_1^{ki/ki}$  and sGC $\alpha_1^{-/-}$  mice (impairment of the response to 10  $\mu$ mol/L BAY 41-2272: 25.05%<sup>2</sup> vs. 29.78%). A significant part of the relaxing influence of BAY 41-2272 in the CC can be attributed to (an) sGC-independent mechanism(s), since BAY 41-2272 elicited a substantial response in the sGC $\beta_1^{ki/ki}$  CC. The sGC $\beta_1^{ki/ki}$  aortic rings, on the other hand, only slightly responded to 10  $\mu$ mol/L BAY 41-2272. This was also the case in the aortic rings from the sGC $\alpha_1^{-/-}$ , however the 10  $\mu$ mol/L BAY 41-2272-induced relaxation was less impaired in the sGC $\alpha_1^{-/-}$  aortas (impairment of the response to 10  $\mu$ mol/L BAY 41-2272: 36.83%<sup>1</sup> vs. 82.81%). This indicates that at a concentration of 10  $\mu$ mol/L, BAY 41-2272 mainly activates the sGC $\alpha_2\beta_1$  isoform whereas at lower concentrations, sGC $\alpha_1\beta_1$  is the main target for BAY 41-2272. As in the sGC $\beta_1^{ki/ki}$  mice, the basal sGC-activity is not abrogated and BAY 41-2272 is known to inhibit the PDE-5 mediated hydrolysis of cGMP at concentrations of 10  $\mu$ mol/L or higher<sup>26</sup>, the remaining effect of BAY 41-2272 in the sGC $\beta_1^{ki/ki}$  preparations may be in part due to the accumulation of basally produced cGMP.

The specificity of the impaired responses we found in the sGC $\beta_1^{ki/ki}$  preparations was assessed by a concentration-response curve to the cell permeable cGMP-analogue 8-pCPT-cGMP, to forskolin, which acts via activation of AC and/or to HA-1077, an Rho kinase inhibitor. The femoral artery and CC preparations from both sGC $\beta_1^{+/+}$  and sGC $\beta_1^{ki/ki}$  mice responded to 8-pCPT-cGMP in a similar way, indicating that the signaling cascade downstream of sGC functions normally. This can not be concluded for the aortic rings as the 8-pCPT-cGMP-induced response is somewhat reduced in the sGC $\beta_1^{ki/ki}$  aortic rings. Moreover, the vasorelaxing effect of forskolin is also significantly impaired in the aortic rings from the sGC $\beta_1^{ki/ki}$  mice, whereas in the femoral arteries the response to forskolin is not altered. In addition, HA-1077 relaxed the CC preparations from both sGC $\beta_1^{+/+}$  and sGC $\beta_1^{ki/ki}$  mice to a similar extent.

The marked contractile activity of the sGC $\beta_1^{ki/ki}$  aortic rings, which will be discussed next, may be responsible for the somewhat impaired sGC-independent relaxation seen in the sGC $\beta_1^{ki/ki}$  aortas.



A number of reports to date have described the effect of the genetic background on the results of transgenesis and targeted gene disruption<sup>27-29</sup>. Therefore, possible strain-specific differences between the sGC $\alpha_1$ <sup>-/-</sup> (mixed background: Swiss-129/SvJ) and sGC $\beta_1$ <sup>ki/ki</sup> (mixed background: 129/SvJ-C57BL/6J) mice must be considered for a correct interpretation and comparison of the data obtained from the two mice models.

The absence of sGC that can be activated by NO, significantly influences the contractile activity of vascular smooth muscle. In the different subset of experiments, contraction induced by NOR, was significantly greater in both the aorta and femoral artery from male and female sGC $\beta_1$ <sup>ki/ki</sup> mice, but not in CC. Particularly in the sGC $\beta_1$ <sup>ki/ki</sup> aortic rings, the increased sensitivity towards NOR was very pronounced. NOR is known to induce arterial vasoconstriction through interaction with a mixed population of postjunctional vascular  $\alpha_1$ - and  $\alpha_2$ -adrenergic receptors. Stimulation of  $\alpha_2$ -adrenergic receptors can induce either an increase or decrease of vascular tone depending on the kind of vessel<sup>30,31</sup> and on the location of the receptors<sup>32</sup>. Since activation of endothelial  $\alpha_2$ -adrenergic receptors can induce the release of NO<sup>33,34</sup>, it could be that the augmented NOR-induced tone in the sGC $\beta_1$ <sup>ki/ki</sup> preparations is due to the fact that NO produced in response to NOR, can no longer exert its sGC-dependent vasorelaxing influence in the sGC $\beta_1$ <sup>ki/ki</sup> mice. Moreover, contractions to NOR in the aortic rings from  $\alpha_{2AD}$ -adrenoreceptor knockout mice have been shown to be significantly increased<sup>35</sup>. However, this can not be the sole cause as also the response to the selective  $\alpha_1$ -adrenoreceptor agonist methoxamine was significantly increased in the aortic rings of the sGC $\beta_1$ <sup>ki/ki</sup> mice. This observation is in line with the fact that the  $\alpha_1$ -adrenergic receptors play a predominant role in the NOR-induced contraction of large noninnervated conductance arteries<sup>36,37</sup> as well as small innervated vessels<sup>38</sup>. Whatsoever, the significant higher NOR-evoked contraction in the sGC $\beta_1$ <sup>ki/ki</sup> aortic rings can not be selectively linked to the  $\alpha_1$ -adrenergic receptors, as the sGC $\beta_1$ <sup>ki/ki</sup> aortic rings are also more sensitive to PGF<sub>2 $\alpha$</sub> . Additionally, depolarization of the vascular smooth muscle cells by increasing the external K<sup>+</sup> concentration, also resulted in a significantly higher tone of the sGC $\beta_1$ <sup>ki/ki</sup> aortic rings compared to the sGC $\beta_1$ <sup>+/+</sup> preparations. In contrast to the aortic rings, the femoral arteries from both sGC $\beta_1$ <sup>+/+</sup> and sGC $\beta_1$ <sup>ki/ki</sup> mice responded to methoxamine, PGF<sub>2 $\alpha$</sub>  and K<sup>+</sup> in a similar way. Hence, the  $\alpha_2$ -adrenoreceptor-mediated NO release and relaxation is more likely to be involved in the NOR specific higher force development of the sGC $\beta_1$ <sup>ki/ki</sup> femoral arteries. However, the fact that following treatment with the Rho-kinase inhibitor HA-1077, the contractile response to NOR in sGC $\beta_1$ <sup>ki/ki</sup> femoral arteries closely resembled that of the sGC $\beta_1$ <sup>+/+</sup> femoral arteries, suggests a higher activity of the Ca<sup>2+</sup> sensitization pathway as

underlying case for the increased NOR-induced contraction in the  $sGC\beta_1^{ki/ki}$  femoral arteries. However, as prostanoid receptor agonists like  $PGF_{2\alpha}$  have been reported to be preferentially involved in  $Ca^{2+}$  sensitization through activation of RhoA<sup>39, 40</sup>, one would expect then that also the  $PGF_{2\alpha}$ -induced response is significantly higher in the femoral arteries from the  $sGC\beta_1^{ki/ki}$  mice. Since this is not the case, the hypothesis of a higher  $Ca^{2+}$  sensitization pathway in  $sGC\beta_1^{ki/ki}$  femoral arteries remains debatable. The data of the aorta rings, on the other hand, are more consistent. Preincubation with HA-1077 resulted in a stronger reduction of the NOR-induced contraction in the  $sGC\beta_1^{+/+}$  aortic rings compared to the  $sGC\beta_1^{ki/ki}$  aortic rings, which does not support the involvement of the  $Ca^{2+}$  sensitization pathway. This is in line with the smaller efficiency of  $\alpha$ -adrenoreceptor agonists compared to prostanoid receptor agonists to activate RhoA and thereby to induce  $Ca^{2+}$  sensitization of  $MLC_{20}$  phosphorylation<sup>39, 40</sup>. Moreover, this corresponds with the finding that the increased tone of the  $sGC\beta_1^{ki/ki}$  aortic rings compared to the  $sGC\beta_1^{+/+}$  aortic rings, is more pronounced in response to NOR than in response to  $PGF_{2\alpha}$ .

