

In vitro studies on the effect of red wine polyphenols and cyclic guanosine monophosphate export in the regulation of vascular and erectile tone

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List of abbreviations

4-AP	4-aminopyridine
8-SPT	8-(p-sulfophenyl)-theophylline
All	angiotensin II
AA	ascorbic acid
AC	adenylyl cyclase
Ach	acetylcholine
ADRF	adipocyte-derived relaxing factor
AMP	adenosine monophosphate
apoE	apolipoprotein E
BH ₄	tetrahydrobiopterin
CaM	calcium/calmodulin complex
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
CSE	cystathionine gamma-lyase
DAG	diacylglycerol
DMSO	dimethylsulfoxide
EAT	epicardial adipose tissue
ED	erectile dysfunction
EDCFs	endothelium-derived contracting factors
EDHF	endothelium-derived hyperpolarizing factor
EDRFs	endothelium-derived relaxing factors
EFS	electrical field stimulation
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
ET-1	endothelin 1
FFA	free fatty acid
GDP	guanosine diphosphate
Glib	glibenclamide

GMP	guanosine monophosphate
GPx	glutathione peroxidases
GS-NO	S-nitroso-L-glutathione
GTP	guanosine triphosphate
H ₂ O ₂	hydrogen peroxide
H-89	dihydrochloride hydrate
HDL	high-density lipoprotein
HG	high glucose
HGF	hepatocyte growth factor
ICAM	intercellular adhesion molecule 1
IL-6	interleukin 6
Indo	indomethacin
iNOS	inducible nitric oxide synthase
IP ₃	inositol triphosphate
K _{ATP}	ATP-sensitive potassium channel
KRB	Krebs-Ringer bicarbonate
K _v	voltage dependent potassium channel
L-NAME	N ^ω -nitro-L-arginine methyl ester hydrochloride
MGO	methylglyoxal
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MRP	multidrug resistance protein
NADPH	nicotinamide adenine dinucleotide phosphatase
NANC	non-adrenergic non-cholinergic
NCX	sodium/calcium exchanger
NHE ₁	sodium/hydrogen exchanger
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOR	norepinephrine
NOS	nitric oxide synthase
NOX	nicotinamide adenine dinucleotide phosphate oxidase

O ₂ ^{•-}	superoxide anion
ODQ	1 H-[1,2,4]oxadiazolo[4,3-A]quinoxalin-1-one
OH [•]	hydroxyl
ONOO ^{•-}	peroxynitrite
Orx	orchidectomized
PA	palmitic acid
PAI-1	plasminogen activator inhibitor-1
PDEs	phosphodiesterases
pGC	particulate guanylyl cyclase
Phe	phenylephrine
PIP2	phosphatidyl-inositol-4,5-biphosphate
PKA	cyclic adenosine monophosphate-dependent protein kinase
PKC	protein kinase C
PKG	cyclic guanosine monophosphate-dependent protein kinase
PLC	phospholipase C
PMCA	plasma membrane calcium ATPase
PPAR γ	peroxisome proliferator-activated receptor γ
PVADCF	perivascular adipocyte-derived contractile factor
PVADRF	perivascular adipocyte derived relaxing factor
PVAT	perivascular adipose tissue
RhoGAP	RhoA GTPase activating proteins
RhoGDI	RhoA guanosine dissociation inhibitor
RhoGEF	RhoA guanosine exchange factor
ROS	reactive oxygen species
SERCA	sarco/endoplasmic reticulum calcium ATPase
sGC	soluble guanylyl cylase
SIRT1	sirtuin 1
SNEDDS	self-nano-emulsifying drug delivery systems
SNP	sodium nitroprusside
SOD	superoxide dismutase
TEA	tetraethylammoniumchloride

Temp	tempol
TCA	trichloroacetic acid
TNF α	tumor necrosis factor alpha
TXA $_2$	thromboxane A $_2$
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VOCs	voltage-dependent calcium channels
VSMCs	vascular smooth muscle cells
XO	xanthine oxidase
ZnPPIX	zinc protoporphyrin IX

Chapter I

Introduction

Chapter I Introduction

I.1 Vascular function

Blood flow to different organs and tissues is continuously adapted according to their metabolic needs. This is ensured by the pumping heart, which creates an appropriate blood pressure at the entrance of the tissues. Subsequently, local blood flow within the tissues is regulated by intrinsic contractile and relaxing factors that originate from the blood vessel itself or from surrounding tissues. Intrinsic factors include myogenic (i.e. changes in transmural pressure), neurogenic and metabolic (i.e. changes in the concentration of local metabolites) mechanisms. Ultimately these mechanisms will regulate the tone of the vascular smooth muscle cells, mainly by interfering with the intracellular Ca^{2+} levels. Impaired regulation of the vascular tone is an important factor in the pathogenesis of vascular diseases such as hypertension, atherosclerosis, erectile dysfunction and others.

I.1.1 Mechanism of vasoconstriction (Fig. I.1)

Several stimuli can evoke contraction of vascular smooth muscle cells. Regardless of the stimulus, smooth muscle cells use cross-bridge cycling between actin and myosin to develop force which is initiated by an increase in intracellular Ca^{2+} concentration.

In the smooth muscle, stimuli can increase intracellular Ca^{2+} concentration and thus elicit contraction through two processes. The first process is called 'electromechanical coupling' and refers to stimuli (for example KCl or cell stretch) that elicit a contraction by directly initiating depolarization of the smooth muscle membrane. This membrane depolarization leads to opening of voltage-dependent Ca^{2+} channels (VOCs), allowing Ca^{2+} to diffuse into the cell down its concentration gradient. The second process by which intracellular Ca^{2+} concentration can be elevated is called 'pharmacomechanical coupling'. Here, instead of evoking contraction by changing the membrane potential, contractions are induced by the binding of an agonist to a membrane receptor. Subsequently, intracellular molecules or second messengers are produced which elicit the release of Ca^{2+} [1, 2].

Vascular contractile tone is mainly regulated by sympathetic neurons that innervate the arterial wall. One of the key pathways modulating vessel tone is the release of norepinephrine (NOR) from these nerve endings. Upon arrival of the action potential at the

terminal axon, the open probability of plasma membrane Ca^{2+} channels increases resulting in elevated intraneuronal Ca^{2+} concentration [3]. This in turn will trigger the release of NOR, which will diffuse across the neuromuscular junction and will bind predominantly to α_1 -adrenergic receptors on the smooth muscle membrane. Binding of the α_1 -receptors, which are coupled to a heterotrimeric G protein, stimulates the activity of phospholipase C (PLC). This enzyme is specific for the membrane phospholipid phosphatidyl-inositol-4,5-bisphosphate (PIP_2) which catalyzes the formation of two potent second messengers: inositol triphosphate (IP_3) and diacylglycerol (DAG). The binding of IP_3 to IP_3 receptors on the sarcoplasmic reticulum stimulates the release of Ca^{2+} into the cytosol. DAG also increase intracellular Ca^{2+} content by activating protein kinase C (PKC), which phosphorylates specific target proteins such as VOCs or other proteins that regulate cross-bridge cycling [1, 2, 4-6]. Besides NOR, other agonists such as endothelins, prostaglandins, neuropeptide Y and angiotensin II can also elicit vascular contraction through the aforementioned PIP_2 pathway when binding to their respective G-protein coupled receptors [4, 7].

Elevation of free intracellular Ca^{2+} does not directly stimulate smooth muscle cell contraction. Instead Ca^{2+} acts indirectly by binding to a cytoplasmatic protein, calmodulin, to form a Ca^{2+} /calmodulin complex (CaM). This complex then activates the myosin light chain kinase (MLCK) to phosphorylate the light chains on the myosin heads. This phosphorylation enables the molecular interaction of myosin with actin. In addition, actin activates myosin's magnesium-dependent ATPase activity. This ATPase cleaves high-energy phosphate bonds in ATP thus providing the energy needed for the actin-myosin cross-bridge cycling that shortens the muscle cell and produces force [1, 2, 4-6].

Initially smooth muscle contraction is thus induced by the elevation of intracellular Ca^{2+} . This results in a phasic (rapid) contraction. However, contractile responses also involve a tonic (slow) contraction phase allowing further force generation and is independent of a rise in intracellular Ca^{2+} levels [2, 5, 6, 8-10]. This pathway, called the Ca^{2+} sensitization mechanism, allows vascular smooth muscle cells to maintain contractile force for a longer period of time at a low cost of energy [2, 5, 6, 8-10]. The Ca^{2+} sensitizing pathway is dependent on the activation of the small G-protein RhoA and its downstream target, Rho kinase. RhoA is a small GTPase that acts as a switch by cycling between an active (guanosine triphosphate (GTP)-bound) and an inactive (guanosine diphosphate (GDP)-bound) conformation. The

activity of RhoA is tightly regulated by three proteins; RhoA guanine exchange factor (RhoGEF), RhoA GTPase activating proteins (RhoGAP) and RhoA guanine dissociation inhibitor (RhoGDI) [8, 10-14]. In resting cells, RhoA-GDP is generally trapped in the cytosol by GDIs, which binds to RhoA-GDP and extracts it from the membrane to the cytosol. Activation of RhoGEF, by binding of various agonists to their G-protein coupled receptors, promotes the exchange of GTP for GDP on RhoA and the dissociation from RhoGDI. The activated RhoA-GTP is then able to translocate from the cytosol to the plasma membrane, where it can initiate signal transduction by interacting with Rho kinase. RhoA is inactivated by RhoGAP, which enhances the intrinsic GTPase activity of RhoA, leading to the hydrolysis of GTP to GDP and subsequent inactivation of RhoA. RhoA then re-associates with RhoGDI and relocates to the cytosol [8, 10-14]. In addition inhibition of RhoA-mediated functions can also occur by phosphorylation of RhoA which increases its association with GDIs [15]. Binding of RhoA-GTP with Rho kinase, a serine/threonine kinase, induces a conformational change leading to activation of Rho kinase towards specific substrates. In smooth muscle cells, Rho kinase induces vasoconstriction through the phosphorylation and subsequent inhibition of myosin light chain phosphatase (MLCP), an enzyme that dephosphorylates the myosin light chains and thus promotes smooth muscle relaxation. Due to the Rho kinase inhibition of MLCP, myosin light chains remain phosphorylated and interaction between actin and myosin is favored [16, 17]. In addition, Rho kinase has been shown to directly phosphorylate the myosin light chains [18], allowing interaction with actin. Taken together, the RhoA-Rho kinase pathway results in MLC phosphorylation to promote smooth muscle contraction independent of changes in intracellular Ca^{2+} concentration or MLCK activity but by increasing Ca^{2+} sensitivity.

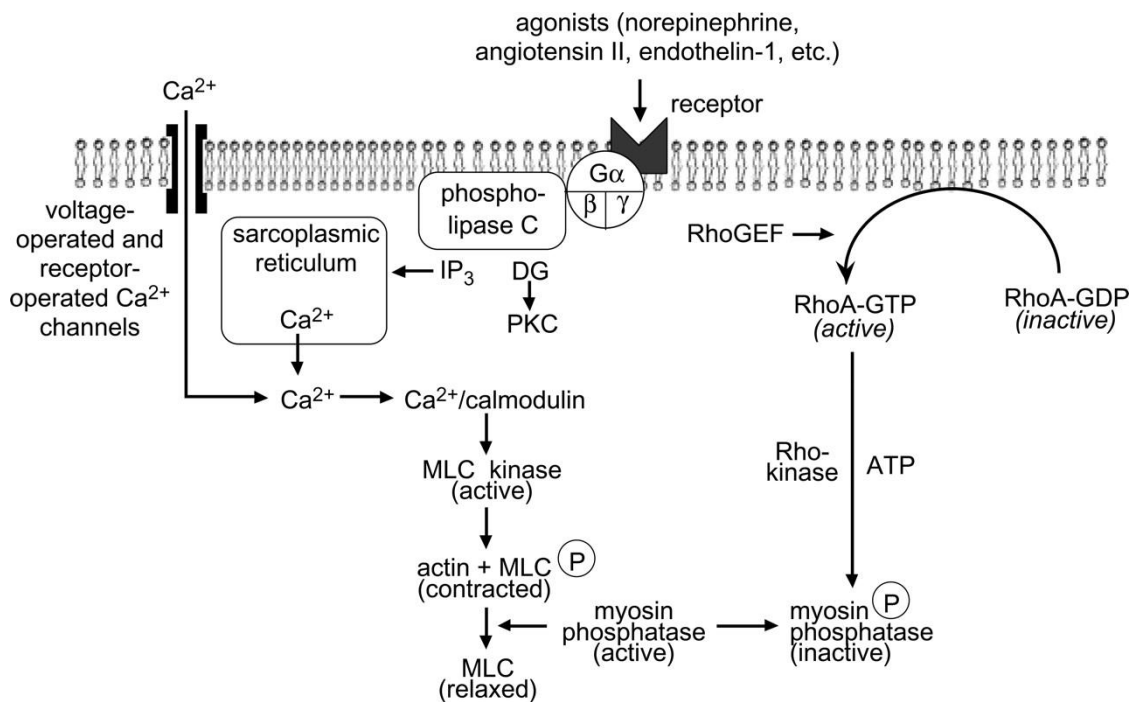


Figure I.1 Regulation of smooth muscle contraction. Various agonists bind to their specific receptors to initiate smooth muscle contraction. Activation of these G protein-coupled receptors increases PLC activity which catalyzes the hydrolysis of PIP_2 to produce two potent second messengers: DAG and IP_3 . IP_3 binds to specific receptors on the sarcoplasmic reticulum, yielding the release of Ca^{2+} . DAG together with Ca^{2+} activates PKC, which phosphorylates different target proteins in order to further sustain the increase in intracellular Ca^{2+} levels. Free Ca^{2+} binds to calmodulin, resulting in the activation of MLC kinase and subsequent phosphorylation of the light chain of myosin. Subsequently cross-bridge cycling between actin and myosin occurs and the shortening of the smooth muscle cell is initiated. As the elevation in Ca^{2+} levels within the cell is transient, contractile response is maintained by a Ca^{2+} sensitizing mechanism. This Ca^{2+} -sensitizing mechanism is initiated at the same time that PLC is activated, and it involves the activation of the small GTP-binding protein RhoA by RhoGEF. Upon activation RhoA enhances Rho kinase activity. This will lead to phosphorylation and inhibition of MLCP, favoring the maintenance of phosphorylated myosin light chain. (Webb et al. [6].)

I.1.2 Mechanism of vasorelaxation (Fig. I.2)

Smooth muscle relaxation occurs when the contractile stimulus is removed or by direct action of a substance that inhibits the contractile mechanism. In all cases, the process of relaxation requires a decrease in intracellular Ca^{2+} concentration and/or increased MLCP activity.

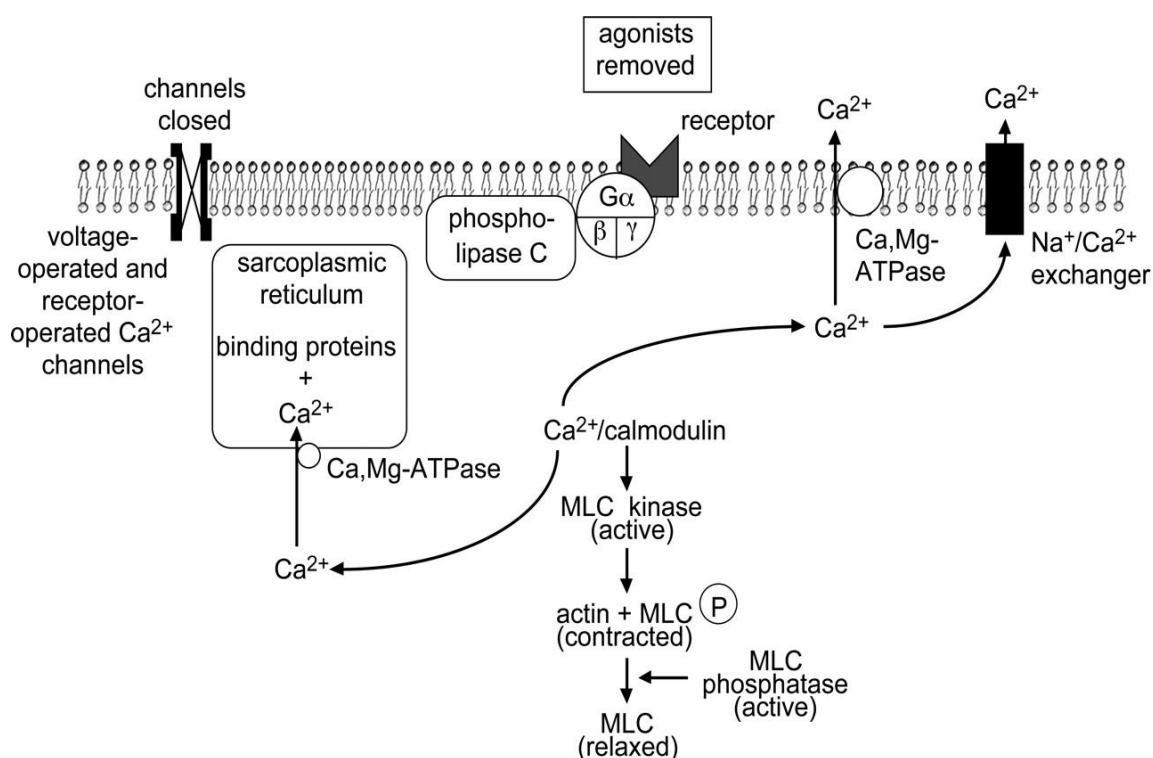


Figure 1.2 Regulation of smooth muscle relaxation. Removal of the contractile stimulus or direct action of a substance stimulates inhibition of the contractile mechanism elicit vasorelaxation. A decrease in intracellular Ca^{2+} levels and/or increased MLCP activity is needed to initiate the relaxation process. Ca, Mg-ATPase in the sarcoplasmic reticulum and the plasma membrane and $\text{Na}^+/\text{Ca}^{2+}$ exchangers in the plasma membrane all reduce intracellular Ca^{2+} levels by removing Ca^{2+} from the cytosol. Receptor- and voltage-operated Ca^{2+} channels in plasma membrane close during relaxation, leading to a decreased Ca^{2+} entry into the cell. (Webb et al.[6])

There are several ways to lower intracellular Ca^{2+} concentrations and thus elicit vasorelaxation. The first way is removal of the stimulus that is responsible for the increased intracellular Ca^{2+} levels. For example, if the stimulus for contraction is an agonist that binds to its specific membrane receptor, such as NOR, then dissociation of the agonist will end the production of second messengers such as IP_3 and DAG that led to increased intracellular Ca^{2+} levels [1, 2, 4, 6]. If contraction is initiated by an agonist that induces membrane depolarization, such as elevated extracellular K^+ levels, then removal of this agonist will result in membrane repolarization. As this repolarization is accompanied with the closure of VOCs, influx of extracellular Ca^{2+} will stop. Similarly hyperpolarization of the smooth muscle membrane, due to activation of K^+ channels and subsequent K^+ efflux, results in the closure

of VOCs and thus reduced Ca^{2+} influx. As a consequence both repolarization and hyperpolarization of the smooth muscle membrane are associated with vascular smooth muscle relaxation [2].

Another way to lower intracellular Ca^{2+} concentrations involves activation of ATP-dependent Ca^{2+} pumps which are located in the membrane of sarcoplasmic reticulum as well as in the plasma membrane. In both cases, the pump has an ATPase activity providing the energy needed for moving Ca^{2+} against a concentration gradient. When phosphorylated the Ca,Mg-ATPase in the sarcoplasmic reticulum (SERCA), binds two Ca^{2+} ions, which are then translocated to the luminal side of the sarcoplasmic reticulum where they are stored until they are released again. Mg^{2+} is required for the activity of the ATPases as it binds to the catalytic site of ATPase to mediate the reaction. This Ca^{2+} uptake into the sarcoplasmic reticulum is reversibly inhibited by phospholamban. Phosphorylation of phospholamban relieves its inhibitory effect, therefore increasing SERCA activity and the rate of Ca^{2+} uptake [2, 4, 9]. The plasma membrane also contains Ca,Mg-ATPases (PMCA) which provide an additional way to reduce the intracellular Ca^{2+} levels as it will transport Ca^{2+} out of the cell. This Ca,Mg-ATPase differs from the ATPase in the sarcoplasmic reticulum in that it has an autoinhibitory domain that can be bound by calmodulin, causing stimulation of the plasma membrane Ca^{2+} pump. In addition, $\text{Na}^+/\text{Ca}^{2+}$ exchangers (NCX) are also located on the plasma membrane. This exchanger transports one Ca^{2+} ion out of the cell in exchange for two sodium ions brought into the cell [1, 2, 4, 6].

A third way by which smooth muscle relaxation can occur is by elevation of levels of the second messengers, cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) within the smooth muscle cell. cAMP is synthesized from intracellular ATP by adenylyl cyclase (AC), which can be activated by several agonists that bind to their G-protein-coupled receptor. cGMP may be synthesized in the vascular smooth muscle cells from GTP by a particulate guanylyl cyclase (pGC), located at the plasma membrane and activated by natriuretic peptides, or by soluble guanylyl cyclase (sGC), located in the cytosol and mainly activated by NO and NO donors [1, 19] (Fig. I.3). Upon formation, cAMP activates cAMP-dependent protein kinase (PKA), while cGMP activates cGMP-dependent protein kinase (PKG). However each cyclic nucleotide can activate both protein kinases. For instance, cAMP can also activate PKG, but it requires nearly a 10-fold

higher cAMP concentration compared to the cGMP levels that activate this kinase [1, 2, 19]. The signals evoked by cAMP and cGMP are mainly ceased and controlled by phosphodiesterases (PDEs). These enzymes catalyze the hydrolysis of cAMP and cGMP to their inactive metabolites adenosine monophosphate (AMP) and guanosine monophosphate (GMP) respectively [1, 19].

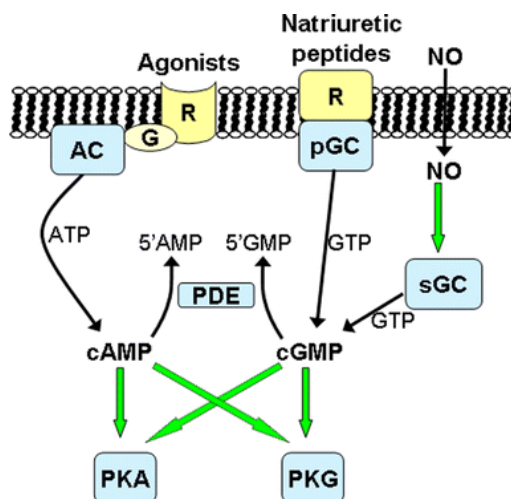


Figure I.3 Regulation of levels of cyclic nucleotides and activation of kinases. Green arrows indicate stimulation. (Morgado et al. [1])

Both PKA and PKG will phosphorylate numbers of targets which all lead to a decrease in intracellular Ca^{2+} concentrations and thus smooth muscle relaxation (Fig. I.4). Several targets for PKA and PKG have been suggested, including K^+ channels, different types of Ca^{2+} channels (such as those located on the sarcoplasmic reticulum and VOCs), SERCA, PMCA, NCX, phospholamban, IP_3 , RhoA and many others. The net result of all of these PKA- and PKG-mediated effects is a decrease of intracellular Ca^{2+} concentration, leading to a decrease in MLCK activity and/or increase in the MLCP activity through inhibition of RhoA. Subsequently dephosphorylation of the myosin light chains is favored and thus smooth muscle relaxation occurs [1, 4, 19].

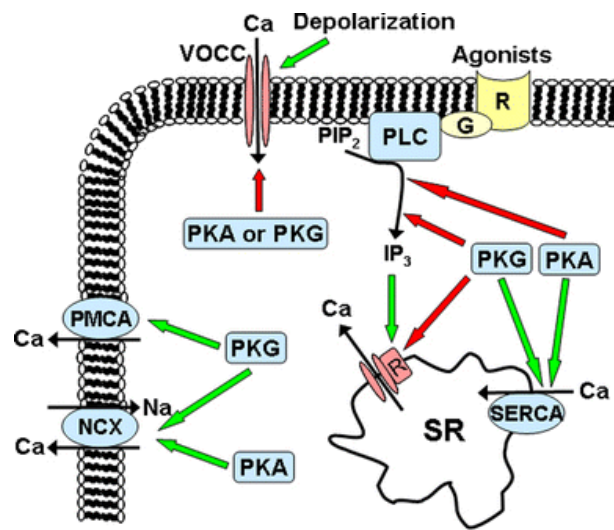


Figure I.4 Mechanisms involved in the decrease of intracellular Ca^{2+} concentrations induced by cyclic nucleotide-dependent protein kinases. Green arrows indicate stimulation, red arrows indicate inhibition. (Morgado et al. [1])

Nitric oxide

Although many endogenous vasodilators have been shown to exert relaxant effects on different vascular beds, nitric oxide (NO) is believed to be the principal mediator of vascular relaxation. NO is a gaseous messenger molecule with a short life-time, that is enzymatically formed together with L-citrulline from L-arginine, molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) by NO synthases (NOS) [20, 21]. Constitutive NOS enzymes are present in endothelial cells (eNOS) and in non-adrenergic non-cholinergic (NANC) neurons (nNOS). In addition, an inducible NOS (iNOS) isoform exists, which is up-regulated in response to various stimuli [21]. eNOS and nNOS are activated in response to receptor stimulation (e.g. acetylcholine) causing intracellular Ca^{2+} increase and subsequent formation of CaM complexes, which will activate eNOS or nNOS. Subsequent increase in blood flow causes shear stress, which further stimulates eNOS through phosphorylation at the serine 1177 residue, resulting in sustained activation of eNOS [20, 21]. Upon release, from neurons and endothelial cells, NO diffuses to the smooth muscle cell and activates sGC, resulting in an increase of cGMP concentration and thus smooth muscle relaxation. Apart from NO, other vasodilators such as vasoactive intestinal peptide, prostaglandin E_1 , prostaglandin I_2 (prostacyclin), substance P, calcitonin gene related

peptide, H₂S have been shown to influence smooth muscle tone [4, 22-27]. However their contribution in vessel tone regulation remains rather vague.

I.2 Paracrine modulation of vascular tone

Tissues or cells surrounding arteries can influence the contractile degree of the vascular smooth muscle cells through the release of vasoactive substances. These factors that are released by neighboring cells are often called 'paracrine modulators'. The term 'paracrine' refers to a kind of hormone function in which the effects of the hormone are restricted to the local environment. Nowadays, besides the nervous system, the vascular endothelium is a well-known paracrine regulator of vascular smooth muscle tone. More recently it was discovered that also the perivascular adipose tissue (PVAT) functions as a paracrine organ in the regulation of vascular tone.

I.2.1 Endothelium

In the past the endothelium was believed to be just a simple semipermeable membrane lining the luminal surface of all blood vessels. However, from the 1980s it has become clear that the vascular endothelium is a fundamental regulator for the homeostasis of the underlying layer of smooth muscle cells by controlling the balance between vasoconstriction and vasorelaxation [28]. This is accomplished through the release of several relaxing and contracting factors under basal conditions and in response to many substances (drugs, circulating hormones and cytokines) as well as to physical and chemical stimuli (e.g. changes in pressure, shear stress and pH) [29]. Endothelium-derived factors with vasodilatory effects, collectively called 'endothelium derived relaxing factors (EDRFs)', include NO, prostacyclin and endothelium-derived hyperpolarizing factors (EDHF). These latter refer to molecules causing endothelium-dependent relaxations that are resistant to NOS and cyclooxygenase (COX) inhibitors and that result in endothelium-dependent hyperpolarization of the vascular smooth muscle cells [30, 31]. Hyperpolarization of the vascular smooth muscle cells leads to a decrease in intracellular Ca²⁺ concentration and in this way EDHF can promote vasorelaxation. The contribution of EDHF to endothelium-dependent relaxations appears to be inversely related to vessel diameter since EDHF seems to play a more prominent role in smaller (resistance) arteries compared to larger (conduit) arteries [32]. From its discovery in the late 1980s it has become clear that EDHF is likely not a single diffusible 'factor'.

Numerous identified putative EDHFs have been reported and the acronym 'EDHF' has become an overall term applied to the various endothelium-derived mechanisms that mediate hyperpolarization and relaxation of the vascular smooth muscle cells [30, 31, 33]. Of the most frequently reported mechanisms underlying EDHF activity are that of K^+ -efflux from the endothelial cells, generation of diffusible epoxyeicosatrienoic acids, hydrogen peroxide and electrical coupling between endothelial cells and vascular smooth muscle cells through myoendothelial gap junctions [30, 31, 33].

Endothelin-1 (ET-1), angiotensin II (AII), thromboxane A_2 (TXA₂) and reactive oxygen species (ROS) are among the factors that exert vasoconstrictor effects and are collectively known as 'endothelium-derived contracting factors (EDCFs)' [7, 29, 31, 33] (Fig. 1.5).

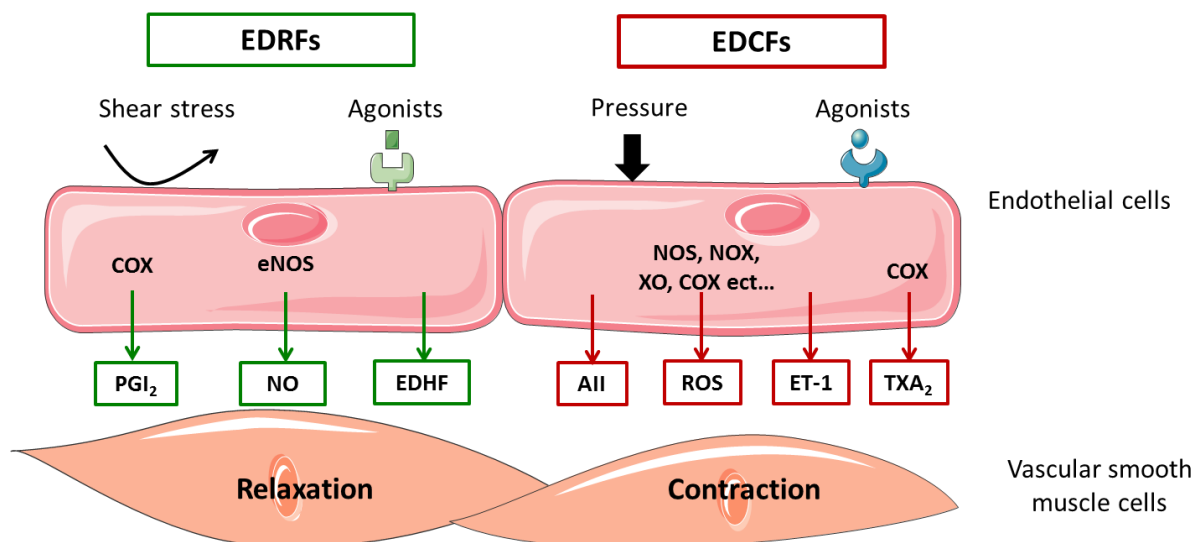


Figure 1.5 Endothelial cells release various vasoactive factors and therefore maintain the balance between vasodilation and vasoconstriction.

Endothelial dysfunction and oxidative stress

Normal endothelial function regulates the balance between the release of EDRFs and EDCFs which is vital for maintaining vascular smooth muscle relaxation, blood pressure and blood flow. Disturbance of this tightly regulated balance leads to endothelial dysfunction [29, 34]. There is considerable evidence showing that endothelial dysfunction is an early event in the development of cardiovascular diseases such as diabetes, hypertension, hypercholesterolemia, atherosclerosis and heart failure [35-38]. Although the pathogenesis

of endothelial dysfunction is multifactorial, the hallmark seems to be impaired NO bioavailability which is functionally reflected in decreased endothelium-dependent relaxation [39, 40]. Impaired NO bioavailability can be the result of either decreased NO production or accelerated NO degradation. However in both cases oxidative stress seems to play a pivotal causative role, limiting NO to exert its effect [29]. Oxidative stress refers to a condition in which cells are exposed to excessive amounts of chemical oxygen derivatives, the so called ROS [41, 42]. ROS include free radicals such as superoxide anion ($O_2^{\bullet-}$), peroxynitrite ($ONOO^{\bullet-}$), and hydroxyl (OH^{\bullet}), and non-radicals such as hydrogen peroxide (H_2O_2). $O_2^{\bullet-}$ is considered as the 'primary' ROS and is formed after addition of one electron to molecular oxygen (dioxygen) [43]. Within mammalian cells, several enzyme systems are capable of transferring electrons to molecular oxygen producing $O_2^{\bullet-}$, the four most important being nicotinamide-adenine-dinucleotide phosphate oxidases (NOX), the mitochondrial electron transport chain, xanthine oxidase (XO) and uncoupled NOS [41]. $O_2^{\bullet-}$ can further interact with other molecules to generate 'secondary' ROS such as H_2O_2 and $ONOO^{\bullet-}$. $O_2^{\bullet-}$ is rapidly converted into H_2O_2 either spontaneously or via the catalytic intervention of superoxide dismutase (SOD). OH^{\bullet} is generated from H_2O_2 in the presence of ferrous iron (Fe^{2+}) by the Fenton reaction, but is also formed by the interaction between $O_2^{\bullet-}$ and H_2O_2 [41, 42] (Fig. I.6).

Oxidative stress contributes markedly to endothelial dysfunction, primarily due to rapid oxidative inactivation of NO by excess $O_2^{\bullet-}$ resulting in the highly reactive $ONOO^{\bullet-}$ and thereby reducing NO bioavailability. In a second step, persisting oxidative stress renders eNOS dysfunctional (eNOS uncoupling), a process whereby eNOS switches from a NO-producing enzyme to a $O_2^{\bullet-}$ -generating molecule [44, 45]. Mechanistically, the major cause for eNOS uncoupling and endothelial dysfunction could be found in the oxidation of tetrahydrobiopterin (BH_4 , a critical eNOS cofactor) by $ONOO^{\bullet-}$ or $O_2^{\bullet-}$ which makes BH_4 unavailable for eNOS generation of NO [20, 21, 41, 45, 46]. In addition depletion of the enzyme substrate L-arginine can also be implicated [20, 21, 41]. Thus uncoupled eNOS not only reduces NO production, but also potentiates the pre-existence of oxidative stress and endothelial dysfunction.

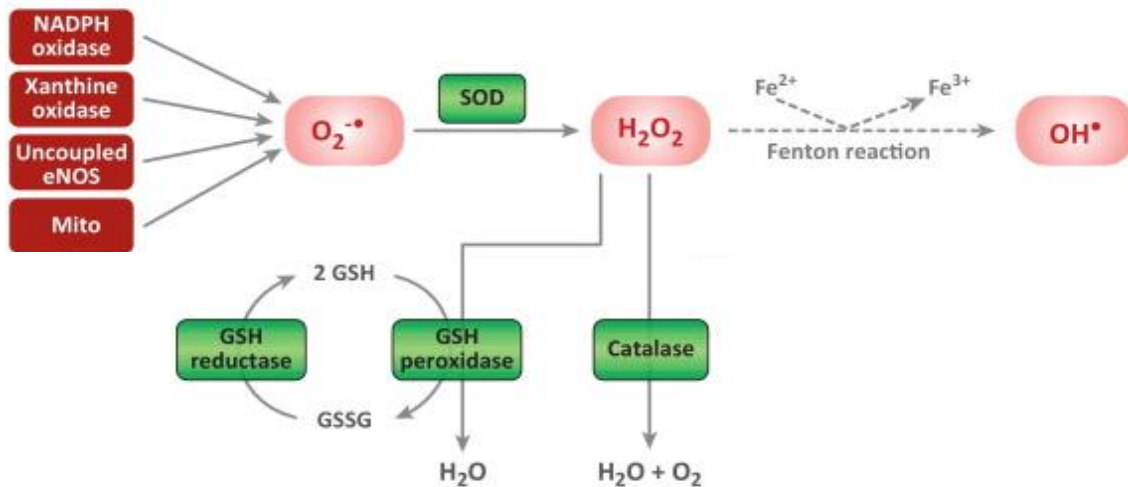


Figure I.6 Enzymes involved in the generation and inactivation of reactive oxygen species (ROS). The superoxide anion ($O_2^{\bullet-}$) can be produced by NADPH oxidase (NOX), xanthine oxidase (XO), uncoupled endothelial nitric oxide synthase (eNOS) and the leakage of activated oxygen (O_2) from mitochondria during respiration. $O_2^{\bullet-}$ can be converted to hydrogen peroxide H_2O_2 by the enzyme superoxide dismutase (SOD). H_2O_2 can undergo spontaneous conversion to the hydroxyl radical (OH^{\bullet}) via the Fenton reaction. H_2O_2 can be detoxified by glutathione (GSH) peroxidase and catalase to H_2O and O_2 . (Adapted from Li et al. [41])

1.2.2 Perivascular adipose tissue

Historically adipose tissue was thought to be simply lipid-rich connective tissue [47]. Similarly, perivascular adipose tissue (PVAT), which is the adipose tissue surrounding nearly all blood vessels, was long considered as a mechanical support and protection to the vessels during contraction of neighboring tissues [48]. Therefore PVAT was routinely removed for isolated blood vessel studies. Soltis and Cassis [49] demonstrated for the first time that PVAT decreases NOR-induced contractions of rat aorta. From then on, it became clear that besides structural support, PVAT also act as an active endocrine organ that releases various substances, called adipo(cyto)kines, which affect the underlying vascular cells[50-53]. Among the plethora of adipo(cyto)kines are pro- and anti-inflammatory cytokines and an adipocyte-derived releasing factor (ADRF), which can influence vascular tone in a paracrine way. These are discussed in a review in **chapter III** (published in *Curr Hypertens Rep* 2012;14:270-278). Under physiological conditions, the release of these vasoactive adipo(cyto)kines results in a net beneficial anticontractile effect on vascular function and this is essential for the maintenance of vascular resistance [49, 54-57]. The anticontractile effect of PVAT is directly dependent on its amount [54, 57, 58]. Therefore it would be conceivable to assume that in

obesity, where the amount of PVAT is increased throughout the vasculature, the anticontractile effect of PVAT is increased. However, the opposite occurs and the anticontractile effect of PVAT is lost in obesity [54, 59-61] (Fig. I.7). Various explanations have been proposed for this surprisingly finding, mostly focusing on the fact that, besides structural changes, functional changes in PVAT are triggered in obesity, which render the adipose tissue dysfunctional. These functional changes imply a dysregulated synthesis of vasoactive adipo(cyto)kines by the adipose tissue in favor of harmful vasoconstrictor and pro-inflammatory substances [62]. Due to this imbalanced pro-inflammatory adipo(cyto)kine production, obesity is characterized as a state of chronic low-grade inflammation [63]. It has been proposed that hypoxia underlies this inflammatory response, as hypoxia occurs in areas of fat depots when the vascular oxygen supply is compromised due to tissue mass expansion [64]. Interestingly, adipose tissue dysfunction is not restricted to obesity, where PVAT mass and adipocyte size increase. In several hypertensive animals models, where PVAT mass and adipocyte size decreases, a similar ‘obesity-like’ impairment of PVAT function was reported [58, 65, 66] (Fig. I.7).