When a receptor coupled to a heterotrimeric GTP binding protein is activated,  $Ca^{2+}$  sensitization as well as  $Ca^{2+}$  mobilization occurs. The  $Ca^{2+}$  reactivity of the  $sGC\beta_1^{ki/ki}$  preparations was explored with the L-type  $Ca^{2+}$  channel agonist Bay k 8644 and by the TEA-induced depolarization. Both substances provoke the influx of extracellular  $Ca^{2+}$  through voltage dependent  $Ca^{2+}$  channels. Bay k 4486 as well as TEA elicited significantly greater contractions in the  $sGC\beta_1^{ki/ki}$  aortic rings than in the  $sGC\beta_1^{+/+}$  aortic rings, suggesting that the  $sGC\beta_1^{ki/ki}$  aortic rings are more sensitive to cytosolic  $Ca^{2+}$ . In contrast with the aortic rings, the femoral arteries do not show an indication towards an increased  $Ca^{2+}$  sensitivity as the femoral arteries from both  $sGC\beta_1^{+/+}$  and  $sGC\beta_1^{ki/ki}$  mice responded to TEA in a similar way. Those data are not completely in line with the effect of HA-1077 on the NOR-induced contraction, from which we suggested that the  $sGC\beta_1^{ki/ki}$  femoral arteries exhibited a higher RhoA kinase activity and the  $sGC\beta_1^{ki/ki}$  aortic rings a lower. Besides Rho kinase also protein kinase C (PKC) is a major determinant for the  $Ca^{2+}$  sensitization in vascular smooth muscles<sup>41</sup>. Therefore it can be hypothesized that the PKC-pathway is responsible for an enhanced contractile activity of the  $sGC\beta_1^{ki/ki}$  aortic rings. However, our data indicate that in the  $sGC\beta_1^{ki/ki}$  aortic rings, the contractile response is rather unselective, whereas in the corresponding femoral arteries it is less marked and rather specific for NOR. This discrepancy is perhaps related to the variable importance of basally released NO among vessel types. There are literature data on C57BL/6J mice showing that basal NO is massively produced in the aorta<sup>42</sup> and carotid artery, whereas in the femoral artery it is far less pronounced<sup>21</sup>.

Knowing that there is cross talk between cAMP and cGMP<sup>43</sup>, this higher amount of spontaneously released NO in the aortic rings could also explain the impaired forskolin-induced response we have observed in the sGC $\beta_1$ <sup>ki/ki</sup> aortic rings. In vitro, basal NO was shown to decrease the sensitivity towards NO-donors and its impact appeared to decline over time<sup>21</sup>. Therefore, its possible influence on the vasomotor responses we have investigated should be considered. Still, preincubation of aortic rings and femoral artery segments (isolated from C57BL/6J mice) with ODQ, resulted in a comparable increase of the NOR-induced response in both vessel types, which is not completely in line with the findings on the heterogeneity in basal NO<sup>21,42</sup>. Furthermore, the findings on C57BL/6J mice can not be fully converted to our sGC $\beta_1$ <sup>ki/ki</sup> mice, which have a mixed background (129/SvJ-C57BL/6J). Hence, we suggest that the enhanced contractile activity of the sGC $\beta_1$ <sup>ki/ki</sup> preparations can not completely attributed to the abolished vasorelaxing influence of basal NO.

## **VI.6. Conclusions**

This study provides evidence that in the aortic rings the response to NO is completely sGC-dependent, whereas in the femoral arteries, also (an) sGC-independent mechanism(s) are involved. In contrast to endogenous NO and the NO-independent sGC stimulator BAY 41-2272, that mainly activate the predominant sGC $\alpha_1\beta_1$  isoform, exogenous NO mainly activates the minor sGC $\alpha_2\beta_1$  isoform. In the CC, on the other hand, sGC $\alpha_1\beta_1$  is the main target for endogenous NO, BAY 41-2272 as well as exogenous NO. Furthermore, we found that the arteries from the sGC $\beta_1$ <sup>ki/ki</sup> mice exhibited a higher contractile activity, which to some extent could be explained by the incapability to respond to basal endothelial NO.

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# Chapter VII

Discussion and future perspectives



## **VII.1. General discussion and future perspectives**

Although both sGC and its product cGMP, were identified in the 1960's, it has been only within the last 15 years that the outline of the NO/cGMP signaling pathway has been understood. Ever since, many good examples of signal transduction by this system have emerged. They appeared to be most notably in the cardiovascular system (smooth muscle relaxation and blood pressure regulation <sup>1</sup>, platelet aggregation and disaggregation <sup>2</sup>) and the nervous system (neurotransmission both peripherally, in non-adrenergic-non-cholinergic nerves <sup>3</sup> and centrally in the process of long-term potentiation and depression <sup>4</sup>). However, also light transduction in the retina <sup>5</sup>, kidney function <sup>6</sup>, suppression of leukocyte adhesion <sup>7</sup> and vascular remodelling <sup>8</sup> may be put on the list of NO/cGMP mediated physiological processes. This extensive record implies of course that impairment of the NO/cGMP system is the underlying cause for many diseases. Despite decades of research, many aspects of sGC, including the physiological relevance of its two isoforms remain to be revealed. The capital aim of this thesis was to gain more insight into the importance of the sGC $\alpha_1\beta_1$  and sGC $\alpha_2\beta_1$  isoforms in vascular and corpora cavernosa smooth muscle relaxation in order to validate the isoforms as potential pharmacological targets. Because of the lack of sGC subunit or isoform specific inhibitors, we made use of genetically engineered mice with a targeted deletion of one of the sGC $\alpha$  genes or a mutation of the sGC $\beta$  gene to obtain our goal.

Two isoforms for each sGC subunit have been described, but only the predominantly expressed heterodimer  $\alpha_1\beta_1$  and the less profusely expressed heterodimer  $\alpha_2\beta_1$  have been reported to be physiologically active <sup>9</sup>. Because the  $\alpha_1\beta_1$  isoform represents the largest part of the total sGC content in most tissues <sup>10</sup>, we first focussed our research on the sGC $\alpha_1^{-/-}$  mice in which the  $\alpha_1\beta_1$  isoform is no longer functionally active. In the CC as well as in the aorta isolated from the sGC $\alpha_1^{-/-}$  mice, we found that the response to endogenous NO was completely or almost completely abolished, which reflects the principal role of the  $\alpha_1\beta_1$  isoform. In order to evaluate the correlation between this functional alteration in the CC and erectile dysfunction, one of the future perspectives would be to determine the erectile hemodynamics of the sGC $\alpha_1^{-/-}$  mice in response to drugs or nerve stimulation by ICP measurements. In contrast with the CC and aorta, the femoral arteries isolated from the sGC $\alpha_1^{-/-}$  mice, still showed a substantial response to ACh, suggesting that besides the sGC $\alpha_1\beta_1$  isoform, also the sGC $\alpha_2\beta_1$  isoform and/or (an) sGC-independent mechanism(s) are involved. In addition to NO, ACh is known to induce the release of prostacyclin (PGI<sub>2</sub>) and of the endothelium-derived hyperpolarising factor (EDHF) <sup>11</sup>. However, the contribution of these

endothelium-derived substances could be excluded by the fact that the relaxing influence of ACh was completely blocked by the NO-synthase inhibitor N<sup>0</sup>-nitro-L-arginine (L-NA). Furthermore, this finding suggests that the remaining effect of ACh seen in the sGC $\alpha_1^{-/-}$  mice is completely mediated by NO. To investigate whether this effect of NO is sGC-dependent, we explored the response to ACh in sGC $\beta_1^{ki/ki}$  mice, in which the NO-mediated activation of sGC is abolished. The data obtained from these mice gave us also conclusive information on the involvement of the sGC $\alpha_2\beta_1$  isoform. As compared with the sGC $\alpha_1^{-/-}$  mice, ACh had less effect in the sGC $\beta_1^{ki/ki}$  femoral arteries. This suggests that the minor sGC isoform,  $\alpha_2\beta_1$ , is also activated by endogenous NO in this preparation. However, endogenous NO is also able to exert a sGC-independent relaxant effect, since vasorelaxation induced by ACh is not completely abolished in the sGC $\beta_1^{ki/ki}$  femoral arteries. On the other hand, also EDHF could be responsible for the remaining endothelium-dependent relaxation of those arteries. In contrast to our findings in mice with a 129/SvJ-Swiss background, there is evidence in literature that EDHF is an important endothelium-dependent vasodilator in femoral arteries of C57BL/6J mice <sup>12</sup>.

It should however be noted that the responsiveness to ACh as well as to the NO-donor SNP were characterised by some variability, as in one third of the preparations, the relaxant effect of the NO-related substances was completely abolished in the sGC $\beta_1^{ki/ki}$  femoral arteries. We suggest that this variation is due to variation in the available sGC $\beta_1^{ki/ki}$  mice. At the beginning only young mice were available because most of the sGC $\beta_1^{ki/ki}$  mice died at an early age. Later on, the survival rate of the sGC $\beta_1^{ki/ki}$  mice increased due to an adapted diet. A study performed by the research unit of Prof. Dr. P. Brouckaert of the Department of Molecular Biomedical Research and Flanders Interuniversity Institute of Biotechnology, who bred the genetically engineered mice, demonstrated that the lifespan of the sGC $\beta_1^{ki/ki}$  mice markedly increased when they receive expanded food, with a higher dose of vitamins.

Besides the diet, also the mixed background of the sGC $\beta_1^{ki/ki}$  mice can have influenced the results. The sGC $\beta_1$  knockin was created in a 129/SvJ-derived embryonic stem (ES) cell line and crossed to C57BL/6J mice to test the germ line transmission. C57BL/6J x 129/SvJ F2 mice were then intercrossed to produce the homozygous mutant mice. Subsequently the sGC $\beta_1$  mutant strain was being backcrossed to a more uniform inbred strain (C57BL/6J strain), in order to eliminate phenotypic variations caused by the mixed background and thus to more accurately evaluate the specific gene phenotype. However, the time involved to create fully backcrossed congenic strains, defined as 10 generations of backcrossing <sup>13</sup>, combined

with scientific curiosity, led to characterization of  $sGC\beta_1^{ki/ki}$  mice with a background that was still segregating for genes from the progenitor strains. Numerous reports illustrate the importance of an appropriate genetic background<sup>14</sup>. Heterozygous insulin receptor knockout mice for example, showed a variable hyperinsulinemia on a mixed background, a mild hyperinsulinemia on a C57BL/6 congenic background and a severe hyperinsulinemia on a 129S6 background<sup>15</sup>.

Like the  $sGC\beta_1^{ki/ki}$  mice, also the  $sGC\alpha_1^{-/-}$  mice were generated on a mixed background; targeted ES cells derived from a 129/SvJ strain were injected into Swiss blastocysts to form the chimeric mice. The vascular as well as the cavernosal responsiveness of the  $sGC\alpha_1^{-/-}$  mice was obtained on this mixed background. As illustrated above, this creates a potential source of variability in the results<sup>16</sup>, especially since the Swiss strain is known as an outbred strain (in contrast to the 129/SvJ and C57BL/6J strains). Still, these F2 mice obtained by crossing the chimera to Swiss and then intercrossing their heterozygous offspring, offered a reasonable compromise between the demands of time for scientific progress on the one hand and the rigorous control of background on the other hand. In the long term, it is however recommended that mutants are analysed on a more defined background<sup>17</sup>. Therefore, we also characterised  $sGC\alpha_1^{-/-}$  mice which were successfully backcrossed to a pure C57BL/6J background (see caption VIII.2: Influence of the genetic background on the phenotype of the  $sGC\alpha_1^{-/-}$  mice). The aortic ring segments of these mice, showed a significantly reduced response to ACh, though the remaining effect of ACh in these arteries was more than twice as big compared with the remaining effect of ACh in the aortic rings of the  $sGC\alpha_1^{-/-}$  mice on the mixed background. This phenotypic difference has severe implications for the interpretation of our results, as the  $sGC\alpha_1\beta_1$  and the  $sGC\alpha_2\beta_1$  isoform are physiologically more important in respectively the  $sGC\alpha_1^{-/-}$  mice on the mixed background and the  $sGC\alpha_1^{-/-}$  mice on the pure background. The exclusive sGC-dependency of endogenous NO was suggested by the complete absence of ACh-induced relaxation in the aortic rings from the  $sGC\beta_1^{ki/ki}$  mice. Furthermore, this clearly shows that for a correct comparison of the data obtained from the  $sGC\alpha_1^{-/-}$  and  $sGC\beta_1^{ki/ki}$  mice, both mutant mice should be characterised when fully backcrossed to the same inbred strain. Moreover, to identify genetic modifiers of the gene of interest, minimum two congenic strains of every mutation should be examined<sup>17</sup>. In this perspective, it could be interesting to examine the vascular and cavernosal responsiveness in  $sGC\alpha_1^{-/-}$  mice on a pure 129/SvJ background and in the  $sGC\beta_1^{ki/ki}$  mice on a C57BL/6J and 129/SvJ background. Additionally, the phenotypic evaluation of a tissue-specific targeted

disruption (e.g. in the smooth muscle cells) of the  $sGC\alpha_1$  gene, may provide more detailed information on the role of the  $sGC\alpha_1\beta_1$  isoform in smooth muscle relaxation. Furthermore, a temporal regulation of the  $sGC\beta_1$  mutation can perhaps circumvent the developmental retardation of the  $sGC\beta_1^{ki/ki}$  mice and in that way lead to an increased life span.

Under physiological conditions, the endothelium acts as an inhibitory regulator of vascular contraction through the continuous release of basal NO<sup>18</sup>. We suggest that in the aorta,  $sGC\alpha_1\beta_1$  is the predominant target for basally produced NO. After all, the aortic rings isolated from the  $sGC\alpha_1^{-/-}$  mice were characterised by a significant lower basal cGMP level, an almost completely abolished response to the phosphodiesterase type 5 inhibitor T-1032 and a significant smaller increase of the precontraction tonus upon addition of the sGC-inhibitor ODQ. In the  $sGC\alpha_1^{-/-}$  femoral arteries and CC, on the other hand, more basally produced cGMP was accumulated as T-1032 still had a substantial relaxant effect in these preparations. This suggests that besides  $sGC\alpha_1\beta_1$ , also  $sGC\alpha_2\beta_1$  is a target for basally released NO.

A lot of diseases, in which an impaired bioavailability and/or responsiveness to endogenous NO has been implicated, are currently treated with organic nitrates and other NO-donor drugs that release NO by spontaneous decomposition or bioconversion<sup>19</sup>. Given its clinical relevance, we examined the responsiveness towards exogenously applied NO in both  $sGC\alpha_1^{-/-}$  and  $sGC\beta_1^{ki/ki}$  mice. First of all, SNP, which releases exogenous NO upon biotransformation and NO-gas, which represents the purest form of exogenous NO, still had a substantial relaxing effect in the aortic and femoral artery segments isolated from the  $sGC\alpha_1^{-/-}$  mice. Furthermore, the sGC inhibitor ODQ had a strong inhibitory influence on the exogenous NO-induced vasorelaxation and on the SNP-induced cGMP production. ODQ is described as a potent and highly selective inhibitor of sGC and has therefore been used widely to probe for the involvement of cGMP in a given pharmacological response and to discriminate between cGMP-dependent and -independent effects of NO. However, there are also reports on the influence of ODQ on the redox state of other heme containing proteins such as the cytochrome P-450 system, thereby inhibiting the reductive bioactivation of organic nitrates and SNP and thus vasorelaxation induced by these compounds<sup>20</sup>. Moreover, as targets for ODQ, myoglobin and hemoglobin can positively (e.g. in the aorta<sup>21</sup>) or negatively (e.g. in cardiomyocytes<sup>22</sup>) influence the effectiveness of ODQ depending on their concentrations in the tissues. Therefore, a transgenic approach, such as the NO-insensitive sGC mutation is of great value. Whatsoever, our results obtained with ODQ are in line with the results obtained from the  $sGC\beta_1^{ki/ki}$  mice. In the femoral arteries of the  $sGC\beta_1^{ki/ki}$  mice, the

response to SNP was far more reduced compared to the SNP-induced response in the  $sGC\alpha_1^{-/-}$  femoral arteries. Moreover, the aortic rings of the  $sGC\beta_1^{ki/ki}$  mice did not respond to SNP at all. Even though all those findings favour the  $sGC\alpha_2\beta_1$  isoform as an important target for exogenous NO, the cGMP-measurements in the  $sGC\alpha_1^{-/-}$  aortic rings are not convincing, since there is only a small, not significant increase in the production of cGMP upon addition of SNP. Moreover, the absence of the  $sGC\alpha_1$  subunit is not compensated by an upregulation of the  $sGC\alpha_2$  subunit as suggested by the QPCR measurements in the ring segments of both  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_1^{+/+}$  mice. However, the study of Mergia et al. in which the role of the sGC isoforms was explored using  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_2^{-/-}$  mice, suggests that the small amount of cGMP produced by the  $sGC\alpha_2\beta_1$  isoform, would be sufficient to exert a profound effect on vascular tone<sup>23</sup>. They claim that the  $sGC\alpha_1\beta_1$  isoform has a predominant role in NO-induced vasorelaxation, but that the less abundantly expressed  $sGC\alpha_2\beta_1$  isoform is able to induce an equivalent response, although higher NO concentrations are needed. As the transgenic mouse models used in the study of Mergia et al. were developed on a mixed 129/SvJ-C57BL6/J background it is not surprising that their observations are more in line with our data from the  $sGC\alpha_1^{-/-}$  mice on the C57BL/6J background (see caption VIII.2: Influence of the genetic background on the phenotype of the  $sGC\alpha_1^{-/-}$  mice) than those of the  $sGC\alpha_1^{-/-}$  mice on the 129/SvJ-Swiss background. This also suggests that the NO origin-dependent difference we observed in  $sGC\alpha_1^{-/-}$  vasorelaxation is related to the Swiss background of the  $sGC\alpha_1^{-/-}$  mice. Overall, we can state that despite its lower expression, the  $sGC\alpha_2\beta_1$  isoform, plays a very important role in NO-mediated vasorelaxation. To know whether the vasorelaxing effect of  $sGC\alpha_2\beta_1$  is a compensation for the absence of the  $sGC\alpha_1\beta_1$  isoform or the  $sGC\alpha_2\beta_1$  is actually the main physiologically target for NO, NO-induced vasorelaxation should be explored in  $sGC\alpha_2^{-/-}$  mice. Such experiments were scheduled in our work program, but  $sGC\alpha_2^{-/-}$  mice are not yet available due to problems with the germline transmission of the mutation and infertility of the chimeric mice. As in the Transgene Core Facility of the research unit of Prof. Dr. P. Brouckaert from the Department of Molecular Biomedical Research and Flanders Interuniversity Institute of Biotechnology, the  $sGC\alpha_2$  knockout generation has been restarted from newly electroporated ES, it could perhaps be possible to characterise those mice in the future. Notwithstanding, Mergia and collaborators already explored the responsiveness to NO in  $sGC\alpha_2^{-/-}$  mice, it would still be interesting, as there are some important differences between the transgenic mouse models, and this can lead to variable results as was observed with the  $sGC\alpha_1^{-/-}$  mice.