Taken together, in addition to endothelial dysfunction, adipose tissue dysfunction might create an environment for the development of vascular inflammation and dysfunction [53, 67-69].

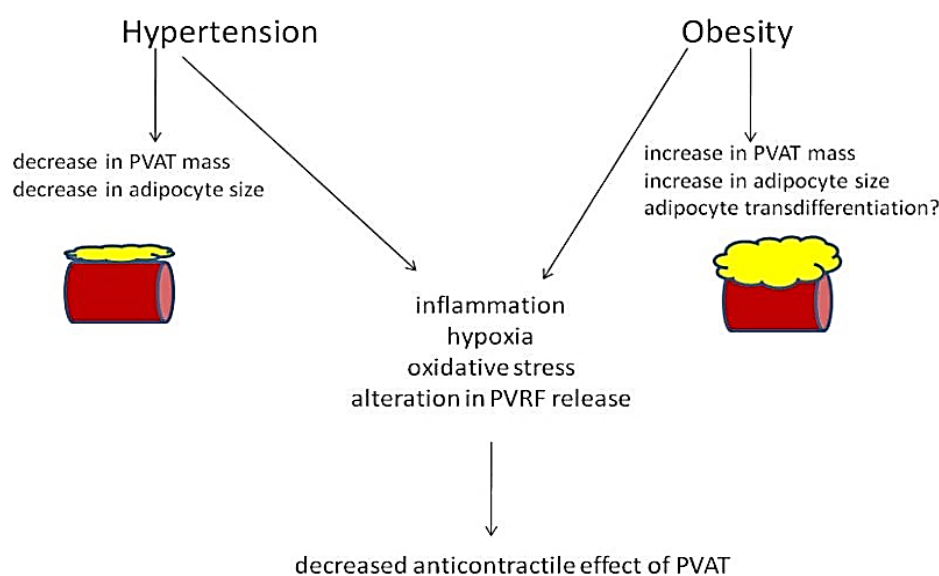


Figure I.7 Alterations of PVAT structure and function during obesity and hypertension lead to decreased anticontractile effects of PVAT. (Adapted from [48]).

I.3 Erectile function

I.3.1 Anatomy of the penis (Fig. I.8)

The penis in man and in most other mammalian species consists of three corpora: dorsally paired corpora cavernosa, which comprise the erectile tissue and the ventral corpus spongiosum that surrounds the urethra and expands distally to form the glans penis. A thick fibrous sheath, the tunica albuginea, surrounds each of the corpora cavernosa and fuses in the midline to form a perforated septum.

The corpora cavernosa resemble a sponge as it is composed of a meshwork of interconnected hollow cavernous spaces or sinusoids, which are lined with endothelial cells and are separated by trabeculae. The trabeculae contain bundles of smooth muscle cells in a framework of elastic fibers and connective tissue. The arterial blood supply is ensured by the resistance helicine arteries which branch from the deep penile cavernosal artery. Subtunical veins drain the sinusoidal spaces and pierce the tunica to drain into the circumflex veins, and then join the deep dorsal vein of the penis. The cavernosal arteries and the trabecular smooth muscles are under control of sympathetic and parasympathetic innervation [70, 71].

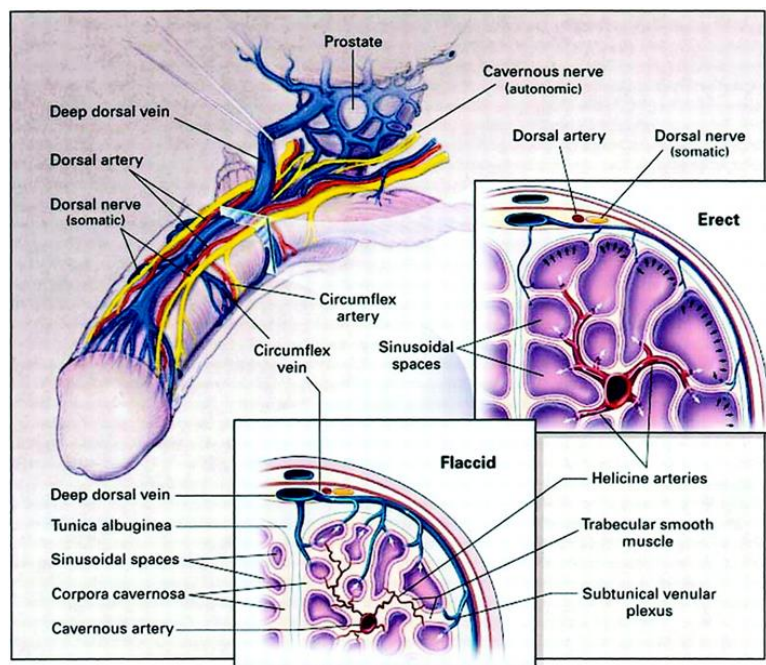


Figure I.8 Anatomy and hemodynamics of the corpora cavernosa. The figure is adapted from Kandeel et al. [71].

I.3.2 Regulation of penile erection

Penile erection is basically a spinal reflex that involves central nervous processing and integration of tactile, olfactory, auditory, and mental stimuli [72]. The generated nervous signals will influence the balance between the contracting and relaxing factors, which determine the contractile tone of the cavernosal and arterial smooth muscle cells and thus the functional state of the penis. As with vascular smooth muscle contraction and relaxation, penile smooth muscle tone is determined by the levels of intracellular free Ca^{2+} as well as by the Rho kinase pathway.

Under basal conditions, NOR is released from adrenergic neurons and binds to α_1 adrenoceptors on the smooth muscle membrane eliciting contraction of arterial and cavernosal smooth muscle. In addition, the RhoA/RhoA kinase signaling pathway has been suggested to be involved in the maintenance of contraction of the cavernosal smooth muscle cells [70, 73, 74]. Subsequently only a small blood volume is delivered to the sinuses and this volume is readily drained out through subtunical veins. As a consequence intracavernosal pressure remains low and the penis maintains a flaccid state.

Nowadays, NO is accepted as the key mediator of penile erection [75]. Upon sexual stimulation, NO is released directly from the parasympathetic NANC neurons which innervate the penis (Fig. I.9). The subsequent increase in blood flow-induced shear stress and the release of acetylcholine from the parasympathetic cholinergic nerve endings will activate eNOS and stimulate NO release from the arteriolar and sinusoidal endothelial cells [75-77] (Fig. I.9). Neurogenic-derived NO is believed to be responsible for the initiation and the majority of the smooth muscle relaxation, while NO originating from endothelial cells contributes to the maintenance of the erection [77, 78]. Like in vascular relaxation, NO release will result in relaxation of the arterial and cavernosal smooth muscle cells through the activation of the sGC/cGMP pathway. As a consequence increased arterial blood flow to and the expansion of the cavernosal sinusoids is allowed [70, 75, 76, 78, 79]. As the corpora cavernosa are surrounded by the tunica albuginea, which has a limiting stretching capability, the expansion of the cavernosal sinusoids will compress subtunical veins, causing a decreased venous outflow (veno-occlusion). The increased inflow and decreased outflow results in the high intracavernosal pressures which are characteristics of penile erection [70,

79]. Degradation of cGMP by PDE 5, the predominantly form present in penile tissue, is primarily responsible for detumescence and returning the penis to the flaccid state [71, 74, 80].

Although NO/cGMP is considered the most important agent responsible for penile erection, other regulatory mechanisms such as VIP/cAMP may also be involved [70, 75, 76, 78, 79].

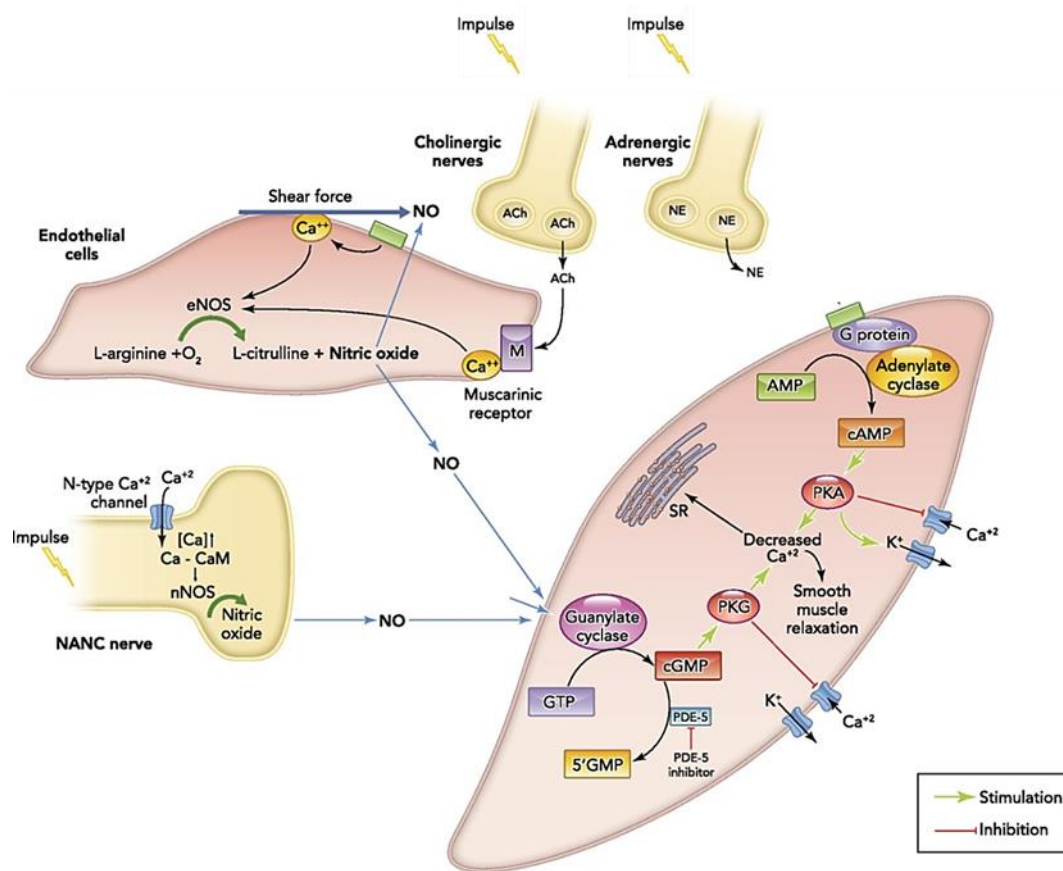


Figure 1.9 Regulation of cavernosal smooth muscle relaxation by NO released from the non-adrenergic non-cholinergic (NANC) nerves and sinusoidal endothelium. Stimulation of the NANC nerves and the endothelial cells in the penis cause Ca^{2+} influx which promotes the production of NO. Binding of NO on sGC in the cavernous smooth muscle catalyzes the conversion of GTP to cGMP. Due to activation of PKG and PKA, increased levels of cGMP and cAMP induce relaxation of the arterial and cavernosal smooth muscle cells by decreasing intracellular levels of Ca^{2+} , which eventually results in the expansion of the cavernosal sinusoids and thus in penile erection (adapted from [81]).

I.3.3 Erectile dysfunction

Normal erectile function is a complex neurovascular process which depends on a tightly regulated balance between vasoconstrictor agents, causing limited blood inflow, and vasodilators, allowing increased blood inflow and erection. Hence, alterations favoring contractile responses and/or impeding relaxant responses of the cavernosal smooth muscle may lead to erectile dysfunction (ED). ED is generally defined as the inability to achieve and maintain an erection sufficient to permit satisfactory sexual intercourse [82]. It is estimated that ED affects approximately 20 % of adult males over the age of 20 [83], and by 2025 it is thought that ED will affect 332 million men worldwide [84]. Although ED is not a life threatening dysfunction, it seriously affects man's quality of life as well as interpersonal well-being.

The cause of ED can be psychological or organic (vascular, neurologic, hormonal, structural or drug-induced) or mixed psychological and organic. Vascular disease is however by far the most common cause of ED [72, 85]. Several conditions such as type II diabetes and hypertension as well as lifestyle factors such as tobacco use, obesity, frequency of exercise are highly associated with ED [86, 87]. The common characteristic of many of these vascular risk factors is the presence of (oxidative stress-induced) endothelial dysfunction [88, 89]. As the endothelium is a major source of NO, the main mediator of penile erection, impaired NO release and subsequent decreased corporal smooth muscle relaxation inevitably results in ED [85, 90]. However, besides NO several other mediators might also be involved in endothelial and erectile dysfunction. For instance, changes in prostaglandin synthesis and signaling may contribute to imbalanced penile eicosanoid homeostasis which can result in contraction of penile arteries and corporal smooth muscles. In addition, elevated levels of All or ET-1 can further promote (vaso)constriction and thus ED [90].

I.4 Polyphenols

Hippocrates, the father of modern medicine, said many centuries ago: “let food be thy medicine”. Today this ancient quote still shows its value as the popular thought “you are what you eat” is nowadays a well-established fact. Indeed it is known that diet is one of the most important lifestyle risk factors and that it can strongly influence the incidence of cardiovascular disease [91, 92]. In search for understanding how increased intake of certain foods can lead to a better health, polyphenols have gained a lot of interest. Polyphenols are naturally occurring compounds found in diverse plant-derived foods that are synthesized as secondary metabolites as defensive responses against stress due to UV radiation, pathogens and physical damage [93, 94]. According to the number of phenolic rings and on the basis of the structural elements that bind these rings to one another, polyphenols are classified into four categories: phenolic acids, flavonoids, stilbenes and lignans (Table I.1). Flavonoids are further classified into six subclasses: flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols [93].

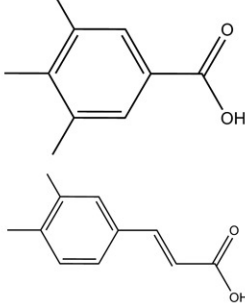
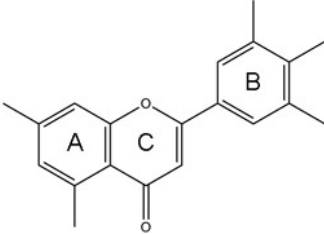
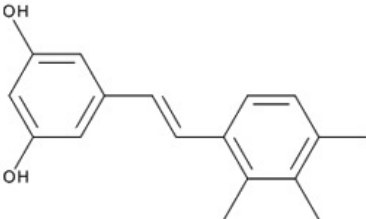
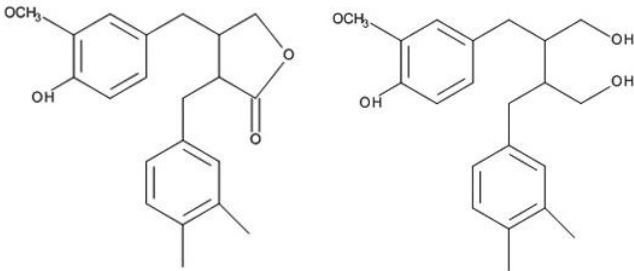
Classes of polyphenols	Chemical structure
Phenolic acids (C6-C1 and C6-C3)	
Flavonoids (C6-C3-C6)	
Stilbenes (C6-C2-C6)	
Lignans (C6-C3-C3-C6)	

Table I.1. Chemical structures of the main classes of polyphenols

The diversity of the chemical structures of dietary polyphenols makes it difficult to estimate their total content in foods. A typical diet rich in fruits, vegetables and plant beverages has been estimated to provide about 1 g of polyphenols/day, with significant variations depending on the amount of consumption of drinks rich in polyphenols [95]. Fruits like grapes, apple, pear, cherries and berries contain up to 200-300 mg polyphenols per 100 grams fresh weight [96]. The manufactured products of these fruits, also contain polyphenols in significant amounts. Typically a glass of red wine contains about 100 mg polyphenols [94].

From some decades, these polyphenols have been the focal point of extensive research on their capacity to improve health. This research includes a wide variety of clinical and nutritional epidemiological studies that indicate that for instance populations who consume a diet rich in polyphenols are less susceptible to cardiovascular diseases and their complications [91, 97].

1.4.1 Resveratrol and quercetin

In 1992 the concept of the 'French paradox' was first introduced based on the observation that the French population had the lowest mortality and a low risk of cardiovascular disease despite a high consumption of saturated fat and prevalence of other risk factors [97]. Moderate red wine consumption was thought to explain this paradoxical finding [97]. Moreover, the polyphenolic content of red wine has been assumed to be responsible for cardiovascular benefits associated with moderate red wine consumption. The polyphenols present in red wine vary extremely due to differences in variety and grape sources as well as due to the grape processing. Red wine contains a higher concentration of polyphenols than white wine, because red wine is matured with the skin and seeds during the wine-making process and these contain most of the polyphenols [98-100].

The main polyphenols present in red wine are resveratrol (a stilbene) and to a lower extent quercetin (a flavonol) [100-103] (Fig. 1.10). Concentrations of resveratrol in red wine vary depending upon grape variety and geographic cultivation sites but concentrations up to 9 mg/L have been found, making red wine the major source of resveratrol in human diet [100, 101, 103]. Besides the skin of grapes, resveratrol and quercetin are found in high abundance

in many fruits and vegetables that are components of the human diet such as mulberries, cranberries, blueberries, onions and peanuts [93, 102]. As a consequence daily intake of quercetin is in the range of <5 mg to about 40 mg quercetin/day [104-107]. However daily amounts of quercetin as 200-500 mg may be reached by high-end consumers of fruits and vegetables, notably when individuals ingest the peel of quercetin-rich fruits and vegetables such as apples and onions [108]. For resveratrol, daily intake, including safe wine drinking, is estimated between 6 and 8 mg resveratrol/day [109].

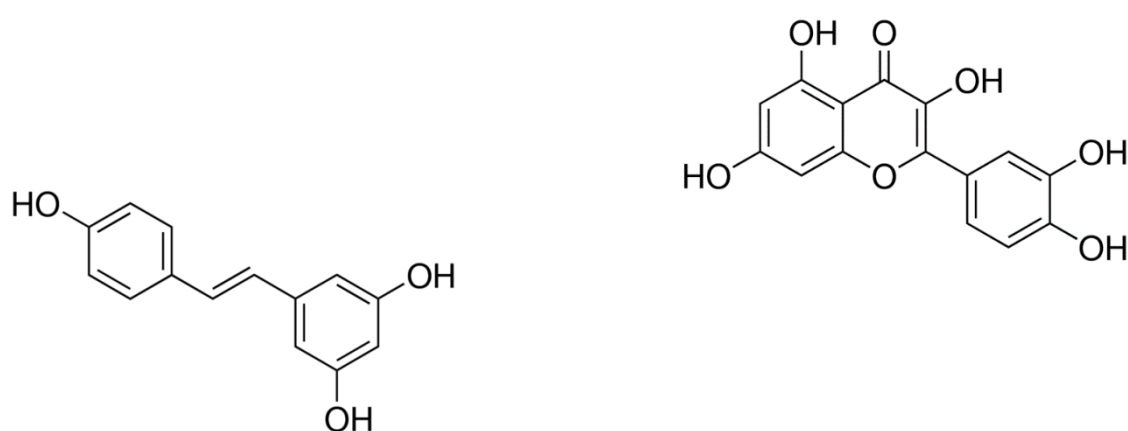


Figure I.10 Chemical structure of resveratrol (left) and quercetin (right)

Both polyphenols have gained a lot of attention for their plethora of beneficial effects against cardiovascular disease, neurodegenerative disease, obesity, diabetes and cancer [110-114]. The beneficial effects of resveratrol and quercetin on the cardiovascular system have been attributed to their ability to improve endothelial function as well as their ability to reduce vascular oxidative stress and their anti-inflammatory properties [110-112, 114-116] (Fig. I.11).

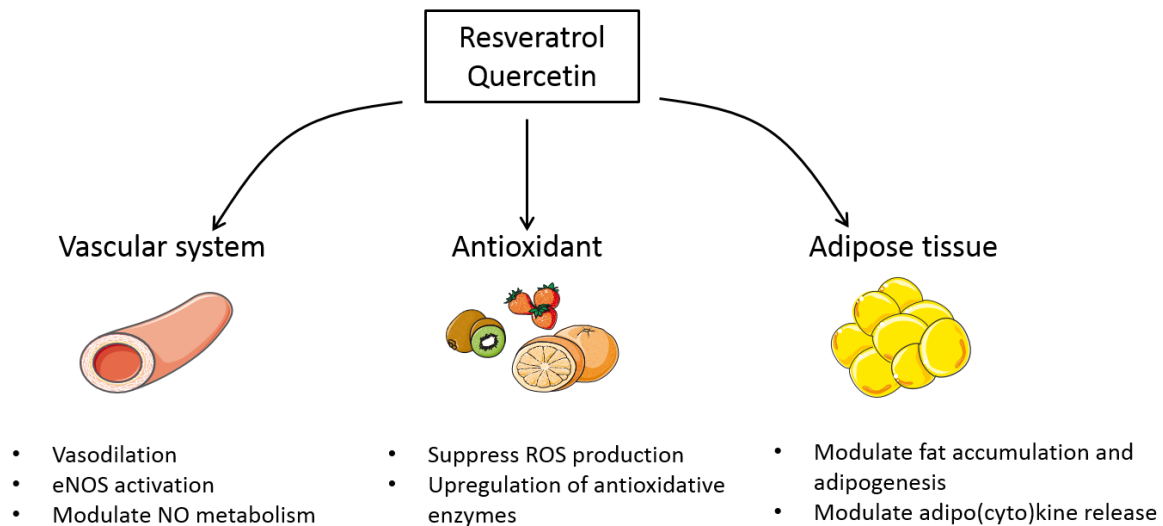


Figure I.11 Potential mechanism(s) in the cardiovascular effects of resveratrol and quercetin.

Vascular activities

Hypertension is one of the major risk factors of cardiovascular disease, which is associated with an increased risk of stroke, myocardial infarction, heart failure, etc. [117]. Adequate management of blood pressure helps to reduce the risk of cardiovascular disease. Several in vitro and in vivo studies in different animals suggested that resveratrol or quercetin may provide an alternative therapeutic option for managing blood pressure as both polyphenols have been demonstrated to exert acute and chronic effects on the cardiovascular system. First of all, resveratrol and quercetin acutely cause endothelium-dependent relaxations in different vascular beds [118-125]. It should however be noted that a part of the relaxant effect of resveratrol and quercetin occurs independently of the endothelium. Activation of different types of K^+ channels may account for this endothelium-independent relaxing effect [119, 122, 123, 125].

Besides a direct relaxant effect, resveratrol ameliorates impaired endothelial function of aortic rings in different disease states. For instance improved vascular responses to acetylcholine were demonstrated after acute or chronic resveratrol administration in hypertensive rats [126-129] or in hypercholesterolemic rabbits [130]. Similarly acute or chronic quercetin treatment enhances endothelial-dependent responses to acetylcholine in various models of experimental hypertension [131-136]. Moreover, it was found that chronic treatment with resveratrol or quercetin lowers blood pressure in hypertensive animal models [126, 127, 133-137]. The improved endothelial function and the direct relaxant effect

of both polyphenols is largely attributed to enhanced NO production as treatment of isolated arteries with eNOS inhibitors decreases the relaxing influence of resveratrol and quercetin. These studies thus indicate that activation of the NO-pathway seems to be involved in their relaxing effect [118, 120, 121, 124].

Resveratrol and quercetin are known to enhance NO bioactivity by acutely increasing enzymatic activity of eNOS. For instance both polyphenols have been found to stimulate eNOS phosphorylation in endothelial cells and thereby increasing eNOS activity [120, 138, 139]. In addition to their acute effect on eNOS activity, which occurs within minutes, resveratrol and quercetin can also enhance eNOS expression (within hours) [136, 140]. At physiological relevant concentrations of 0.1 μ M resveratrol and quercetin increase eNOS and mRNA expression in cultured endothelial cells [140-143]. It is thought that the effects of resveratrol on eNOS expression may be attributed to activation of protein deacetylase sirtuin 1 (SIRT 1). SIRT 1 activation results in deacetylation of eNOS at lysine residues, thereby stimulating eNOS activity [144]. It was demonstrated that pretreating human umbilical vein endothelial cells with resveratrol significantly reduced oxidant-induced decrease in SIRT 1 levels and eNOS acetylation. As a consequence NO production and endothelial function was enhanced [145]. Furthermore knock down of endogenous SIRT 1 by RNAi significantly limited resveratrol-induced increases in eNOS mRNA and protein expression [146] and reduced resveratrol-induced increases in NO levels [144].

Besides activation of SIRT1, activation of estrogen receptors (ER) has been suggested to be involved in the vascular effects of resveratrol as it is structurally similar to a synthetic estrogen, diethylstilbestrol. Indeed, polyphenol extracts induced endothelium-dependent relaxations in wild type but not in ER alpha knockout mice, providing evidence that the vasorelaxant properties of polyphenols are exerted through ER alpha activation [147]. Moreover it has been shown that resveratrol is an agonist for ER alpha [148] and that wine polyphenols, including resveratrol are responsible for eNOS activation by acting on ERs [149]. Furthermore, it has been demonstrated that depletion of ER alpha by siRNA attenuated resveratrol eNOS phosphorylation [138].

Taken together, the ability of resveratrol and quercetin to improve NO production and thus endothelial function may contribute to a great extent to their protective effects in the cardiovascular system.

Anti-oxidant effects

Living organisms have developed several effective mechanisms to protect themselves from ROS-induced damage [41]. These antioxidant defense systems include enzymes such as superoxide dismutase, catalase and glutathione peroxidase but also non-enzymatic reactions with glutathione, ascorbic acid and alpha-tocopherol. However in several diseases the available antioxidant defense systems are exceeded by excessive formation of ROS resulting in an oxidative stress state [41]. The polyphenols resveratrol and quercetin may have an additive effect to the endogenous antioxidant defense mechanisms as they can interfere with several free radical producing systems.

First, quercetin is reported to act as a potent direct scavenger of ROS. Quercetin is known to scavenge $O_2^{\bullet-}$ [150, 151] and $ONOO^{\bullet-}$ [150, 152, 153]. Moreover quercetin also acts as an indirect antioxidant through the inhibition of key ROS-producing enzymes such as XO [154] and NOX [136]. In in vitro systems resveratrol, at high concentrations ($> 100 \mu M$), is reported to directly scavenge a variety of oxidants such as $O_2^{\bullet-}$ [155-157], H_2O_2 [158], OH^{\bullet} [157] and $ONOO^{\bullet-}$ [159]. However the direct antioxidant effects of resveratrol are rather weak compared to those of well-established antioxidants such as ascorbate and cysteine. Thus, the protective effects of resveratrol against ROS-induced damage are likely to be attributed to the upregulation of the endogenous cellular antioxidant systems or to inhibition of ROS forming enzymes rather than its direct ROS scavenging activity [160, 161]. Indeed, resveratrol regulates the expression and activity of ROS-producing or ROS-eliminating enzymes, such as SOD. SOD catalyzes the dismutation of $O_2^{\bullet-}$ into H_2O_2 , which is further inactivated by glutathione peroxidases (GPx) and catalase [162]. In human endothelial cells resveratrol increased mRNA and protein levels of SOD1, SOD2 and SOD3 [163-165]. In addition resveratrol also upregulates GPx1 and catalase in aortic segments or in cultured aortic smooth muscle [158]. Furthermore, treatment of apolipoprotein E (apoE) knockout mice, a model for oxidative stress, with resveratrol (30 – 100 mg/kg per d for 7 d) upregulated SOD1, SOD2, SOD3, GPx1 and catalase in the heart [166]. In addition resveratrol

is reported to decrease ROS by reducing NOX2 and NOX4 expression in the heart of apoE-knockout mice [165] and NOX2 expression in the aorta of diabetic mice [129].

Due to the (direct) antioxidant properties of both polyphenols, enhanced ROS production and thus oxidative stress can be limited. Hence by reducing ROS levels, the phenomenon of 'eNOS uncoupling' can be prevented. Resveratrol and quercetin have both been shown to prevent eNOS uncoupling in cardiovascular tissues [46, 165, 167]. Untreated apoE-knockout mice have an increased oxidation of BH₄ [46] and ROS production in their aorta [46, 168] and heart [165]. As the NOS inhibitor, L-NAME, decreased O₂^{•-} production in both the aorta and heart, eNOS is suggested to be in an uncoupled state and thus producing ROS in this pathological model. Treatment of the apoE-KO mice with resveratrol reduced cardiac O₂^{•-} production and reversed eNOS uncoupling [165]. In addition, also quercetin seems to impede eNOS uncoupling as quercetin was found to prevent endothelial dysfunction by impeding ET 1-induced superoxide formation and eNOS uncoupling in aortic ring segments of male Wistar rats [167].

As previously mentioned, increased ROS production and subsequent oxidative stress causes decreased NO bioavailability and thus impairment of endothelial function. Therefore, the antioxidant capacity of resveratrol and quercetin can prevent oxidative stress-induced inactivation of NO and thus improve vascular function.

Effects on adipose tissue

Obesity, insulin resistance and type 2 diabetes are characterized by chronic state of 'inflammation' which is the result of abnormal cytokine production and activation of a network of inflammatory signaling pathways [63, 169]. The primary source that triggers and where this inflammatory process which emerges in obesity is located, seems to be the adipose tissue [63]. Indeed, both in obese mice and human an imbalanced release/production of pro- and anti-inflammatory adipo(cyto)kines can be found [170-172]. Therefore, as polyphenols, including resveratrol and quercetin, are known to affect adipogenesis and have anti-inflammatory properties, these bioactive compounds could be beneficial in preventing or limiting obesity and its associated complications [173].

It has been shown that the differentiation of swine preadipocytes, mouse mesenchymal stem cells and 3T3/L1 fibroblasts into adipocytes was inhibited by resveratrol in a SIRT1-

dependent manner [174-176]. In addition, resveratrol has been shown to reduce lipid accumulation by both inhibiting lipogenesis and promoting lipolysis [177, 178]. For instance, treatment of isolated rat adipocytes with 125 μM and 250 μM resveratrol reduced insulin activity, which induces lipogenesis (glucose to lipid conversion) in adipose tissue, by 16 % and 25 % respectively [178]. In addition to resveratrol, quercetin was found to inhibit 3T3/L1 adipocyte differentiation and to induce apoptosis in mature adipocytes, suggesting that also quercetin can reduce mature adipocyte mass [179].

The effect of both polyphenols on adipose tissue reaches beyond their anti-adipogenesis activities since resveratrol and quercetin are known to potently reduce adipose tissue inflammation by interfering with the adipo(cyto)kine expression/secretion [180]. Resveratrol ameliorates inflammation by modulating the adipo(cyto)kine expression and secretion profile in different adipocyte models: resveratrol treatment decreases the levels of pro-inflammatory adipo(cyto)kines (such as TNF_α , IL-6 and resistin) and increases the adiponectin expression/secretion [181-186]. Similarly, quercetin supplementation increased adiponectin plasma levels and adiponectin mRNA levels in adipose tissue from high fat- or high fructose-fed rats [187, 188]. Like resveratrol, quercetin increased levels of secreted adiponectin from TNF_α treated of 3T3/L1 adipocytes [185]. Moreover, in primary human adipocytes treated with TNF_α , quercetin has been shown to prevent insulin resistance and to reduce inflammation by attenuating the expression of adipo(cyto)kines such as IL-6 and IL-8, even more effectively than resveratrol [182].

Taken together, studies indicate that the release of adipo(cyto)kines, implicated in increasing the risk of cardiovascular diseases, can be successfully inhibited, at least in vitro, by resveratrol or quercetin. This is accomplished through the reduction of mature adipocyte mass, with concomitant reductions observed in the secretion/expression of inflammatory adipo(cyto)kines.

1.4.2 Resveratrol and quercetin as therapeutic agents

The huge amount of in vitro and animal (preclinical) studies within the field of polyphenols revealed a plethora of beneficial effects of polyphenols administration in different disease states. These preclinical studies suggests that polyphenols can be useful or show their value in the treatment and/or alleviation of the consequences of several diseases such as

cardiovascular disease, diabetes, cancer... [113]. In some studies using diabetic or obese animal models as well as in humans positive effects of resveratrol and quercetin on the vascular system have been described [102, 111, 150, 189-191]. For instance resveratrol and quercetin supplementation were shown to lower systolic blood pressure, flow mediated dilatation and/or the production of inflammatory markers, while they increased eNOS and adiponectin production [102, 111, 150, 189-191]. However, it should be noted that a lot of animal and clinical studies could not confirm these vascular effects [102, 111, 150, 189-191]. It is suggested that the problem for this might be related to the low bioavailability of both polyphenols.

Bioavailability

The bioavailability and pharmacokinetics of resveratrol and quercetin have been studied in experimental animals and humans. Oral administration of high doses of resveratrol or quercetin appears to be safe in humans. No major adverse effects were seen when healthy volunteers were provided with up to 5 g resveratrol [192]. Similarly high doses of quercetin up to 5 g/day (for 28 days) seem to be well tolerated without major adverse events in hepatitis C patients [193].

Upon oral administration, the polyphenols are extensively metabolized in the small intestine and later in the liver yielding glucuronide- and sulfate-conjugates [93].

Pignatelli et al. [194] reported that moderate red wine consumption, which was defined as intake of 300 mL red wine during 15 days, increased total (free and conjugated) plasma levels of resveratrol to 1.72 μM . Similarly total resveratrol in human plasma peaked in the range of 416 – 491 ng/ml (or 2 μM) 30-60 minutes following administration of a single dose of 25 mg resveratrol with a plasma half-life of 9.2 h [195, 196]. Administration of doses up to 5 g increased plasma levels of unchanged resveratrol to about 539 ng/ml (2.4 μM) [192]. However only low levels or even trace amounts (<5 ng/ml) of unchanged resveratrol could be found in the plasma following a single or multiple 25 mg oral dose [195-197]. Likewise, in rodents, oral intake of milligrams yields only low nanomolar plasma levels of unmetabolized resveratrol [198, 199]. An overview of these bioavailability studies is represented in Table I.2.

Dose	Species	Peak plasma concentration of total and/or unmetabolized resveratrol	Reference
300 mL red wine during 15 days	Human	Total: 385 ng/mL (~1.72 μ M)	[194]
25 mg (oral)	Human	Total: 416 - 471 ng/mL (~1.86 - 2 μ M) Unmetabolized: 7.1 - 8.5 ng/mL (~0.03 - 0.04 μ M)	[195]
25 mg (oral)	Human	Total: 491 ng/mL (~2 μ M) Unmetabolized: <5 ng/mL (~0.02 μ M)	[196]
Single dose of 25 mg (oral) 13 doses of 25 mg (oral)	Human	Unmetabolized: 1 ng/mL - 4 ng/mL (~0.004 - 0.018 μ M)	[197]
5 g (oral)	Human	Unmetabolized: 539 ng/mL (~2.4 μ M)	[192]
50 μ mol/kg (11.4 mg/kg) (oral)	Rat	Unmetabolized: 157 ng/mL (~0.7 μ M)	[199]
5 mg/kg (oral)	Rat	Total: 342 ng/mL (~1.5 μ M) Unmetabolized: 18.24 ng/mL (~0.08 μ M)	[198]

Table I.2. Overview of several studies on bioavailability of resveratrol in human and rodents.

Thus both in humans and animals oral administration of resveratrol yields plasma levels of the parent molecule that are either not detectable or some orders of magnitude below the micromolar concentrations that are used in *in vitro* experiments i.e. 5 - 100 μM [200]. The enterohepatic cycle, in combination with the rapid metabolism in the liver explains this low concentration of native compounds in the blood stream. Nonetheless, some of the biological effects of resveratrol have been observed at very low concentrations [201, 202]. Some mechanisms may contribute to this phenomenon i.e. that low resveratrol concentrations are effective, despite the rapid metabolisation into conjugates.

First, due to the lipophilic character of resveratrol, resveratrol and its metabolites can accumulate in tissues and thus tissue levels might be higher than those found in plasma [203]. Accumulation of resveratrol has been found in several tissues including the heart, liver, kidney and skeletal muscle from rats [204-209]. Therefore, plasma levels might be useful in evaluating endothelial exposure, however it might not be an accurate indicator of resveratrol potential bioactivity on tissue level [203]. Second, some metabolites themselves exert biologically beneficial effects. Resveratrol metabolites were shown to reduce fat accumulation in adipocytes, influence adipo(cyto)kine expression and secretion [210, 211] and exhibit anti-inflammatory potential [212, 213]. However no effect of resveratrol-sulfates and β -glucuronides was found on eNOS activity, NO release or ROS levels [214]. Third, some metabolites can be converted back to resveratrol in target cells via glucuronidases and sulfatases [215]. Hence, resveratrol metabolites can also indirectly contribute to the beneficial effects associated with resveratrol administration.

Total quercetin (= free and metabolized) derived from the diet is normally present in plasma in the nanomolar range (<100 nM), however this can be increased to low μM range by quercetin supplementation [216, 217]. For example, in healthy volunteers plasma levels of quercetin increased to 1.5 μM after 28 days supplementation with a high dose (>1 g/d) of quercetin [216]. Interestingly, elimination of quercetin and its metabolites is quite slow, as reported plasma half-lives ranges between 11 – 28 h. This suggests that maintenance of high plasma concentrations of quercetin could be achieved upon repeated intake [93, 111, 150]. Similar to resveratrol, quercetin is extensively metabolized into its sulfate and glucuronide-conjugates. As a result, quercetin in human plasma is mainly found as glucuronide and sulfate conjugates, with very little (<1 μM) unconjugated quercetin present [217, 218]. In

humans, 30 minutes following consumption of 10 mg/70 kg body weight quercetin (dissolved in 100 mL white wine), plasma of total quercetin (free and conjugated) reaches a maximum of 126.8 ng/mL (0.4 μ M) of which around 20 % was unmetabolized quercetin [195]. However, quercetin metabolites can be biologically active as for instance glucuronidated quercetin metabolites were shown to have antioxidant effect *in vitro* and *in vivo* [219, 220]. Yet, glucuronidated and sulfated metabolites lack a direct acute vasodilator effect in isolated arteries and they have only a partial effect in preventing acute endothelial dysfunction [221]. Interestingly, like resveratrol a deconjugation process for quercetin metabolites has been described [222, 223]. It has been shown that glucurono-quercetin conjugates can be deconjugated in the vascular wall of mesenteric arteries, yielding the parent quercetin molecule which accumulates in the tissue [222]. Studies in rats and pigs have shown that quercetin is distributed to several tissues, especially to lung, kidney, colon and liver [224].