In contrast with the aortic rings in which the sGC $\alpha_1$ -independent relaxation is proposed to be completely mediated by sGC $\alpha_2\beta_1$ , our observations on the femoral arteries and CC demonstrate the participation of sGC-independent mechanisms in the response to exogenous NO. Although many actions of NO have been attributed to its ability to activate sGC and to increase cGMP, also cGMP-independent effects of NO have been described. One of the proposed mechanisms underlying cGMP-independent reduction of the intracellular Ca<sup>2+</sup> concentration by NO is the opening of potassium channels<sup>24-26</sup> in the cell membrane, resulting in membrane hyperpolarisation and subsequent closure of the voltage-gated Ca<sup>2+</sup>-channels. Additionally, NO has also been shown to extrude Ca<sup>2+</sup> from the cell by directly stimulating the Na<sup>+</sup>/K<sup>+</sup> ATPase activity<sup>27, 28</sup>. Moreover, it accelerates the sequestration of Ca<sup>2+</sup> into the internal stores by activating the sarco-endoplasmic reticulum Ca<sup>2+</sup>-pumping ATPase (SERCA)<sup>29</sup>. Especially in the CC, only exogenously applied NO seems to have an sGC-independent effect. It is possible that the route of administration of NO may influence the actions of NO and thus also the involvement of an sGC-independent effect. After all, NO has direct effects on the endothelium<sup>30</sup> and differential responses to luminal versus adluminal application of NO have been reported<sup>31</sup>. Thus, endogenous NO released at the smooth muscle/ endothelium interface and exogenous NO administered to the whole vessel may have different effects. Moreover, also the type of NO-donor and related with that the form of NO generated may play a role. NO can exist in a variety of forms, viz. as free radical (NO<sup>•</sup>) or as the ions nitroxyl (NO<sup>-</sup>) or nitrosium (NO<sup>+</sup>), and the form(s) predominating may vary depending on the source of NO<sup>32</sup>.

Besides its direct cGMP-independent actions, NO may also regulate ion channels and transporters by S-nitrosylation<sup>33</sup> or S-glutathiolation<sup>34</sup> of reactive thiols, through the secondary generation of reactive oxygen species. Adachi et al. showed that NO and superoxide anion, through the formation of peroxynitrite, activate SERCA by reversible S-glutathiolation<sup>35</sup>. Additionally, various other proteins involved in metabolism, signaling, cell shape and DNA transcription may be regulated by NO through S-glutathiolation<sup>36</sup>. These non-specific interactions are, together with the development of tolerance<sup>37</sup> and insufficient biometabolism<sup>38</sup>, severe limitations associated with the use of NO releasing drugs for the treatment of NO/cGMP implicated pathologies. Additionally, cGMP-specific phosphodiesterase inhibitors used for the treatment of erectile dysfunction have been shown to be significantly less potent in patients with a reduced bioavailability of endogenously produced NO<sup>39</sup>. Because of these limitations, it was of interest to determine the physiological



relevance of the different sGC isoforms in smooth muscle relaxation, in order to provide information needed for the development of more selective drugs.

Also substances like BAY 41-2272 that can activate sGC in a NO-independent way, might offer considerable therapeutic advantages over the NO-based therapies<sup>19</sup>. Knocking out the sGC $\alpha_1\beta_1$  isoform caused a significant reduction of the response to BAY 41-2272 in the femoral arteries and CC; elimination of the heme-dependent activation of both sGC isoforms did however, not further reduce this BAY 41-2272-induced response. This suggests that in the femoral artery and CC, sGC $\alpha_1\beta_1$  is the only sGC-dependent target of BAY 41-2272. In the aortic rings, on the other hand, only the highest concentration of BAY 41-2272 we applied could induce a substantial relaxation in the sGC $\alpha_1^{-/-}$  mice. In the sGC $\beta_1^{ki/ki}$  aortic rings, the remaining effect of 10  $\mu\text{mol/L}$  BAY 41-2272 was significantly smaller, suggesting that at higher concentrations also sGC $\alpha_2\beta_1$  is being activated. Furthermore, from its relaxant effect in the sGC $\beta_1^{ki/ki}$  mice, we can conclude that in neither of the tissues explored, BAY 41-2272 exerts its effect only through the activation of sGC, at least, if the sGC-activating quality of BAY 41-2272 is completely heme-dependent. It is known that BAY 41-2272 can inhibit  $\text{Ca}^{2+}$  entry by mechanisms that do not involve cGMP<sup>40</sup>. Furthermore, Mullerhausen et al. demonstrated a PDE-5 inhibitory action of BAY 41-2272<sup>41</sup>. However, this has been refuted by a study of Bischoff and Stasch, who observed that BAY 41-2272 was devoid of any inhibitory effect on PDE-5<sup>42</sup>.

From the comparable reaction in sGC $\alpha_1^{-/-}$  and sGC $\alpha_1^{+/+}$  mice towards the cGMP-analogue 8-pCPT-cGMP and the cAMP-producing substance forskolin, we concluded that the impaired responses we observed in the sGC $\alpha_1^{-/-}$  preparations are related to sGC. This could not be concluded for the sGC $\beta_1^{ki/ki}$  mice, at least not in aortic ring segments. In contrast with the femoral arteries and CC, the aortic ring segments from the sGC $\beta_1^{ki/ki}$  mice showed a significantly reduced response to 8-pCPT-cGMP, pointing to an additional mechanism beyond the level of sGC as underlying cause for the reduced relaxations. Moreover, also the AC-activator forskolin did not show an equal relaxant effect in the aortic rings from both sGC $\beta_1^{ki/ki}$  and sGC $\beta_1^{+/+}$ .

Another difference with the sGC $\alpha_1^{-/-}$  mice, is that the arteries of the sGC $\beta_1^{ki/ki}$  mice showed a significant higher response to NOR than the corresponding sGC $\beta_1^{+/+}$  preparations. The elevated sensitivity towards NOR was most pronounced in the aortic rings and its relevance was confirmed by a high reproducibility. These surprising observations were however not reported in the sGC $\beta_1^{-/-}$  mice, generated by the research group of Koesling and

coworkers <sup>43</sup>, who also generated  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_2^{-/-}$  mice <sup>23</sup>. It should however be noted that in their  $sGC\beta_1$  transgenic mice model, the expression of NO-sensitive sGC is completely abrogated, whereas in the  $sGC\beta_1$  mutant mice we characterised, the sGC enzyme is present, yet without a prosthetic heme-group. This is also the case for the  $sGC\alpha_1^{-/-}$  mice, as the model we used expressed a catalytically inactive  $sGC\alpha_1$  protein, whereas in the one of Mergia et al.,  $sGC\alpha_1$  expression was completely eliminated <sup>23</sup>. This implies that in the knockout mice generated by Koesling and coworkers, the influence of enzyme structural functions on the phenotype, as demonstrated for the PI3K $\gamma$  knockout mice <sup>44</sup>, can not be ruled out.

Further explorations of the higher NOR-induced response in the  $sGC\beta_1^{ki/ki}$  arteries, revealed that this effect is rather specific for NOR in the femoral arteries. The addition of other contractile substances such as the selective  $\alpha_1$ -adrenoreceptor agonist methoxamine, the prostanoid receptor agonist prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) and  $K^+$  did not result in a higher response of the  $sGC\beta_1^{ki/ki}$  femoral arteries. In the aortic rings, on the other hand, all tested contractile agents induced a significant higher response in the  $sGC\beta_1^{ki/ki}$  preparations, although with a different extent. The  $sGC\beta_1^{ki/ki}$  aortic rings had a greater sensitivity towards  $PGF_{2\alpha}$ , although the maximum response was not significantly increased. The contractile effect of a G-protein coupled receptor agonist has been shown to result from  $Ca^{2+}$  sensitization as well as  $Ca^{2+}$  mobilization <sup>45</sup>. The importance of both processes was explored in the contractile responsiveness of the  $sGC\beta_1^{ki/ki}$  preparations.