Enhancing bioavailability

Given the low bioavailability and extensive metabolism of orally administrated resveratrol and quercetin, efforts have been made to identify novel strategies to ameliorate the bioavailability of both polyphenols in order to achieve plasma concentrations that have been shown to have biological activity.

The composition of the food matrix can impact the bioavailability of resveratrol and quercetin. Trans-resveratrol is better absorbed when ingested through wine or grape juice than from tablets [225]. Similarly quercetin bioavailability is greater when quercetin is consumed as an integral food component compared to quercetin-filled capsules [226]. Several studies suggest that the dietary fat content also influences polyphenolic bioavailability. When resveratrol is added to diets with fat contents equal to or greater than 40 %, significant improvement in energy metabolism and aerobic exercise endurance is seen in mice. This was not the case when resveratrol was added to a standard composition diet [227, 228]. Likewise, resveratrol increases brain antioxidant enzyme activities by approximately 2-fold when given in a high-fat diet, while the same dose in a standard mouse diet did not have a significant effect [229]. Dietary fat-dependent improvements in polyphenolic bioavailability has also been found for quercetin. In pigs, co-ingesting quercetin with a meal containing 32 % fat enhanced quercetin bioavailability with 50 % compared to

its ingestion with a 3 % fat meal [230]. Moreover, in rats resveratrol bioavailability is enhanced by combining it with piperine, a natural product from black pepper [231]. Thus, the in vivo bioavailability could be increased by the food matrix because of the presence of other natural compounds, such as other polyphenols that might play a synergic role [232].

In search of how metabolic breakdown of resveratrol and quercetin could be bypassed or how the absorption of polyphenols could be improved, new delivery strategies are under development. Encapsulation into lipid nanoparticles or liposomes are investigated as potential carriers of resveratrol and quercetin to delay their metabolism and maintain free polyphenol levels in blood and other tissues for a longer period [233-236]. For instance, several formulations have been used to generate quercetin-containing lipid nanoparticles such as self-nano-emulsifying drug delivery systems (SNEDDS). The bioavailability and absorption of quercetin, delivered as quercetin-SNEDDS, was improved in rats after oral administration of these quercetin-SNEDSS [237].

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Chapter II

Aims and main research questions

Chapter II Aims and main research questions

II.1 General aims

Part I of this thesis will focus on the perivascular adipose tissue (PVAT), which is now considered as an important paracrine modulator of vascular tone through the release of various adipo(cyto)kines. These vasoactive substances, which will be discussed in a review in **chapter III**, elicit a net beneficial anticontractile effect on vascular function. Normal functioning of PVAT is thus essential for normal vascular function. Hence interfering with PVAT (dys)function might alleviate or aggravate vascular complications seen in several pathophysiologic conditions. Therefore **chapter IV** will explore whether the normal functioning of PVAT i.e. its relaxing influence, can be affected under several circumstances such as resveratrol addition and/or testosterone depletion.

Part II of this thesis will focus on one of the vascular complications, which is frequently associated with pathophysiologic conditions, such as obesity, diabetes and hypertension: erectile dysfunction (ED). Like vascular function, normal erectile function and thus penile erection relies on a tightly regulated balance between contraction and relaxation. The pivotal role of the NO/cGMP pathway in the regulation of normal penile erection is nowadays well established [1]. Disturbance or impairment will therefore inevitably result in erectile dysfunction [1]. Due to its central role for normal penile erection, current available therapies for ED often aim at increasing cGMP concentrations. Indeed, nowadays the golden standard for treating ED is the use of phosphodiesterase-5 (PDE5) inhibitors. However, since 30-35 % of the patients fail to respond to current PDE-5 inhibitors [2] and due to the adverse effects of current therapies to treat ED, the search for alternative and better strategies is a necessity. Potential alternative options for the treatment of (diabetic) ED will be explored in **chapters V, VI and VII**.

II.2 Main research questions

II.2.1 Chapter IV - Can the relaxing influence of PVAT be positively or negatively modulated by resveratrol treatment and/or testosterone depletion?

In pathophysiologic conditions, such as obesity, diabetes and hypertension the relaxing effect of PVAT is lost due to dysfunctional adipose tissue which is characterized by an unbalanced secretion of relaxing adipo(cyto)kines [3-5]. Since adipogenesis, body fat distribution and/or adip(cyto)kine secretion can be modulated by (i) resveratrol [6-8] or (ii) (low levels of) testosterone [9-11], **chapter IV** will investigate whether resveratrol and testosterone depletion (orchidectomy) also affect the vasorelaxing influence of PVAT using in vitro tension measurements.

II.2.2 Chapter V & VI - Do resveratrol and/or quercetin exert positive effects on corpora cavernosa? How do they work? Do they work under in vitro-diabetic conditions?

Since numerous cardiovascular positive effects such as a direct relaxant and antioxidant capacity, have been ascribed to red wine polyphenols, resveratrol and quercetin, they might be useful for improving erectile function. **Chapter V** will investigate the ability of resveratrol and quercetin to relax mouse corpora cavernosa as well as the underlying mechanism using in vitro tension measurements. In addition obesity and type 2 diabetes are characterized by elevated levels of free fatty acids, including palmitic acid [12]. Therefore the ability of resveratrol and quercetin to improve palmitic acid-induced impairments of corporal responses will be studied.

For our next study, we wondered whether the positive effects of resveratrol and quercetin on erectile tissue would still account in pathophysiologic conditions. As in the previous study, palmitic acid was found to impair the corporal relaxant responses only to a limited extent, other more potent compounds were sought to create an in vitro model for oxidative stress. Therefore our next study, described in **chapter VI**, will evaluate the effect of in vitro diabetic mimicking conditions using high concentrations of glucose in combination with methylglyoxal, a glucose metabolite which is found in high concentrations in diabetes, on vascular and corporal responses. In addition it will be explored whether resveratrol and quercetin could be useful in the prevention of in vitro induced diabetic deficits to the relaxant responses of mouse arteries and corpora cavernosa.

II.2.3 Chapter VII - Does inhibition of cGMP export elicit positive relaxant responses in mouse corpora cavernosa?

In addition to metabolic degradation of cGMP by PDE5, it has recently been suggested that cGMP can also be actively transported across the plasma membrane by members of the multidrug resistance protein (MRP) 4 and 5 [13-17], both being expressed in smooth muscle cells of the corpora cavernosa [18, 19]. Prevention of cGMP-transport out of the cavernosal smooth muscle cells might thus represent a new and alternative approach to elevate intracellular cGMP levels and thus to treat ED. In the last study, described in **chapter VII**, the functional effect of MRP 4 inhibition as well as the role of MRP 4-mediated cGMP export in mouse corpora cavernosa will be investigated in vitro and in vivo.

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Chapter III

Adipose tissue as regulator of vascular tone

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Chapter III Adipose tissue as regulator of vascular tone

III.1 Abstract

Adipokines secreted by visceral, subcutaneous and perivascular adipocytes are involved in the regulation of vascular tone by acting as circulatory hormones (leptin, adiponectin, omentin, visfatin, angiotensin II, resistin, tumor necrosis factor alpha, interleukin-6, apelin) and/or as local paracrine factors (perivascular adipocyte-derived relaxing and contractile factors). Vascular tone regulation by adipokines is compromised in obesitas and obesity-related disorders. Hypoxia created in growing adipose tissue dysregulates synthesis of vasoactive adipokines in favor of harmful proinflammatory adipokines, while the levels of the cardioprotective adipokines adiponectin and omentin decrease. Considering the role of adipokines in obesity-related vascular diseases potential, strategies to counter these diseases by targeting the adipokines are discussed.

III.2 Introduction

The tone of vascular smooth muscle cells importantly determines blood pressure and regional blood flow. This vascular tone is regulated by myogenic mechanisms and by vasoactive mediators released either by nerves, or endocrine and paracrine tissues. In last decennia the cardinal influence of endothelial cells on vascular smooth muscle tone has become evident. More recently evidence emerged that also adipocytes are involved [1, 2]. Adipokines secreted by adipocytes include circulating hormones, inflammatory cytokines and local acting paracrine factors and are involved in various physiological processes [3]. The role of the vasoactive adipokines in vascular physiology will be highlighted in view of the worldwide pandemic character of obesity with its associated cardiovascular diseases [4].

III.3 Sources of vasoactive adipokines

Fat depots are interspersed in many different body locations and includes visceral, subcutaneous and perivascular adipose tissues. These depots constitute a variety of mini-organs with unique proteomics fingerprint. The total number of secreted components of adipocytes approaches 100 distinct proteins, a number that is likely to increase [5]. Several adipokines possess vasoactive properties and some of them are well characterized as circulating hormones (eg adiponectin, leptin ...). More recently it has become clear from in vitro studies on isolated blood vessels that vascular tone is also importantly influenced by paracrine mediators from perivascular adipose tissue.

III.4 Adipocytes in obesity

Obesity is associated with a state of chronic low-grade inflammation involving the production of pro- and anti-inflammatory cytokines by adipocytes, including those surrounding the vasculature [6]. It has been proposed that local hypoxia may be the fundamental determinant in the development of this inflammatory response. Hypoxia occurs in areas of the fat depots when the vascular oxygen supply is compromised due to tissue mass expansion [7]. Direct evidence that growing adipose tissue becomes hypoxic has been obtained in mice [8]. Furthermore, cell-culture studies using murine and human adipocytes strongly support the modulatory role of hypoxia in the production of several pro-inflammatory adipokines [9]. A recent study demonstrates that even modest changes in O₂-level already induce specific changes in gene expression and metabolism of human

adipocytes [10] indicating that adipocytes are highly sensitive to the prevailing level of O_2 . This is an important observation considering that most studies in vitro are performed using cells oxygenated with a gas containing 21 % O_2 , which is actually well above the O_2 -tension prevailing in tissues where oxygen levels are much lower than in arterial blood.

It can be noted that hypoxia also promotes angiogenesis [11]. Hypoxia upregulates inducible transcription factors, which trigger the expression of angiogenic adipokines such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and plasminogen activator inhibitor-1 (PAI-1). Novel vascularisation can be considered as an automatic fail-safe in order to counter hypoxia and to ensure sufficient nutrient and oxygen supply to the different tissues. Despite the great angiogenic potential, rapidly expanding fat tissue still experiences hypoxia. Also aging is associated with hypoxia and oxidative stress in adipose tissue, similar to what is seen in obesity. Aging is associated with altered function, size and number of adipose cells and altered distribution of adipose tissues in the body [12].

III.5 Vasoactive adipokines (Fig.III.1)

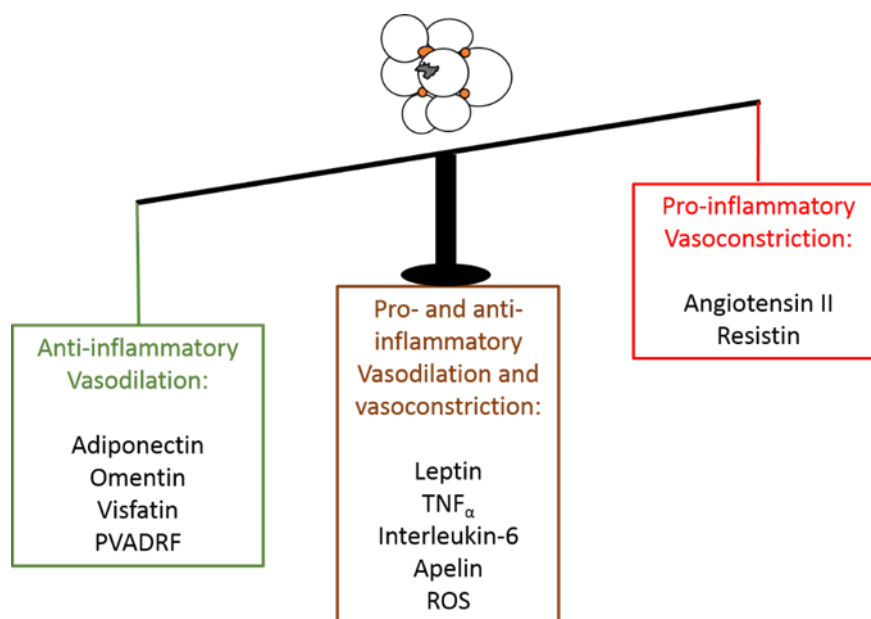


Figure III.1 Adipose tissue releases several vasoactive adipokines. Some of them have vasorelaxing or anti-inflammatory properties, some have vasocontractile or pro-inflammatory properties and others share both. PVADRF: perivascular adipocyte derived relaxing factor, TNF_{α} : tumor necrosis factor alpha, ROS: reactive oxygen species.

III.5.1 Leptin

Leptin is best known as the adipokine regulating feeding behavior. However, leptin also has vasorelaxing and vasocontractile effects and in that way contributes to a balanced blood pressure homeostasis. While the contractile effect of leptin is attributed to activation of the sympathetic nervous system, various mechanisms seem to be responsible for the leptin-induced vasorelaxation including endothelium-dependent release of nitric oxide (NO). Leptin levels are markedly increased during obesity [13] and this hyperleptinemia is believed to dysregulate blood pressure, resulting in hypertension. Sustained hyperleptinemia leads to endothelial dysfunction affecting endothelium-dependent vasorelaxation [14]. This might be the result of several leptin-induced effects: increase of vasoconstrictor endothelin-1 [15], a leptin-induced expression of endothelin type A receptors in vascular smooth muscle cells [16], a leptin-induced depletion of NO and increase of cytotoxic reactive oxygen species (ROS) [17]. Leptin also promotes smooth muscle cell proliferation contributing to the increased peripheral vascular resistance [18]. Furthermore, it stimulates the release of pro-inflammatory cytokines from macrophages which may further elevate blood pressure and exacerbate the inflammatory process [19]. Significant associations have been found between plasma leptin levels and hypertension in both males and females, which makes leptin a potential predictor of hypertension [20, 21].

III.5.2 Adiponectin

Adiponectin is one of the most abundant adipokines secreted in the circulation [22]. While many adipokines are pro-inflammatory and contribute to vascular dysfunction, adiponectin has favorable cardiovascular effects and possess potential as a therapeutic target for combating obesity-associated vascular disease.

Both in vivo and in vitro animal studies as well as clinical data consistently support the role of adiponectin as vasodilator that induces endothelial NO-synthase (eNOS) activation and consequently endothelial NO production. In addition adiponectin protects the endothelium from oxidative stress by decreasing ROS production, prevents endothelial cell activation (a key pathological event in vascular inflammation and atherosclerosis) and monocyte adhesion. Adiponectin also improves endothelial progenitor cell function, leading to improved repair after vascular injury. It also inhibits macrophage activation and foam cell

formation and it reduces proliferation and migration of vascular smooth muscle cells. Adiponectin thus inhibits almost every pathological event involved in vascular disease, ranging from endothelial injury and dysfunction to atherosclerotic lesion formation. These favorable effects are mediated through its pleiotropic actions on several types of cells in the vasculature, including mature endothelial cells, endothelial progenitor cells, monocytes and vascular smooth muscle cells [23, 24].

Epidemiological studies on different ethnic groups often have identified low level of circulating adiponectin as an independent risk factor for type-2 diabetes, hypertension, atherosclerosis and myocardial infarction. Hypoadiponectinemia causes endothelial dysfunction by increasing superoxide anion production [25, 26], by promoting the production of adhesion molecules in endothelial cells and the proliferation of vascular smooth muscle cells. Decreased plasma adiponectin concentrations are found in patients with essential hypertension [27]. However, it should be noted that after the establishment of atherosclerosis, this association may become weaker, especially in the presence of conditions inducing a hyper-catabolic state (such as heart or renal failure) which are associated with increased plasma adiponectin, accelerated progression of atherosclerosis and worse clinical outcome. In fact, several data show that high circulating adiponectin levels are associated with increased cardiovascular mortality in patients with coronary artery disease [28]. Therefore, hypoadiponectinemia may have a clinical value at the early stages of atherogenesis, but at more advanced disease stages its role as a meaningful biomarker is questioned. An abundance of epidemiological studies demonstrate that circulating levels of adiponectin correlate with increased mortality and severity of cardiac heart failure. It is still inconclusive whether the increased adiponectin production plays a detrimental role or a protective role in cardiac heart failure as it could be that increased adiponectin production in patients with cardiac heart failure serves as a means of compensatory upregulation against oxidative stress and inflammation [29].

Considering the beneficial effects of adiponectin on vascular system, strategies to increase adiponectin levels could be of great therapeutic value. It has already been demonstrated in obese adiponectin knockout mice with hypertension that adiponectin replenishment lowers the elevated blood pressure [30]. Results from a recent study in mice indicate that exogenous adiponectin supplementation might have a prophylactic value against secondary

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myocardial ischemic injury after a primary non-lethal mechanical trauma [31]. Existing drugs like peroxisome proliferator-activated receptor γ (PPAR γ) agonists (thiazolidinediones), some angiotensin type 1 receptor blockers (telmisartan), angiotensin converting enzyme inhibitors and cannabinoid type 1 receptor blockers (rimonabant, taranabant) have been shown to increase circulating adiponectin levels [28]. This also occurs with weight loss and physical exercise, along with the adaptations of a Mediterranean-type diet [32]. Recent studies showed that the antioxidant resveratrol increases the adiponectin expression [33] and that the antioxidants NO-acetylcysteine and allopurinol synergistically enhance cardiac adiponectin content and reduce myocardial reperfusion injury in diabetic rats [34].

Whatsoever, future strategies may focus on up-regulation of adiponectin's expression (and/or its receptors) or on targeting adiponectin's receptors through the development of specific agonists. As yet direct evidence that adiponectin as such protects against vascular disease in humans is still lacking. Adiponectin-based therapeutics are not available partly due to difficulties in converting the full size adiponectin protein into a viable drug. A recent study reports on the design and initial preclinical development of the first adiponectin receptor agonist which may be promising to substitute for the low adiponectin levels in obesity-related diseases [35].

III.5.3 Omentin

Omentin is a recently identified adipose tissue-derived cytokine expressed in visceral rather than in subcutaneous adipose tissue and exists in 2 isoforms of which omentin-1 is the major circulating isoform in human plasma. In isolated arteries, omentin induces relaxations [36]. As yet only in vitro studies on isolated blood vessels have been performed. In vivo studies are required to explore the influence of omentin on blood pressure and its chronic influence on vascular reactivity.

Omentin plasma levels and the adipose tissue gene expression are decreased in obesity [36] and even more when overweight is combined with type-2 diabetes [37]. Furthermore, decreased omentin-1 levels are associated with low plasma adiponectin and high-density lipoprotein (HDL) levels. In addition, omentin-1 levels are negatively correlated with leptin levels, waist circumference, body mass index and insulin resistance [38]. Like adiponectin, circulating omentin-1 concentrations increase after weight loss-induced improvement of

insulin sensitivity [39]. Although further research is necessary, elevating the omentin levels might be an interesting therapeutic strategy in obesity and obesity-related disorders.

III.5.4 Visfatin

Visfatin is another novel identified cytokine with multiple functions in the vasculature. Visfatin relaxes isolated arteries, stimulates growth of vascular smooth muscle cells [40 and endothelial angiogenesis [41]. Most studies, but not all, showed an increase in visfatin levels in obesity [42]. It has been reported that the expression of visfatin is high at plaque rupture sites in patients with coronary artery disease [43]. Visfatin accelerates monocyte adhesion to endothelial cells by up-regulating intercellular (ICAM-1) and vascular (VCAM-1) cell adhesion molecule-1 due to ROS overproduction, suggesting a possible role for visfatin in the development of atherosclerosis [44]. Further studies are necessary to clarify the atherogenic and vasoactive effects of visfatin and its potential clinical relevance.

III.5.5 Angiotensinogen/Angiotensin II

Adipocytes are rich sources of angiotensinogen, the precursor protein of a major vasocontractile peptide called angiotensin II [45]. They possess all the enzymes necessary to produce angiotensin II [46], suggesting the existence of a local renin-angiotensin system in adipose tissue. An important effect of angiotensin II is that this peptide enhances the metabolism of NO into ROS, which damage the vascular tissue [47]. An imbalance between angiotensin II and NO leads to endothelial dysfunction resulting in a loss of vasodilator capacity. This results in an increased expression of adhesion molecules and pro-inflammatory cytokines in endothelial cells promoting monocyte and leukocyte adhesion plus migration to the vessel wall [48]. Furthermore angiotensin II exerts detrimental effects on progression and destabilization of atherosclerotic plaque due to an increased release of plasminogen activator inhibitor (PAI-1) causing thrombosis and an increased expression of growth factors leading to smooth muscle cell proliferation and migration [48].

Most data support an elevation of angiotensinogen mRNA expression in adipose tissue during obesity [49]. Several studies highlight a contribution of adipose tissue-derived angiotensinogen and/or angiotensin peptides to obesity-related hypertension [49]. High angiotensin II levels may deteriorate obesity-related hypertension due to an increased

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secretion of pro-inflammatory cytokines [50], decreased adiponectin secretion [51] or increased leptin production in adipocytes [52].

III.5.6 Resistin

Resistin is secreted into the medium by cultured adipocytes and circulates in plasma. However, especially circulating monocytes and macrophages seem to be responsible for resistin production in humans [41]. Although resistin does not directly affect the contractility of isolated blood vessels [41, 53], coronary blood flow, mean arterial pressure or heart rate [54], it has been associated with endothelial dysfunction and coronary heart disease [55]. It has also been shown that high plasma resistin levels independently associate with an increased risk for hypertension among non-diabetic women [56].

III.5.7 Tumor necrosis factor alpha (TNF α)

The cytokine TNF α is an adipokine showing both vasoconstrictor and vasodilator activity. TNF α -mediated vasoregulation can occur through both endothelium-dependent [57] and endothelium-independent mechanisms [58]. An increased adipose tissue expression of TNF α mRNA has been reported in different rodent models of obesity as well as in clinical studies involving obese patients [13]. TNF α is considered to be a molecule that links inflammation to obesity [13]. Moreover, the infiltration of macrophages in adipose tissue during obesity contributes to increased TNF α production [59]. Increased TNF α expression induces the production of ROS, resulting in endothelial dysfunction in obesity and obesity-related disorders like hypertension, atherosclerosis and type-2 diabetes [60].

III.5.8 Interleukin-6 (IL-6)

Acute exposure to IL-6 in vitro relaxes aortas [61]. However, a sustained increase of IL-6 plasma levels is associated with high blood pressure [62, 63]. In obesity an increase in cytokine IL-6 has been observed at mRNA and protein level in white adipose tissue [64]. IL-6 has been shown to be a predictor of future myocardial infarction [63] and is highly associated with cardiovascular mortality [65]. Some studies have suggested that IL-6 is rather an indirect marker of vascular dysfunction, while others have suggested a more active role for IL-6 in vascular dysfunction. Long term elevation of IL-6 in mice has shown to impair endothelial function by increasing angiotensin II-stimulated production of ROS and by

reducing eNOS mRNA expression [66]. In addition, IL-6 enhances vascular smooth muscle cell proliferation [67], a key event in the genesis of atherosclerotic lesions. Genetic deletion of IL-6 attenuates angiotensin II-induced hypertension in mice [62], suggesting that elevated IL-6 in obesity might contribute to hypertension via angiotensin II. In addition, IL-6 inhibits adiponectin gene expression in cultured adipocytes [64] which may exacerbate obesity-related hypertension.

III.5.9 Apelin

Apelin causes NO-dependent vasorelaxation of human arteries both in vitro and in vivo [68, 69]. Exogenous apelin administration causes a rapid NO-dependent fall in blood pressure in a rodent model, confirming its powerful vasorelaxing effect [70]. However, some reports associate apelin with an increase in arterial pressure [71]. In contrast to acute exposure, long term exposure to apelin does not affect blood pressure [72].

Apelin production in adipose tissue is strongly up-regulated by insulin and plasma concentrations are increased in obese and hyperinsulinemic mice and humans [73]. In atherosclerosis, apelin might have beneficial effects as apelin stimulates endothelial NO-production and antagonizes the angiotensin II-induced formation of atherosclerotic lesions and aortic aneurysm in mice [74].

III.5.10 Perivascular adipocyte-derived relaxing and contractile factors (PVADRF and PVADCF)

Virtually all blood vessels are surrounded by adventitial adipose tissue. Adipocytes in perivascular adipose tissue (PVAT) are very inhomogeneous and differ from subcutaneous and visceral adipocytes. Because PVAT encroaches into the adventitia without a fascial layer as barrier, humoral factors secreted by PVAT have easy access to the blood vessel wall, indicating that PVAT can function as a paracrine organ transducing metabolic signals directly to the vascular cells.

Several in vitro studies have shown that the presence of adhering PVAT reduces contractility of isolated blood vessels. Verlohren et al. even showed a positive correlation between the vasorelaxing influence and the amount of perivascular adipose tissue [75]. Whether NO formation and endothelium are involved in the vasorelaxation effect by PVADRF is still a matter of debate [75, 76]. The vasorelaxing effect of PVADRF is likely mediated by the

opening of different K⁺ channels in vascular smooth muscle cells, depending on the tissue and species studied [75-80]. These divergent observations suggest a different distribution of K⁺ channels in different vessels and/or species or the existence of different PVADRFs. This relaxing effect is mediated by (a) factor(s) which are as yet not fully established although several candidates have already been proposed.

Reactive oxygen species (ROS) are a class of oxygen-derived molecules including superoxide anion and hydrogen peroxide, both modulators of vascular tone and released from PVAT. Hydrogen peroxide is a more likely paracrine ROS as it is not a free radical and therefore more stable and less reactive with other tissue radicals [81]. Hydrogen peroxide is known to induce both vasorelaxation and –constriction depending on species, type of vascular bed, concentration, membrane potential and degree of obesity [81, 82]. In general, superoxide and hydrogen peroxide production in adipose tissue is increased in obese mice, which promotes endothelial dysfunction. Hydrogen peroxide produced from the NAD(P)H oxidase has been described to be involved in the endothelium-independent pathway of the PVADRF [76]. Superoxide anions impair endothelium-dependent vasorelaxation by decreasing NO bioavailability via formation of another ROS peroxynitrite [27, 83]. Furthermore, ROS contributes to endothelial dysfunction by up-regulating the expression of adhesion and chemotactic molecules in endothelial cells, which promote monocyte adhesion and migration to the vessel wall [84]. The adhesion of these circulating blood cells to vascular endothelium is a key element in the development of inflammation and thrombosis within the vasculature in vascular diseases associated with oxidative stress like atherosclerosis [84]. Ketonen et al. [82] showed that endothelium-dependent vasodilation is impaired in aorta of mice fed a high fat diet. The impaired endothelium-dependent vasodilation was restored by removal of PVAT or by quenching ROS indicating that PVAT-derived oxidative stress impairs endothelial function [82].

Another PVADRF candidate is the peptide angiotensin (1-7) which is a vasodilator released by adipose tissue surrounding rat aorta [85]. Blocking this particular peptide inhibits the vasorelaxing effect of PVAT and induces endothelium-dependent relaxation via NO-release [85]. In a recent study Lu et al. reported that besides arteries also veins are surrounded by PVAT and that it attenuates venous contractile tone by releasing Ang-(1-7). PVAT thus may also modulate venous function [86]

Also hydrogen sulfide has been proposed as a PVADRF or at least as a mediator in the ADRF effect [80, 87]. Hydrogen sulfide is generated by cystathionine gamma-lyase (CSE) in PVAT tissue [88, 89]. Blocking CSE inhibits the vasorelaxing effect of PVAT in rat aorta and mice mesenteric arteries [80, 87]. Moreover, hydrogen sulfide-induced vasorelaxation of rat aorta was inhibited by a blocker of a particular ADRF-related K^+ -channel (KCNQ) blocker [80].

A recent study suggested that one of the PVADRFs could be identified as methylpalmitate [90], an enigmatic factor which according to the same authors is also released from superior cervical ganglia and retina. It should however be noted that the relaxing capacities of methylpalmitate could not be confirmed by Takir et al.[91].

Adipokines from PVAT also seem to be involved in the vasorelaxing effects of insulin. Insulin dilates vasculature in muscle tissue with high glucose uptake. This insulin-mediated diversion of blood flow to active tissues is disturbed in obesity. Adipokines might be involved, as TNF_{α} and IL-6 impair insulin-induced vasodilation while adiponectin increases sensitivity to insulin and increases glucose uptake [92, 93].

Factors from PVAT can also stimulate vasoconstriction. Perivascular nerve activation by electric stimulation in rat aorta induces vasoconstriction dependent on the presence of PVAT. In dogs PVAT releases a vasocontractile factor that impairs coronary endothelial NO production. Lu et al. recently showed that adipocyte-derived angiotensin II is critically involved in PVAT-mediated potentiation of perivascular neuronal stimulation-evoked contraction in rat mesenteric arteries [94].

Lu et al. recently showed that PVAT-associated inhibition of a contractile response to agonist was impaired in SHR and that this impairment is not due to reduced PVAT mass in SHR [95]. Using a bio-assay cascade technique Lee et al. also found a decreased release of PVADRF from PVAT of hypertensive SHR rats. This supports the hypothesis that continuously released PVADRF from PVAT protects against the development of hypertension [90].

Obesity is characterized by a decrease in vasorelaxing effect of PVAT [82, 96, 97]. In a study in which rats were fed a fructose-rich diet, inducing metabolic alterations resembling the profile of the human metabolic syndrome, it was found that vascular smooth muscle response was unaffected but that the PVAT-dependent vascular relaxant influence was diminished, illustrating the strong association between adipose tissue and vascular

dysfunction [98]. This might imply a decrease in ADRF release or an imbalance in adipose tissue-derived relaxing and vasocontractile factors during obesity leading to hypertension. In obese patients there is an increased production of TNF_{α} in PVAT and TNF_{α} inhibits the relaxing influence of PVAT. However, incubation of PVAT from obese patients with an anti- TNF_{α} antibody did not restore the anti-contractile response, suggesting that TNF_{α} is just one of many perivascular adipokines with complex effects on vascular function. On the other hand, hypoxia, which develops within adipose tissue during obesity, has recently been shown to enhance (acute hypoxia) [78] or to inhibit (chronic hypoxia) the anti-contractile properties of PVAT [97]. Interesting is the finding that macrophage activation might be responsible for loss of anti-contractile function in inflamed PVAT [99].

Also the heart is surrounded by fat depots. Risk factors for cardiovascular disease associate with the expansion of the fat depot surrounding the heart and coronary vessels. Epicardial adipose tissue (EAT) is located between the myocardium and the visceral pericardium and secretes adipokines like adiponectin, adrenomedullin and omentin that may have protective effects on the myocardium and vasculature. Experimental evidence for this is limited since very small amount of EAT is present in laboratory rodents compared to larger mammals and human beings. Several studies reported alterations in adipokine expression in EAT during pathological states like coronary artery disease, metabolic syndrome or following cardiac surgery. Resistin secretion is enhanced from EAT in patients with coronary artery disease. Resistin may directly interfere with cardiac function as overexpression of resistin was found to impair contractile function in rat cardiomyocytes [100].

III.6 Conclusions

Under normal circumstances, vascular tone is influenced by adipokines. Arterial tone can be controlled through the release of ROS, leptin, adiponectin, TNF_{α} , IL-6, Ang II, omentin, resistin, visfatin, apelin, PVADRF and PVADCF from adipose tissues. Maintenance of a normal amount of adipose tissue is essential. It is thought that vascular tone regulation is compromised in obesity and obesity-related disorders, in which the amount of adipose tissue has grown out of proportion and hypoxic conditions are created. This eventually leads to a dysregulated synthesis of vasoactive adipokines by dysfunctional adipose tissue in favour of harmful pro-inflammatory adipokines. Circulating levels of adiponectin and

omentin are decreased while levels of leptin, resistin, apelin and pro-inflammatory cytokines are increased. The dysregulated synthesis/secretion of adipokines and the infiltration of macrophages into adipose tissue lead to a state of inflammation. A pro-inflammatory state in adipose tissue can not only induce a dysregulation of vascular tone but also local insulin resistance, adhesion of monocytes, vascular remodelling, foam cell formation in the arterial wall and endothelial dysfunction. Endothelial dysfunction is reflected as a decrease in NO bioavailability and endothelium-dependent relaxation and as impaired ability of the endothelium to respond to circulating hormones. All these changes clearly promote the development of cardiovascular diseases and type-2 diabetes.

Whatsoever, increasing adiposity is associated with adipose tissue inflammation and dysregulation of adipokines. The anatomic proximity of PVAT to the vasculature suggests that this dysregulation of adipokines in obesity and other disease states may have local pathogenic effects on blood vessels. Because PVAT likely contributes to vascular diseases it is suggested that PVAT may be a novel target for the treatment of atherosclerosis and restenosis after coronary intervention. The emerging knowledge of the regulatory influence of the PVAT and its contribution to diseases associated with metabolic syndrome brings hope that better medications become available to control the complications associated with obesity, as adjuncts to what remains the mainstay of management of this condition namely exercise and a low fat intake.

One therapeutic strategy to counter the progression of obesity-related vascular diseases is elevating adiponectin and omentin levels. Adiponectin levels are already elevated when using thiazolidinediones, telmisartan, angiotensin-converting enzyme inhibitors, rimonabant and taranaban. On the other hand, development of specific agonists to target adiponectin and omentin receptors or inhibition of detrimental adipokines signaling pathways may be new and promising to attenuate the pro-inflammatory effects and ultimately to reduce the progression of obesity-related vascular diseases.

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Chapter IV

Effect of resveratrol and orchidectomy on the vasorelaxing influence of perivascular adipose tissue

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Chapter IV Effect of resveratrol and orchidectomy on the vasorelaxing influence of perivascular adipose tissue

IV.1 Abstract

INTRODUCTION. Perivascular adipose tissue (PVAT) releases several adipo(cyto)kines. Some are vasoactive substances that elicit a net beneficial anticontractile effect. Resveratrol and testosterone are known to modulate adipo(cyto)kine release from adipose tissue and could therefore influence the anticontractile effect of PVAT.

MATERIALS AND METHODS. In vitro tension measurements were performed using thoracic aorta segments with and without adipose tissue from sham-operated or orchidectomized male Swiss mice. Concentration-response curves to norepinephrine (NOR) were constructed in the presence and absence of resveratrol (10 μ M, 15 min) or the relaxant effect of resveratrol (10-100 μ M) was investigated after inducing tone with NOR (5 μ M).

RESULTS. Aortas with PVAT displayed significantly attenuated contractions to NOR compared to aortas without PVAT. In aortas without PVAT, resveratrol (10 μ M) significantly decreased NOR responses and elicited concentration-dependent (10-100 μ M) relaxations. However in aortas with adherent PVAT, resveratrol (10 μ M) neither decreased NOR responses, nor did resveratrol (10-100 μ M) induce arterial relaxations. The anticontractile effect of PVAT was less pronounced in presence of resveratrol and unaltered by orchidectomy. Orchidectomy did not influence contractions induced by NOR.

CONCLUSION. Orchidectomy does not modulate the anticontractile capacity of PVAT, while resveratrol decreases the vasorelaxing influence of PVAT. The positive effects associated with resveratrol addition are neutralized by the presence of PVAT. This is thought to result from a dual effect of resveratrol: (i) inhibition of the influence of vasodilatory adipo(cyto)kines and (ii) a direct relaxant effect on the vascular smooth muscle. Overall the beneficial relaxing effect of resveratrol is lost in mice thoracic aorta surrounded by PVAT.

Keywords perivascular adipose tissue, resveratrol, orchidectomy, vascular

IV.2 Introduction

Until recently adipose tissue was considered as only being involved in total body lipid and energy homeostasis. However nowadays it is well accepted that adipose tissue also acts as a major endocrine and paracrine organ through the release of a variety of inflammatory adipo(cyto)kines and other factors which also influence vascular tone [1]. Several studies revealed that also perivascular adipose tissue (PVAT) is not just structural support for blood vessels, but plays an important role in the regulation of vascular function. Under normal physiological circumstances PVAT releases several vasoactive substances that elicit a net beneficial and protective anticontractile effect on vascular tone [1-6], which directly depends on the amount of PVAT [2]. Surprisingly in case of increased amounts of PVAT the anticontractile effect of PVAT is not enhanced. In contrast, it has been reported that excessive amounts of PVAT are deleterious as in obesity the protective anticontractile capacity of PVAT is lost [7]. Although the exact underlying mechanism remains unclear, imbalanced release of adipo(cyto)kines in favour of pro-inflammatory ones due to hypoxia-induced dysfunctional adipose tissue has been proposed [8, 9]. It thus seems that the release of adipo(cyto)kines can be influenced under certain circumstances. In this perspective it has been shown that also resveratrol, a naturally occurring polyphenol found in the skin of grapes and thus abundant in red wine, reverses the adipocyte secretion profile towards a less inflammatory phenotype by for instance decreasing the expression of detrimental adipo(cyto)kines and increasing the production of anti-inflammatory ones [10-16]. Together with other beneficial properties associated with resveratrol administration, such as its relaxant and antioxidant [17] and anti-atherogenic capacity [18], resveratrol could be a promising therapeutic target to alleviate obesity-induced metabolic complications. Besides resveratrol treatment also sex hormones can influence adipo(cyto)kine secretion from adipose tissue. In female mice, estrogen depletion by ovariectomy increases adiponectin levels [19] and the production of IL-6 and TNF α , two pro-inflammatory adipo(cyto)kines [20]. This enhanced adipo(cyto)kine secretion can be reversed by estradiol replacement or by administration of estrogens [19, 20]. Moreover, it has been reported that in ovariectomized rats the relaxing effect of PVAT is attenuated [21]. The effect of orchidectomy (testosterone depletion) on the anticontractile effect of PVAT is unknown. This could be of interest as also an interrelationship between testosterone and adipo(cyto)kine levels has been reported in

different studies. In rodents testosterone injection decreases adiponectin levels [22], hypogonadal men have increased adiponectin levels compared to eugonadal subjects, which can be reduced by testosterone replacement therapy [23]. Moreover, testosterone therapy reduces serum leptin concentration in subjects with low testosterone levels [24]. Therefore, as adipo(cyto)kine secretion can be influenced by resveratrol and testosterone, the aim of the present study was to find out whether (i) resveratrol or (ii) orchidectomy can modulate the anticontractile effect of PVAT in mice aorta.

IV.3 Materials and methods

IV.3.1 Animals and orchidectomy

Adult (8-12 weeks) male Swiss mice were obtained from Janvier (Saint-Berthevin, France). Food and water was provided ad libitum and all animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. This study was also approved by the local Ethical Committee for Animal Experiments, Faculty of Medicine and Health Sciences, Ghent University, Belgium. Under 2-4 % isoflurane inhalation, mice were sham-operated (Sham, n=10) or orchidectomized (Orx, n=10). Four weeks post orchidectomy the effectiveness of the orchidectomy was confirmed by atrophy of the testosterone-dependent seminal vesicles.

IV.3.2 Tissue preparation

After cervical dislocation, thoracic aortas surrounded by adipose tissue were isolated and kept in cooled and oxygenated (5 % CO₂, 95 % O₂) Krebs-Ringer bicarbonate (KRB) solution. The aortas were mounted into a wire myograph for isometric tension recording. Two stainless steel wires (40 μM diameter) were guided through the lumen of the segments. In order to measure changes in isometric tension, one wire was fixed to a force-displacement transducer and the other was connected to a micrometer. Two different types of aortic segments were prepared: segments cleaned of adipose tissue ((-)PVAT) and segments with adherent adipose tissue ((+)PVAT). In some experiments the removed adherent adipose tissue was kept in cooled and oxygenated (5 % CO₂, 95 % O₂) KRB solution. Just before starting the experimental protocol it was placed in the organ bath but not in close proximity of cleaned aortic segments. In addition some aortic segments were used from which 50 % of the adherent adipose tissue was removed ((+)½ PVAT). All preparations were equilibrated

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for approximately 30 min in oxygenated KRB solution at 37°C (pH 7.4) and gradually stretched until a stable preload of 0.5 g was obtained. At the start of each experiment, aorta segments were activated with 120 mM K⁺ and 5 μM norepinephrine (NOR). Thereafter the tissues were rinsed until their basal tension was reached again. Subsequently segments were precontracted with 5 μM NOR and after obtaining a stable contraction, 10 μM acetylcholine (Ach) was added to evaluate the functionality of the endothelium. Only tissues that relaxed more than 50 % to Ach, were included in this study. Then preparations were washed and allowed to relax again to their basal tension before starting the experimental protocol.

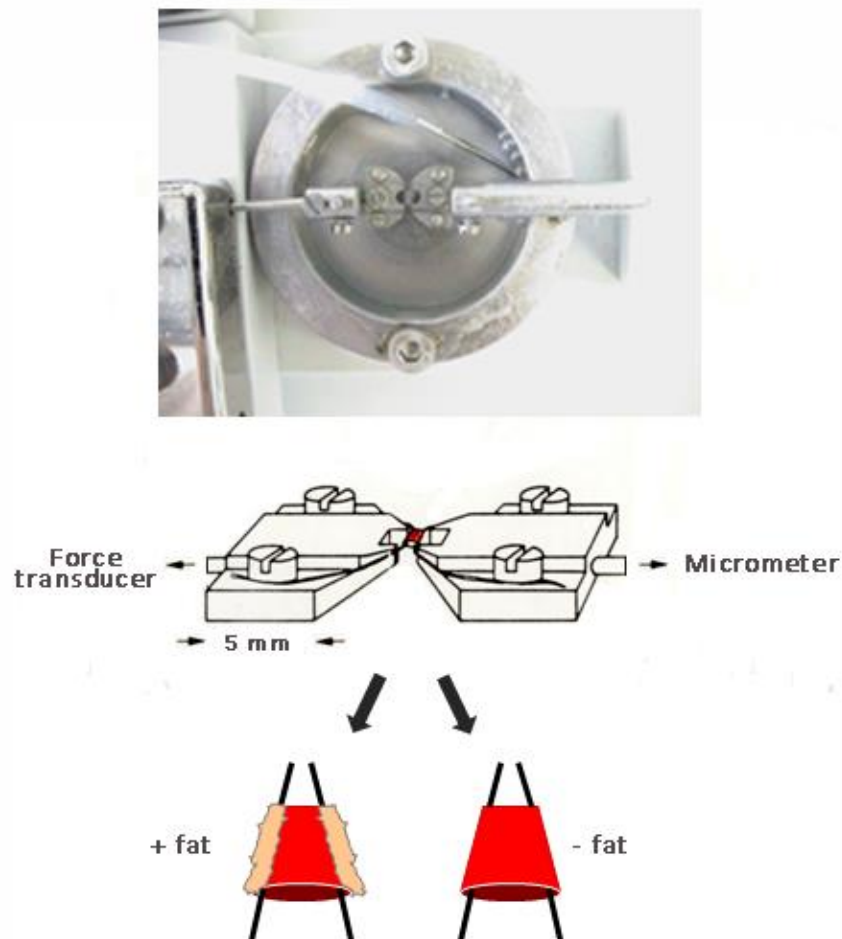


Figure IV.1 A photograph of the organ bath for the wire myograph, a schematic detail of a pair of holders and vessel segments with and without adherent adipose tissue. Vessel segments are mounted on two stainless steel wires (40 μm), fixed on the two holders in the organ bath. One holder is connected to a micrometer which is used to change the distance between the wires. The other holder is connected to a force transducer which measures isometric tension changes in the vessel segment.

IV.3.3 Experimental protocol

Cumulative concentration-response curves to NOR were constructed in presence and absence of resveratrol (10 μ M, 15 min) or in aorta from sham-operated or orchidectomized mice. In all experiments the basal influence of cyclooxygenase metabolites and NO was excluded by incubating the preparations with the cyclooxygenase (COX) inhibitor indomethacin (indo, 10 μ M, 20 min) and the NO-synthase inhibitor N ω -nitro-L-arginine methyl ester hydrochloride (L-NAME, 0.1 mM, 20 min), except in the first series. In another set of experiments concentration-response curves to resveratrol (10-100 μ M, 15 min) were obtained after precontracting the aortas with NOR 5 μ M.

IV.3.4 Drugs and chemicals

The experiments were performed in a KRB solution of the following composition (mM): NaCl 135, KCl 5, NaHCO₃ 20, glucose 10, CaCl₂ 2.5, MgSO₄ 1.3, KH₂PO₄ 1.2 and EDTA 0.026 in H₂O. KRB solution containing 120 mM K⁺ was prepared by equimolar replacement of NaCl by KCl. Resveratrol, dimethylsulfoxide (DMSO), NOR, Ach, L-NAME and indo were obtained from Sigma (St. Louis, MO). Stock solution of 100 mM of resveratrol was made in DMSO, but was further diluted in distilled water (10 mM) before adding to the organ baths. Other stock solutions were made in water, except for indomethacin (dissolved in ethanol). The final concentration of DMSO or ethanol in the organ bath never surpassed 0.1 %.

IV.3.5 Statistics

The data were computed as means \pm S.E.M. and evaluated statistically using one-way ANOVA with Bonferroni post hoc test. Two groups of data were considered significantly different when $P < 0.05$. Contractions are expressed as mN contractions, relaxations as % decrease in precontractile tone. N is the number of tissues used.

IV.4 Results

IV.4.1 Influence of resveratrol on PVAT modulated contractility

Concentration-response curves to NOR in absence of indo and L-NAME showed cumulative contractions in both (-)PVAT and (+)PVAT segments (Fig. IV.2). (+)PVAT preparations displayed attenuated overall and maximal contractile responses to NOR which significantly

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differed from (-)PVAT controls. (E_{max} : 9.18 ± 0.67 mN for (-)PVAT vs. 5.33 ± 1.04 mN for (+)PVAT, $p < 0.05$) (Fig. IV.2a). In presence of resveratrol ($10 \mu\text{M}$, 15 min) the anticontractile effect of PVAT was only moderately observed (Fig. IV.2b). Pre-incubating the (-)PVAT preparations with resveratrol ($10 \mu\text{M}$, 15 min) significantly decreased maximal NOR responses (E_{max} : 9.18 ± 0.67 mN in absence vs. E_{max} : 5.76 ± 1.05 mN in presence of resveratrol, $p < 0.05$) (Fig. IV.2c). In contrast incubation with resveratrol ($10 \mu\text{M}$, 15 min) did not influence contractility of (+)PVAT segments (E_{max} : 5.33 ± 1.04 mN; pEC_{50} : 6.75 ± 0.22 in absence vs. E_{max} : 4.59 ± 0.67 mN; pEC_{50} : 6.56 ± 0.08 in presence of resveratrol, $p > 0.05$) (Fig. IV.2d).

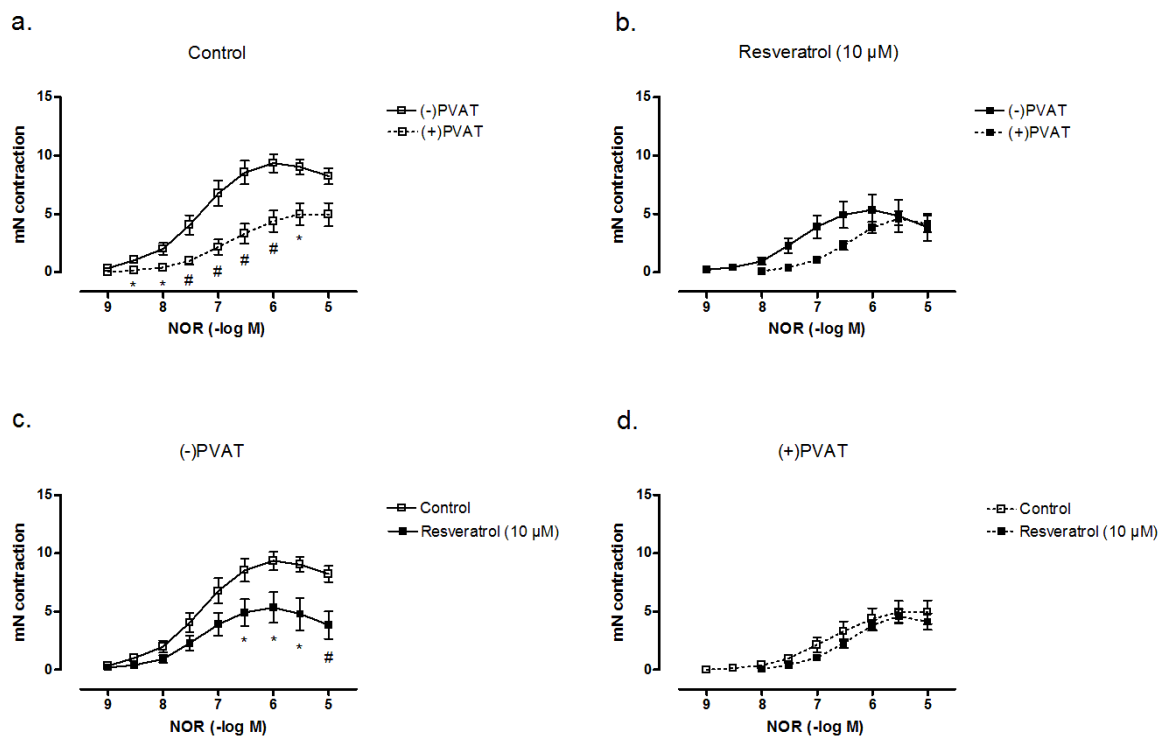


Figure IV.2 shows that norepinephrine (NOR, $1 \text{ nM} - 10 \mu\text{M}$) evoked concentration-dependent contractions in aorta without ((-)PVAT) and with ((+)PVAT) adherent adipose tissue. The presence of PVAT (a) significantly attenuated contraction responses to NOR in control conditions but not (b) in presence of resveratrol ($10 \mu\text{M}$, 15 min). Incubation with resveratrol ($10 \mu\text{M}$, 15 min) significantly decreased NOR responses in (c) (-)PVAT segments, but not in (d) (+)PVAT preparations. Data are expressed as mN contraction; one-way ANOVA with Bonferroni post hoc test; # $p < 0.01$, * $p < 0.05$; $n = 6-8$.

In presence of indo ($10 \mu\text{M}$, 20 min) and L-NAME (0.1 mM , 20 min), the contractions induced by NOR were more stable compared to the control conditions. In presence of both inhibitors (+)PVAT segments displayed significantly lower contraction responses to NOR compared to (-

)PVAT segments (pEC50: 7.87 ± 0.08 for (-)PVAT vs. 6.73 ± 0.13 for (+)PVAT, $p < 0.01$) (Fig. IV.3a). The maximal contractile response however was comparable for (-)PVAT (11.40 ± 1.45 mN) and (+)PVAT (11.21 ± 1.65 mN, $p > 0.05$) vessels. Pre-incubating the (-)PVAT preparations with resveratrol ($10 \mu\text{M}$, 15 min) significantly decreased maximal as well as overall NOR responses (E_{max} : 11.40 ± 1.45 mN; pEC50: 7.87 ± 0.08 in absence vs. E_{max} : 5.61 ± 1.11 mN; pEC50: 7.54 ± 0.11 in presence of resveratrol, $p < 0.05$) (Fig. IV.3b). In presence of indo ($10 \mu\text{M}$, 20 min) and L-NAME (0.1 mM , 20 min) incubation with resveratrol ($10 \mu\text{M}$, 15 min) did not modify the contractility of (+)PVAT segments (E_{max} : 11.21 ± 1.65 mN; pEC50: 6.73 ± 0.13 in absence vs. E_{max} : 9.82 ± 0.66 mN; pEC50: 6.60 ± 0.18 in presence of resveratrol, $p > 0.05$) (Fig. IV.3c).

Taken together, the presence of indo and L-NAME yielded similar differences in contractile responses to NOR between (-)PVAT and (+)PVAT segments with or without resveratrol as observed in absence of indo and L-NAME.

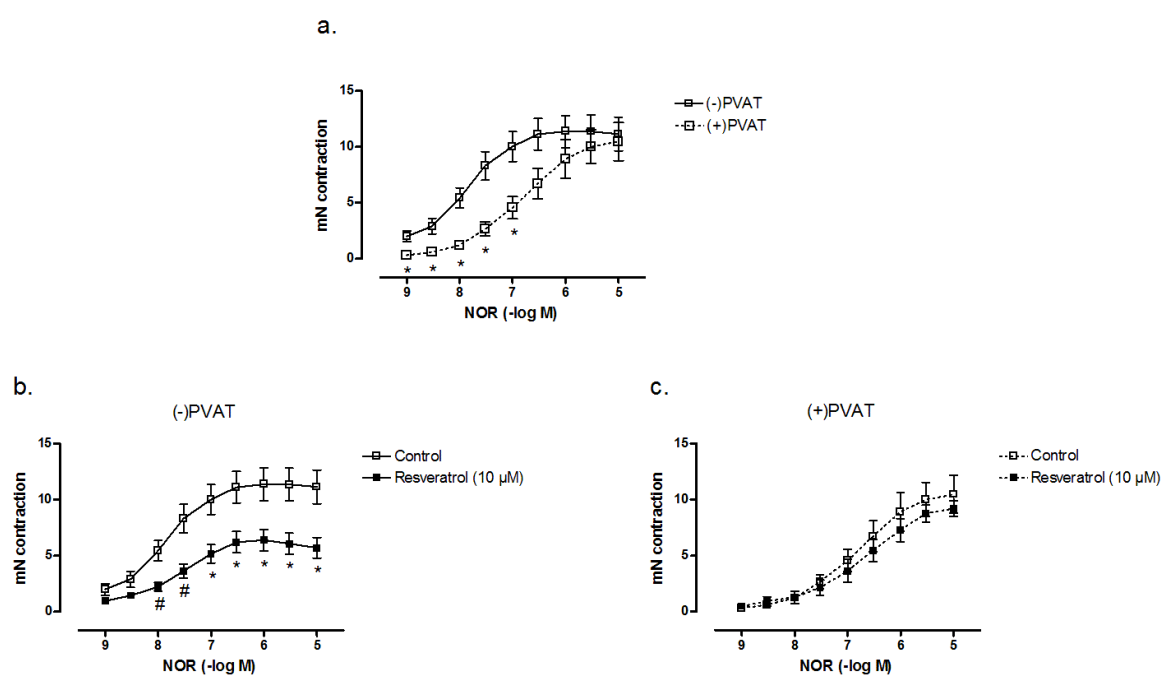


Figure IV.3 shows that norepinephrine (NOR, 1 nM – 10 μM) elicited concentration-dependent contractions in aorta without ((-)PVAT) and with ((+)PVAT) adherent adipose tissue in presence of indomethacin (indo, 10 μM , 20 min) and N ω -nitro-L-arginine methyl ester hydrochloride (L-NAME, 0.1 mM, 20 min). (a) The presence of PVAT significantly attenuated contraction responses to NOR. Incubation with resveratrol (10 μM , 15 min) significantly decreased NOR responses in (b) (-)PVAT segments, but not in (c) (+)PVAT preparations. Data are expressed as mN contraction; one-way ANOVA with Bonferroni post hoc test; # $p < 0.01$, * $p < 0.05$; $n = 6-7$.

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IV.4.2 Concentration-response curves of resveratrol in (-)PVAT and (+)PVAT preparations

In (-)PVAT segments resveratrol (10-100 μM , 15 min) evoked concentration-dependent relaxations, after incubation with indo (10 μM , 20 min) and L-NAME (0.1 mM, 20 min) (Fig. IV.4). However, in presence of PVAT resveratrol-induced relaxations were significantly reduced. The lowest concentration of resveratrol (10 μM) even induced contractions. In aortas with 50 % PVAT, resveratrol was able to evoke concentration-dependent relaxations, however these were clearly reduced compared to cleaned aortas. In contrast, when placing PVAT in the organ bath, but not in direct contact with the vascular smooth muscle cells (VSMCs), full response to resveratrol was seen (Fig. IV.4).

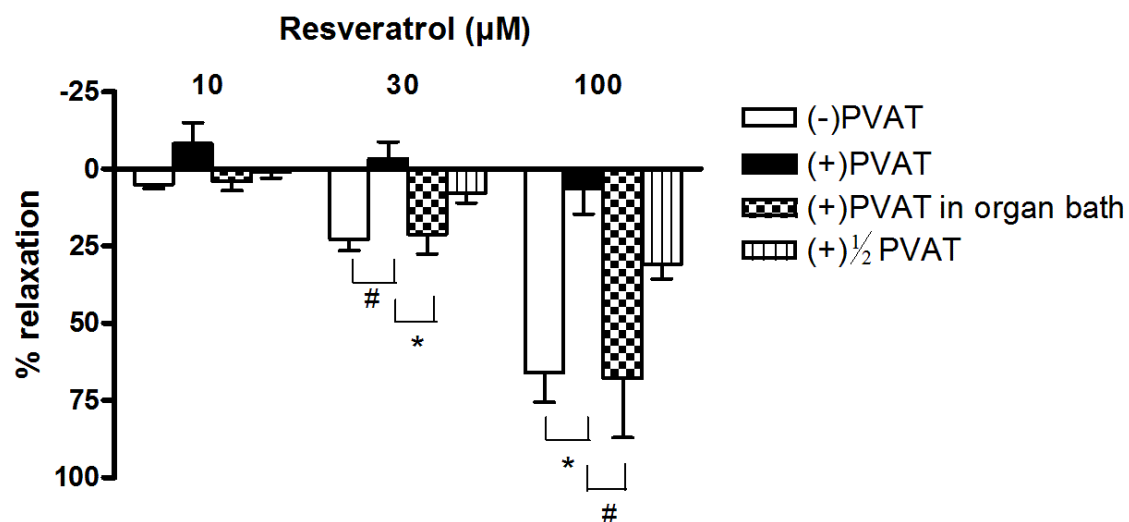


Figure IV.4 shows that after incubation with indomethacin (10 μM , 20 min) N ω -nitro-L-arginine methyl ester hydrochloride (0.1 mM, 20 min) resveratrol (10-100 μM , 15 min) evoked concentration-dependent relaxations in mice aorta without adipose tissue ((-)PVAT, but not in aorta surrounded by adipose tissue ((+)PVAT). In (+)PVAT preparations 10 μM resveratrol caused small contractions. Full response to resveratrol was seen, when placing adipose tissue in the organ bath, but not in direct contact with the vascular smooth muscle cells. In presence of 50 % adipose tissue ((+)½ PVAT), resveratrol elicited reduced relaxations. Data are expressed as % relaxation of NOR-induced tone; one-way ANOVA with Bonferroni post hoc test; # $p < 0.01$, * $p < 0.05$, $n = 6$.

IV.4.3 Influence of orchidectomy on PVAT modulated contractility.

Atrophy of seminal vesicles (Sham: 337.78 mg \pm 21.31 versus Orx: 44.86 mg \pm 4.29; $p < 0.01$; $n = 10$) indicated that orchidectomy was successfully performed. After pretreatment with indo (10 μM , 20 min) and L-NAME (0.1 mM, 20 min) NOR elicited concentration-dependent contractions in aortic tissues from all groups (Sham/(-)PVAT, Sham/(+)PVAT, Orx/(-)PVAT and

Orx/(+)PVAT) (Fig. IV.5). The presence of PVAT attenuated NOR responses in the sham-group (pEC_{50} : 8.17 ± 0.25 in absence vs. 6.24 ± 0.13 in presence of PVAT, $p < 0.01$) (Fig. IV.5a). In the Orx-group PVAT clearly shows a trend to decrease contractile responses, however this was only found significant at two concentrations (pEC_{50} : 7.94 ± 0.31 vs. 6.85 ± 0.38 , $p > 0.05$) (Fig. IV.5b). Maximal contractions were not influenced by the presence of PVAT neither in the sham-group (12.44 ± 2.13 mN in absence vs. 8.81 ± 1.06 mN in presence of PVAT, $p > 0.05$) nor in the Orx-group (12.39 ± 1.66 mN in absence vs. 7.81 ± 1.37 mN in presence of PVAT, $p > 0.05$) (Fig. IV.5a and IV.5b). Orchidectomy neither changed the contractility of the aorta (Fig. 4c), nor did it influence the anticontractile effect of PVAT (Fig. IV.5d).

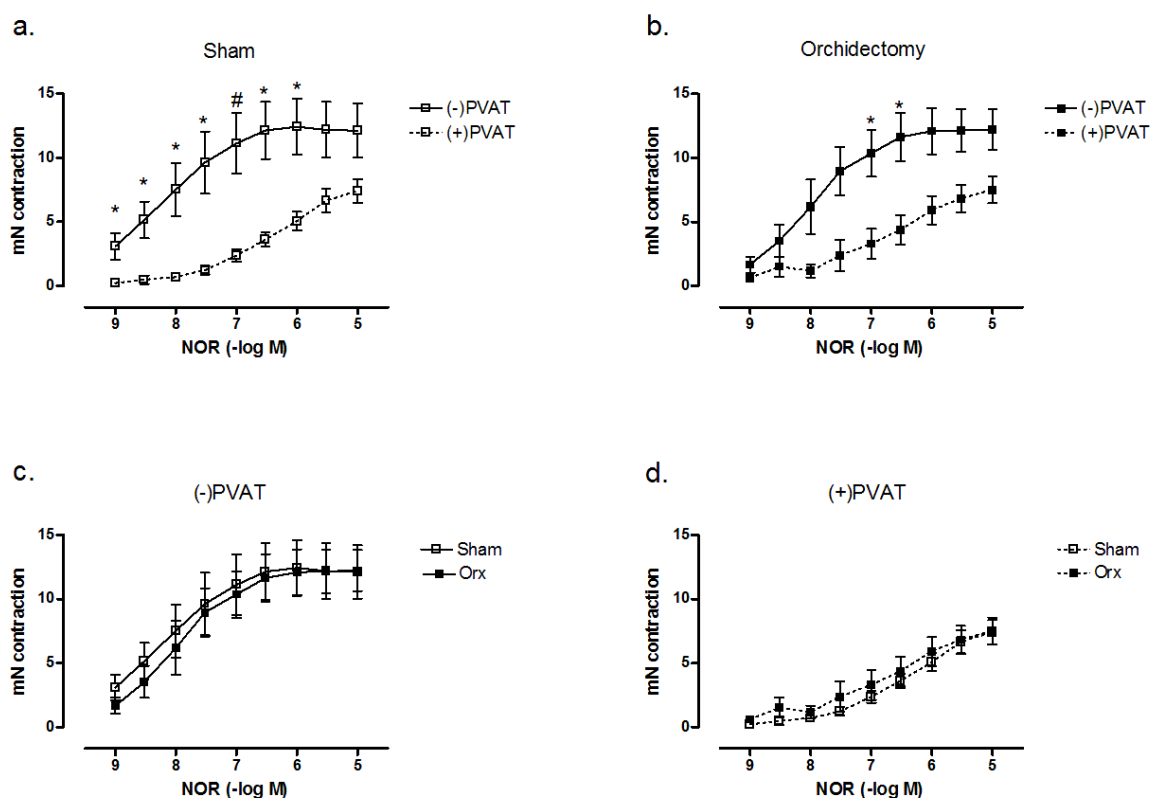


Figure IV.5 illustrates the effect of orchidectomy on contractions in response to norepinephrine (NOR 1 nM – 10 μ M) in the presence and absence of adipose tissue, after incubation with indomethacin (10 μ M, 20 min) N ω -nitro-L-arginine methyl ester hydrochloride (0.1 mM, 20 min). NOR elicited concentration-dependent contractions in aortic tissue of (a) sham and (b) orchidectomized (Orx) mice with ((+)PVAT) and without ((-)PVAT) adherent adipose tissue. (a, b) Both in the sham-group and the Orx-group PVAT attenuated responses to NOR. However in the Orx-group this was only found significant at two concentrations of NOR. Orchidectomy neither changed the contractility of the aorta (c), nor did it influence the anticontractile effect of PVAT (d). Data are expressed as mN contraction; one-way ANOVA with Bonferroni post hoc test; # $p < 0.01$, * $p < 0.05$; $n = 10$.

IV.5 Discussion

Since nowadays PVAT is recognized as a highly active endocrine and paracrine organ being able to influence vascular tone, it has become clear that functional PVAT is necessary to maintain vascular physiology [1]. Under normal physiological circumstances PVAT exerts a net beneficial anticontractile effect through the release of adipo(cyto)kines [1-5]. In the present study, aortas without PVAT also displayed a stronger contraction in response to NOR than aortas with adherent PVAT. These results confirm that PVAT indeed releases relaxation factors. As the relaxing effect of PVAT was still observed even in presence of indo and L-NAME, our results furthermore suggest that this anticontractile effect of PVAT is not mediated by COX products or NO, which is in line with others [3, 6]. However it should be noted that several studies did report a role for NO or COX products in the relaxing influence of adipose tissue [7, 25-27].

Resveratrol is a naturally occurring polyphenol, found in the skin of grapes and is thus abundant in red wine. Resveratrol is believed to contribute to the cardiovascular benefits associated with moderate red wine consumption (the 'French paradox') [17]. For example it has been reported that resveratrol exerts a direct relaxant effect in several vascular beds [28-31]. Indeed, also in our study resveratrol elicits concentration-dependent relaxations in mice aorta without PVAT, which are independent of K⁺ channels (own unpublished observations). Furthermore, in aortas without PVAT, incubation with resveratrol clearly attenuated NOR responses. This might explain the less pronounced anticontractile effect of PVAT after resveratrol treatment. This could be explained by resveratrol's direct relaxant effect on the smooth muscle cells of the arteries. Resveratrol has been reported to target NO [28, 30, 31] or COX products to exert its effect [32]. However in our study both can be excluded as possible mediators of resveratrol's relaxant effect, as even in presence of indomethacin and L-NAME resveratrol induced relaxations and attenuated NOR responses of aortas without PVAT.

Taken together, both PVAT and resveratrol thus independently elicit a protective effect on the vascular tone: PVAT through its anticontractile effect, resveratrol through its direct relaxant effect. Therefore it would be conceivable to think that in presence of both PVAT and resveratrol, an enhanced protective effect would be observed. Surprisingly, in aortas with

adherent PVAT resveratrol neither attenuated NOR responses, nor did it relax the aortas. Moreover, in aortas with 50 % PVAT, resveratrol only partly evoked relaxations. It thus seems that the presence of PVAT inhibits resveratrol's protective effect. Different hypotheses could explain this observation. First, the presence of adipose tissue surrounding the arteries could act as a structural barrier that limits resveratrol to reach the VSMCs. As resveratrol is lipophilic it is assumable that resveratrol would accumulate in PVAT and therefore be unable to induce relaxation of the VSMCs. However, our results indicate that this is not the case as in experiments where PVAT is present in the organ bath, but not in close contact with VSMCs, resveratrol relaxed arteries to a similar extent as arteries without PVAT. These results suggest that the presence of adipose tissue, does not impede resveratrol to reach the VSMCs to evoke relaxations. This is not surprising since the arteries are mounted in the organ bath with their lumen stretched. Hence compounds added to the organ bath, even in presence of PVAT, can easily reach the VSMCs through the lumen. In such way, resveratrol could still directly relax VSMCs, while it does not necessarily has to pass through the PVAT to reach the VSMCs.

Second, PVAT could release factors which inactivate resveratrol. Besides secretion of anti-inflammatory adipo(cyto)kines PVAT also releases several pro-inflammatory adipo(cyto)kines such as TNF_{α} and IL-6 [1, 8]. It is known that TNF_{α} and IL-6 can reduce the vasorelaxing influence of PVAT due to increased reactive oxygen species (ROS) production [7]. Moreover it has been reported that in adipocytes oxidative stress, as a result from increased ROS production, can be reduced or restored by resveratrol [33, 34]. Though, the amount of oxidative stress could be too high for resveratrol to cope with, so that resveratrol no longer exerts its protective effect. However increased ROS production seems to be unlikely as tissue from 'healthy' mice was used which in resting conditions is protected against oxidative stress by antioxidants.

Third, perhaps one has to consider the relationship between PVAT and resveratrol's action in an inverse way as assumed above. Indeed the presence of PVAT seems to limit resveratrol's relaxing effect, but maybe one should consider the idea that also resveratrol might limit the protective effect of PVAT. In this perspective, it is known that resveratrol can influence the secretion of adipo(cyto)kines released from adipose tissue [10-16]. For instance resveratrol decreases the production of visfatin and leptin [11, 13] two adipo(cyto)kines able to cause

vasorelaxation [1]. So, considering that resveratrol can decrease the secretion of some vasodilatory adipo(cyto)kines, this would mean that the relaxing influence of PVAT, caused by vasodilatory adipokines, would partly be limited by resveratrol. This in turn would cause an increased contractility and would eventually result in an upward shift of the NOR response curve in arteries with PVAT. However, since resveratrol also has a direct pronounced relaxing effect on arteries, this upward shift of the NOR response curve is counteracted by resveratrol's direct action on the VSMCs. In summary, resveratrol can act on two levels (i) decreasing the relaxing influence of PVAT by diminishing the secretion of vasodilatory adipo(cyto)kines, and thus causing an upward shift of the NOR response curve and (ii) a relaxing effect on the arteries, causing a downward shift of the NOR response curve. Taken together, the net effect of resveratrol on the contractility of aortas with adherent adipose tissue will be zero. Hence the display of a NOR response curve similar to those from aortas with PVAT without resveratrol. This third hypothesis, albeit complicated, would explain all of our results, including the small contractions observed with resveratrol 10 μ M in presence of PVAT. In this case the concentration of resveratrol was too low to elicit arterial relaxations, but sufficient to inhibit vasodilatory adipo(cyto)kines. This leads to imbalanced release of vasodilatory and vasocontractile adipo(cyto)kines, favouring secretion of the latter ones, without resveratrol being able to counteract this effect by eliciting direct arterial VSMCs relaxation. The net effect in this case is therefore a small contraction.

Our results suggest that, with respect to adipose tissue functioning, acute resveratrol administration decreases the relaxing influence of PVAT. However it has been shown that long-term treatment with resveratrol can improve adipose tissue function [13, 35, 36]. It is important to mention that in these studies, in contrast to our study, the ability of resveratrol to improve adipose tissue function (i) was investigated in obesity-mimicking conditions and (ii) did not focus on the function of PVAT. However, it cannot be excluded that the overall neutral effect of acute resveratrol administration in presence of PVAT is shifted to a protective effect when administered chronically.

Besides resveratrol also testosterone has been reported to influence the levels of some adipo(cyto)kines such as adiponectin and leptin [22-24]. Moreover androgen deprivation therapy, as treatment of prostate cancer, is a risk of coronary heart disease, diabetes and cardiovascular death [37]. In animal models castration accelerates aortic plaque build-up

[38-40] and can increase vasoreactivity [41]. Furthermore low testosterone levels promote an increase in fat deposition and adipocyte number [42]. As testosterone depletion plays a role in the impairment of vascular function and induces changes in the adipose tissue, it is not surprising to assume that orchidectomy could modulate the anticontractile capacity of PVAT resulting in a higher contractility of arteries. However, our study suggests that 4 weeks of testosterone depletion by orchidectomy, does not affect the protective influence of PVAT. It should be noted that, changes in adipose tissue by low testosterone levels are often displayed in white or visceral adipose tissue [42-44]. The fact that thoracic aorta is in contrast surrounded by brown adipose tissue [45], could explain the inability of orchidectomy to influence anticontractile effect of PVAT.

In conclusion it was shown that orchidectomy does not modulate the vasorelaxing influence of PVAT while resveratrol treatment might decrease the anticontractile effect of PVAT. Furthermore our results indicate that the positive effects associated with resveratrol addition are neutralized by the presence of PVAT. The exact underlying mechanism remains unclear but we hypothesize that this might be the result of resveratrol's dual effect (i) inhibition of the release/influence of vasodilatory adipo(cyto)kines and in the meantime (ii) a direct relaxant effect on the VSMCs. Overall resveratrol thus causes a net unchanged contractility of the mice thoracic aorta surrounded by PVAT.

IV.6 Acknowledgments

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Resveratrol, orchidectomy and PVAT

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Chapter V

Relaxant and antioxidant capacity of the red wine polyphenols, resveratrol and quercetin, on isolated mice corpora cavernosa

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V.1 Abstract

INTRODUCTION. The red wine polyphenols resveratrol and quercetin are known for their vasorelaxant and antioxidant capacity which is assumed to rely on the activation of the NO/sGC pathway. Vasodilators as well as antioxidants can regulate penile erection and be beneficial for the treatment of erectile dysfunction (ED).

AIMS. The goal of this study was to evaluate the NO/sGC dependency of the relaxant effect of resveratrol and quercetin on mice aorta and corpora cavernosa as well as to explore their influence on oxidative stress-induced ED.

METHODS. Isolated mice aorta and corpora cavernosa were mounted for isometric tension recordings into organ baths. Cumulative concentration-response curves were constructed for resveratrol and quercetin in the absence/presence of inhibitors of the NO/sGC pathway. In addition, in corpora cavernosa the effect of resveratrol and quercetin was studied on NO-mediated relaxations using acetylcholine (Ach), sodium nitroprusside (SNP) and electrical field stimulation (EFS). In certain experiments corporal tissues were exposed to oxidative stress using palmitic acid (PA, 0.5 mM).

MAIN OUTCOME MEASURES. Corporal responses to resveratrol and quercetin were measured in the presence/absence of inhibitors of different molecular pathways. The effect of resveratrol and quercetin incubation on Ach-, SNP- or EFS-mediated responses was explored in presence/absence of PA.

RESULTS. While both polyphenols are potent vasodilators of mice aorta, only resveratrol relaxes mice corpora cavernosa. The relaxation response to resveratrol on aorta was diminished in sGC $\alpha_1^{-/-}$ mice, but not on corpora cavernosa. The polyphenols did not influence Ach-, SNP- or EFS-mediated relaxations as such. Resveratrol, but not quercetin, was able to significantly reverse PA-induced decrease of EFS relaxations.

CONCLUSION. The red wine compound resveratrol, but not quercetin, relaxes isolated mice corpora cavernosa concentration-dependently through mechanisms independent of the

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NO/sGC pathway. Resveratrol is a more potent antioxidant than quercetin being able to restore decreased neuronal NO responses in mice corpora cavernosa.

Keywords. Corpora cavernosa; resveratrol; quercetin; antioxidant; erectile function

V.2 Introduction

Resveratrol and quercetin are naturally occurring polyphenols present in the skin of grapes and are thus abundant in red wine. These compounds are thought to contribute to the cardiovascular benefits associated with moderate red wine consumption (often referred to as 'the French paradox') [1, 2]. Both resveratrol and quercetin are known to relax arteries in different vascular beds, mainly by activation of the NO/sGC pathway [3-7]. However some part of the relaxation induced by these polyphenols occurs NO/sGC-independent, through activation of different types of K⁺ channels [7-10]. Several studies showed that, besides their vasorelaxant effect, resveratrol and quercetin can reduce oxidative stress [11-17]. This could be of interest in the field of ED as several pathophysiological conditions (diabetes, hypercholesterolemia and atherosclerosis), which are linked to enhanced oxidative stress due to elevated ROS levels [18], show a higher prevalence of ED [18, 19]. Nowadays there is growing evidence for the role of ROS in the aetiology of ED as the interaction of superoxide anion with NO impairs cavernosal smooth muscle cell relaxation [18, 20]. Thus elimination of ROS using antioxidants may have a beneficial therapeutic effect on ED [18, 19]. Considering that vasodilators and antioxidants possess pro-erectile effects, resveratrol and quercetin may be useful for the treatment of ED.

V.3 Aim

The aim of this study was to evaluate the extent of NO/sGC dependency in the relaxant capacity of resveratrol and quercetin on isolated mice corpora cavernosa. In addition the antioxidant capacity of both polyphenols was explored in presence and absence of palmitic acid (PA).

V.4 Materials and methods

V.4.1 Animals

All experiments were performed on adult (8-12 weeks) male Swiss mice (n=143), obtained from Janvier (Saint-Berthevin, France) or on 129SvEvS7 sGC wild type (n=15, sGC $\alpha_1^{+/+}$) or sGC alpha1 knock-out (n=15, sGC $\alpha_1^{-/-}$) mice. These 129SvEvS7 mice were bred in the SPF facility of the Inflammation Research Center, VIB, Ghent, Belgium [21]. Food and water was provided ad libitum and all animals were treated in accordance with the Guide for the Care

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and Use of Laboratory Animals published by the US National Institutes of Health. This study was approved by the local Ethical Committee for Animal Experiments, Faculty of Medicine and Health Sciences, Ghent University, Belgium.

V.4.2 Tissue preparation

After cervical dislocation, thoracic aorta was carefully dissected and corpora cavernosa were separated from each other and excised at the base. Tissues were kept in cooled Krebs Ringer bicarbonate solution (KRB). Ring segments of the aorta were mounted in a wire myograph organ bath filled with 10 mL KRB solution. Two stainless steel wires (40 μM diameter) were guided through the lumen of the segments. In order to measure changes in isometric tension, one wire was fixed to a force-displacement transducer and the other was connected to a micrometer. The corpora cavernosa were mounted horizontally for isometric tension measurements in 10 mL myograph chambers, containing KRB solution. Changes in isometric force of the corpora cavernosa were recorded as one end was fixed to a force displacement transducer and the other to a micrometer. After mounting, the tissues were allowed to equilibrate for 30 minutes in KRB solution that was frequently replaced (37°C, pH 7.4; bubbled with 95 % O_2 –5 % CO_2). Next, the aorta and the corpora cavernosa were gradually stretched until a stable preload of 0.5 g (aorta) and 0.45 g (corpora cavernosa) was obtained and allowed to equilibrate during 30 or 60 minutes. At the end of the equilibration period aortic rings and corpora cavernosa were repeatedly activated in order to obtain maximal and stable contractions and relaxations. Aortic rings were activated using 120 mM K^+ and 5 μM norepinephrine (NOR), corpora cavernosa were repeatedly activated using two times 5 μM NOR. Thereafter the tissues were washed and allowed to relax to the basal tension before starting the actual protocol. The tissues were precontracted with 5 μM NOR and after obtaining a stable contraction, 1 μM (corpora cavernosa) or 10 μM (aorta) acetylcholine (Ach) was added to evaluate the functionality of the endothelium. Only tissues that relaxed more than 50 % to Ach, were included in this study.