As preincubation with the Rho kinase inhibitor HA-1077, abolished the difference in the NOR-induced response between the  $sGC\beta_1^{ki/ki}$  and  $sGC\beta_1^{+/+}$  femoral arteries, we suggest that the  $Ca^{2+}$  sensitization pathway, in which Rho kinase plays a key role, has a more profound action in the  $sGC\beta_1^{ki/ki}$  mice. However, in the aortic rings from the  $sGC\beta_1^{ki/ki}$  mice, the Rho kinase activity is not suggested to be enhanced, as even in the presence of HA-1077, the NOR-induced response was still significantly higher in the  $sGC\beta_1^{ki/ki}$  preparations. Still, this does not exclude a possible higher sensitivity towards  $Ca^{2+}$ , as besides Rho kinase, also protein kinase C (PKC) is a major determinant for the  $Ca^{2+}$  sensitization in vascular smooth muscles <sup>46</sup>. The influx of extracellular  $Ca^{2+}$  induced by Bay k 8644 (an L-type  $Ca^{2+}$  channel agonist) and/or TEA (a non-selective  $K^+$  channel inhibitor), caused a significant higher force development in the aortic rings, but not in the femoral arteries from the  $sGC\beta_1^{ki/ki}$  mice. As in contrast with the  $sGC\beta_1^{ki/ki}$  arteries, the vessel segments from the  $sGC\alpha_1^{-/-}$  mice showed an equal contractile activity compared with the  $sGC\alpha_1^{+/+}$  mice, we hypothesize that in these mice, the presence of the  $sGC\alpha_2\beta_1$  isoform suppresses the NOR-induced contraction. As this

compensation by the sGC $\alpha_2\beta_1$  isoform is not possible in the sGC $\beta_1^{ki/ki}$  arteries, one would speculate that the insensitivity towards basal endothelium-derived NO of the sGC $\beta_1^{ki/ki}$  vessel segments is responsible for their higher contractile activity. The stimulating effect of ODQ on the NOR-induced response in C57BL/6J mice also points into that direction. The importance of basally released NO has been shown to depend on the vessel type studied<sup>12, 47</sup>. However, this heterogeneity does not explain the discrepancy in contractile activity between the sGC $\beta_1^{ki/ki}$  aorta and sGC $\beta_1^{ki/ki}$  femoral artery as we could not observe a significant difference in the ODQ-induced increase in tone between the C57BL/6J aorta and femoral artery. Therefore, we suggest that besides basal endothelium-derived NO, also other mechanisms are responsible for the higher tone of the sGC $\beta_1^{ki/ki}$  arteries. Additional experiments should further address this issue, for example simultaneous measurements of  $[Ca^{2+}]_i$  and force.

## **VII.2. Conclusions**

Our results show that the sGC $\alpha_1\beta_1$  isoform, which represents the largest fraction of the total sGC protein concentration, is the main target for NO and NO-independent sGC stimulators, like BAY 41-2272. However, the sGC $\alpha_2\beta_1$  isoform can despite its limited expression, produce sufficient cGMP to largely compensate for the lack of the sGC $\alpha_1\beta_1$  isoform. Contrary to the aorta, in which sGC is the sole NO-receptor, the femoral artery and corpora cavernosa, also support the participation of cGMP-independent mechanisms. The latter can also be concluded from our experiments with BAY 41-2272. The selective targeting of the sGC $\alpha_1\beta_1$  isoform might offer a therapeutic approach to compensate for the depressed NO/cGMP pathway, which is the underlying cause of many vascular disease states.

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# Chapter VIII

Addendum



## **VIII.1. NO-dependent corpus cavernosum smooth muscle relaxation in Wnt-1 conditional sGC $\alpha_1$ <sup>-/-</sup> mice.**

### **VIII.1.1. Introduction**

Peripheral nitrergic nerves have a widespread distribution, and are particularly important in that they produce relaxation of smooth muscle in the gastrointestinal, respiratory, vascular and urogenital systems <sup>1</sup>. Despite the importance of NO as neurotransmitter, little is known about the regulation of the release of NO from the peripheral nitrergic nerves. In rabbit colon <sup>2</sup> and corpus cavernosum <sup>3</sup>, sGC/cGMP has been shown to exert a positive feedback on the release of NO from the autonomic neurons in the gastrointestinal tract and penis. How this proposed feedback is mediated is however unclear. The immunohistochemical detection of sGC in the nerve fibers of the corpus cavernosum (CC) <sup>4</sup> and neuromodulatory role of presynaptical sGC in the central nervous system <sup>5</sup>, suggest a possible involvement of sGC in the neurons. Therefore, in the present study, using CC isolated from Wnt-1 conditional sGC $\alpha_1$ <sup>-/-</sup> and sGC $\alpha_1$ <sup>+/+</sup> mice, we investigated if neuronal sGC modulates the nerve-induced NO release from autonomic neurons in the penis.

### **VIII.1.2. Animals**

Through homologous recombination in mice embryonic stem (ES) cells, the sixth exon of the sGC $\alpha_1$  gene was flanked with loxP sites. These ES cells gave rise to chimeric mice, which were then used to produce mice homozygous for the floxed allele (see caption materials and methods: mice models: sGC $\alpha_1$  knockout mice). In the next step those sGC $\alpha_1$ <sup>flox/flox</sup> mice were mated with transgenic mice expressing Cre recombinase under the control of the Wnt-1 promoter and enhancer. The Wnt-1 protooncogene is found to be expressed in the dorsal neural tube of the vertebrate embryo, from which the pluripotent neural crest cells originate <sup>6</sup>. Therefore, by utilizing the Wnt-1 promoter, Cre-expression and thus deletion of the sGC $\alpha_1$ <sup>flox</sup> allele, was restricted to the derivatives of the neural crest cells <sup>7</sup>, which include all peripheral autonomic neurons <sup>8</sup>.

### VIII.1.3. Tissue collection

From male Wnt-1 conditional  $sGC\alpha_1^{-/-}$  and  $sGC\alpha_1^{+/+}$  mice (genetic background: C57BL/6J), CC was isolated and transferred to cooled KRB solution (see caption III.4.1. Dissection).

### VIII.1.4. Tension measurements

Of each mouse, one corpus cavernosum was mounted horizontally in a myograph (see caption III.4.3. Mounting of the penile tissue) and preloaded with 0.45 g of tension (see caption III.4.4. Preparation of the tissue before the experiment). In order to increase and stabilize the subsequent submaximal pre-contraction response to 5  $\mu\text{mol/L}$  norepinephrine (NOR), the preparations were 3 times contracted with 5  $\mu\text{mol/L}$  NOR, washed, and allowed to relax to resting tension before starting the protocol. When the pre-contraction response (5  $\mu\text{mol/L}$  NOR) reached a stable level, electrical field stimulation (EFS), 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. The CC were washed and allowed to recover for 20 min between every response curve.

### VIII.1.5. Calculations and statistics

Data are presented as mean values  $\pm$  SEM;  $n$  represents the number of arteries (each obtained from a different mouse). Statistical significance was evaluated by using Student's  $t$ -test for unpaired observations (SPSS, version 12).  $P < 0.05$  was considered as significant.

### VIII.1.6. Results

The endothelium-dependent vasodilator acetylcholine (ACh) relaxed the CC preparations from both  $sGC\alpha_1^{+/+}$  and Wnt-1 conditional  $sGC\alpha_1^{-/-}$  mice to a similar extent (response to 10  $\mu\text{mol/L}$ :  $71.70\% \pm 6.07$  vs.  $72.77\% \pm 5.00$  ( $n=6$ ,  $P > 0.05$ )).

The CC from both  $sGC\alpha_1^{+/+}$  and Wnt-1 conditional  $sGC\alpha_1^{-/-}$  mice responded to EFS-induced and thus neuronal derived NO in a similar way (response to 8Hz:  $79.94\% \pm 13.50$  vs.  $72.82\% \pm 4.69$  ( $n=5$ ,  $P > 0.05$ )).

Exogenous NO delivered by the NO-donor sodium nitroprusside (SNP) and NO-gas, had a concentration-dependent and similar relaxant effect in the CC preparations of  $sGC\alpha_1^{+/+}$  and Wnt-1 conditional  $sGC\alpha_1^{-/-}$  mice (response to 10  $\mu\text{mol/L}$  SNP:  $99.72\% \pm 5.18$  vs.  $96.41\% \pm 5.84$

(n=5, P>0.05); response to 0.1 mmol/L NO-gas: 89.71%±7.89 (n=4) vs. 88.05%±7.69 (n=5, P>0.05)).

### VIII.1.7. Discussion

It is widely accepted that the release of NO from autonomic neurons in the CC results in an increase of the cGMP-level in the penile tissue and will subsequently lead to corporal smooth muscle relaxation. Hallen et al. showed that the nerve-induced release of NO from the rabbit CC is subject to modulation by the sGC/cGMP pathway<sup>3</sup>. It is however not clear whether this modulation occurs in the nitrenergic neuron or in some other cell type. As there are no reports on the colocalisation of NOS and sGC in the neurons of the CC, Hallen et al. suggested that it is more likely that sGC upregulates the formation of NO within a cell located close to the nitrenergic neuron<sup>3</sup>. Our data however, give no indication towards a role for sGC as neuromodulator in the autonomic neurons of the penis. As the pelvic neurons arise from the sacral neural crest<sup>9</sup>, the nerve fibers of the penis of the Wnt-1 sGC $\alpha_1$ <sup>-/-</sup> mice lack the functional sGC $\alpha_1$  enzyme. The neural crest origin of the pelvic neurons is strengthened by the fact that they have been reported to express neurotrophic factors<sup>10, 11</sup> that promote the migration of the neural crest cells to their correct site. The CC preparations from both Wnt-1 sGC $\alpha_1$ <sup>-/-</sup> and sGC $\alpha_1$ <sup>+/+</sup> mice, responded to EFS in a similar way, suggesting that sGC $\alpha_1\beta_1$ , present in the cavernosal neurons, had no influence on the nerve-induced release of NO. As expected, the responses to ACh, SNP and NO-gas were not influenced by the targeted deletion of the sGC $\alpha_1$  subunit in the nervous system.