V.4.3 Experimental protocol

Direct relaxant effect of resveratrol and quercetin

Cumulative concentration-response curves to resveratrol or quercetin (10-100 μM , 15 min) were obtained in corpora cavernosa or aorta precontracted with 5 μM NOR. In parallel, responses to resveratrol were examined in the presence/absence of inhibitors of different molecular pathways.

Antioxidant capacity

In another set of experiments the effect of resveratrol or quercetin was studied on NO-mediated relaxations in corpora cavernosa. In the absence and presence of resveratrol, quercetin (100 μM , 15 min) or tempol (100 μM , 20 min) concentration-response curves to Ach or sodium nitroprusside (SNP) were constructed or electrical field stimulation (EFS; parameters: train duration 40 s; 1, 2, 4 and 8 Hz; pulse duration 5 ms and 80 V) was applied to the corpora cavernosa. In order to obtain comparable precontraction levels between control conditions and resveratrol conditions, the precontractile tone of the resveratrol-treated tissues was adjusted by adding 10 μM NOR instead of 5 μM NOR. In some experiments acute oxidative stress was induced by pretreating the corpora cavernosa with palmitic acid (PA, 0.5 mM, 30 min) or its solvent ethanol before constructing Ach-, SNP-, or EFS curves. Between the response-curves, the corpora cavernosa were washed and allowed to recover for 20-30 min.

V.4.4 Drugs and chemicals

The experiments were performed in a KRB solution of the following composition (mM): NaCl 135, KCl 5, NaHCO_3 20, glucose 10, CaCl_2 2.5, MgSO_4 1.3, KH_2PO_4 1.2 and EDTA 0.026 in H_2O . KRB solutions containing 30 mM (K_{30}) and 120 mM K^+ (K_{120}) were prepared by equimolar replacement of NaCl by KCl. Resveratrol, quercetin, dimethylsulfoxide (DMSO), norepinephrine (NOR), acetylcholine (Ach), sodium nitroprusside (SNP), 1 H-[1, 2, 4]oxadiazolo[4,3-A]quinoxalin-1-one (ODQ), N ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME), tetraethylammoniumchloride (TEA), glibenclamide (Glib), 4-aminopyridine (4-AP), palmitic acid (PA), tempol, indomethacin (indo), dihydrochloride hydrate (H-89), 8-(p-sulfophenyl)-theophylline (8-SPT) and zinc protoporphyrin IX (ZnPPiX) were obtained from Sigma (St. Louis, MO). Stock solutions of 100 mM of resveratrol and quercetin were made in

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DMSO, but were further diluted in distilled water (10 mM) before adding to the organ baths. Other stock solutions were made in water, except for ODQ and PA (dissolved in ethanol), Glib (dissolved in DMSO). The final concentration of DMSO in the organ bath never surpassed 0.1 %.

V.4.5 Statistics

The data were computed as means \pm S.E.M. and evaluated statistically using the Mann-Whitney U test, Wilcoxon test or repeated measures ANOVA with Bonferroni post hoc test, when appropriate. Two groups of data were considered significantly different when $P < 0.05$. Relaxations are expressed as % decrease in precontractile tone. N is the number of tissues used.

V.5 Main outcome measures

Organ bath experiments demonstrate that resveratrol but not quercetin relaxes isolated corpora cavernosa in a concentration-dependent manner through mechanisms independent of NO/sGC. Resveratrol is more potent than quercetin to reverse the PA-induced impairment of neuronal NO-mediated responses.

V.6 Results

V.6.1 Effect of resveratrol and quercetin on precontracted mice aorta and corpora cavernosa

Resveratrol as well as quercetin (10 nM - 100 μ M, 15 min) relaxed Swiss mice aorta (Fig. V.1A and V.1B). Time and vehicle control aortic segments showed a spontaneous, nonvehicle related steady further increase in tone (Fig. V.1A and V.1B). Contrary in corpora cavernosa, only resveratrol was able evoke concentration-dependent relaxations. Although time and vehicle controls demonstrate the presence of a spontaneous loss of tone, resveratrol (10 μ M and 100 μ M) still induced significantly greater corporal relaxations compared to vehicle and time controls (Fig. V.1C). To elucidate the mechanisms involved in resveratrol-induced corporal relaxations, responses to resveratrol (10 - 100 μ M) were further studied in the presence of inhibitors of specific molecular pathways. As quercetin did not evoke significant corporal relaxations (Fig.V.1D), no further efforts were made examining the effects of inhibitors.

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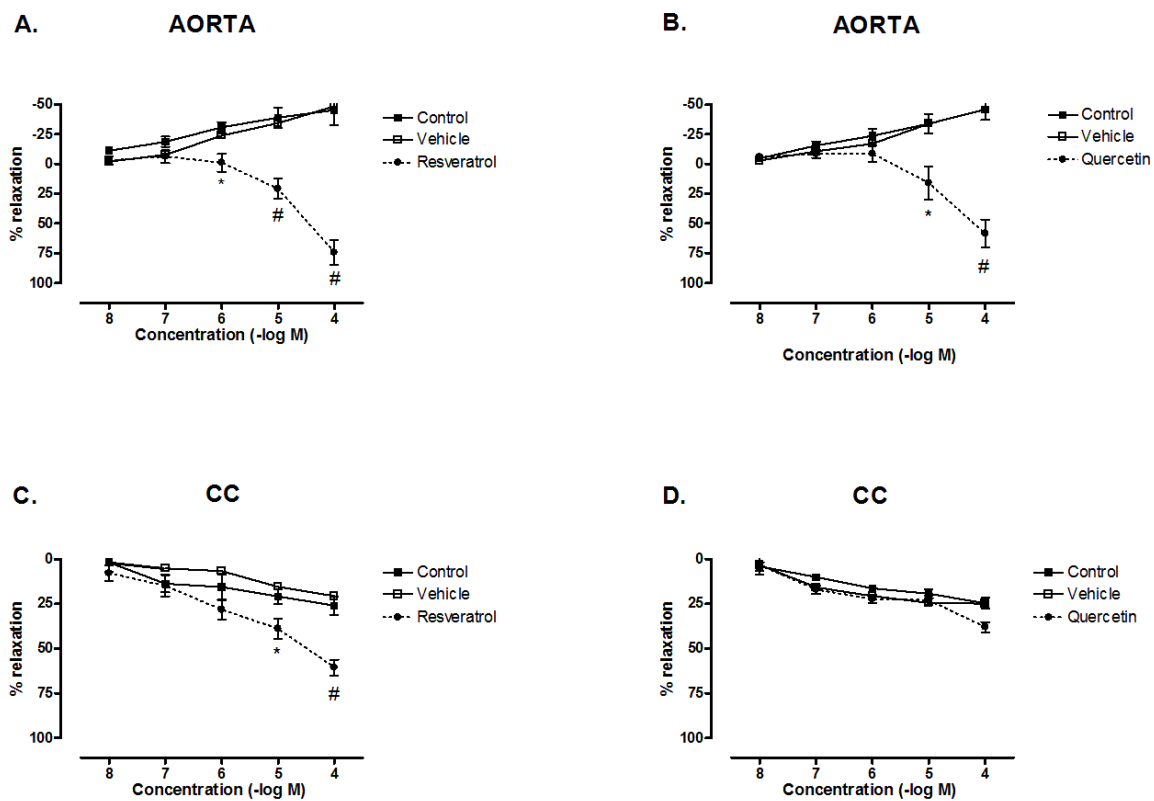


Figure V.1 shows the effect of resveratrol (10 nM-100 μ M, 15 min) and quercetin (10 nM-100 μ M, 15 min) on mice corpora cavernosa (CC) and aorta versus time and vehicle controls. (A, B) indicate that (A) resveratrol as well as (B) quercetin evoke concentration-dependent relaxations in aorta after precontraction with norepinephrine (NOR) 5 μ M. (C) shows that resveratrol elicits concentration-dependent relaxations of mice corpora cavernosa after precontraction with NOR 5 μ M, whilst (D) quercetin does not cause significant relaxations. Data are expressed as % relaxation of the NOR-induced tone; Mann-Whitney U test; # p <0.01 * p <0.05; n =7-8.

V.6.2 Involvement of NO/sGC pathway

The involvement of the NO/sGC pathway in the vasodilatory effect of quercetin and resveratrol on aorta and in the relaxant effect of resveratrol on corpora cavernosa, was evaluated using aorta and corpora cavernosa from sGC $\alpha_1^{-/-}$ mice. Quercetin- and resveratrol-induced relaxations in aorta from sGC $\alpha_1^{-/-}$ mice were both significantly diminished compared to sGC $\alpha_1^{+/+}$ controls (Fig. V.2A and V.2B), indicating the NO/sGC dependency of the polyphenols relaxant effect in aorta. In contrast, resveratrol-induced relaxations of corpora cavernosa from sGC $\alpha_1^{-/-}$ mice were not decreased compared to sGC $\alpha_1^{+/+}$ controls (Fig. V.2C). In Swiss mice, the presence of the sGC inhibitor ODQ (10 μ M, 10 min), or the NOS-inhibitor L-NAME (100 μ M, 20 min) did not significantly influence resveratrol-induced corporal

relaxations. L-NAME only significantly reduced resveratrol relaxation induced at a concentration of 10 μM (Fig. V.3A and V.3B).

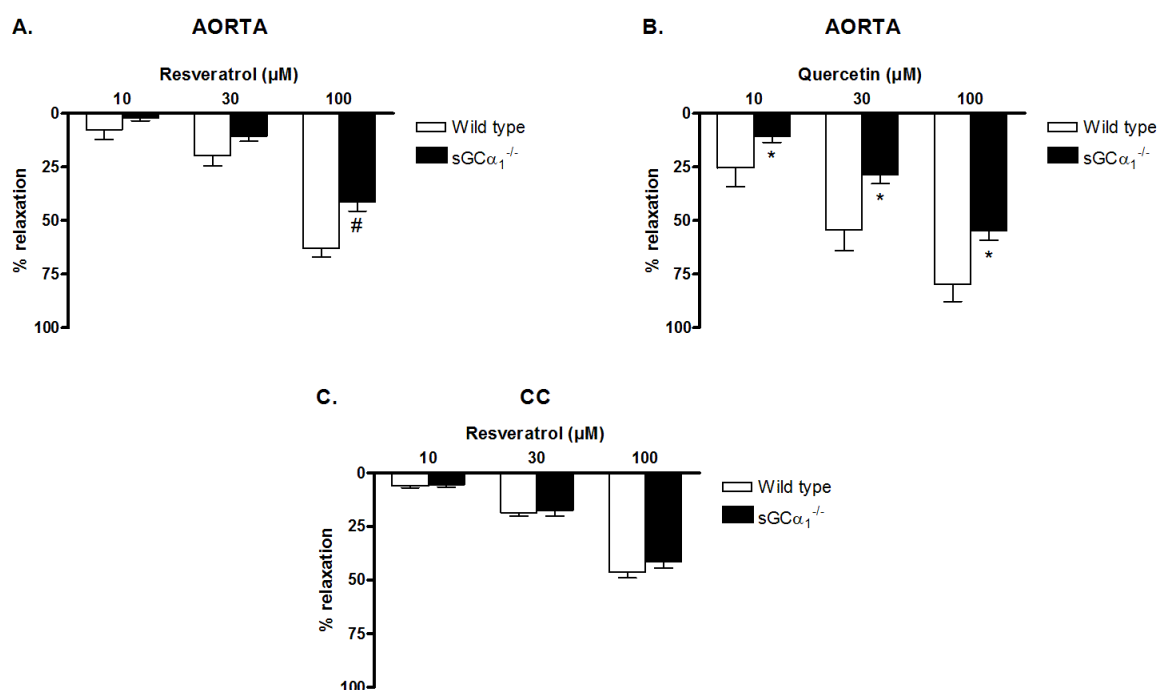


Figure V.2. indicates the effect of resveratrol (10–100 μM , 15 min) and quercetin (10–100 μM , 15 min) on aorta and corpora cavernosa (CC) from $s\text{GC}\alpha_1^{-/-}$ mice after precontraction with norepinephrine 5 μM . (A, B) show that (A) resveratrol- as well as (B) quercetin-induced relaxations in aorta are significantly decreased in aorta from $s\text{GC}\alpha_1^{-/-}$ mice. In contrast resveratrol-induced corporal relaxations in corpora cavernosa from $s\text{GC}\alpha_1^{-/-}$ mice did not differ from control segments (C). Data are expressed as % relaxation of the NOR-induced tone; Mann-Whitney U test; # $p < 0.01$ * $p < 0.05$; $n = 6-15$.

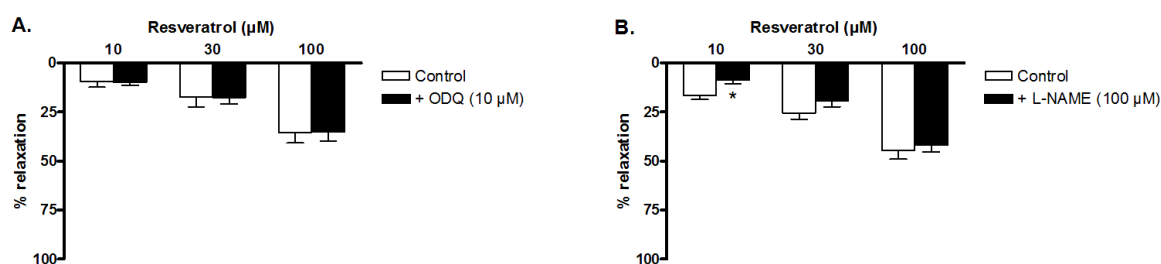


Figure 3. illustrates that resveratrol (10–100 μM , 15 min) causes concentration-dependent relaxations in precontracted (NOR 5 μM) mice corpora cavernosa that is similar in presence and absence of (A) ODQ (10 μM , 10 min) or (B) L-NAME (100 μM , 20 min). Incubation with L-NAME (100 μM , 20 min) slightly inhibited corporal relaxations induced by resveratrol 10 μM but not by resveratrol 30 μM or 100 μM (B). Data are expressed as % relaxation of the NOR-induced tone; Mann-Whitney U test; * $p < 0.05$; $n = 8-10$.

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V.6.3 Involvement of other possible mediators

To study the involvement of potassium channels, the relaxation effect of resveratrol was examined in corporal tissues precontracted using 5 μM NOR in combination with a high K^+ (30 or 120 mM) solution. However the presence of these higher K^+ concentrations did not alter the corporal responses to resveratrol (Table V.1). In addition, the resveratrol-induced corporal relaxations remained similar in the presence and absence of the non-selective K^+ channel blocker TEA (3 mM, 15 min) as well as the selective K^+ channel blockers such as the ATP-sensitive K^+ channel blocker (K_{ATP}) Glib (3 μM , 10 min) or the voltage-dependent K^+ channel blocker (K_{V}) 4-AP (3 mM, 20 min) (Table V.1). The involvement of other mediators was explored by pretreating the corpora cavernosa with the COX inhibitor, indomethacin (10 μM , 20 min), heme oxygenase inhibitor, ZnPPiX (10 μM , 60 min), the adenosine receptor antagonist, 8-SPT (100 μM , 10 min) or the cAMP-dependent protein kinase inhibitor, H-89 (10 μM , 20 min). None of these inhibitors altered the relaxant effect of resveratrol on mice corpora cavernosa.

Table V.1. % Relaxation (\pm SEM) induced by 10, 30 and 100 μ M resveratrol in mice corpora cavernosa in absence and presence of several inhibitors of molecular pathways.

	Resveratrol 10 μ M	Resveratrol 30 μ M	Resveratrol 100 μ M
Control	13.7 \pm 3.7	27.0 \pm 6.9	41.4 \pm 7.8
+ K₁₂₀	13.3 \pm 3.1	23.6 \pm 10.4	38.1 \pm 11.4
Control	12.2 \pm 5.6	27.0 \pm 10.3	45.0 \pm 10.0
+ K₃₀	8.6 \pm 2.3	17.4 \pm 6.6	42.3 \pm 7.9
Control	11.4 \pm 2.1	28.3 \pm 4.1	36.8 \pm 6.7
+ TEA (3 mM)	10.7 \pm 3.3	26.6 \pm 7.3	40.3 \pm 9.9
Control	9.5 \pm 3.0	18.1 \pm 3.8	37.7 \pm 7.1
+ Glib (3 μM)	15.2 \pm 4.2	23.3 \pm 5.2	41.9 \pm 6.7
Control	13.9 \pm 3.1	28.6 \pm 5.1	52.0 \pm 5.0
+ 4-AP (3 mM)	14.9 \pm 7.7	31.8 \pm 4.3	61.8 \pm 4.1
Control	4.8 \pm 4.9	22.2 \pm 5.6	53.3 \pm 6.2
+ Indo (10 μM)	12.1 \pm 4.5	27.8 \pm 7.6	44.2 \pm 10.8
Control	18.3 \pm 5.9	39.7 \pm 7.4	67.4 \pm 11.9
+ ZnPPIX (10 μM)	11.4 \pm 5.6	33.8 \pm 11.1	57.6 \pm 7.3
Control	18.7 \pm 4.2	28.6 \pm 5.9	38.4 \pm 4.5
+ 8-SPT (100 μM)	12.4 \pm 2.2	20.4 \pm 4.1	37.9 \pm 5.1
Control	10.5 \pm 2.4	19.0 \pm 3.6	39.1 \pm 4.0
+ H-89 (10 μM)	15.6 \pm 2.1	24.9 \pm 3.0	38.6 \pm 3.3

K₁₂₀, Krebs-Ringer bicarbonate solution with 120 mM K⁺; K₃₀, Krebs-Ringer bicarbonate solution with 30 mM K⁺, TEA, tetraethylammoniumchloride; Glib, glibenclamide; 4-AP, 4-aminopyridine; Indo, indomethacin; ZnPPIX, zinc protoporphyrin IX; 8-SPT, 8-(p-sulfophenyl)-theophylline; H-89, dihydrochloride hydrate

V.6.4 Effect of resveratrol and quercetin on NO-mediated relaxation of corpora cavernosa

Ach and SNP induced concentration-dependent relaxations of Swiss corpora cavernosa preparations (Fig. V.4A-V.4F). These relaxations were not or only to a low extent influenced by preincubation with resveratrol (Fig. V.4A and V.4B), quercetin (100 μ M, 15 min) (Fig. V.4C and 4D) or PA (0.5 mM, 30 min) (Fig. V.4E and V.4F).

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The effect of neuronal NO was examined by stimulating the nerves with EFS. EFS relaxed corpora cavernosa in a frequency-dependent manner (Fig. V.5A-V.5E). These relaxations were not influenced by the presence of resveratrol or quercetin (100 μ M, 15 min) (Fig. V.5A and V.5C). Pretreatment with PA (0.5 mM, 30 min) significantly reduced EFS evoked relaxations (Fig. V.5E). Co-administration of quercetin showed a clear trend in reversing PA-decreased EFS relaxations (Fig. V.5D). Co-administration of resveratrol however completely restored PA-induced impaired EFS responses to the level of controls (Fig. V.5B). In comparison, pretreatment with a known antioxidant, tempol, only partly reversed PA-impaired EFS relaxations (Fig. V.5F).

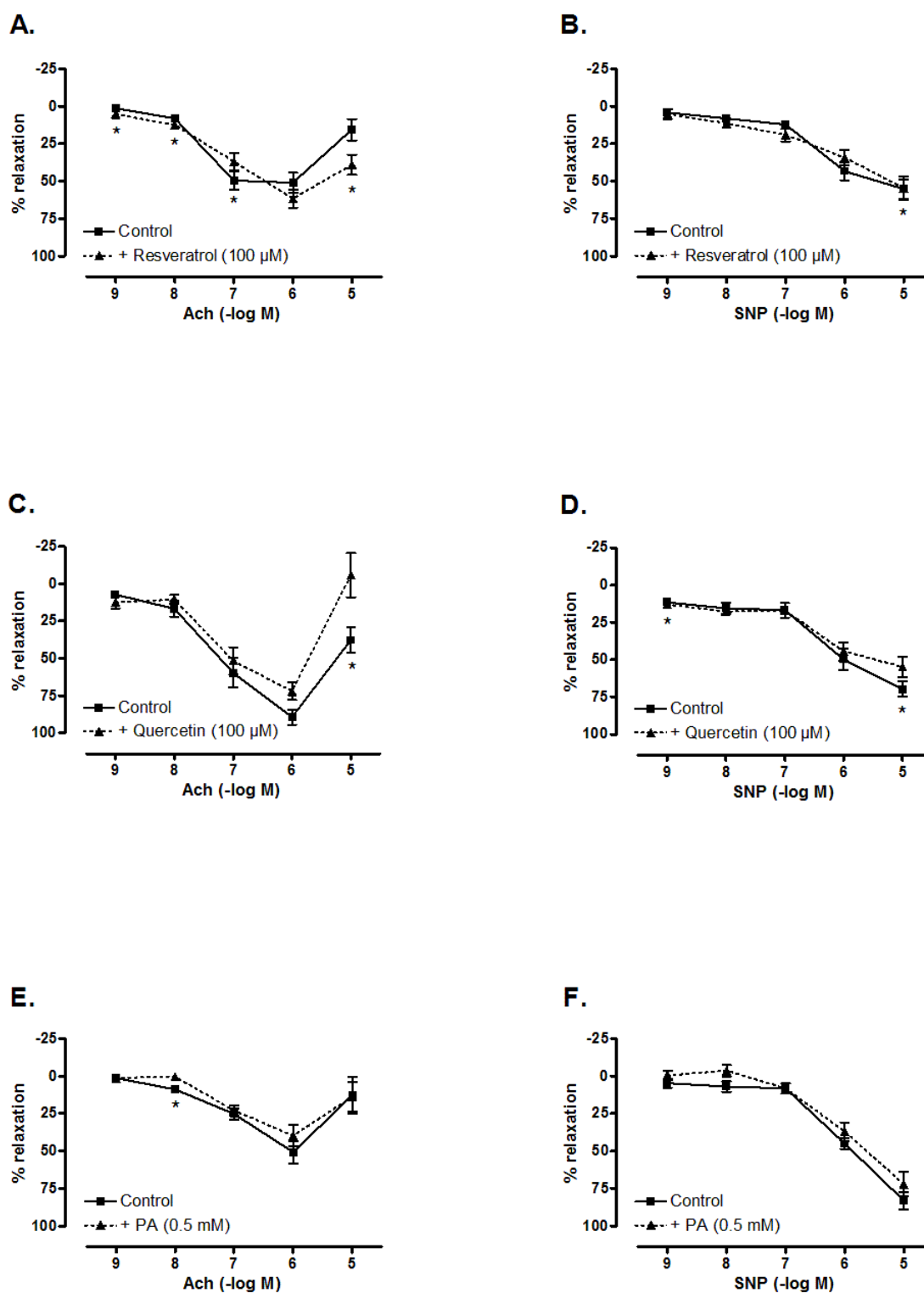


Figure V.4 demonstrates that Ach and SNP (1 nM-10 μ M) evoke concentration-dependent relaxations of precontracted (NOR 5 μ M or 10 μ M (resveratrol)) mice corpora cavernosa. Incubation with (A, B) resveratrol (100 μ M, 15 min), (C, D) quercetin (100 μ M, 15 min) or (E, F) PA (0.5 mM, 30 min) did not or hardly influence Ach- or SNP-induced corporal relaxations. Data are expressed as % relaxation of the NOR-induced tone; Wilcoxon test; * $p < 0.05$; $n = 6-9$.

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Relaxant and antioxidant capacity of RWPs on mice corpus cavernosum

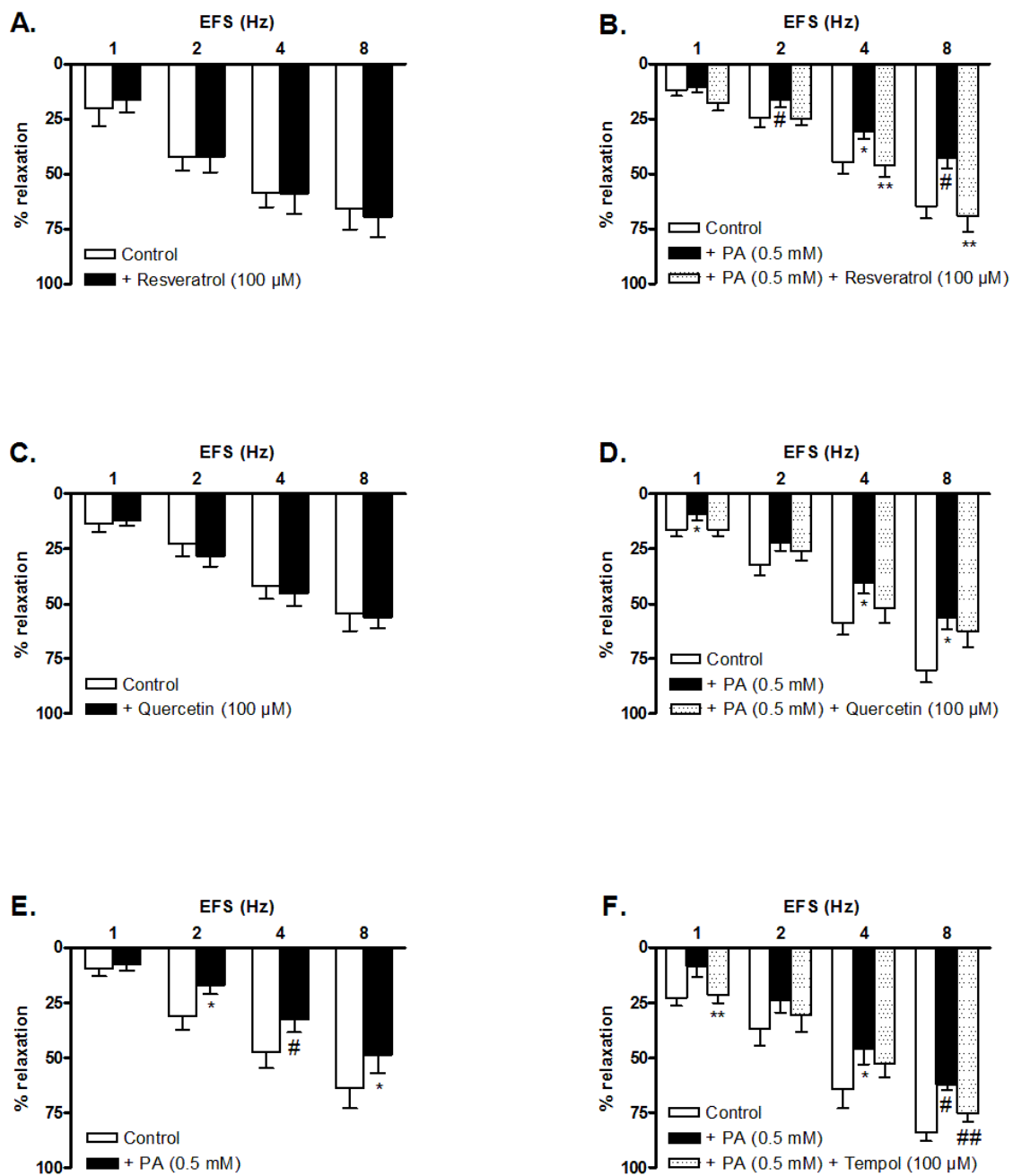


Figure V.5. shows the effect of electrical field stimulation (EFS; parameters: train duration 40s; 1, 2, 4 and 8 Hz; pulse duration 5 ms and 80V) on precontracted (NOR, 5 μM or 10 μM (resveratrol)) mice corpora cavernosa. EFS induced frequency-dependent corporal relaxations, which were not influenced by the presence of (A) resveratrol or (C) quercetin (100 μM, 15 min). Incubation with PA (0.5 mM, 30 min) significantly reduced EFS evoked relaxations. Co-administration of PA (0.5 mM, 30 min) with (B) resveratrol (100 μM, 15 min) but not (D) quercetin (100 μM, 15 min) significantly reversed PA diminished EFS responses, whereas co-administration of PA with (F) tempol (100 μM, 20 min) only partly restored PA-impaired EFS relaxations. Data are expressed as % relaxation of the NOR-induced tone; (A,C,E) Wilcoxon test; * $p < 0.05$; $n = 7-8$; (B,D,F) repeated measures ANOVA with Bonferroni post hoc test; # $p < 0.01$ * $p < 0.05$ (as compared to controls); ## $p < 0.01$ ** $p < 0.05$ (as compared to PA treatment); $n = 8-9$.

V.7 Discussion

The main finding of this study is twofold: (i) Resveratrol but not quercetin relaxes isolated mice corpora cavernosa in a concentration-dependent manner through mechanisms independent of NO/sGC. (ii) Resveratrol is more potent than quercetin in reversing PA-induced decrease in nNOS responses.

As resveratrol and quercetin are believed to contribute to the cardiovascular benefits of moderate red wine consumption, numerous studies have shown that both polyphenols can induce vasorelaxation [3-10]. Even in corporal tissues positive effects to resveratrol and quercetin administration have been reported [22-27]. However, this study is the first to report a relaxant capacity of resveratrol on isolated mice corpora cavernosa. Surprisingly, our results demonstrate that both polyphenols are potent vasodilators of mice aorta, but only resveratrol is able to induce corporal relaxation. The failure of quercetin to relax mice corpora cavernosa in contrast to human corpora cavernosa [25] could be due to species differences. In addition in mice aorta, in contrast to corpora cavernosa, a specific molecular target for quercetin could exist or this target could have a higher activity/sensitivity in mice aorta. Moreover, as resveratrol and quercetin belong to a different subclass of the polyphenol family (resveratrol being a stilbene, quercetin being a flavonoid), also structural features could take account for the inability of quercetin to relax mice corpora cavernosa. The idea that the vasorelaxant capacity of polyphenols could be related to structural features has already been proposed [28]. Therefore it is rational to assume that for instance the presence of the flavan moiety may limit the relaxant capacity of quercetin in corporal tissue.

It is well established that resveratrol and quercetin can relax different types of arteries through activation of the NO/sGC-dependent pathway [3-7]. In contrast, the contribution of the NO/sGC-pathway in the relaxant effect of resveratrol on mice corpora cavernosa is unknown. Therefore this was evaluated using sGC $\alpha_1^{-/-}$ mice. sGC exists as an $\alpha\beta$ heterodimer of which two isoforms for each subunit have been characterized (α_1/α_2 and β_1/β_2). However, only the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ heterodimers are catalytically active [29]. Studies suggested that sGC $\alpha_1\beta_1$ is predominantly expressed in corpora cavernosa and is the most important sGC heterodimer responsible for corporal smooth muscle relaxation [30-32]. In aorta from

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sGC $\alpha_1^{-/-}$ mice the resveratrol- and quercetin-induced relaxations were significantly inhibited, confirming the NO/sGC dependency by which polyphenols relax arteries. On the other hand, in corpora cavernosa the resveratrol-induced relaxations were comparable in sGC $\alpha_1^{-/-}$ and sGC $\alpha_1^{+/+}$ mice. Moreover, pretreatment with the NOS inhibitor L-NAME or the sGC inhibitor ODQ did not influence the resveratrol-induced corporal relaxations. In contrast to mice aorta, these results suggest a NO/sGC-independent mechanism of resveratrol to relax mice corpora cavernosa. Surprisingly Fukuhara et al. [24] reported that resveratrol acts NO-dependently to elevate cGMP levels in corpora cavernosa smooth muscle cells. Species differences and different experimental set-ups may explain this discrepancy as in the study of Fukuhara et al. [24] no functional experiments were performed. Moreover, it is well known that the resveratrol-induced relaxations do not solely depend on the activation of the NO/sGC pathway. Other mechanisms have been suggested as well, including activation of K⁺ channels [7, 8, 10]. Within this respect, Dalaklioglu et al. [22] reported that resveratrol could relax rat corpora cavernosa independent of the NO/sGC pathway by activation of different types of K⁺ channels. In our study, neither the presence of high K⁺ concentrations nor pretreatment of the corpora cavernosa with the non-selective (TEA) as well as the selective K_{ATP} (Glib) or K_v (4-AP) channel blockers modified resveratrol-induced corporal relaxations. Therefore, it is unlikely that opening of K⁺ channels is involved in the mice corporal relaxant effect of resveratrol. Other mediators such as prostaglandins, heme oxygenase, adenosine receptors or cAMP pathway, which have been reported to be involved in the resveratrol's mechanism of action [33-36], can also be excluded since pretreatment of de corpora cavernosa with indomethacin, ZnPPIX, 8-SPT or H-89 respectively did not alter corporal relaxations induced by resveratrol. The exact mechanism by which resveratrol relaxes mice corpora cavernosa remains elusive.

It has been shown that resveratrol and quercetin increase endothelium-dependent relaxations to Ach in different rat models with endothelial dysfunction [14, 27, 37]. Interestingly, resveratrol can improve Ach-induced relaxations even in aortic strips from normal mice [39]. In our study neither resveratrol nor quercetin enhanced endogenously (Ach), exogenously (SNP) or neuronal (EFS) NO-mediated relaxation responses of corpora cavernosa. However when oxidative stress was enhanced using PA, resveratrol was able to reverse the decreased EFS-induced relaxations. This finding is in line with what was observed

by Ertug et al. [23] who found that quercetin restored exogenous NO responses when mice corpora cavernosa were exposed to free radicals. This could imply that a certain amount of oxidative stress is required in order for quercetin or resveratrol to exert their beneficial effect. As in resting conditions corpora cavernosa are exposed to a limited amount of oxidative stress, resveratrol and quercetin failed to improve NO-mediated corporal relaxations. Quercetin treatment clearly shows a trend to reverse PA-related decreases in corporal EFS responses. However, resveratrol completely reversed PA-induced impairment of EFS responses. In addition, as incubation of the corpora cavernosa with a known antioxidant, tempol, only partly restored PA-induced impaired EFS relaxations, it seems that on corpora cavernosa resveratrol exerts a greater beneficial effect than tempol.

PA is one of the most abundant free fatty acids (FFA) found in plasma. In addition elevated FFA are a common feature in obesity and diabetes [38], two conditions which are frequently associated with ED [18]. Furthermore, PA incubation can stimulate ROS generation in vitro and impair endothelium-dependent relaxation responses [39-41]. As increased ROS could result in ED, elevated PA levels could be a cause of ROS-related ED. In our study however PA incubation did not cause endothelial or vascular damage since Ach and SNP responses of the corpora cavernosa were not influenced by PA incubation. Therefore it seems that PA causes only a low degree of oxidative stress, which was too low to impair Ach or SNP responses. Despite the mild oxidative stress, EFS-evoked relaxations were significantly decreased by PA, indicating that even mild oxidative stress is sufficient to cause lower neuronal NO bioavailability. In corpora cavernosa this could be physiological relevant as neuronal NO is the primary trigger for erection. If the primary trigger to induce erection is decreased, this might lead to ED since NO derived from other sources cannot completely serve as back-up for the decrease in primary NO source [18, 42]. Regardless of the severity of oxidative stress induced by PA, our results suggest that in mice corpora cavernosa resveratrol is more potent than quercetin to reverse PA-related decreases in neuronal NO-mediated responses.

It should be noted that our study has limitations hampering to draw firm conclusions. First, our study represents a strictly in vitro approach. Therefore our results cannot be extrapolated as such to the in vivo situation or be compared to the effect of orally ingested polyphenols. This latter should be taken into account since polyphenols are rapidly metabolized and thus have a poor oral bioavailability [43]. This raises the question whether

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the beneficial effects observed *in vitro* can be reproduced *in vivo*. However considering the recent study by Yu et al. [44] it seems that resveratrol even *in vivo* has positive effect on corporal tissues as resveratrol improved erectile function in streptozocin-induced diabetic rats by preventing oxidative stress. In addition as low testosterone levels are more often associated with ED [45] also hormonal status can influence the responsiveness of corporal tissues and thus the responsiveness to resveratrol. A second limitation is the lack of direct measurements of the effect of resveratrol and quercetin on oxidative stress. However substantial data is available indicating that resveratrol is able to reduce ROS production *in vitro* [12, 15-17] as well as *in vivo* [11, 44] even in corporal tissue.

V.8 Conclusions

In conclusion, our study shows that the wine compounds resveratrol and quercetin are both potent vasodilators of mice aorta, however only resveratrol exerts a relaxant effect in mice corpora cavernosa which is independent of the NO/sGC pathway. The exact mechanism underlying resveratrol's relaxant effect on mice corpora cavernosa remains elusive. Furthermore, our study indicates that resveratrol is a more potent antioxidant than quercetin being able to restore decreased neuronal NO responses in mice corpora cavernosa.

It is necessary to consider back-up therapeutic approaches in the treatment of ED as specific patient populations are refractory to first-line therapy with PDE5 inhibitors [19]. In this perspective, our results demonstrate that resveratrol, a naturally occurring polyphenol with direct relaxant and antioxidant effects on mice corpora cavernosa, is potentially beneficial for patients suffering from ED. Still, additional pharmacological and toxicological research is needed to elucidate whether resveratrol also exerts these relaxant and antioxidant properties *in vivo* and thus possesses strong therapeutic value for patients suffering from ED.

V.9 Acknowledgements

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Chapter VI

Protective effect of resveratrol and quercetin on in vitro-induced diabetic mouse corpus cavernosum

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VI.1 Abstract

BACKGROUND. Hyperglycemia and increased levels of methylglyoxal (MGO) can trigger the development of vascular complications in diabetes. Resveratrol and quercetin are red wine polyphenols with known beneficial cardiovascular properties, including an antioxidant capacity. This study evaluated whether resveratrol and/or quercetin could prevent *in vitro*-induced diabetic changes in neurogenic and vascular relaxant responses of mouse arteries and corpora cavernosa.

METHODS Isometric tension of isolated aorta, mesenteric arteries and corpora cavernosa was measured using organ bath systems. Diabetic conditions were mimicked *in vitro* by co-incubating the tissues for 2 h with high glucose (HG, 30 mM) and MGO (120 μ M).

RESULTS. The presence of HG and MGO significantly blunted acetylcholine (Ach)-induced relaxations in corpora cavernosa and mesenteric arteries but not in aorta. Electrical field stimulated (EFS) responses of corpora cavernosa were also significantly inhibited by these diabetic conditions. In corpora cavernosa 2 h co-incubation with resveratrol (30 μ M) or quercetin (30 μ M) significantly attenuated HG and MGO-induced deficits in Ach- and EFS-responses.

CONCLUSIONS. Our study demonstrates that in mouse arteries, HG and MGO rather affect endothelium derived hyperpolarizing factor-mediated than nitric oxide (NO)-mediated relaxations. In corpora cavernosa HG and MGO interfere with NO release. Resveratrol and quercetin protect mouse corpora cavernosa from diabetic-induced damage to NO-mediated relaxant responses. This might rely on their antioxidant capacity.

Keywords: resveratrol, quercetin, diabetes, erectile dysfunction

VI.2 Introduction

Hyperglycemia and increased levels of methylglyoxal (MGO), a reactive metabolite produced during glycolysis, are two hallmarks of diabetes mellitus which can trigger the development of vascular complications in diabetes [1-5]. These vascular events are presumed to underlie the pathogenesis of diabetic-related erectile dysfunction (ED). Indeed, in different models of diabetes it has been demonstrated that neurogenic- and endothelium-mediated relaxation of isolated corpora cavernosa [6-11] or arteries [5, 12, 13] is impaired. Moreover, *in vitro* research showed that acute exposure of isolated arteries to high glucose or MGO affects endothelium-dependent relaxations in different vascular beds [1, 3, 14-16]. Oxidative stress may play a causative role in the damaging effect of hyperglycemia or MGO on the endothelium [1, 3, 5, 15, 16]. Therefore, antioxidant therapy might offer an option for treating diabetic complications, including ED. Resveratrol and quercetin are two naturally occurring polyphenols, mainly present in the skin of grapes and thus abundant in red wine. Both polyphenols are suggested to have beneficial cardiovascular properties, including a relaxant [17, 18] and antioxidant [17, 19, 20] capacity. Recently, our group reported a relaxant and antioxidant effect of resveratrol in isolated mouse corpora cavernosa [21]. Considering their antioxidant properties resveratrol and/or quercetin could potentially be of value in the treatment of diabetic ED. However, the antioxidant effect of these polyphenols in mouse mesenteric arteries and corpora cavernosa in diabetic conditions is as yet unexplored. Therefore, the aim of this study was to evaluate whether resveratrol and/or quercetin could protect mouse mesenteric artery and/or corpora cavernosa from high glucose and MGO-induced defects in endothelium- and neurogenic-mediated relaxations.

VI.3 Materials and methods**VI.3.1 Animals**

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

VI.3.2 Tissue preparation

After cervical dislocation, thoracic aorta, mesenteric arteries (1st and 2nd order) and corpora cavernosa were carefully isolated and dissected free from surrounding structures. All tissues were kept in cold Krebs-Ringer bicarbonate (KRB) solution. Aorta and mesenteric arteries were cut into rings of about 3 mm in length and mounted in a wire myograph for isometric tension measurements. Of each mouse, one corpus cavernosum was mounted horizontally in a myograph with one end fixed to a transducer and the other to a micrometer. The tissue chambers were filled with 10 mL KRB solution at 37 °C (pH 7.4) equilibrated with 95 % O₂–5 % CO₂. The preparations were allowed to equilibrate for 30 minutes in KRB solution that was frequently replaced. In order to obtain maximal, stable contractions and relaxations, mesenteric arteries were stretched to their optimal lumen diameter that gives a maximum response, as calculated on the basis of the passive wall tension-internal circumference relationship [22]. Aorta segments and corpora cavernosa were gradually stretched until a stable preload of 0.5 g (aorta) or 0.45 g (corpora cavernosa) was obtained. Subsequently, tissues were repeatedly activated using different protocols: (i) aorta with 120 mM K⁺ and 1 μM phenylephrine (Phe) (ii) mesenteric arteries 3 times with 120 mM K⁺ and 10 μM Phe and (iii) corpora cavernosa two times with 5 μM Phe. The integrity of the endothelium was examined by precontracting the tissues with submaximal Phe concentrations (1 μM for aorta, 5 μM for corpora cavernosa and 10 μM for mesenteric arteries) and adding acetylcholine (ACh) (10 μM for arteries or 1 μM for corpora cavernosa). Only tissues that relaxed more than 50 % to ACh, were included in this study.

VI.3.3 Experimental protocol

Tissues were incubated for 2 h with: (i) control KRB solution containing 10 mM glucose or (ii) KRB solution containing 30 mM glucose (high glucose) and 120 μM MGO. Every 30 minutes the KRB solution in the organ baths was refreshed. After 2 h incubation, tissues were precontracted with Phe (1 – 10 μM). Firstly, the relaxant effect of resveratrol (1 – 100 μM) and quercetin (1 – 100 μM) was evaluated. Secondly, the protective effect of resveratrol and quercetin on endothelium-(in)dependent relaxations were evaluated. Relaxant responses to ACh (1 nM – 1 or 10 μM) and sodium nitroprusside (SNP) (1 nM – 1 μM), were examined after 2 h pretreatment with 30 mM glucose and 120 μM MGO in absence or presence of ascorbic acid (100 μM), tempol (100 μM), resveratrol (30 μM) or quercetin (10 or 30 μM). In

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corpora cavernosa, neurogenic-mediated relaxations were also studied by applying electrical field stimulation (EFS; parameters: train duration 40 s; 1, 2, 4 and 8 Hz; pulse duration 5 ms and 80 V).

VI.3.4 Drugs and chemicals

The experiments were performed in a KRB solution of the following composition (mM): NaCl 135, KCl 5, NaHCO₃ 20, glucose 10 (control) or 30 (high glucose), CaCl₂ 2.5, MgSO₄ 1.3, KH₂PO₄ 1.2 and EDTA 0.026 in H₂O. L-phenylephrine hydrochloride (Phe), acetylcholine chloride (Ach), sodium nitroprusside (SNP), N ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME), indomethacin (indo), methylglyoxal solution (MGO), 4-hydroxy-tempo (Temp), L-ascorbic acid (AA), resveratrol, quercetin and dimethylsulfoxide (DMSO) were obtained from Sigma (St. Louis, MO). Stock solutions were made in water except for indo (ethanol). The final concentrations of vehicle solution in the organ bath never exceeded 0.1 %. Stock solutions of 100 mM of resveratrol and quercetin were made in DMSO, but were further diluted in distilled water (10 mM) before adding to the organ baths.

VI.3.5 Statistics

The data are presented as mean values \pm S.E.M. Relaxations are expressed as % decrease in precontractile tone. N is the number of individual strips/rings used. Sensitivity (pEC₅₀) and maximum response (E_{max}) were calculated from the concentration-response curves to resveratrol, quercetin or acetylcholine. pEC₅₀ was defined as the negative logarithm to base 10 of the EC₅₀ values and E_{max} was defined as the maximal relaxation. The data in our study was analysed using SPSS, version 22; IBM Corporation, Armonk, NY, USA. Statistical significance was evaluated using the Mann-Whitney U test. Two groups of data were considered significantly different when p<0.05. Graphs were created using Graphpad Prism, version 4.00, GraphPad Software, San Diego California USA.

VI.4 Results

VI.4.1 Relaxant capacity of resveratrol and quercetin in presence of high glucose and MGO

Both resveratrol and quercetin (1 – 100 μ M, 15 min) relaxed Phe-precontracted aortic and mesenteric rings concentration dependently (Fig. VI.1a and VI.1b). Maximal relaxation for resveratrol was 74.4 \pm 5.1 % (aorta) and 101.8 \pm 2.0 % (mesenteric arteries); pEC₅₀ values

were 4.4 ± 0.1 and 4.9 ± 0.1 for aortic and mesenteric rings respectively. Maximal relaxation for quercetin was 59.0 ± 11.1 % (aorta) and 102.8 ± 4.3 % (mesenteric arteries); pEC50 values were 4.5 ± 0.1 and 5.4 ± 0.1 for aortic and mesenteric rings respectively. In contrast, only resveratrol evoked concentration dependent relaxations in corpora cavernosa (E_{\max} : 50.6 ± 9.6 % and pEC50: 4.9 ± 0.5) (Fig. VI.1c). Based on these results, following concentrations of resveratrol and quercetin were chosen for our subsequent experiments: $10 \mu\text{M}$ resveratrol and $3 \mu\text{M}$ quercetin for mesenteric arteries, $30 \mu\text{M}$ resveratrol and $30 \mu\text{M}$ quercetin for corpora cavernosa.

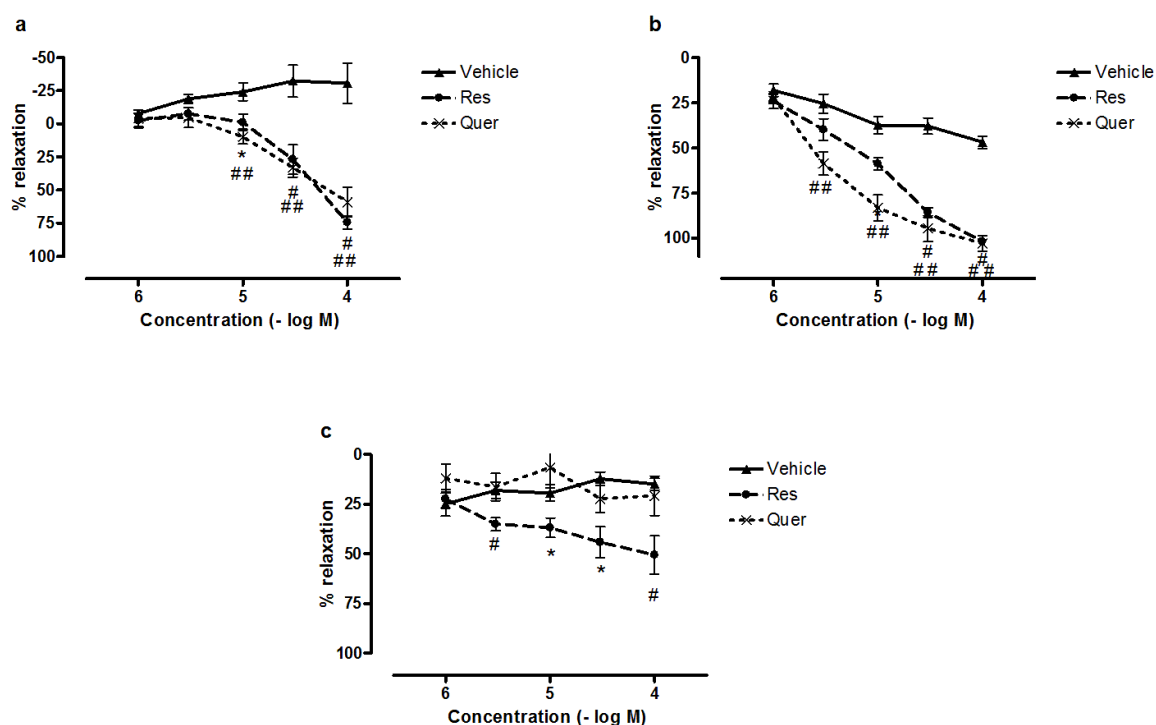


Figure VI.1: (Vaso)relaxant effect of resveratrol or quercetin in presence of high glucose and methylglyoxal. (a) Aorta, (b) mesenteric artery and (c) corpora cavernosa. Tissues were incubated for 2 h with 30 mM glucose (HG) and $120 \mu\text{M}$ methylglyoxal (MGO) before constructing concentration-response (1-100 μM) curves to resveratrol (Res, 15 min) or quercetin (Quer, 15 min). Data are expressed as % decrease of Phe-induced tone; Mann-Whitney U test; # $p < 0.01$ and * $p < 0.05$ (vehicle control vs. Res) ($n = 5-6$); ## $p < 0.01$ and ** $p < 0.05$ (vehicle control vs. Quer) ($n = 5-6$).

VI.4.2 High glucose and MGO impair Ach- and EFS-relaxations

Endothelium-dependent relaxations were evaluated using Ach (1 nM – 1 or 10 μ M), which induced concentration dependent relaxations in all tissues. To explore the role of endothelium derived hyperpolarizing factor (EDHF), Ach-curves were constructed in presence of the NO synthase inhibitor, L-NAME (0.1 mM, 20 min), and the cyclooxygenase blocker, indo (10 μ M, 20 min). The presence of L-NAME and indo completely abolished Ach-relaxations in aorta and corpora cavernosa, whereas these inhibitors did not affect Ach-relaxations of mesenteric arteries (data not shown).

After 2 h incubation with high glucose (30 mM) and MGO (120 μ M), Ach-induced relaxations were significantly blunted in mesenteric arteries (Fig. VI.2a, VI.2b and VI.3a) and corpora cavernosa (Fig. VI.2c, VI.2d and VI.3b). EFS-relaxations of corpora cavernosa were significantly reduced by 2 h exposure to high glucose (30 mM) and MGO (120 μ M) (Fig. VI.2e, VI.2f and VI.3c).

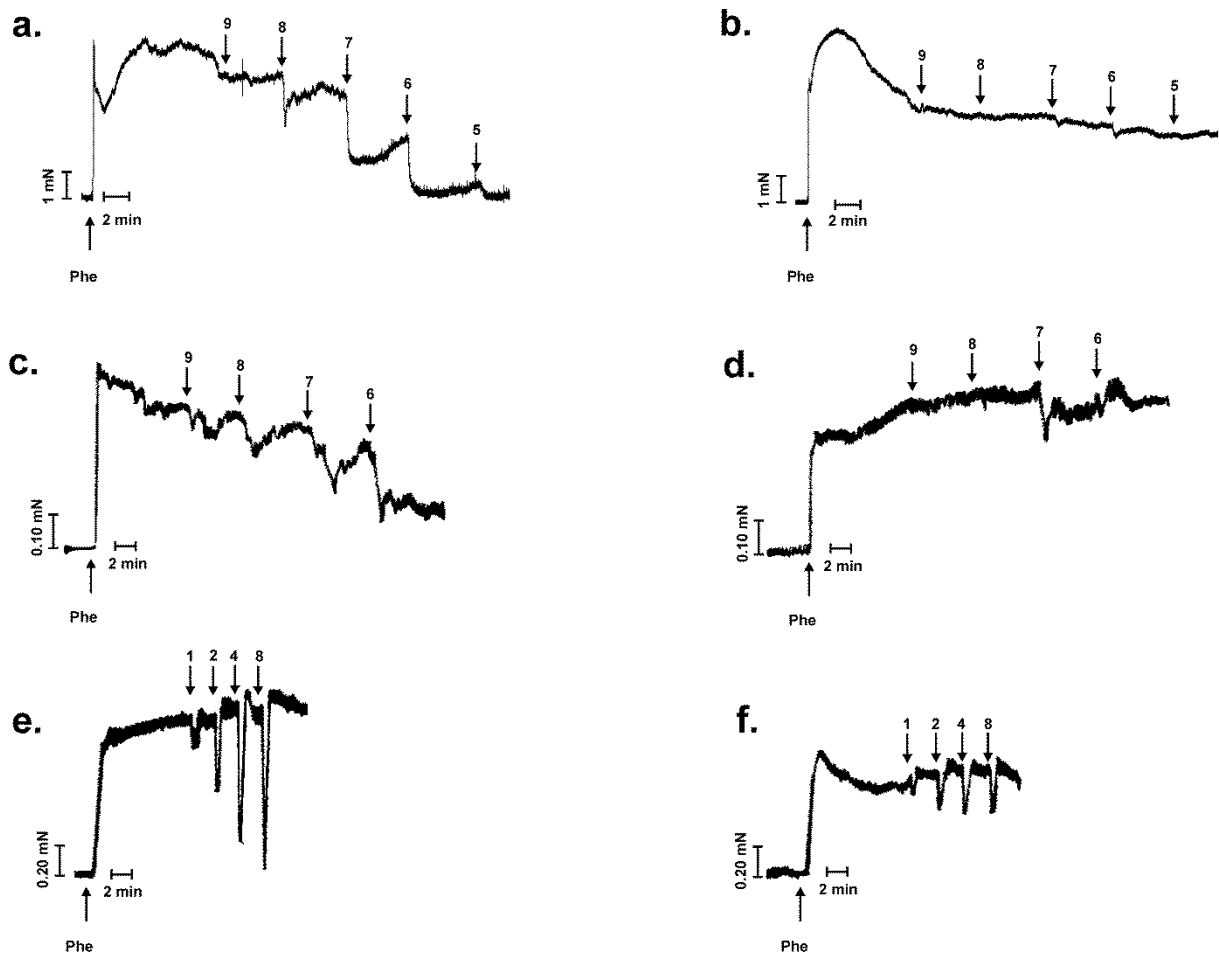


Figure VI.2: Effect of in vitro-diabetic conditions on acetylcholine and electrical field stimulated responses of mesenteric artery and corpora cavernosa. Original tracings showing the effect of 2 h incubation with high glucose (HG, 30 mM) and methylglyoxal (MGO, 120 μ M) on (a-d) acetylcholine (Ach, $-\log$ M) and (e and f) electrical field stimulated (Hz) relaxations of mesenteric artery and corpora cavernosa after precontraction with phenylephrine (Phe; 10 μ M for mesenteric arteries; 5 μ M for corpora cavernosa). Response curve to Ach in mesenteric artery in (a) absence and (b) presence of HG and MGO; in corpora cavernosa in (c) absence and (d) presence of HG and MGO. EFS-relaxations in corpora cavernosa in (e) absence and (f) presence of HG and MGO.

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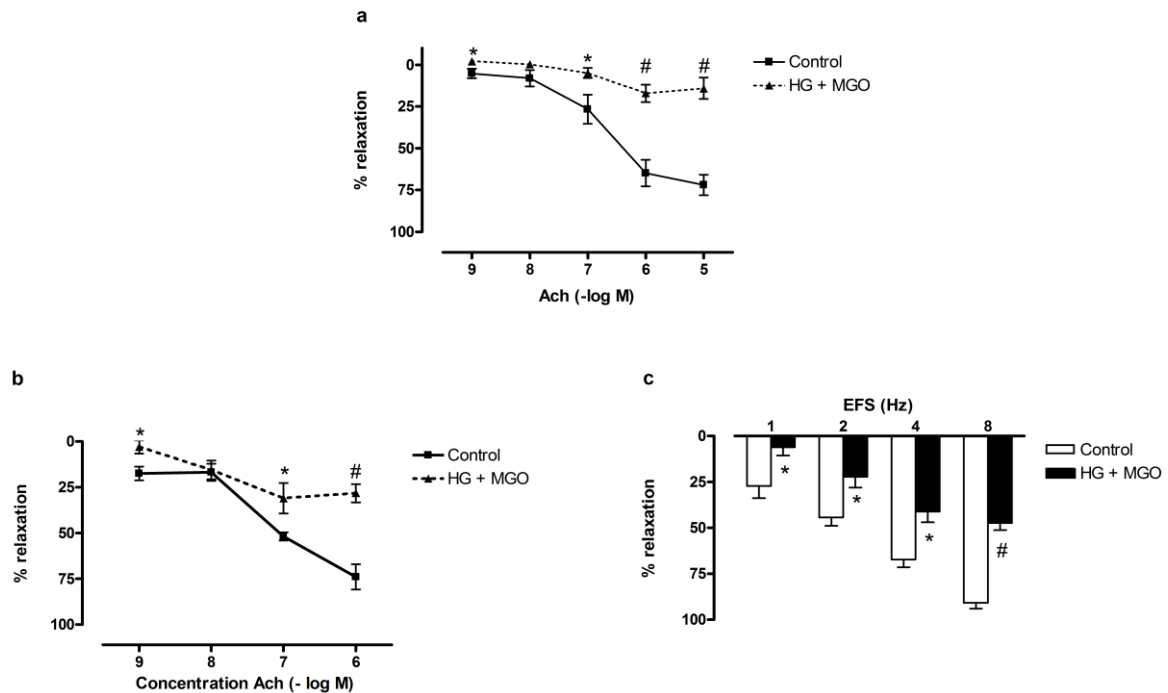


Figure VI.3: Effect of in vitro-diabetic conditions on acetylcholine and electrical field stimulated responses of mesenteric artery and corpora cavernosa. Acetylcholine (Ach)-induced relaxations of (a) mesenteric artery and (b) corpora cavernosa after 2 h incubation with high glucose (HG, 30 mM) and methylglyoxal (MGO, 120 μ M) after precontraction with Phe (10 μ M for mesenteric arteries; 5 μ M for corpora cavernosa). (c) Electrical field stimulated (EFS) relaxations of 5 μ M Phe-precontracted mouse corpora cavernosa after 2 h incubation with high glucose (HG, 30 mM) and methylglyoxal (MGO, 120 μ M). Data are expressed as % decrease of Phe-induced tone; Mann-Whitney U test; # p <0.01 and * p <0.05 (n =6-7).

In contrast, the presence of 30 mM glucose and 120 μ M MGO neither affected Ach-relaxations of aorta (Fig VI.4a) nor SNP-relaxations of aorta, mesenteric arteries or corpora cavernosa (Fig. VI.4b, VI.4c and VI.4d).

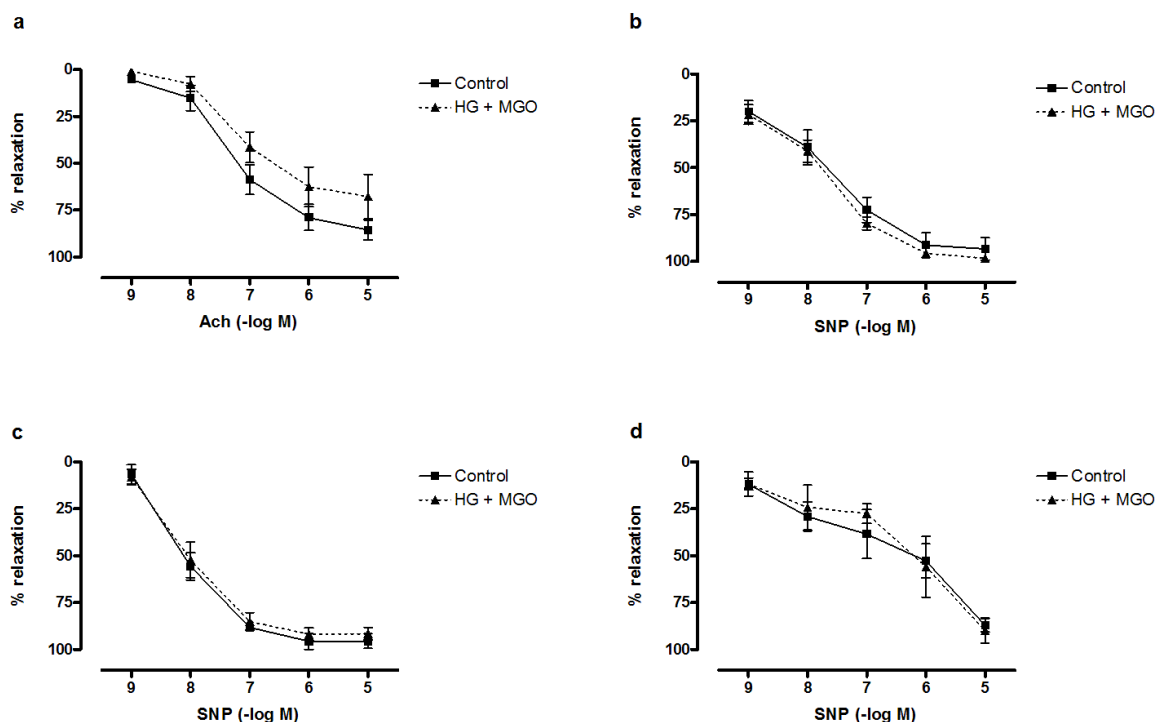


Figure VI.4: Effect of in vitro-diabetic conditions on acetylcholine and sodium nitroprusside responses of aorta, mesenteric artery and corpora cavernosa. Acetylcholine (Ach)-induced relaxations of mouse (a) aorta and on sodium nitroprusside (SNP)-induced relaxations of (b) aorta, (c) mesenteric artery and (d) corpora cavernosa after 2 h incubation with high glucose (HG, 30 mM) and methylglyoxal (MGO, 120 μ M) after precontraction with Phe (1 μ M for aorta, 10 μ M for mesenteric arteries and 5 μ M for corpora cavernosa). Data are expressed as % decrease of Phe-induced tone; Mann-Whitney U test; (n=7-8 for Ach-curves) (n=4-6 for SNP-curves).

VI.4.3 Role of oxidative stress in glucose and MGO-impaired relaxations

Mesenteric arteries and corpora cavernosa were pretreated for 2 h with 30 mM glucose and 120 μ M MGO in combination with the antioxidant AA (100 μ M) or the superoxide anion scavenger Temp (100 μ M). In mesenteric arteries both AA (Fig. VI.5a) and Temp (Fig. VI.6a) prevented glucose and MGO impairment of Ach-relaxations. Neither AA nor Temp altered sensitivity to Ach, whereas maximal Ach-responses were improved in presence of AA or Temp (Table 1). Similarly, in corpora cavernosa, both antioxidants counteracted the effect of glucose and MGO incubation on Ach- and EFS-responses (Fig. VI.5b and VI.5c, VI.6b and VI.6c). pEC₅₀ values were unaltered by antioxidant treatment. Maximal Ach-responses were however improved in presence of AA or Temp (Table VI.1).

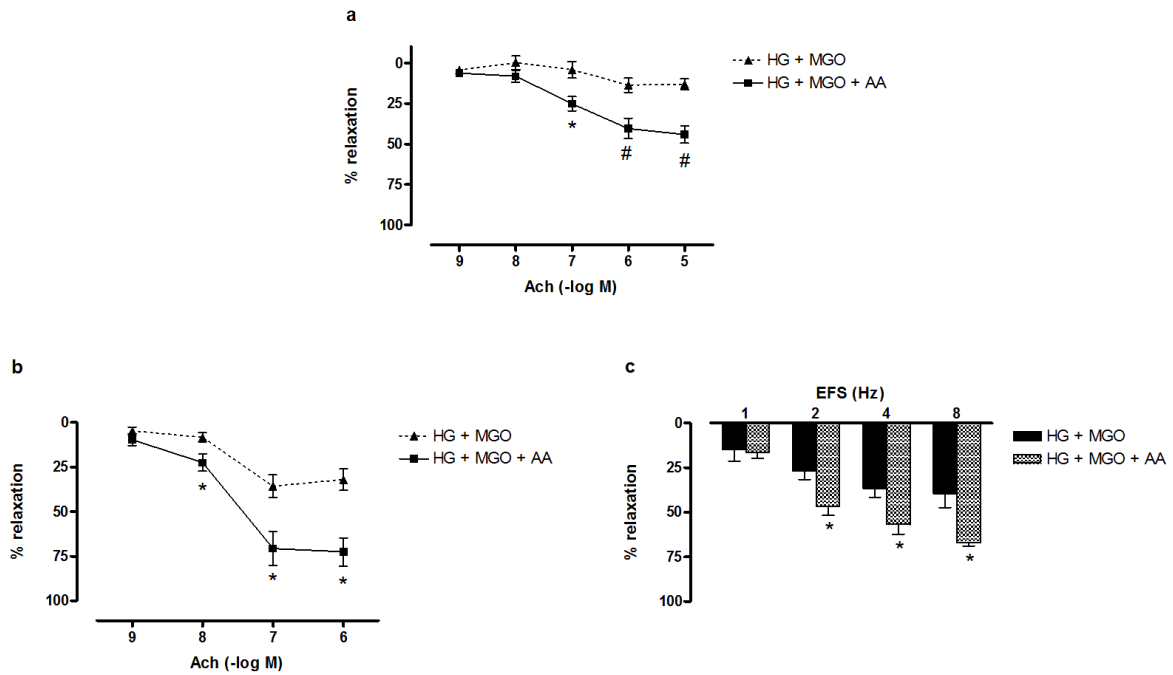


Figure VI.5: Ascorbic acid prevents high glucose and methylglyoxal-induced deficits in mesenteric artery and corpora cavernosa. Acetylcholine (Ach)-induced relaxations of (a) mesenteric artery and (b) corpora cavernosa after 2 h co-incubation with high glucose (HG, 30 mM) and methylglyoxal (MGO, 120 μ M) in absence and presence of ascorbic acid (AA, 100 μ M) after precontraction with Phe (10 μ M for mesenteric arteries; 5 μ M for corpora cavernosa). (c) Electrical field stimulated (EFS) relaxations of corpora cavernosa after 2 h incubation with high glucose (HG, 30 mM) and methylglyoxal (MGO, 120 μ M) in absence and presence of ascorbic acid (AA, 100 μ M). Data are expressed as % decrease of Phe-induced tone; Mann-Whitney U test; # p <0.01 and * p <0.05 (n=5-7).

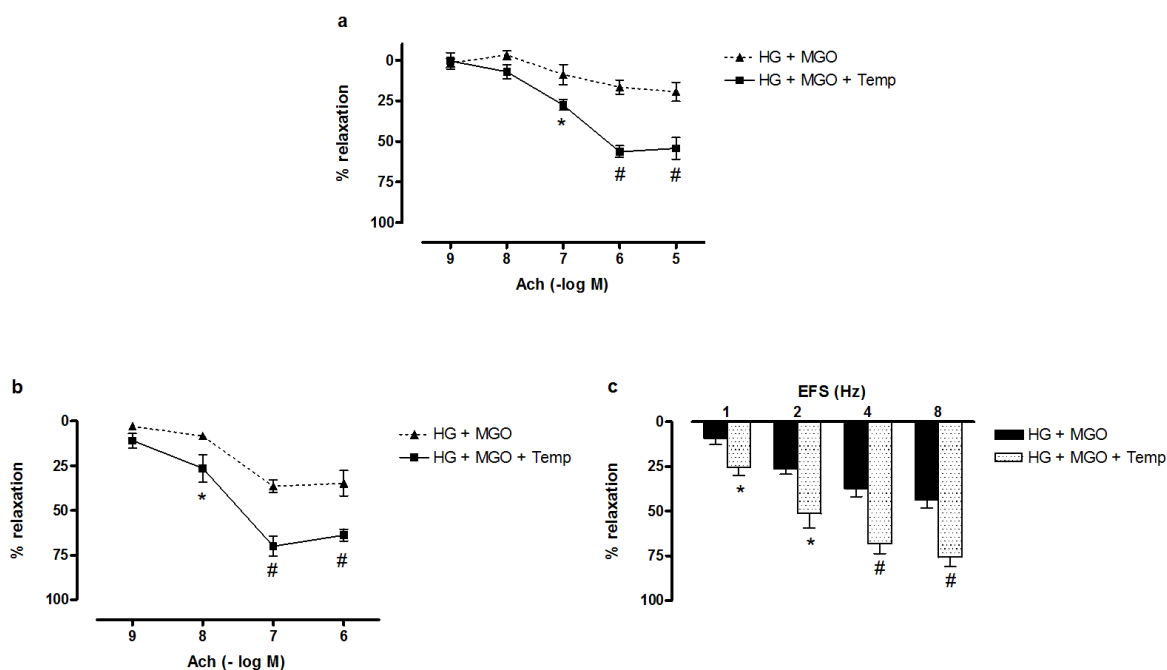


Figure VI.6: Tempol prevents high glucose and methylglyoxal-induced deficits in mesenteric artery and corpora cavernosa. Acetylcholine (Ach)-induced relaxations of (a) mesenteric artery and (b) corpora cavernosa after 2 h incubation with high glucose (HG, 30 mM) and methylglyoxal (MGO, 120 μ M) in absence and presence of tempol (Temp, 100 μ M) after precontraction with Phe (10 μ M for mesenteric arteries; 5 μ M for corpora cavernosa). (c) Electrical field stimulated (EFS) relaxations of 5 μ M Phe-precontracted mouse corpora cavernosa after 2 h incubation with high glucose (HG, 30 mM) and methylglyoxal (MGO, 120 μ M) in absence and presence of tempol (Temp, 100 μ M). Data are expressed as % decrease of Phe-induced tone; Mann-Whitney U test; # p <0.01 and * p <0.05 (n=5-6).

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Table VI.1. Effect of antioxidants and polyphenol treatment on acetylcholine-relaxations of mesenteric arteries and corpora cavernosa.

	Mesenteric arteries		Corpora cavernosa	
	pEC50	E _{max} (%)	pEC50	E _{max} (%)
HG + MGO	7.1 ± 0.4	19.8 ± 2.6	7.8 ± 0.1	35.8 ± 6.4
HG + MGO + AA	7.0 ± 0.2	46.8 ± 4.8 [#]	7.8 ± 0.2	83.5 ± 6.1 [*]
HG + MGO	7.0 ± 0.3	23.9 ± 5.04	7.7 ± 0.1	38.7 ± 4.2
HG + MGO + Temp	7.1 ± 0.1	58.3 ± 4.4 [#]	7.9 ± 0.2	74.3 ± 3.5 [#]
HG + MGO	6.3 ± 0.9	23.2 ± 5.0	7.7 ± 0.3	47.6 ± 4.3
HG + MGO + Res	6.7 ± 0.5	34.8 ± 3.7	7.6 ± 0.2	83.8 ± 4.6 [#]
HG + MGO	7.4 ± 1.0	24.4 ± 4.4	7.9 ± 0.3	44.9 ± 3.5
HG + MGO + Quer	6.7 ± 0.2	31.3 ± 6.3	7.9 ± 0.02	78.4 ± 13.3 [#]

pEC50, $-\log(\text{EC}_{50})$; E_{max}: maximal relaxation response; HG: high glucose (30 mM), MGO: methylglyoxal (120 μM); AA: ascorbic acid (100 μM); Temp: tempol (100 μM); Res: resveratrol (10 μM for mesenteric arteries; 30 μM for corpora cavernosa); Quer: quercetin (3 μM for mesenteric arteries; 30 μM for corpora cavernosa). Data are means ± SEM. *p<0.05; #p<0.01 (compared to HG + MGO group).

VI.4.4 Effect of resveratrol and quercetin on glucose and MGO-impaired relaxations

In mesenteric arteries, Ach-induced relaxations were explored after 2 h pretreatment with 30 mM glucose and 120 μM MGO in combination with resveratrol (Res, 10 μM, 2 h) or quercetin (Quer, 3 μM, 2 h). Neither resveratrol (Fig. VI.7a) nor quercetin (Fig. VI.8a) prevented glucose and MGO impairment of Ach-relaxations as neither E_{max} nor pEC50 values were affected by resveratrol or quercetin treatment (Table VI.1). On the contrary, exposing the corpora cavernosa for 2 h to 30 mM glucose and 120 μM MGO in combination with resveratrol (30 μM, 2 h) or quercetin (30 μM, 2 h) significantly corrected blunted Ach- and EFS- relaxations (Fig. VI.7b and VI.7c, VI.8b and VI.8c). E_{max} values were significantly corrected by resveratrol as well as by quercetin treatment (Table VI.1). No effect of both polyphenols was found on the potency of Ach as pEC50 values were unchanged by resveratrol or quercetin addition (Table VI.1).

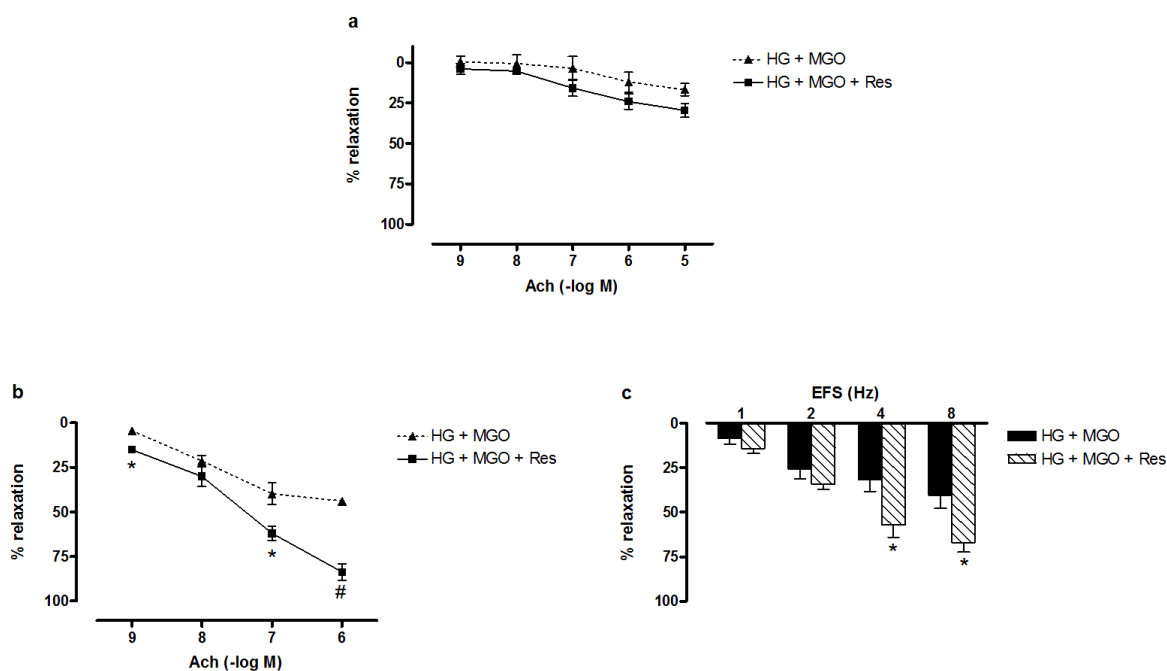


Figure VI.7: Effect of resveratrol on high glucose and methylglyoxal-induced deficits in mesenteric artery and corpora cavernosa. Acetylcholine (Ach)-induced relaxations of (a) mesenteric artery and (b) corpora cavernosa after 2 h incubation with high glucose (HG, 30 mM) and methylglyoxal (MGO, 120 μ M) in absence and presence of resveratrol (Res, 10 μ M for (a); 30 μ M for (b)) after precontraction with Phe (10 μ M for mesenteric arteries; 5 μ M for corpora cavernosa). (c) Electrical field stimulated (EFS) relaxations of 5 μ M Phe-precontracted mouse corpora cavernosa after 2 h incubation with high glucose (HG, 30 mM) and methylglyoxal (MGO, 120 μ M) in absence and presence of resveratrol (Res, 30 μ M). Data are expressed as % decrease of Phe-induced tone; Mann-Whitney U test; # p <0.01 and * p <0.05 (n =5-7).