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## **VIII.2. Influence of the genetic background on the phenotype of the sGC $\alpha_1$ <sup>-/-</sup> mice.**

### **VIII.2.1. Introduction**

As the predominant intracellular target for NO, sGC regulates vascular smooth muscle relaxation, which is a very important process in the cardiovascular system <sup>1</sup>. In order to get insight into the relevance of the two physiological active sGC isoforms in vasorelaxation, NO-induced relaxation was measured in vessel segments isolated from sGC $\alpha_1$ <sup>-/-</sup> and sGC $\beta_1$ <sup>ki/ki</sup> mice. The sGC $\alpha_1$ <sup>-/-</sup> mice have a mixed Swiss-129/SvJ background, whereas the sGC $\beta_1$ <sup>ki/ki</sup> mice have a mixed C57BL/6J-129/SvJ background. A mixed genetic background is known to produce a potential source of variability in the experiments and the phenotypes of mutant mice have been reported to be influenced by their genetic background <sup>2</sup>. Moreover, a genetic background effect has been put forward as hypothesis for the gender-specific development of hypertension that was found in the sGC $\alpha_1$ <sup>-/-</sup> mice on the mixed background but not in the sGC $\alpha_1$ <sup>-/-</sup> mice backcrossed to a C57BL/6J background. In order to recognize the importance of the genetic background, we examined the relaxing influence of NO on the aortic rings from sGC $\alpha_1$ <sup>-/-</sup> and sGC $\alpha_1$ <sup>+/+</sup> mice with a pure C57BL/6J background and compared this with the results obtained from sGC $\alpha_1$ <sup>-/-</sup> and sGC $\alpha_1$ <sup>+/+</sup> mice with a mixed Swiss-129/SvJ background.

### **VIII.2.2. Animals**

We used sGC $\alpha_1$ <sup>-/-</sup> mice which were generated on a mixed Swiss-129/SvJ background and then backcrossed to a C57BL/6J background for more than 12 generations while selecting for the mutation of the sGC $\alpha_1$  gene.

### **VIII.2.3. Tissue collection**

Aortic rings from female sGC $\alpha_1$ <sup>-/-</sup> and sGC $\alpha_1$ <sup>+/+</sup> mice with a pure C57BL/6J background, were isolated and transferred to cooled KRB solution (see caption III.3.1. Dissection).

### **VIII.2.4. Tension Measurements**

Aortic ring segments were mounted in a small vessel myograph (see caption III.3.2.2. Mounting of the ring segment) and gradually stretched until a stable preload of 0.5 g is obtained (see caption III.3.2.3. Preparation of the aortic segments before the experiment).

Subsequently, the preparations were contracted 3 times with a KRB solution containing 120 mmol/L  $K^+$  and 5  $\mu\text{mol/L}$  norepinephrine (NOR), washed, and allowed to relax to basal tension before starting the protocol. When a stable precontraction (30  $\mu\text{mol/L}$   $\text{PGF}_{2\alpha}$ ) was obtained, cumulative concentration-response curves to ACh and SNP were obtained.

### VIII.2.5. Calculations and statistics

Data are presented as mean values  $\pm$  SEM;  $n$  represents the number of arteries (each obtained from a different mouse). Statistical significance was evaluated by using Student's  $t$ -test for unpaired observations (SPSS, version 12).  $P < 0.05$  was considered as significant.

### VIII.2.6. Results

The endothelium-dependent vasoactive substance ACh (Fig. 1A, B), induced a concentration-dependent relaxation of the aortic rings from both  $\text{sGC}\alpha_1^{+/+}$  and  $\text{sGC}\alpha_1^{-/-}$  mice. However, the ACh-induced response was significantly reduced in the aortic rings from  $\text{sGC}\alpha_1^{-/-}$  mice with a pure background and almost completely blocked in the aortic rings from  $\text{sGC}\alpha_1^{-/-}$  mice with a mixed background (response to 10  $\mu\text{mol/L}$  ACh: mixed background:  $67.8\% \pm 3.5$  for  $\text{sGC}\alpha_1^{+/+}$  vs.  $16.4\% \pm 5.2$  for  $\text{sGC}\alpha_1^{-/-}$  ( $n=6$ ,  $P < 0.05$ ); pure background:  $71.5\% \pm 4.9$  for  $\text{sGC}\alpha_1^{+/+}$  vs.  $41.4\% \pm 10.2$  for  $\text{sGC}\alpha_1^{-/-}$  ( $n=4$ ,  $P < 0.05$ ))

The aortic rings from both  $\text{sGC}\alpha_1^{+/+}$  and  $\text{sGC}\alpha_1^{-/-}$  mice responded to the NO-donor SNP (Fig. 1C, D) in a concentration-dependent way. However, the relaxing effect of SNP was significantly reduced in the aortic rings of the  $\text{sGC}\alpha_1^{-/-}$  mice with a pure and a mixed background (response to 10  $\mu\text{mol/L}$  SNP: mixed background:  $77.9\% \pm 4.0$  for  $\text{sGC}\alpha_1^{+/+}$  vs.  $48.6\% \pm 3.6$  for  $\text{sGC}\alpha_1^{-/-}$  ( $n=15$ ,  $P < 0.05$ ); pure background:  $58.0\% \pm 2.4$  for  $\text{sGC}\alpha_1^{+/+}$  vs.  $35.2\% \pm 5.5$  for  $\text{sGC}\alpha_1^{-/-}$  ( $n=4$ ,  $P < 0.05$ )).

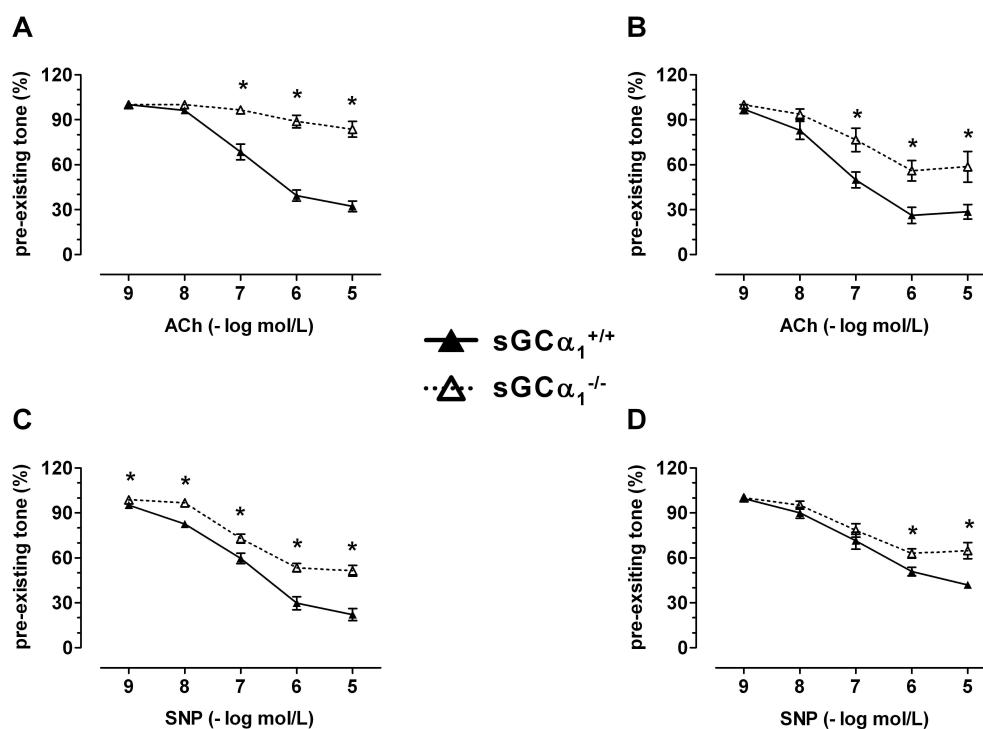
### VIII.2.7. Discussion

Induced mutations are often generated and maintained on a mixed segregating background. Further backcrossing to another inbred strain may result in a phenotype different from the phenotype initially reported on the mixed genetic background<sup>3</sup>. To look for possible phenotypic differences with the  $\text{sGC}\alpha_1^{-/-}$  mice on a mixed background, the NO-induced response was obtained on aortic rings from  $\text{sGC}\alpha_1^{-/-}$  and  $\text{sGC}\alpha_1^{+/+}$  mice with a pure C57BL/6J background. We found that the genetic background modulates the response to endogenous NO in the  $\text{sGC}\alpha_1^{-/-}$  mice, as the ACh-induced relaxation was more reduced in the  $\text{sGC}\alpha_1^{-/-}$



mice on the mixed background compared to the  $sGC\alpha_1^{-/-}$  mice on the pure background (reduction of the response to 10  $\mu\text{mol/L}$  ACh: 75.83% vs. 42.17%). This also implies a background-related difference in the importance of the  $sGC\alpha_1\beta_1$  isoform and the participation of the  $sGC\alpha_2\beta_1$  isoform and/or (an) sGC-independent mechanism(s). In case of the response to exogenous NO, we found far less supporting evidence for a genetic background effect, as only the responses to the lowest concentrations of SNP were somewhat more reduced in the  $sGC\alpha_1^{-/-}$  mice on a mixed background. The relaxing influence of 10  $\mu\text{mol/L}$  SNP was however, similarly reduced in the  $sGC\alpha_1^{-/-}$  mice on both a mixed background and a pure background (reduction of the response to 10  $\mu\text{mol/L}$  SNP: 37.59% vs. 39.41%).

In conclusion, we found that the physiological importance of the  $sGC\alpha_1\beta_1$  isoform in the response to endogenous NO is clearly dependent of the genetic background. This implies the presence of genetic modifiers<sup>4</sup>, which have to be taken into consideration for the correct interpretation and comparison of the phenotypes of the genetically engineered mice.



**Figure VIII.1:** Relaxation effect of ACh (A, B) and SNP (C, D) on precontracted (30  $\mu\text{mol/L}$   $\text{PGF}_{2\alpha}$ ) aortas from female  $sGC\alpha_1^{+/+}$  and  $sGC\alpha_1^{-/-}$  mice on a mixed background (Swiss-129/SvJ) (A, C) and a pure background (C57BL/6J) (B, D). \*( $sGC\alpha_1^{+/+}$  vs.  $sGC\alpha_1^{-/-}$  mice):  $P < 0.05$ .

VIII.2.8. References

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# Summary

Soluble guanylyl cyclase (sGC) plays a key role in the transduction of inter- and intracellular signals conveyed by NO; as such its physiological importance is invaluable. To fulfil this role, sGC has a unique heme coordination which makes it suitable as receptor for NO. Its product, cGMP, regulates a wide range of biological processes, including vascular and non-vascular smooth muscle relaxation, peripheral and central neurotransmission, platelet reactivity and phototransduction. Due to its widespread nature, signaling via the NO/cGMP pathway is depressed in many disease states (e.g. erectile dysfunction (ED)) providing a rationale for enhancing sGC activity by drugs.

In 1998, the first oral treatment for ED, a phosphodiesterase (PDE) type 5 inhibitor (blocks the breakdown of cGMP) called sildenafil (Viagra<sup>®</sup>), became available. The assumption that Viagra<sup>®</sup> was a "cure" for erectile dysfunction raised high expectations for the treatment, sustained by the high interest of the media. However, treatment with PDE-5 inhibitors turns out to be significantly less efficient in patients with a reduced bioavailability of endogenously generated NO such as in diabetics or patients with severe neurologic damage. Because sildenafil has a low selectivity for PDE-6, which is found in the retina, transient visual disturbances including blurring of vision, increased light sensitivity and impaired blue/green color discrimination, are common side effects. Furthermore, the concomitant use of Viagra and nitrovasodilators is absolutely contraindicated, since PDE-5 inhibitors potentiate the systemic hypotensive effect of NO.

Nitrovasodilators have been used for more than a century to induce vasodilatation via the NO/cGMP pathway, however the development of tolerance and potentially adverse cGMP-independent actions of nitrovasodilators such as promoting oxidative stress and protein modification are functionally important limitations for their use.

This illustrates the interest for the design and development of more selective and NO-independent therapeutic strategies, in which the understanding of the functional importance of the physiologically active sGC isoforms (the main sGC $\alpha_1\beta_1$  and minor sGC $\alpha_2\beta_1$ ) can be of great value. Therefore, the aim of this thesis was to gain more insight into the physiological relevance of the sGC $\alpha_1\beta_1$  and sGC $\alpha_2\beta_1$  isoform in vascular and corpus cavernosum (CC) smooth muscle relaxation. As there are no sGC isoform specific inhibitors available, we made use of a mouse transgenic approach to study the function of each isoform. From genetically engineered mice with a targeted deletion of the sGC $\alpha_1$  gene or a mutation of the sGC $\beta_1$  gene,

vascular and corporal tissues were isolated and mounted in a myograph to measure changes in isometric forces. These experiments were supplemented with measurements of cGMP concentrations in the aortic rings. **Chapter III** encloses a detailed description of those techniques.

As sGC $\alpha_1\beta_1$  is considered to be the "universal heterodimer" with the highest expression level, we first draw our attention to the sGC $\alpha_1^{-/-}$  mice in which the sGC $\alpha_1\beta_1$  isoform is not longer functionally active. In **chapter IV**, we demonstrated that the sGC $\alpha_1\beta_1$  isoform is of functional importance in vasorelaxation induced by endogenous NO (released from the endothelium in response to acetylcholine (ACh)), exogenous NO (delivered by NO-donors (sodium nitroprusside (SNP) and SNAP) and NO-gas, which represents NO as such) as well as basal NO (examined by the accumulation of basally produced cGMP caused by the PDE-5 inhibitor T-1032). Also the vasorelaxing effect of NO-independent sGC stimulators (BAY 41-2272 and YC-1), which have been forwarded as potential new drugs for the treatment NO/cGMP related pathologies, was found to involve the sGC $\alpha_1\beta_1$  activity. However, the observation that the responsiveness of the arteries from the sGC $\alpha_1^{-/-}$  mice is diminished but not completely abolished, suggests that besides sGC $\alpha_1\beta_1$ , also the less abundantly expressed isoform sGC $\alpha_2\beta_1$  and/or (an) sGC-independent mechanism(s) play a substantial role.

Subsequently, we performed a study on the CC isolated from sGC $\alpha_1^{-/-}$  mice (**chapter V**). CC are defined as two spongelike regions in the penis which run the length of the organ and which will become engorged with blood upon erection. The importance of CC smooth muscle relaxation in penile perfusion (and thus in erection) is illustrated by the fact that impaired responsiveness of CC smooth muscle cells is a common cause of ED or impotence. Our findings in the CC were overall comparable to the observations in the aorta and femoral artery. We demonstrated the involvement of the sGC $\alpha_1\beta_1$  isoform in CC smooth muscle relaxation in response to NO (in addition to endothelium-derived NO, the release of endogenous NO from the nitrenergic neurons was induced by electrical field stimulation) and NO-independent sGC-stimulators. Furthermore, also the participation of the sGC $\alpha_2\beta_1$  isoform as well as (an) sGC-independent mechanism(s) could be argued.

To get further insight into the mechanism responsible for the remaining relaxation in the sGC $\alpha_1^{-/-}$  mice, the relaxant properties of vessel and CC smooth muscle, was investigated in sGC $\beta_1^{ki/ki}$  mice (**chapter VI**). Those mice express a catalytically active but NO-insensitive sGC enzyme, as the axial ligand of the prosthetic heme group (histidine 105 of the sGC $\beta_1$  subunit) is substituted. By comparing the impairment of the responses in the sGC $\beta_1^{ki/ki}$  mice

with those in the  $sGC\alpha_1^{-/-}$  mice, we concluded that the cGMP producing capacity of the minor  $sGC\alpha_2\beta_1$  isoform is sufficient to largely compensate the absence of the  $sGC\alpha_1\beta_1$  isoform. Furthermore, we confirmed the unique role of sGC as target for NO in the aorta, whereas in the femoral artery and CC also sGC/cGMP-unrelated mechanisms are involved.

Moreover, we also observed a higher contractile activity in the arteries from the  $sGC\beta_1^{ki/ki}$ , which in the femoral artery appears to be specific for norepinephrine. The aortic rings from the  $sGC\beta_1^{ki/ki}$ , on the other hand, contracted significantly stronger to a wide range of  $Ca^{2+}$  increasing substances. We suggest that this increased contractility to some extent may be explained by the unresponsiveness to basal endothelial NO.

Characterization of genetically engineered mice requires also a consideration of the genetic background on which the mutation is maintained, as numerous reports illustrate the influence of the genetic background on the phenotype. Also we found evidence for the presence of genetic modifiers (alleles present in the background strain genome that alter the expression of the gene of interest) since the contribution of the  $sGC\alpha_1\beta_1$  isoform in the response to endogenous NO was dependent on the genetic background (**addendum VIII.2**). This was taken into account by the interpretation and comparison of the phenotypes.

In conclusion, the results obtained from the studies from this thesis demonstrate the physiological importance of  $sGC\alpha_1\beta_1$  in vascular and CC smooth muscle relaxation and in that way promote this isoform as a potential more selective therapeutic target for the treatment of cardiovascular diseases and ED.



## Samenvatting

Het feit dat oplosbaar guanylaatcyclase (sGC) een hoofdrol speelt in de signaaltransductie tussen de boodschappermoleculen stikstofmonoxide (NO) en cyclisch guanosine 3', 5'-monofosfaat (cGMP), illustreert het fysiologisch belang van dit enzym. sGC wordt gekenmerkt door een unieke heem-bindingsplaats die als receptor voor NO fungeert. Daarbij wordt cGMP gevormd, welke een brede waaier van biologische processen regelt, waaronder relaxatie van vasculaire en niet-vasculaire gladde spiercellen, neurotransmissie in perifere neuronen, aggregatie van bloedplaatjes en fototransductie. De NO/cGMP signaaltransductie is in heel wat ziektebeelden onderdrukt (bv. bij erectiele dysfunctie (ED)). Vandaar het veelvuldig gebruik van farmaca die de activiteit van sGC trachten te verhogen.