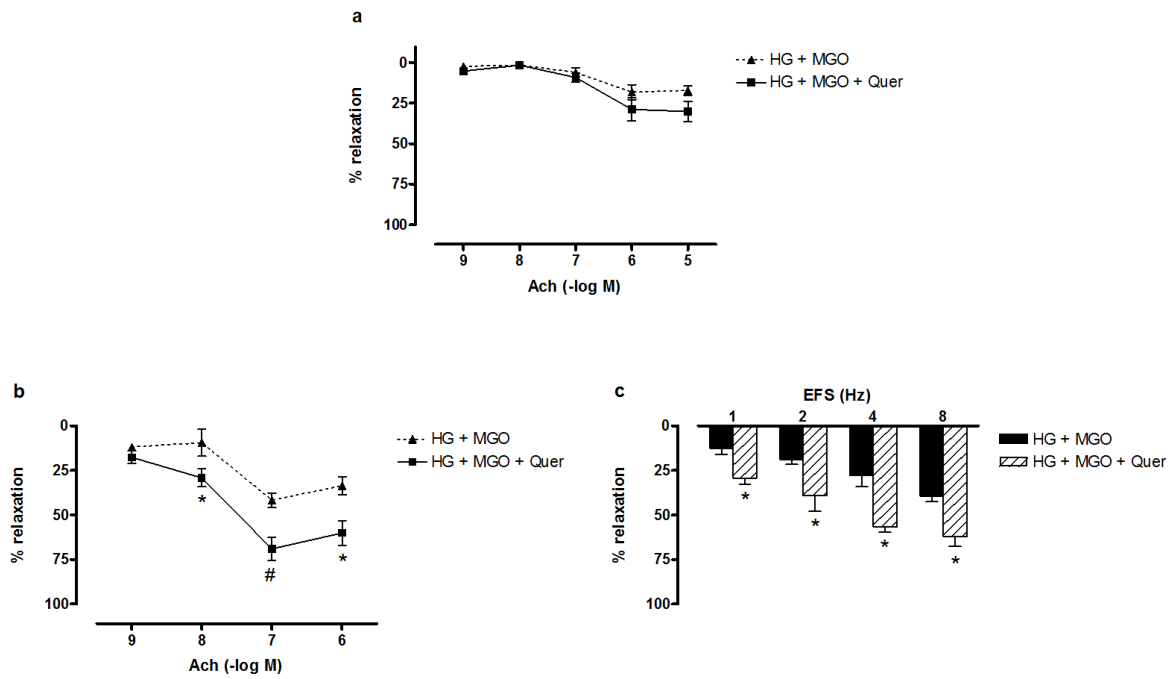


Figure VI.8: Effect of quercetin on high glucose and methylglyoxal-induced deficits in mesenteric artery and corpora cavernosa. Acetylcholine (Ach)-induced relaxations of (a) mesenteric artery and (b) corpora cavernosa after 2 h incubation with high glucose (HG, 30 mM) and methylglyoxal (MGO, 120 μ M) in absence and presence of quercetin (Quer, 3 μ M for (a); 30 μ M for (b)) after precontraction with Phe (10 μ M for mesenteric arteries; 5 μ M for corpora cavernosa). (c) Electrical field stimulated (EFS) relaxations of 5 μ M Phe-precontracted mouse corpora cavernosa after 2 h incubation with high glucose (HG, 30 mM) and methylglyoxal (MGO, 120 μ M) in absence and presence of quercetin (Quer, 30 μ M). Data are expressed as % decrease of Phe-induced tone; Mann-Whitney U test; # p <0.01 and * p <0.05 (n=4-7).

VI.5 Discussion

The present study was the first to demonstrate that co-incubation of mouse mesenteric arteries and corpora cavernosa with 30 mM glucose and 120 μ M MGO causes deficits in endothelium- and neurogenic-mediated relaxations. In addition, resveratrol and quercetin were effective in preventing diabetic-induced deficits in corpora cavernosa.

VI.5.1 Diabetic (neuro)vascular damage induced by *in vitro* high glucose and MGO treatment

The concentrations of glucose and MGO used in our study were chosen based on literature. 30 mM glucose is a level that is found or even exceeded in the blood of db/db mouse model of type 2 diabetes that is associated with severe endothelial dysfunction in the small mesenteric arteries [23]. In addition, in patients with poorly controlled diabetes, the MGO level in blood can raise up to 400 μ M [24]. Moreover, exogenous MGO is not fully absorbed intracellularly [25]. Therefore the use of 30 mM glucose and 120 μ M MGO in this study seems to be justified as these concentrations seem pathophysiologically relevant.

The effect of either glucose or MGO on endothelium-dependent relaxations has been investigated in several studies [1, 3, 5, 14, 16], however no study has evaluated the joint effect of MGO and elevated glucose in mouse mesenteric arteries or corpora cavernosa. Since both glucose and MGO levels are increased in diabetes mellitus [4, 24], diabetic conditions are more precisely mimicked *in vitro* by co-incubating the tissues with both compounds. Our results are in line with previous studies showing a negative effect of high glucose or MGO on endothelium-dependent relaxations in mesenteric arteries of rats or mice [1, 14-16]. In addition, the impaired endothelium- and neurogenic-mediated relaxations observed in corpora cavernosa are in agreement with studies using other animal models for diabetes [7-11] or in diabetic men [6]. It thus seems that the *in vitro* glucose/MGO treatment used in this study indeed yields neurovascular damage as observed *in vivo* in diabetes mellitus. Confirming most of these studies, relaxant responses to SNP, an endothelium-independent nitric oxide (NO) donor, were unaltered by exposing the tissues to high glucose and MGO. Thus neither a diminished response at the level of the smooth muscle cells nor defects in the cyclic guanosine monophosphate pathway can explain the observed diabetic-induced deficits of Ach- and EFS-relaxations. Instead, these deficits will

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thus rather result from decreased synthesis and/or increased quenching of endothelium- or neuronal-derived relaxing factors by high glucose and MGO.

VI.5.2 Aorta versus mesenteric arteries

No effect of diabetic conditions was found on Ach-relaxations in aorta, which is in contrast to Dhar *et al.* [3], who found a decreased Ach response in rat aorta after incubation with 25 mM glucose. As in our study a trend in reduced Ach-relaxations of aorta could be observed, it is possible that the incubation time of glucose/MGO was too short to cause endothelial damage. With respect to the arteries used in this study, our results indicate that diabetic conditions affect endothelium-mediated relaxations in mesenteric arteries more rapidly than in aorta. This implies that the type of artery and/or the lumen diameter determines the effect of glucose/MGO on Ach-induced relaxations. Ach stimulates the release of endothelium derived relaxing factors: NO, prostacyclin and EDHF. Different vascular beds exhibit a marked heterogeneity in the relative contribution of these factors to endothelium-dependent relaxations [26]. Indeed, inhibition of NO and prostacyclin production (using L-NAME and indo respectively) did not affect Ach-relaxations in mesenteric arteries whereas the presence of these inhibitors completely abolished Ach-responses in aorta. This demonstrates that Ach-relaxations in mesenteric arteries are EDHF-mediated [12, 26] while in aorta these are NO-mediated. Thus, in mouse arteries high glucose and MGO seem to interfere more rapidly with EDHF- than with NO-dependent relaxations.

VI.5.3 Corpora cavernosa

Like in aorta, Ach- and EFS-relaxations of corpora cavernosa are NO-mediated (own observations and [10, 27]). However, unlike in aorta, these NO-mediated relaxations are inhibited by co-incubating the corpora cavernosa with 30 mM glucose and 120 μ M MGO. So, besides the lumen diameter of the vessels, the type of tissue also contributes to the effect of diabetic conditions on relaxant responses to Ach or EFS. Since unaltered aortic Ach-relaxations were observed in presence of high glucose and MGO, it can be assumed that (i) these diabetic conditions induce less damage in aorta or (ii) the aorta is more resistant to changes evoked by glucose/MGO. As previously mentioned, our results cannot rule out that longer exposure to high glucose/MGO could yield similar endothelial damage in aorta as seen in corpora cavernosa or mesenteric arteries. However, based on our results it seems

that both corpora cavernosa and mesenteric arteries are more prone to diabetic-induced endothelial and/or neurogenic changes compared to large arteries such as aorta.

VI.5.4 Oxidative stress as underlying cause of in vitro-diabetic damage

A hyperglycemia or MGO-dependent increase in reactive oxygen species (ROS) [1, 3, 5, 15, 16] seems to be an important contributor to the pathogenesis of the diabetic-(neuro)vascular complications. After all, antioxidant administration can limit oxidative damage and restore diabetes-induced defects in endothelial- and/or neurogenic responses of arteries [28-30] or corpora cavernosa [8-11]. Here we demonstrate that the antioxidant AA or the superoxide anion scavenger Temp markedly inhibit glucose/MGO-induced damage to endothelium- and neurogenic-mediated responses of mesenteric arteries and corpora cavernosa. Despite the large amount of evidence for high glucose or MGO-induced oxidative stress, the mechanisms whereby ROS formation is induced, are not fully understood. Recently it was reported that MGO-stimulated ROS production activates the endothelial plasma membrane transport protein, Na⁺/H⁺ exchanger (NHE1) [31]. Moreover high glucose-induced vascular deficits are related to hyperactivity of NHE1 [32]. Activation of NHE1 may thus be a critical step for ROS-induced damage. Regardless of the mechanism by which glucose/MGO generate oxidative stress, our results indicate that ROS, including superoxide anion, interfere with the NO-pathway, in corpora cavernosa, or the EDHF-pathway, in mesenteric arteries, leading to impaired Ach- and EFS-relaxations. Although not addressed in this study, ROS interference with the NO-pathway involves either decreased NO synthesis or increased NO degradation. Increased oxidative stress leads to the oxidation of tetrahydrobiopterin (BH₄), a cofactor that tightly regulates NO production, resulting in the uncoupling of endothelial NO synthase (eNOS) [33, 34]. In the presence of reduced concentrations of BH₄, eNOS transfers electrons to molecular oxygen to produce superoxide rather than NO [33, 34]. In addition superoxide itself can react and consume NO, forming peroxynitrite and thus enhancing oxidative stress. Hence, NO-mediated relaxations are further impaired. On the contrary, less is known about the interference of oxidative stress with the EDHF-pathway. Impaired EDHF-responses due to increased oxidative stress does not result from altered K⁺ channel function [16]. ROS-induced altered interaction between cAMP and gap junctions communication might represent another explanation [16].

VI.5.5 Resveratrol and quercetin in diabetic conditions

Numerous health benefits have been ascribed to the red wine polyphenols resveratrol and quercetin [17]. Even in diabetic rats these polyphenols restore impaired endothelium-dependent relaxations of aorta [35-37] and improve erectile function [38-41]. In addition, studies have reported a direct anti-diabetic effect of resveratrol and quercetin as they were able to lower blood glucose levels in diabetic rats and mice [42, 43]. In this study resveratrol and quercetin administration prevented glucose/MGO-induced damage to endothelium- and neurogenic-induced relaxations in corpora cavernosa. This is in line with Murat et al. [44] who found that resveratrol protects and restores endothelium-dependent relaxation in hypercholesterolemic rabbit corpus cavernosum. On the contrary, in mesenteric arteries no protective effect of resveratrol or quercetin was found. Different explanations could clarify this tissue specific effect of both polyphenols. First, studies pointed out that resveratrol and quercetin stimulate endothelial NO production [17, 18, 20]. As endothelial- and neurogenic relaxant responses in corpora cavernosa are NO-mediated, enhanced endothelial or neuronal NO synthesis by the polyphenols could clarify the improved Ach-relaxations. In contrast this could not explain the inability of resveratrol and quercetin to protect mesenteric arteries from diabetic-damage to Ach-responses. We have shown that Ach-induced relaxations in mesenteric arteries are EDHF mediated. Nonetheless, one could expect that if resveratrol and quercetin enhance endothelial NO production this could compensate for decreases in EDHF-responses, resulting in improved Ach-relaxations. However this was not the case. Moreover in a previous study [21] we found that resveratrol relaxes mouse corpora cavernosa independent of the endothelium or NO. In addition we also demonstrated that neither resveratrol nor quercetin stimulated Ach-relaxations of mouse corpora cavernosa as such [21]. Therefore improved NO production cannot explain why the protective effect of resveratrol and quercetin against glucose/MGO-induced damage is limited to the corpora cavernosa.

Recently it was suggested that K^+ channel activation could also be involved in resveratrol's positive effects on the cardiovascular system [45], including in resveratrol's relaxant effect on corpora cavernosa [46]. In our study there is no evidence to strengthen this idea, as we previously found that resveratrol relaxes mouse corpora cavernosa independent of K^+ channel activation [20]. Furthermore this could not explain the protective effect of quercetin

on EFS- and Ach-relaxations in corpora cavernosa, since quercetin was not able to relax mouse corpora cavernosa.

A second explanation for the positive effects of resveratrol and quercetin in corpora cavernosa and not in mesenteric arteries, could be found in the different amount of oxidative stress that is induced by the glucose/MGO treatment. Since in mesenteric arteries Ach-relaxations were almost completely abolished by high glucose and MGO, it is possible that the degree of generated oxidative stress is too high for resveratrol or quercetin to cope with. It is for instance suggested that the protective effects of resveratrol against oxidative injury are attributed to up-regulations of the endogenous cellular antioxidant system rather than to its direct ROS scavenging activity, as it is known that the direct antioxidant effect of resveratrol is rather low [17]. However both polyphenols are as efficacious as known antioxidants to attenuate diabetic-induced oxidative endothelial damage. In addition, in diabetic-mimicking conditions resveratrol has a direct relaxant effect as in the present study resveratrol relaxed corpora cavernosa concentration-dependently in presence of glucose/MGO. Taken together, these results thus suggest a potential therapeutic role for both polyphenols, but especially for resveratrol, in the treatment of diabetes- and oxidative stress-associated ED.

VI.5.6 Limitations

As this study represents a strictly pharmacological *in vitro* approach, it is hard to extrapolate our results to *in vivo* circumstances. Although some studies already pointed out that polyphenols *in vivo* have positive effects on vascular [17] or erectile function [38, 40, 41], further research in this field is definitely required. Furthermore, in our study only the acute effect of glucose/MGO and resveratrol and quercetin administration could be evaluated. Further research will have to confirm whether a similar positive effect on erectile function can be obtained after chronic treatment.

VI.6 Conclusions

This study demonstrates that high glucose in combination with MGO impairs endothelium- and neurogenic-mediated relaxations in mouse corpora cavernosa and mesenteric arteries which is prevented by antioxidants. In arteries, these diabetic conditions primarily affect the EDHF-pathway, while in corpora cavernosa high glucose and MGO interfere with NO release.

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Furthermore it was shown that resveratrol and quercetin are effective in preventing high glucose and MGO deficits of endothelium- and neurogenic-mediated relaxations of mouse corpora cavernosa. Therefore our study further strengthens the strategy of using polyphenols, especially resveratrol, as possible alternative option in the treatment of diabetic ED.

VI.7. Acknowledgements

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Chapter VII

Inhibition of cyclic GMP export by multidrug resistance protein 4: a new strategy to treat erectile dysfunction?

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Chapter VII Inhibition of cyclic GMP export by multidrug resistance protein 4: a new strategy to treat erectile dysfunction?

VII.1 Abstract

BACKGROUND. Intracellular cGMP concentrations are regulated by degradation enzymes (phosphodiesterases, PDEs) as well as by active transport across the plasma membrane by multidrug resistance protein (MRP) 4 and 5.

AIM. This study evaluated the functional effect of MRP 4 inhibition and the role of MRP 4-mediated cGMP export in mouse corpora cavernosa.

METHODS. Isometric tension of mouse corpora cavernosa was measured after cumulative addition of MK-571, an inhibitor of MRP 4, or sildenafil, a PDE-5 inhibitor (PDE5i). In addition the effect of MRP 4 inhibition on cGMP-(in)dependent relaxations was studied. In vivo intracavernosal pressure (ICP) and mean arterial pressure (MAP) measurements were performed after intracavernosal injection (i.c.) of MK-571. The effect of MRP 4 inhibition on cGMP content was determined using an enzyme immunoassay kit.

OUTCOMES. Measurement of the effect of MK-571 on cGMP content and relaxant responses of mouse corpora cavernosa to cGMP-(in)dependent vasodilating substances and determination of ICP/MAP after i.c. injection of MK-571.

RESULTS. MK-571 and sildenafil both relaxed the corpora cavernosa concentration dependently with sildenafil being the most potent relaxing compound. Furthermore, MK-571 enhanced relaxing responses to cGMP-dependent substances, such as sodium nitroprusside, sildenafil, acetylcholine and electrical field stimulation, the latter ones even under in vitro diabetic conditions. In contrast cGMP-independent relaxations were not altered by MRP 4 inhibition. Intracavernosal administration of MK-571 significantly increased ICP, with minimal effect on MAP. cGMP analysis revealed that MRP 4 inhibition was accompanied with increased cGMP levels.

CLINICAL TRANSLATION. MRP 4, at least when targeted locally in the penis or when combined with PDE5i, might be a valuable alternative strategy for the treatment of (diabetic) ED.

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STRENGTHS & LIMITATIONS. This study is the first to demonstrate an in vitro direct relaxant and an in vivo pro-erectile effect of the MRP 4 inhibitor, MK-571, on mouse corpora cavernosa. However the functional effect of MRP 5-mediated export in mouse corpora cavernosa was not explored, which is suggested to play the predominant role in cGMP export.

CONCLUSION. Inhibition of MRP 4 increases basal and stimulated levels of cGMP leading to corpora cavernosa relaxation and penile erection. Therefore it is suggested that, in addition to degradation of cGMP, export of cGMP by MRP 4 substantially contributes to regulating the cGMP levels in mouse corpora cavernosa.

Keywords cyclic GMP, multidrug resistance protein, corpora cavernosa, relaxation, erectile dysfunction

VII.2 Introduction

The intracellular signaling molecule cyclic guanosine monophosphate (cGMP) plays an important role in several cardiovascular processes, including in vascular relaxation. Intracellular levels of cGMP are the result of the balance between the rate of cGMP synthesis and the rate of cGMP elimination. In penile tissue, elevation of cGMP levels and subsequent relaxation of vascular and corporal smooth muscle cells is a key event for normal penile erection [1]. Therefore, most currently available treatment strategies for erectile dysfunction (ED) are targeted to increase intracellular levels of cGMP. Many studies focused on how degradation of cGMP could be prevented by phosphodiesterase inhibitors (PDEi). Due to the success of this research PDE5i are nowadays first choice in the treatment of ED. However, in addition to metabolic degradation of cGMP by PDE-5, cGMP can also be actively transported across the plasma membrane by members of the multidrug resistance protein (MRP) family [2-7]. MRPs are transmembrane proteins that use the energy produced by adenosine triphosphate (ATP) hydrolysis to transport a variety of substrates out of the cell. Yet, in contrast to the huge amount of research conducted on cGMP degradation no studies previously evaluated cGMP export in penile tissues. Two MRP family members, MRP 4 and MRP 5, have been shown to transport cGMP [2-8]. MRP 4 and/or MRP 5 expression was found in several tissues including vascular smooth muscle cells, endothelial cells [9-11] and interestingly also in corporal smooth muscle cells [12, 13]. In mouse aortic rings MRP 4-mediated cGMP export has been demonstrated as an important regulator of cGMP-mediated vascular relaxant responses [8]. Hence, MRP 4-mediated cGMP export could play a role in corporal relaxant responses as well and prevention of cGMP-transport out of the cavernosal smooth muscle cells might represent a new and alternative approach to elevate intracellular cGMP levels and thus to treat erectile dysfunction.

VII.3 Aim

The aim of this study was to examine the functional effect of MRP 4 inhibition as well as the role of MRP 4-mediated cGMP export in mouse corpora cavernosa.

VII.4 MethodsVII.4.1 Animals

Adult (8-12 weeks) male Swiss mice were obtained from Janvier (Saint-Berthevin, France). Food and water was provided ad libitum and all animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. This study was approved by the local Ethical Committee for animal experiments, Faculty of Medicine and Health Sciences, Ghent University, Belgium.

VII.4.2 In vitro tension measurements*Tissue preparation*

After cervical dislocation, corpora cavernosa were carefully dissected free from surrounding structures and mounted in organ bath myograph systems for isometric tension measurements. Corpora cavernosa were mounted horizontally in the myograph with one end fixed to a transducer and the other to a micrometer. The organ baths were filled with 10 mL Krebs-Ringer bicarbonate (KRB) solution at 37 °C (pH 7.4) equilibrated with 95 % O₂–5 % CO₂. The tissues were allowed to equilibrate for 30 minutes in KRB solution that was frequently replaced. The corpora cavernosa were gradually stretched during 60 minutes until a stable preload of 0.45 g was obtained. Subsequently, the corpora cavernosa were contracted two times with 5 µM phenylephrine (Phe) and when a stable precontraction was reached the tissues were washed and allowed to relax to the resting tension. Next, the functionality of the endothelium within the corporal strips was examined by precontracting the corpora cavernosa with 5 µM Phe and adding 1 µM acetylcholine (Ach). Preparations that were not able to relax minimally 50 % of the maximum tension were excluded from the study. Thereafter, the corpora cavernosa were washed once again. When reaching a stable resting tension the experimental protocol was started.

Experimental protocol

Cumulative concentration-response curves to the MRP 4 inhibitor, MK-571, (1 nM – 100 µM) or to the PDE5i, sildenafil (1 nM – 100 µM), were constructed in cavernosal strips precontracted with Phe (5 µM). Similarly, the effect of MK-571 (1 nM – 100 µM) was studied in presence of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ).

In other experiments various cGMP–(in)dependent vasodilating substances were added at a stable tension to analyze the relaxing responses of the corpora cavernosa. In some experiments electrical field stimulation (EFS; parameters: train duration 40 s; 1, 2, 4 and 8 Hz; pulse duration 5 ms and 80 V), was applied to study neurogenic-mediated relaxations. After constructing the first response curve, the corpora cavernosa were washed out and allowed to recover for 20-30 minutes. Next MK-571 (10 μ M, 10 min) was added and responses to cGMP-(in)dependent vasodilating substances or EFS were studied again. In order to obtain comparable precontraction levels between control conditions and MK-571, the precontractile tone of the control corporal strips was adjusted by adding 3 μ M Phe instead of 5 μ M Phe.

VII.3 In vivo study

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed on a heated blanket to maintain their body temperature at 37°C. A PE-10 tube filled with heparinized saline (25 U/mL) was inserted into the carotid artery and connected to a pressure transducer to monitor the mean arterial pressure (MAP). A 30-gauge needle attached to another PE-10 tube was introduced into the right corpus cavernosum and also connected to a pressure transducer to measure the intracavernosal pressure (ICP). The ICP response to MK-571 (0.1 – 1 mg/kg) was investigated through intracavernosal (i.c.) administration via a separate cannula (30-gauge needle attached to PE-10 tube) inserted into the left corpus cavernosum (standardized injection volume of 5 μ L). Between each injected dose the cannula was flushed several times with heparinized saline to remove residual agent.

VII.4 cGMP analysis

Corpora cavernosa from Swiss mice were isolated and dissected as described for the in vitro tension measurements. Subsequently, corpora cavernosa were weighed to determine the tissue wet weight. Next the two corpora cavernosa of each mouse were placed for 10 minutes in 10 mL KRB solution at 37°C, bubbled with 95 % O₂–5 % CO₂. Thereafter, the control corpora cavernosa were treated with 5 μ M Phe for 20 minutes. Other corpora cavernosa were treated with 5 μ M Phe for 10 minutes and then MK-571 (10 μ M or 100 μ M) was added. Exactly 20 minutes after adding Phe (controls) or 10 minutes after adding MK-

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571, the corpora cavernosa were snap frozen in liquid nitrogen and stored at -80°C until further processing. cGMP was extracted and quantified using an enzyme immunoassay kit (Cayman Chemical cyclic GMP EIA kit, Michigan, USA). The frozen corpora cavernosa were pulverized by a Mikro-Dismembrator U (B. Braun Biotech International, Germany), homogenized in 6 % trichloroacetic acid (TCA) and centrifuged at 2000 g for 15 min at 4°C to collect the supernatant. The supernatant was washed three times with water-saturated ether to extract the TCA after which it was dried under nitrogen at 60°C . Dried extracts were dissolved in a 10 times volume of the assay buffer. Then, samples, controls and standards were acetylated and were added to the enzyme immunoassay plate to incubate for 18h at 4°C . Optical density was measured with a 96-well plate reader (Biotrak II, Amersham Biosciences) at 405 nm. The concentration of cGMP was expressed as pmol/g tissue wet weight.

Drugs and chemicals

The experiments were performed in a KRB solution of the following composition (mM): NaCl 135, KCl 5, NaHCO_3 20, glucose 10 (control) or 30 (high glucose), CaCl_2 2.5, MgSO_4 1.3, KH_2PO_4 1.2 and EDTA 0.026 in H_2O . L-phenylephrine hydrochloride (Phe), acetylcholine chloride (Ach), sodium nitroprusside (SNP), sildenafil citrate and methylglyoxal solution (MGO) were obtained from Sigma (St. Louis, MO, USA); MK-571, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), forskolin and pinacidil monohydrate from Enzo Life Sciences (Lausen, Switzerland) and trichloroacetic acid (TCA) from Merck (Darmstadt, Germany). Stock solutions were made in water except for forskolin and pinacidil (DMSO) and ODQ (ethanol). The final concentrations of vehicle solution in the organ bath never exceeded 0.1 %. All concentrations are expressed as final molar concentration in the organ bath.

Statistical analysis

The data are presented as mean values \pm S.E.M. Relaxations are expressed as % decrease in precontractile tone. N is the number of individual strips used, or in case of the cGMP analysis n represents the number of animals used. Sensitivity (pEC_{50}) and maximum response (E_{max}) were calculated from the concentration-response curves to MK-571 or sildenafil. pEC_{50} was defined as the negative logarithm to base 10 of the EC_{50} values and E_{max} was defined as the maximal relaxation. The data in our study was analyzed using SPSS, version 22; IBM Corporation, Armonk, NY, USA. Statistical significance was evaluated using the Mann-

Whitney U test or Wilcoxon test when appropriate. Two groups of data were considered significantly different when $p < 0.05$.

VII.5 Results

VII.5.1 In vitro experiments

Relaxant effect of MK-571 on isolated mouse corpora cavernosa

MK-571 and sildenafil evoked concentration-dependent relaxations in corpora cavernosa from Swiss mice (Fig. VII.1A and VII.1B). However, sildenafil was significantly more efficacious compared to MK-571 (E_{max} in presence of sildenafil: 85.95 ± 4.09 % vs in presence of MK-571: 67.02 ± 4.69 %, $p < 0.05$). In addition the potency of sildenafil to relax the corpora cavernosa was significantly higher compared to MK-571 (pEC_{50} of sildenafil: 6.18 ± 0.30 vs MK-571: 5.07 ± 0.10 , $p < 0.05$).

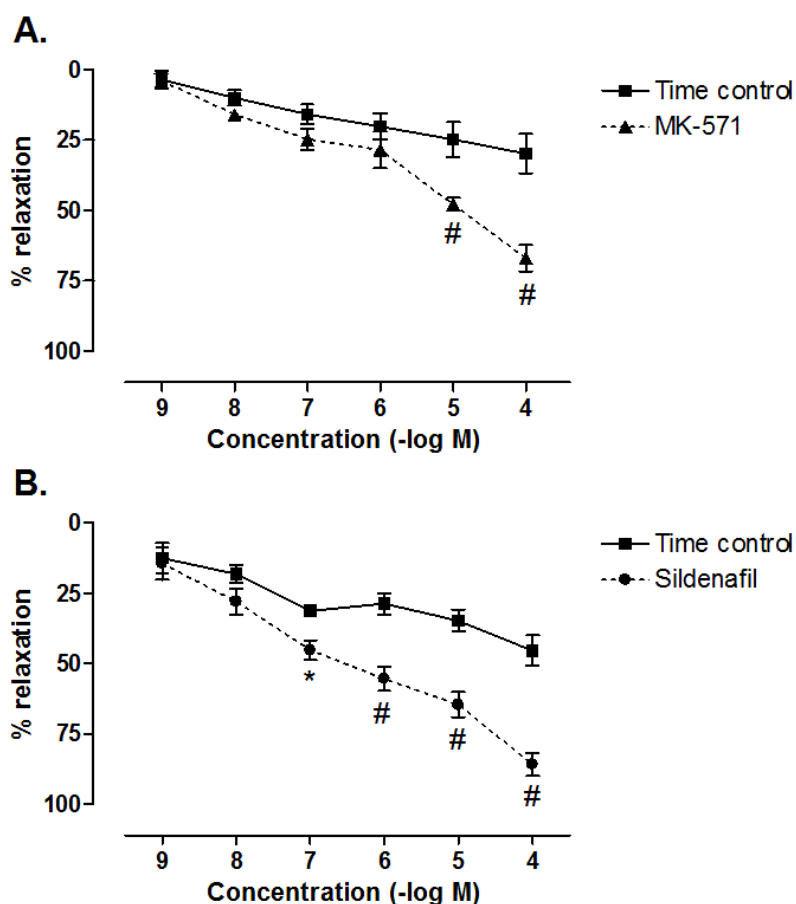


Figure VII.1 Relaxant effect of (A) MK-571 and (B) sildenafil on precontracted ($5 \mu\text{M}$ Phe) corpora cavernosa from Swiss mice versus time controls. Data are expressed as % decrease of Phe-induced tone; Mann-Whitney

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U test; # $p < 0.01$ and * $p < 0.05$ (n=6).

Involvement of the cGMP-pathway in the relaxant effect of MK-571

To explore the involvement of the sGC/cGMP pathway in the relaxant effect of MK-571 on mouse corpora cavernosa, MK-571-induced relaxations were studied in presence of the sGC inhibitor ODQ (10 μ M, 10 min). The presence of ODQ did not alter the relaxant effect of MK-571 in mouse corpora cavernosa (Fig. VII.2).

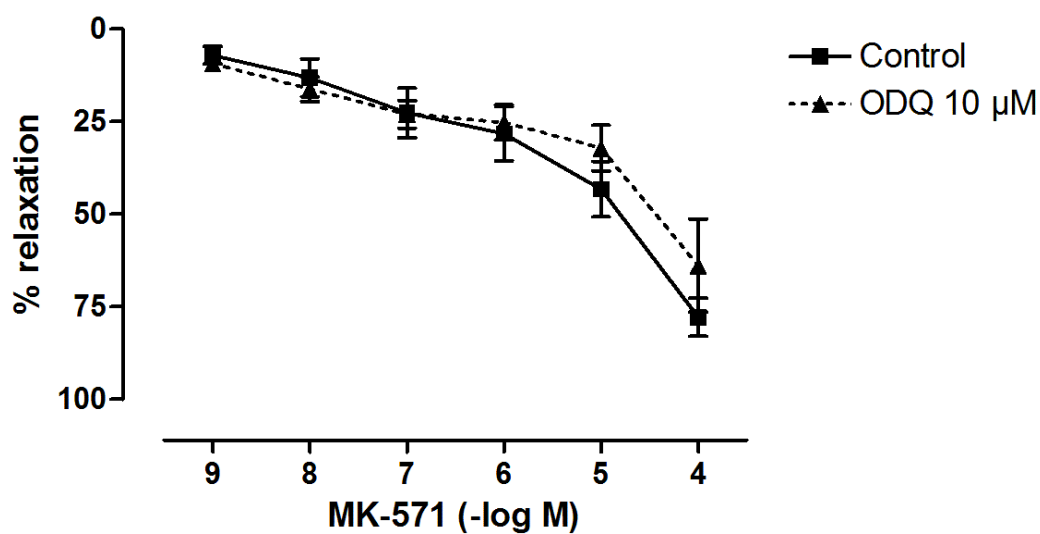


Figure VII.2 Relaxant effect of MK-571 on precontracted (5 μ M Phe) corpora cavernosa from Swiss mice in absence and presence of ODQ (10 μ M, 10 min). Data are expressed as % decrease of Phe-induced tone; Mann-Whitney U test; (n=6).

Effect of MK-571 on cGMP-dependent and cGMP-independent responses

The effect of MK-571 on cGMP-dependent, cAMP-dependent and K^+ channel-mediated responses was studied using SNP, sildenafil, forskolin, an adenylate cyclase activator, and pinacidil, a K_{ATP} channel opener, respectively. SNP- and sildenafil-induced relaxations of the mouse corpora cavernosa were significantly enhanced by preincubating the tissues with MK-571 (10 μ M, 10 min) (Fig. VII.3A and VII.3B). In contrast, MK-571 (10 μ M, 10 min) did not affect the relaxant responses of the corpora cavernosa to forskolin or pinacidil (Fig. VII.3C and VII.3D).

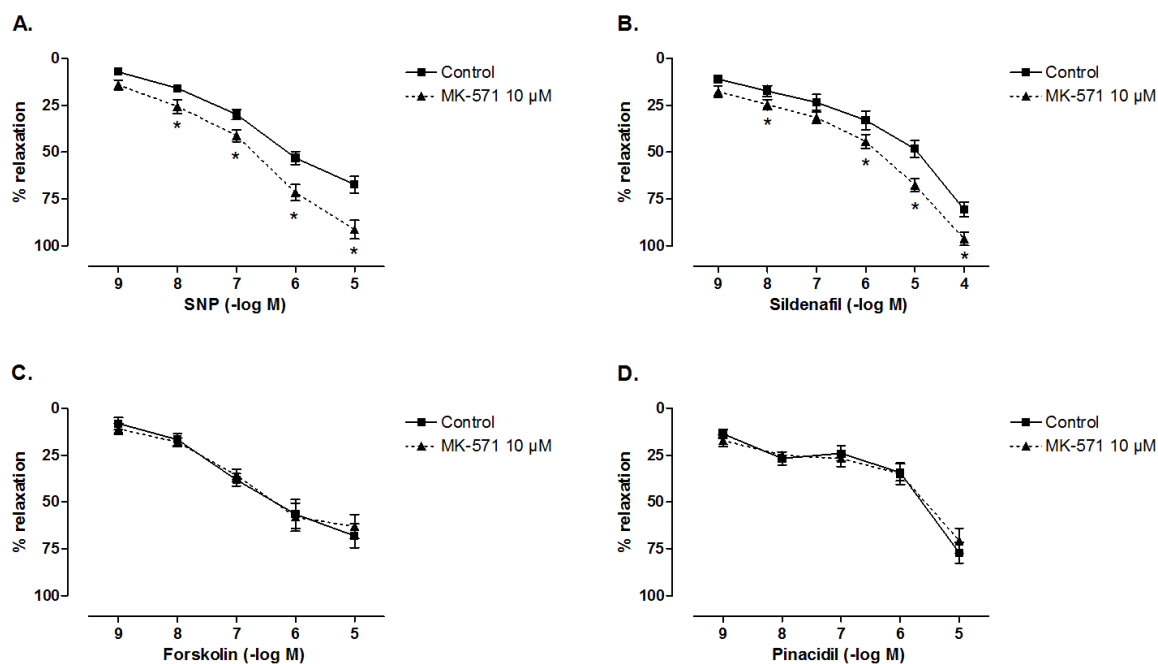


Figure VII.3 Relaxant effect of (A) SNP, (B) sildenafil, (C) forskolin and (D) pinacidil on Swiss corpora cavernosa before and after addition of MK-571 (10 μ M, 10 min). In control conditions corpora cavernosa were precontracted with 3 μ M Phe, while after addition of MK-571 precontraction was ensured by adding 5 μ M Phe. Data are expressed as % decrease of Phe-induced tone; Wilcoxon test; # p <0.01 and * p <0.05 (n=6-8).

Effect of MK-571 on normal and diabetic damaged erectile function

Neurogenic- and endothelial-mediated responses of mouse corpora cavernosa were studied by constructing EFS- and Ach-curves respectively. EFS and Ach evoked frequency- and concentration-dependent relaxations, which were significantly stronger after MK-571 treatment (10 μ M, 10 min) (Fig. VII.4A and VII.4B). 2 h treatment of corpora cavernosa with high concentrations of glucose (HG, 30 mM) in combination with methylglyoxal (MGO, 120 μ M) mimics diabetic damage to neurogenic- and endothelium-mediated corporal responses [14]. MK-571 (10 μ M, 2h) significantly enhanced corporal relaxant responses to EFS and Ach even in diabetic conditions (Fig. VII.4C and VII.4D).

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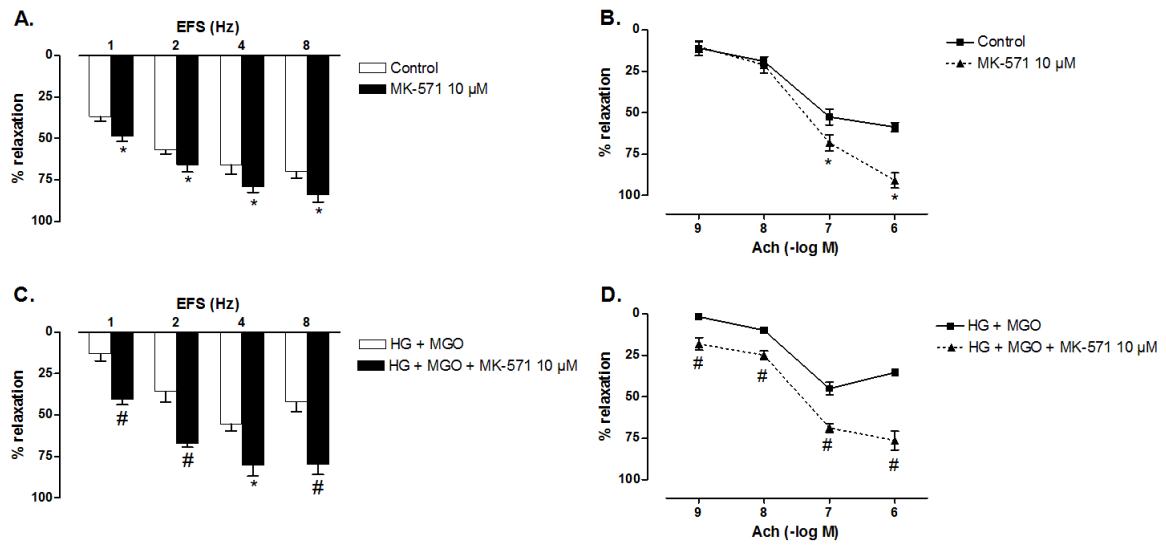


Figure VII.4 Relaxant effect of (A) electrical field stimulation (EFS) and (B) Ach on Swiss corpora cavernosa before and after addition of MK-571 (10 μ M, 10 min). Data are expressed as % decrease of Phe-induced tone; Wilcoxon test; * p <0.05 (n=6-7). Relaxant effect of (C) EFS and (D) Ach on Swiss corpora cavernosa after 2 hours co-incubation with high concentrations of glucose (HG, 30 mM) and methylglyoxal (MGO, 120 μ M) in absence and presence of MK-571 (10 μ M, 10 min). Data are expressed as % decrease of Phe-induced tone; Mann-Whitney U test; # p <0.01 and * p <0.05 (n=5-6). (A-D) In control conditions corpora cavernosa were precontracted with 3 μ M Phe, while after addition of MK-571 was ensured by adding 5 μ M Phe.