In 1998 werd de eerste orale medicatie voor ED op de markt gebracht, namelijk de fosfodiesterase (PDE) type 5 antagonist sildenafil (remt de afbraak van cGMP), beter gekend onder de naam Viagra<sup>®</sup>. De verwachtingen van deze behandeling waren zeer hoog aangezien sildenafil, mede door de enorme media-aandacht, naar voren werd gebracht als het middel tegen erectiestoornissen. PDE-5 remmers blijken echter significant minder doeltreffend te zijn voor de behandeling van ED bij patiënten met een verlaagde biologische beschikbaarheid van endogeen gegenereerd NO, zoals dit het geval is bij diabetici en bij patiënten met ernstige neurologische schade. Bovendien inibeert sildenafil ook de PDE-6 welke betrokken is bij de fototransductie in de retina. Hierdoor zijn tijdelijke effecten op het gezichtsvermogen zoals een wazig zicht, een verhoogde gevoeligheid voor licht en een verstoorde kleurdiscriminatie (blauw/groen) veel voorkomende bijwerkingen. Verder mag sildenafil ook niet gelijktijdig ingenomen worden met nitrovasodilatoren (nitraten) aangezien PDE-5 remmers het systemisch hypotensieve effect van NO versterken.

Van oudsher worden nitrovasodilatoren gebruikt als vaatverwijders via de NO/cGMP signaaltransductiecascade. Nochtans hebben deze geneesmiddelen ook belangrijke beperkingen: de ontwikkeling van tolerantie en de potentieel cGMP-onafhankelijke effecten van nitrovasodilatoren zoals het verhogen van oxidatieve stress en het modifieren van eiwitten.

Dit verklaart de interesse voor het ontwerpen en ontwikkelen van meer selectieve en NO-onafhankelijke therapieën. Hierbij kan informatie over het functioneel belang van de fysiologisch voorkomende sGC isovormen (sGC $\alpha_1\beta_1$ : de hoofd isovorm en sGC $\alpha_2\beta_1$ : de secundaire isovorm) heel nuttig zijn. Het hoofddoel van deze thesis was dan ook meer te weten te komen over het fysiologisch belang van sGC $\alpha_1\beta_1$  en sGC $\alpha_2\beta_1$  bij de relaxatie van

## Samenvatting

vasculaire en corpus cavernosum (CC) gladde spiercellen. Doordat er geen sGC isovorm-specifieke inhibitoren beschikbaar zijn, hebben we gebruik gemaakt van transgene muizen om de functie van elke isovorm te achterhalen. Arteriën (de aorta en de femorale arterie) en CC werden geïsoleerd uit muizen, waarbij door genetisch manipulatie het gen coderend voor de sGC $\alpha_1$  subeenheid uitgeschakeld is of waarin het gen coderend voor de sGC $\beta_1$  subeenheid gemuteerd is. Deze weefsels werden vervolgens opgespannen op een myograaf om isometrische krachtveranderingen te meten. Daarnaast werd ook de cGMP concentratie in aortapreparaten bepaald. Deze technieken werden uitvoerig beschreven in **hoofdstuk II**.

Omdat sGC $\alpha_1\beta_1$  beschouwd wordt als de universele heterodimeer met het hoogste expressie niveau, hebben we in ons onderzoek eerst de aandacht gevestigd op de sGC $\alpha_1$  knockout (sGC $\alpha_1^{-/-}$ ) muizen, waarbij de sGC $\alpha_1\beta_1$  isovorm niet langer functioneel actief is. In **hoofdstuk IV** werd aangetoond dat de sGC $\alpha_1\beta_1$  isovorm van functioneel belang is bij vasodilatatie geïnduceerd door zowel endogeen NO (vrijgesteld uit het endotheel als antwoord op acetylcholine (ACh)), als exogeen NO (geleverd door NO-donoren (natrium nitroprusside (SNP) en SNAP) en NO-gas als dusdanig), als basaal NO (onderzocht aan de hand van de opstapeling van basaal geproduceerd cGMP, veroorzaakt door de PDE-5 blokker T-1032). Verder werd ook vastgesteld dat de sGC $\alpha_1\beta_1$  isovorm een rol speelt bij het relaxerend effect van NO-onafhankelijke sGC stimulators (BAY 41-2272 en YC-1). Door hun vermogen om sGC te activeren zonder NO, worden deze substanties naar voor gebracht als potentiële nieuwe medicamenteuze behandelingen voor NO/cGMP-gerelateerde pathologische aandoeningen. Het antwoord van de arteriën afkomstig van de sGC $\alpha_1^{-/-}$  muizen is nochtans slechts deels verminderd, wat er op wijst dat naast sGC $\alpha_1\beta_1$  ook sGC $\alpha_2\beta_1$  (de isovorm met een lagere expressie) en/of (een) sGC-onafhankelijke mechanisme(n) een substantiële rol spelen.

In **hoofdstuk V** hebben we de CC afkomstig van de sGC $\alpha_1^{-/-}$  muizen onderzocht. CC zijn opgebouwd uit twee cilindrische sponsachtige weefsels die zich uitstrekken in de lengte van de penis en die bij een erectie volstromen met bloed. Het belang van de relaxatie van de CC gladde spiercellen bij de bloeddorstroming in de penis (en dus bij erectie) wordt geïllustreerd door het feit dat ED of impotentie veelal te wijten is aan een verminderd antwoord van de corporale gladde spiercellen. Algemeen gezien waren onze bevindingen in de CC vergelijkbaar met deze in de aorta en femorale arterie. We hebben aangetoond dat sGC $\alpha_1\beta_1$  betrokken is bij de relaxatie van CC gladde spiercellen wanneer deze geïnduceerd wordt door NO (naast NO afkomstig van het endotheel werd ook NO vanuit de nitrege zenuwen vrijgesteld door middel van elektrische veldstimulatie) en NO-onafhankelijke sGC



stimulators. Verder werden argumenten gevonden voor het belang van zowel de sGC $\alpha_2\beta_1$  isovorm als (een) sGC-onafhankelijke mechanisme(n).

Om informatie te verkrijgen over het mechanisme dat verantwoordelijk is voor de resterende gladde spiercel relaxatie in de sGC $\alpha_1^{-/-}$  muizen, werd het relaxatievermogen onderzocht van de vasculaire en CC preparaten afkomstig van sGC $\beta_1^{ki/ki}$  muizen (**hoofdstuk VI**). Het sGC enzym in deze muizen is katalytisch actief maar ongevoelig voor NO door het vervangen van het axiale bindingsresidu voor de prosthetische heme groep (histidine 105 van de sGC $\beta_1$  subunit). De vermindering van de gladde spiercel relaxatie geobserveerd in de preparaten van de sGC $\beta_1^{ki/ki}$  muizen werd vergeleken met deze in de preparaten van de sGC $\alpha_1^{-/-}$  muizen. Hieruit konden we besluiten dat de afwezigheid van een functioneel sGC $\alpha_1\beta_1$  isovorm grotendeels gecompenseerd wordt door cGMP geproduceerd door de minder tot expressie gebrachte sGC $\alpha_2\beta_1$  isovorm. Verder werd in de aorta de rol van sGC als enige receptor voor NO bevestigd. Dit in tegenstelling tot de femorale arterie en de CC, waarin ook niet sGC-gerelateerde mechanismen actief zijn.

Bovendien stelden we vast dat de arteriën geïsoleerd uit de sGC $\beta_1^{ki/ki}$  muizen een grotere contractie vertonen, welke in de femorale arterie specifiek bleek te zijn voor noradrenaline. De aortapreparaten uit de sGC $\beta_1^{ki/ki}$  muizen daarentegen, vertoonden een groter antwoord op een brede waaier van  $[Ca^{2+}]_i$  verhogende substanties. Deze verhoogde contractiliteit zou ten dele kunnen te wijten zijn aan de absentie van het vasodilerend effect van basaal NO in de preparaten afkomstig van de sGC $\beta_1^{ki/ki}$  muizen.

Bij de karakterisering van genetisch gemanipuleerde muizen dient men steeds rekening te houden met de genetische achtergrond van de gemuteerde muizen. In de literatuur is namelijk veelvuldig beschreven welke invloed de genetische achtergrond kan hebben op het fenotype. In **addendum VIII.2** rapporteren we dat de bijdrage van de sGC $\alpha_1\beta_1$  isovorm in het antwoord op endogeen NO varieert naargelang de genetische achtergrond. Dit wijst op de aanwezigheid van ‘genetic modifiers’ (allelen die eigen zijn aan het genoom van de muisstam en die de expressie van het gen van interesse wijzigen), die in rekening dienen te worden gebracht bij de interpretatie en het vergelijken van fenotypes.

Samenvattend tonen de resultaten van dit werk aan dat de sGC $\alpha_1\beta_1$  isovorm van fysiologisch belang is bij de relaxatie van vasculaire en CC gladde spiercellen en dat deze isovorm kan aangegeven worden als een potentieel meer selectief therapeutisch doelwit voor de behandeling van cardiovasculaire aandoeningen en ED.



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