VII.5.2 In vivo experiments

Intracavernosal injection of increasing amounts of MK-571 significantly increased ICP and ICP/MAP while decreasing MAP only minimally (3.74 mmHg \pm 1.02 at 1 mg/kg) (Fig. VII.5 and VII.6)

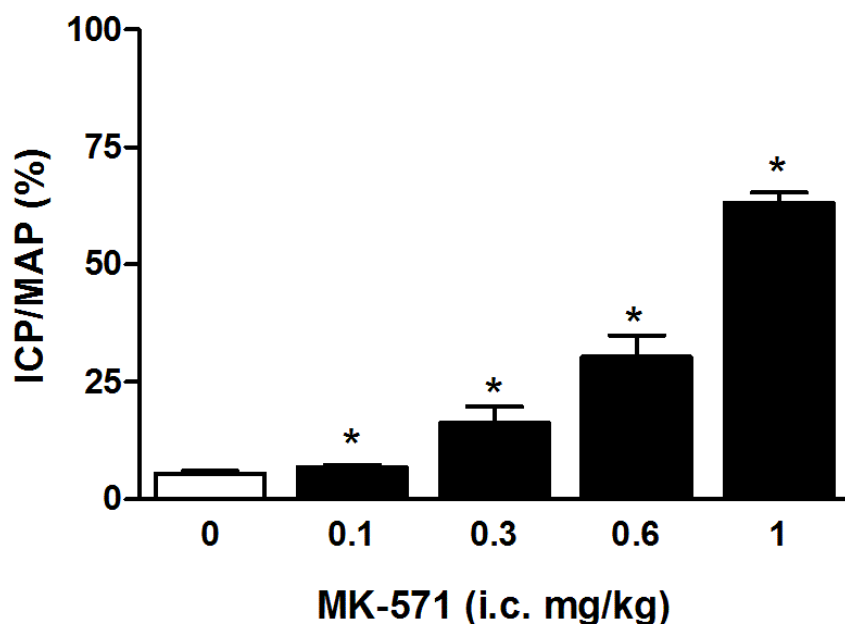


Figure VII.5 The intracavernosal pressure/mean arterial pressure (ICP/MAP) ratio (in %) after intracavernosal administration of MK-571 in Swiss mice; Wilcoxon test; * $p < 0.05$ (n=6).

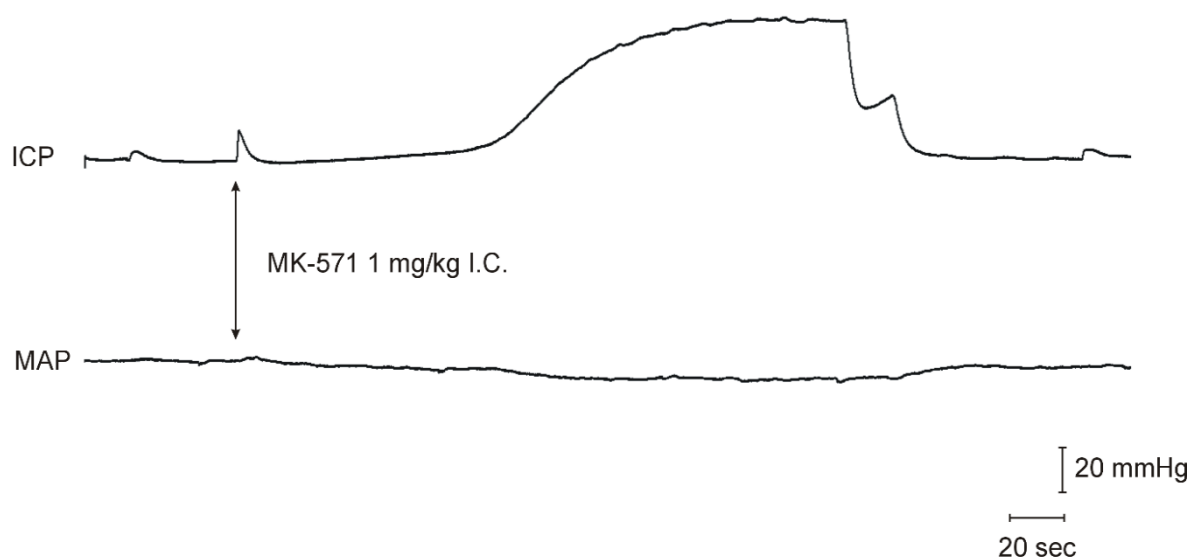


Figure VII.6 Original tracings showing the change in intracavernosal pressure (ICP) as well as mean arterial pressure (MAP) after intracavernosal (I.C.) administration of MK-571 (1 mg/kg) in Swiss mice.

VII.5.3 cGMP analysis

MK-571 (100 μM) significantly increased basal cGMP levels of the mouse corpora cavernosa by approximately 4.5 fold (Fig. VII.7). MK-571 (10 μM) also increased the level of cGMP in the corpora cavernosa by approximately 1.8 fold, however due to high variation of the results, this increase did not significantly differ from controls (Fig. VII.7).

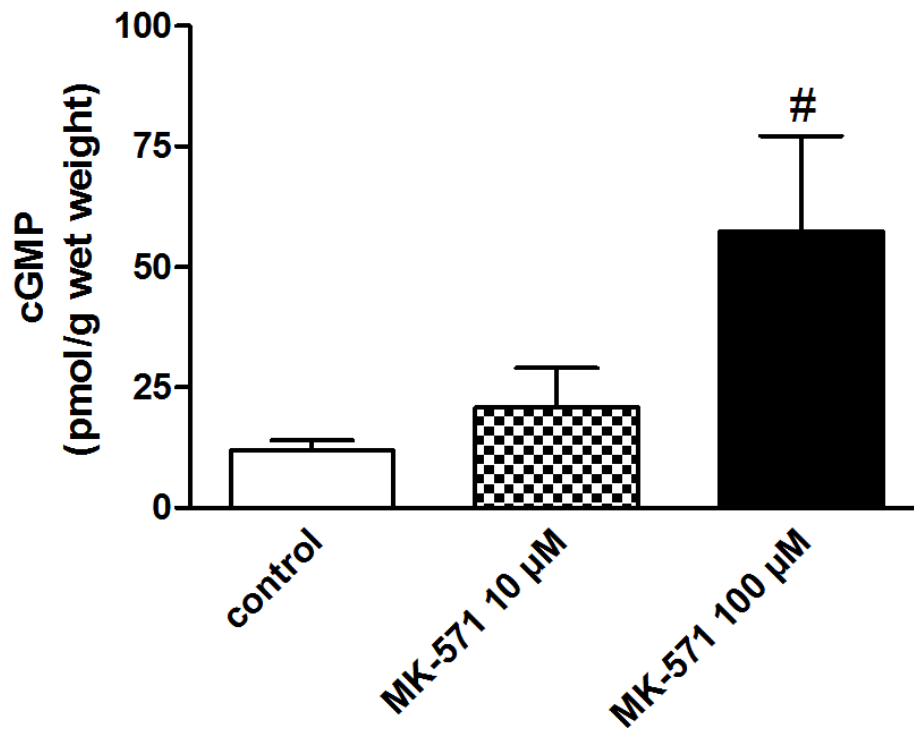


Figure VII.7 cGMP levels in corpora cavernosa from Swiss mice in basal conditions (control) and when incubated with MK-571 (10 or 100 μM , 10 min) in corpora cavernosa from Swiss mice. Data represent the means \pm S.E.M; Mann-Whitney U test; # $p < 0.01$ ($n = 5$ (control), $n = 4$ (MK-571 10 μM) and $n = 6$ (MK-571 100 μM)).

VII.6 Discussion

This study evaluated for the first time the functional effect of inhibiting MRP 4-mediated transport in mouse corpora cavernosa using MK-571. MK-571 relaxes mouse corpora cavernosa concentration dependently and induces erection by increasing basal intracellular cGMP levels. In addition, MRP 4 inhibition enhances cGMP-dependent relaxations in normal as well as under in vitro diabetic conditions.

For some years MRP 4 and MRP 5 have been identified as export pumps for cGMP. Thus MRP 4 and MRP 5 contribute, along with PDEs, to the regulation of intracellular cGMP levels [2-8]. Interestingly, MRP 4 and MRP 5 are also expressed in the smooth muscle cells of human corpora cavernosa [12, 13]. Hence, both MRP 4 and MRP 5 might be relevant in mediating cGMP export in corpora cavernosa. As the affinity of MRP 5 for cGMP is higher ($K_m = 2.1 \mu\text{M}$ [5]) than that of MRP 4 ($K_m = 9.7 \mu\text{M}$ [4]), MRP 5 is suggested to play a predominant role in cGMP export [12]. In the present study MRP-mediated transport was inhibited using MK-571, a known leukotriene receptor antagonist and potent inhibitor of MRP 4- but not MRP 5-mediated cGMP transport [5, 15, 16]. Therefore the results obtained in this study can only be interpreted in relation to MRP 4-mediated cGMP export.

This study demonstrates for the first time that MK-571 relaxes mouse corpora cavernosa concentration dependently albeit somewhat less potent than sildenafil. The in vitro relaxant effect of MK-571 could furthermore be demonstrated in vivo since i.c. administration of MK-571 raised ICP with a minimal effect on MAP. These results suggest that MK-571, at least when applied locally, might have potential in treating ED.

Although a direct relaxant effect of MK-571 was not earlier described, MK-571 administration has been shown to reverse pulmonary hypertension in mice [10]. The protective effect of MRP 4 inhibition was accompanied with increased cGMP levels [10]. Our measurement of intracellular cGMP content in the corpora cavernosa also revealed significantly higher cGMP levels in tissues treated with 100 μM MK-571 compared to controls. Although 10 μM MK-571 induced about 50 % relaxation of mouse corpora cavernosa, surprisingly the increase in cGMP levels was not significantly different from controls after incubating the corpora cavernosa with 10 μM MK-571. Since mouse corpora cavernosa are very tough to homogenize, cGMP levels are not easily determined in these

tissues. Hence, the high variation in our results and the lack of significance in cGMP between controls and tissues incubated with 10 μ M MK-571. However overall (a trend of) increased cGMP levels could be observed in MK-571 treated tissues, indicating that MK-571 relaxes mouse corpora cavernosa by enhancing basal cGMP levels. The relaxant effect of MK-571 was furthermore evaluated in presence of the sGC inhibitor, ODQ. As sGC catalyzes the conversion of guanosine triphosphate (GTP) to cGMP, pharmacological inactivation of sGC will hamper subsequent cGMP production. The presence of ODQ did however not diminish the relaxing effect of MK-571 on the corpora cavernosa. Therefore it might be thought that MK-571 relaxes the corpora cavernosa independently of cGMP, which would be in contrast with the cGMP measurements. Yet, the results with ODQ should be interpreted more carefully. Although the use of ODQ inhibits cGMP production, some basal levels of cGMP are still maintained [17]. Export or degradation of basal cGMP levels is thus unaffected by pharmacological inhibition of sGC. Like MK-571, others reported that the relaxant effect of PDE5i under basal conditions in corpora cavernosa from different animals was not or only partially affected by ODQ treatment [18-20]. Moreover it has been shown that ODQ is an incomplete inhibitor of sGC as it does not fully inhibit aortic relaxation to a NO-donor when compared to aorta from sGC knockout mice [23]. Hence, as suggested by Lies et al. [21], the lack of effect of ODQ does not exclude the involvement of the cGMP-pathway. Therefore even when sGC activation is inhibited, MK-571 could still increase intracellular cGMP by preventing export of basal cGMP levels and thus relax mouse corpora cavernosa.

Our study is the first to demonstrate an in vitro direct relaxant and an in vivo pro-erectile effect of MK-571 on mouse corpora cavernosa, however a hint that MRP 4 inhibition might be of great importance in the (vascular) relaxation process was provided by the experiments recently reported by Krawutschke et al. [8]. They demonstrated that MRP 4 inhibition by MK-571 significantly enhanced relaxations of aortic rings induced by the NO-donor S-nitroso-L-glutathione (GS-NO). This effect was roughly comparable with that of PDE-5 inhibition. In line with Krawutschke et al. [8], MRP 4 inhibition significantly enhanced SNP responses to a comparable degree as PDE-5 inhibition (own unpublished results). Interestingly, MK-571 had a small, but significant, potentiating effect on sildenafil-induced relaxations. This observation suggest a great potential for MK-571 as combination therapy with sildenafil for the treatment of ED. Combination therapy of MK-571 with sildenafil might therefore improve

the efficacy of sildenafil *in vivo*. Moreover, since MRP 4 is widely distributed throughout the body [3], MRP 4 inhibition could evoke unwanted systemic side effects. Therefore, coupling MK-571 to a PDE5i might offer an elegant way to specifically target the corpora cavernosa in order to surpass these side effects.

To explore whether the enhancing relaxant effect of MK-571 was specific to cGMP-dependent relaxations, various other relaxing substances were tested. It has been reported that MRP 4 is able to export cAMP besides cGMP [2-5, 7, 10, 11, 22]. However pretreating the corpora cavernosa with MK-571 did not improve forskolin-induced relaxations. As MRP 4 has a greater affinity for cGMP than for cAMP [5] this lack of effect is however not surprising. To further evaluate the cGMP-specificity of MK-571's enhancing relaxant effect, the effect of MRP 4 inhibition was tested on K_{ATP} -mediated relaxations, using pinacidil. As no effect of MK-571 was observed on pinacidil-induced relaxations, these results indicate that in mouse corpora cavernosa MRP 4 inhibition seems to be specific for cGMP-mediated effects.

Next, we wondered whether the positive effect of MRP 4 inhibition on cGMP-mediated responses, would still occur in pathologic conditions such as diabetes, a disease frequently associated with ED [23, 24]. Previously we demonstrated that diabetic damage to EFS- and Ach-mediated responses in mouse corpora cavernosa could be mimicked *in vitro* by incubating the corpora cavernosa for 2 h with high concentrations of glucose in combination with MGO [14]. Both in normal as well as under *in vitro* diabetic conditions MK-571 significantly enhanced EFS- and Ach-mediated relaxations. This observation makes MRP 4-mediated cGMP export a potential interesting target for the treatment of diabetic ED.

As no specific inhibitor for the other MRP subtypes is available, this study could only investigate the effect of MRP 4 inhibition, which might play a minor role in cGMP export in corpora cavernosa [12]. It should be noticed that in hepatocytes oxidative stress induces MRP 4 expression [25]. Moreover, higher MRP 4 mRNA and protein levels were found in proliferative human coronary smooth muscle cells *in vitro*, compared to quiescent smooth muscle cells [11]. Similarly, MRP 4 was upregulated in pulmonary arteries from patients with idiopathic pulmonary arterial hypertension as well as in mice exposed to hypoxic conditions [10]. These studies indicate that MRP 4 expression in smooth muscle cells is increased under pathologic conditions. Hence the pronounced positive effects of MRP 4 inhibition on the

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functioning of mouse corpora cavernosa seen in this study, might even be greater in in vivo pathological conditions associated with ED.

VII.7 Conclusion

This study demonstrated that in mouse corpora cavernosa two distinct pathways exist to regulate intracellular cGMP levels: (i) cGMP degradation and (ii) cGMP export. Although it has been suggested that MRP 4 plays a minor role in cGMP export [12], inhibition of MRP 4 potentially relaxed mouse corpora cavernosa and enhanced cGMP-dependent relaxations especially in diabetic conditions. Moreover MRP 4 inhibition elicited a pro-erectile effect in vivo. These results suggest that MRP 4, at least when targeted locally in the penis or when combined with a PDE5i, might be a valuable alternative strategy for the treatment of (diabetic) ED.

VII.8 Acknowledgements

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VII.9 References

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Chapter VIII

Discussion and future perspectives

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VIII.1 Modulation of vascular tone by perivascular adipose tissue

Blood flow to the organs and tissues is continuously adapted depending on their metabolic and functional needs. By changing the tone of the vascular smooth muscle cells, blood vessels alter their diameter and as a consequence blood flow to the organs and tissues is regulated. The regulation of blood flow has always been an important research topic, however the focus of vascular research has changed over the years. The earliest functional studies analyzed the effects of agents on vascular smooth muscle tone and identified receptor subtypes. With the discovery in the 1980s of nitric oxide (NO) as an endothelium-derived relaxing factor (EDRF) and its crucial role for normal vascular function, vascular research was reoriented. From then on it became clear that the endothelium is an important paracrine tissue that controls the homeostasis of the underlying vascular smooth muscle cells by producing and releasing several substances that control vascular tone [1]. Nowadays endothelial dysfunction is considered as a pivotal factor in the pathogenesis of several vascular diseases such as hypertension, atherosclerosis and erectile dysfunction which are often associated with obesity and diabetes [2-5]. The past decades, besides the endothelium and the nerve endings, perivascular adipose tissue gained attention as neighboring tissue that can control vascular smooth muscle tone through the release of vasoactive factors (adipo(cyto)kines). It has been shown that PVAT exerts a vasorelaxing ('anticontractile') effect due to the release of the 'adipocyte-derived relaxing factor(s)' (ADRF(s)) [6-10]. Dysfunctional PVAT (i.e. dysregulated adipocytokine release and structural changes in adipose tissue mass) has been observed in various disease states such as obesity and hypertension [11-15]. As a consequence besides the endothelium, PVAT is now considered as an important contributor for the development of obesity-related vascular complications [16-19]. Thus, interference with or prevention of adipose tissue dysfunction might offer a therapeutic option to treat or alleviate vascular complications as seen in for instance obese patients.

VIII.1.1 Can the relaxing influence of PVAT be positively or negatively modulated by resveratrol treatment and/or testosterone depletion?

Previously we have shown that acute hypoxia enhances the anticontractile effect of PVAT surrounding mouse aorta [20]. Since the relaxing influence of PVAT can be altered under acute hypoxia, our interest was driven to seek other circumstances that may modify the relaxing effect of PVAT. As both resveratrol and testosterone are known to influence adipose tissue function, the first part of this project studied the vasorelaxing effect of PVAT and evaluated whether this effect could be modulated by resveratrol or orchidectomy. First of all our study confirmed the importance of PVAT as paracrine regulator of vascular tone. The presence of PVAT clearly attenuated the contractile responses of the aortic segments to norepinephrine (NOR), confirming the beneficial anticontractile (vasorelaxing) effect of PVAT under physiological circumstances [6-10]. Moreover this anticontractile effect was independent of NO and cyclooxygenase (COX), suggesting potential involvement of ADRF(s) as indicated in literature [8]. Next we assessed the influence of acute addition of resveratrol on the vasorelaxing effect of PVAT. Resveratrol is a naturally occurring polyphenol found in the skin of grapes and thus highly abundant in red wine. For some years resveratrol has been broadly studied and diverse physiological actions have been ascribed including antioxidant, anti-inflammatory and anti-carcinogenic effects [21, 22]. In addition resveratrol was shown to exert anti-obesity effects through mechanisms involving down-regulation of adipogenesis and inflammatory processes [23]. Moreover resveratrol has been demonstrated to mimic the positive effects of calorie restriction in obese humans. These include improvement of insulin resistance, decreased levels of blood glucose, triglycerides and cytokines as well as decreased intrahepatic lipid content, and decreased systolic blood pressure [24, 25]. Furthermore several in vitro studies indicated that resveratrol alters the adipocytokine secretion profile of adipocytes towards a less inflamed one [24, 26-32]. Therefore it could be expected that resveratrol potentiates the vasorelaxing influence of PVAT. However, since in our study no enhanced 'anticontractile' effect of PVAT could be observed in the presence of resveratrol, our results could not confirm the beneficial potential of resveratrol addition on adipose tissue functioning as suggested by others. Instead, a lower anticontractile effect of PVAT was seen in the presence of resveratrol. In addition the beneficial relaxing effect of resveratrol was lost in presence of PVAT. These effects could be explained by a probably dual

action of resveratrol: (i) a direct relaxant effect and (ii) an attenuation of vasodilatory adipo(cyto)kine production or secretion.

However, despite our 'negative' results concerning resveratrol addition, the results of this study do not allow us to draw firm conclusions with respect to resveratrol as potential therapeutic option in case of dysfunctional adipose tissue and subsequent vascular diseases. Several factors hamper addressing this question and should therefore be kept in mind.

First, experiments described in chapter IV were performed in normal, healthy conditions. As such, no dysfunctional PVAT was present that could be counteracted by resveratrol treatment. This could be important since resveratrol has been shown to alter adipo(cyto)kine secretion only in dysfunctional adipose tissue [24, 26-32]. In search of a method to render PVAT dysfunctional, we focused on the effect of orchidectomy on PVAT functioning since low levels of testosterone increase the risk of obesity, cardiovascular diseases, and even mortality in men [33, 34] through the increase of body adipose tissue mass deposition [35, 36]. In addition, testosterone depletion increases vasoreactivity of the arteries [37] and testosterone is thought to influence adipocytokine secretion [38-40]. Considering these facts, it was expected that testosterone depletion, using orchidectomy, would affect the normal functioning of PVAT leading to a decreased vasorelaxing effect of PVAT. Despite the orchidectomy that was successfully performed (indicated by atrophy of seminal vesicles), 4 weeks of testosterone depletion surprisingly did not alter the anticontractile effect of PVAT surrounding mouse aorta. The failure of orchidectomy to alter PVAT functioning in our experiments might be explained by a second point that needs to be kept in mind when investigating the paracrine role of PVAT, namely the vascular bed. Vascular beds are surrounded by PVAT that varies with anatomical location and which can be characterized either as white adipose tissue (WAT) or brown adipose tissue (BAT). While both types of PVAT share functions common with adipose tissue, such as the paracrine effects, some specific differences are present. For example, thoracic PVAT is distinct from mesenteric PVAT, as thoracic PVAT resembles BAT. All of the experiments described to evaluate the effect of resveratrol and orchidectomy on the vasorelaxing effect of PVAT, were performed on segments from the thoracic aorta, which is known to be surrounded by BAT [41]. However, unlike white PVAT, surrounding mesenteric arteries [6, 42], brown PVAT appears to be resistant to dysregulated adipocyte biology of obesity and subsequent

inflammation [43]. This surprising regional difference in susceptibility to adipose tissue dysfunction is an interesting field for future research since it is currently unclear whether there are any differences regarding the paracrine (pro- or anti-contractile) effects between thoracic brown PVAT and mesenteric white PVAT, both under normal as well as under pathological conditions.

Since white PVAT surrounding mesenteric arteries, might be a more accurate model to investigate adipose tissue dysfunction as seen in obesity, a first attempt was made to explore whether the functional properties of PVAT vary among vascular beds with distinct PVAT types. Therefore additional experiments were set-up in which mouse mesenteric arteries with and without PVAT were investigated. In line with others [10, 44, 45], the presence of PVAT attenuated the contractile responses of mesenteric arteries to phenylephrine (PHE), indicating that the anticontractile effect of PVAT exists even in mesenteric arteries (**Figure IX.1** in supplementary data). Next, several attempts were made to create an in vitro model for dysfunctional adipose tissue surrounding mesenteric arteries. Since oxidative stress has been found to underlie the functional changes in adipose tissue during obesity [12, 44-47], different protocols were used that are known to induce oxidative stress. Treating the mesenteric arteries with and without PVAT with palmitic acid (PA) or high concentration of glucose in combination with methylglyoxal both resulted in a loss of anticontractile effect of PVAT surrounding the mesenteric arteries. Unfortunately these promising results could no longer be reproduced systematically due to unexplained problems. As a consequence we were not able to investigate the effect of acute resveratrol addition on the functioning of PVAT surrounding mesenteric arteries under obese-like circumstances. The development of a solid in vitro method for adipose tissue dysfunction would therefore be of great value. It was reported that the anticontractile effect of PVAT in rat mesenteric arteries was lost after chronic experimental hypoxia [44, 48]. Therefore chronic hypoxia induction might offer an option to induce adipose dysfunction in our experimental set-up as well. These experiments along with future research should help to clarify whether the different PVAT depots (brown vs white) affect the overall paracrine effect of PVAT in normal as well as under pathological conditions.

Taken together, our first study suggests that acute resveratrol addition attenuates the vasorelaxing effect of PVAT. In contrast our results could not provide evidence for a modulatory role for orchidectomy on the functioning of PVAT.

VIII.2 New strategies to treat erectile dysfunction

The importance of the NO/cGMP as a key pathway for normal penile erection is well established [49]. Dysfunction of the NO/cGMP signaling pathway is believed to contribute to erectile dysfunction (ED) [49]. Currently different treatment options for ED are available, PDE-5 inhibitors such as sildenafil, vardenafil and tadalafil being the first choice [50]. These PDE-5 inhibitors prevent the normal hydrolysis of cGMP by PDE5 and thereby cGMP accumulation is promoted, which facilitates penile smooth muscle relaxation. Therefore, PDE-5 inhibitors can reverse, to a certain degree, deficiencies in the NO/cGMP pathway. PDE-5 inhibitors have a known clinical efficacy in the treatment of ED with various etiologies and a broad range of severity [51-53]. However, the limitation in the efficacy of these agents is that a minimum or 'critical amount' of NO is necessary for these drugs to work. Nerves that are severely damaged will not be able to synthesize NO. In addition in conditions such as diabetes mellitus or in case of severe vascular disease, there is a decreased expression or activity of neuronal nitric oxide synthase (nNOS) or endothelial nitric oxide synthase (eNOS), impaired NO release, or (reactive oxygen species (ROS)-induced) decreased bioavailability of NO which all hamper the production of sufficient amounts of cGMP [54]. If these alterations are severe, they cannot be compensated by PDE-5 inhibition. As a result 30-35 % of the patients suffering from ED fail to respond to PDE-5 inhibitors [54, 55]. Therefore the search for new and better alternatives is necessary and ongoing. Therapeutic strategies favoring NO synthesis, release or bioavailability may improve erectile function or at least enhance the efficacy of PDE-5 inhibitors when applied in combination.

VIII.2.1 Do resveratrol and/or quercetin exert positive effects on corpora cavernosa? How do they work?

The first candidates that we studied as potential new therapeutic option to treat ED, were the red wine polyphenols, resveratrol and quercetin. Both polyphenols are widely distributed in various plants, vegetables and fruit and are known for a variety of biological effects including anti-cancer, anti-inflammatory, and vascular protective effects such as a

vasodilatory and an antioxidant capacity [21, 22, 56, 57]. Therefore these compounds could also be of value for treating erectile dysfunction, however the effect of resveratrol or quercetin on erectile (dys)function is poorly understood. Thus the second and third study of this project evaluated whether resveratrol quercetin could also exert their beneficial vascular and antioxidant effects in erectile tissue. Our study showed that resveratrol, but not quercetin, relaxed mouse corpora cavernosa. Although we could not demonstrate a direct relaxant effect of quercetin on mouse corpora cavernosa, others found that quercetin was able to relax isolated human corpora cavernosa [58]. Several mechanisms have been proposed by which resveratrol relaxes arteries or corpora cavernosa from different species. These include both endothelium (NO/cGMP)-dependent and endothelium-independent mechanisms [59-64]. However in our study the involvement of NO in the relaxant effect of resveratrol on mouse corpora cavernosa could not be confirmed since treatment with the NOS inhibitor, L-NAME, did not decrease the relaxant effect of resveratrol on the corpora cavernosa. Moreover the contribution of cGMP in resveratrol's relaxant effect on mouse corpora cavernosa was further evaluated by focusing on soluble guanylyl cyclase (sGC), the enzyme that generates cGMP. Pharmacological inhibition of sGC using ODC, did however not decrease the relaxant effect of resveratrol in mouse corpora cavernosa. In addition the role of sGC/cGMP in resveratrol-induced relaxations was determined more accurately using genetically modified mice. Despite the existence of two isoforms for each subunit of sGC, only the sGC $\alpha_1\beta_1$ and sGC $\alpha_2\beta_1$ heterodimers are catalytically active and found in vivo [65]. A deletion in the catalytic domain of the α_1 subunit selectively inactivates the sGC $\alpha_1\beta_1$ isoform [66]. Even in the corpora cavernosa from these sGC $\alpha_1^{-/-}$ mice, resveratrol induced concentration-dependent relaxations similar to those in the wild-type control mice. Although sGC $\alpha_1\beta_1$ is the predominant form in most tissues, including in the vascular and penile smooth muscle cells, residual activity of the sGC $\alpha_2\beta_1$ isoform should not be underestimated [67]. Hence, genetically modified knock-in mice (sGC $\beta_1^{ki/ki}$) were developed in which mutation of the histidine 105 residue of sGC β_1 to phenylalanine results in the expression of heme-free NO-insensitive sGC which affects both heterodimeric isoforms [68]. The results of the resveratrol-induced relaxations in these sGC $\beta_1^{ki/ki}$ mice are presented in a supplementary figure (**Figure IX.2**). However, no involvement of the sGC $\alpha_2\beta_1$ isoform could be observed. Taken together, all these results indicate that the relaxant effect of resveratrol on mouse corpora cavernosa occurs independently of NO/sGC/cGMP.

This conclusion is somehow surprising since the limited research on the effect of resveratrol on corporal tissue has provided evidence that resveratrol can elevate cGMP levels in corpora cavernosa smooth muscle cells through a NO-dependent pathway [69]. In addition, it has been reported that resveratrol normalizes the impaired endothelium-dependent relaxations of corpora cavernosa from hypercholesterolemic rabbits [70, 71]. Why our data show no involvement of the NO/cGMP pathway in corporal relaxation to resveratrol in mice, while other studies have clearly demonstrated the contribution of this pathway in the protective effect of resveratrol on corporal tissue could have several reasons. First, species differences could be an explanation. Second, our study focused on the acute effect of resveratrol on corpora cavernosa from 'healthy' mice, whereas the improvement of endothelium-dependent relaxations by resveratrol was observed in 'sick' animals as rabbits were hypercholesterolemic [70, 71]. Therefore it is possible that in resting conditions resveratrol does not induce corporal relaxations through activation of the NO/cGMP pathway or that in resting conditions this pathway and downstream effector mechanisms are not direct targets for the acute effects of resveratrol.

Next, several attempts were made to elucidate the exact underlying mechanism of resveratrol's relaxant effect in mouse corpora cavernosa. In addition to activation of the NO-pathway, multiple modes of action have been identified for resveratrol. Unfortunately the use of several inhibitors of different potential molecular targets of resveratrol failed to prove the involvement of K^+ channels, COX metabolites, heme oxygenase, adenosine receptors, the cAMP pathway or adenosine monophosphate-activated protein kinase (AMPK). The results investigating the involvement of AMPK in the relaxant effect of resveratrol on mouse corpora cavernosa are represented in **Figure IX.3**. Recently a study demonstrated that the resveratrol-induced relaxations in mouse corpora cavernosa is partly dependent on H_2S formation and acts independent of eNOS pathway [72]. However only a small part of the relaxant effect of resveratrol seemed to be mediated by H_2S , suggesting that (an)other mechanism(s) must be involved as well. It thus seems that the exact mechanism(s) by which resveratrol relaxes corpora cavernosa is hard to characterize. The pleiotropic actions of resveratrol and its potential to activate distinct pathways, might explain why the relaxant effect of resveratrol on the corpora cavernosa could never be completely abolished. Maybe a cocktail of several inhibitors of different molecular pathways should be used.

Overall this study found a positive relaxant effect of resveratrol, but not quercetin, on mouse corpora cavernosa. However as yet the underlying mechanism remains elusive.

VIII.2.2 Do resveratrol and/or quercetin exert positive effects on corpora cavernosa under in vitro-diabetic conditions?

In a next step, we wanted to evaluate the effect of the red wine polyphenols on the contractility of the corpora cavernosa and arteries in conditions that are known to be associated with vascular and/or erectile dysfunction. Several reports already indicated that in diabetic or hypercholesterolemic animals resveratrol and quercetin restore impaired endothelium-dependent relaxations of different arteries and/or corpora cavernosa [70, 71, 73-76]. There is growing evidence demonstrating the role of oxidative stress in the pathophysiological mechanism of several vascular diseases including erectile dysfunction [54]. Hence, several protocols were set-up to create an oxidative stress-like environment in order to evaluate the potential positive effects of resveratrol and quercetin on the arteries and corpora cavernosa from mice in diseased conditions.

The first series of experiments were performed using palmitic acid, which is one of the most abundant saturated fatty acids in plasma. In addition, saturated fatty acids are found in elevated concentrations in obesity/diabetes [77]. It is well-documented that high levels of free fatty acid (FFA), including PA, cause oxidative stress [78-82] which is thought to underlie the impairment of endothelium-mediated relaxations after PA incubation [83, 84]. In our study PA significantly attenuated neurogenic mediated relaxations which might reflect the oxidative stress-induced damage. Of the polyphenols, only resveratrol was able to prevent PA-induced neurogenic damage. However, due to the small size of the corpora cavernosa, we were not able to perform direct oxidative stress measurement, therefore no clear conclusions could be made concerning the antioxidant capacity of resveratrol in this set-up. In addition, if PA induced oxidative stress, than it could be expected that the endothelium would be primarily affected considering its crucial role for normal vascular/corporal functioning. However in this study PA did not induce endothelial damage since endothelium-mediated relaxations were not reduced. Moreover, the small restoring effect of tempol, a known antioxidant, and quercetin, which is thought to be a more potent antioxidant than resveratrol [85, 86], suggests that oxidative stress does not completely explain the PA-induced damage in our experimental set-up. Therefore another process must be involved as

well. Recently it has been suggested that in neuronal cells, PA inhibits normal autophagy [87], an important cellular process that removes damaged organelles or cellular constituents and provides energy under starvation conditions or repairs damage under stressed conditions. Interestingly resveratrol was shown to promote autophagy [88, 89] and thus this might explain why resveratrol protected the corpora cavernosa from PA-induced damage to the electrical field stimulation (EFS)-mediated relaxations.

Since PA damaged the contractility of the corpora cavernosa only to a minimal extent, a more effective way to mimick oxidative stress-like damage was sought. Therefore diabetic conditions were mimicked *in vitro* using the combination of high glucose and methylglyoxal (MGO), since both compounds are found in high concentrations in the plasma of diabetic patients. Moreover, both hyperglycemia and increased levels of MGO are thought to contribute to the development of the vascular complications seen in diabetes [90]. Our data with glucose/MGO indeed indicated that these acute diabetic conditions affect the endothelial and neurogenic mediated-relaxant responses of small arteries and the corpora cavernosa. *In vivo* this could eventually be translated into microvascular damage and erectile dysfunction. Tempol, ascorbic acid, resveratrol and quercetin were able to prevent glucose/MGO-induced damage to the corpora cavernosa. Whereas only tempol and ascorbic acid prevented the diabetic damage in the mesenteric arteries. It was suggested that the degree of oxidative stress in the mesenteric arteries might be too high for both polyphenols to counteract, leading to their inability to protect these arteries from *in vitro* diabetic-induced damage.

In summary, in addition to a positive relaxant effect of resveratrol on mouse corpora cavernosa, the studies using PA and glucose/MGO revealed that both resveratrol and quercetin are able to protect mouse corpora cavernosa from obesity or diabetes-like damage to neurogenic and/or endothelium-mediated relaxant responses. These *in vitro* results suggest a potential role of both polyphenols for improving erectile function in diseased conditions. However, the study using glucose/MGO, investigated the effect of acute diabetes induction and acute polyphenol treatment *in vitro* and therefore the results cannot be simply extrapolated to chronic treatment or to an *in vivo* situation. Future experiments should confirm whether the same positive effects of resveratrol and quercetin *in vitro* also occur *in vivo* using for instance diabetic-induced mice. Until now we were unable to

establish a stable *in vivo*-model for diabetes and thus to study these compound in an *in vivo* set-up. However, some reports already demonstrated that chronic treatment of diabetic or hypercholesterolemic animals with resveratrol and quercetin alone or in combination with PDE-5 inhibitors significantly improved erectile function [69, 91-93]. Since we and others investigated the ability of resveratrol and quercetin to prevent diabetic-induced impairment of erectile function, the question still remains whether these polyphenols could be useful as therapeutic option for ED. This should be addressed in future experiments studying the effects of resveratrol and quercetin once erectile dysfunction is already established.

Moreover, we and others demonstrated the positive effects of resveratrol and quercetin in the cardiovascular system using preclinical studies. The next logical step would be to determine whether the observed beneficial effects are transferable to humans. However, evidence regarding the safety, optimal dosage and positive effects of polyphenols in humans is rather scarce. Only few studies demonstrated the cardioprotective activity of resveratrol and quercetin in humans through effects related to the improvement of inflammatory markers, glucose metabolism, endothelial function and the ability the lower blood pressure [94, 95]. As mentioned in the introduction, the therapeutic usage of resveratrol and quercetin is somewhat hampered by their low bioavailability. Therefore future research should focus on methods that are able to increase the bioavailability, delay the metabolism of the polyphenols or target their delivery. Currently, self-nano-emulsifying drug delivery systems (SNEDDS) and liposome- or nanotechnology based resveratrol/quercetin formulations are being developed to improve aqueous solubility and stability and to enhance targetability and the rate and extent of absorption of the polyphenols [96-100]. In addition both compounds could be used as blueprints to design and synthesize novel more potent drugs. For example, a newly developed resveratrol analog, HS-1793, does not contain the unstable double bond found in resveratrol and the positions of two of the three hydroxyl groups in the aromatic ring of HS-1793 are also different. It is found that HS-1793 is metabolically more stable and exerts cardioprotective effects [101]. Furthermore, since metabolites of resveratrol and quercetin were found to exert some biological effects as well [102-107], it would be interesting and physiologically more relevant to study the effects of resveratrol and quercetin glucuronides and sulfates on erectile function.

VIII.2.3 Does inhibition of cGMP export elicit positive relaxant responses in mouse corpora cavernosa?

It is a well-established fact that increasing cGMP is a cardinal event for normal penile erection. Therefore current therapies to treat ED are often targeted towards increasing these cGMP levels. Indeed, PDE-5 inhibitors, which inhibit cGMP degradation, represent an effective way to overcome ED. However, as previously mentioned, the urge for alternatives is necessary. In search for new therapeutic options to treat ED, the last part of this project focused on how cGMP levels could be increased in corpora cavernosa by inhibiting multidrug resistance protein (MRP)-mediated cGMP export. Recently it has become clear that in addition to degradation by PDE, export of cGMP out of the smooth muscle cells by MRP 4 and MRP 5 also determines the intracellular levels of cGMP [108-114]. Since both MRP 4 and MRP 5 expression were found in corporal smooth muscle cells [115, 116], they might represent an alternative way to increase cGMP content and thus to treat ED. Due to the lack of specific inhibitors for the different subtypes of MRPs we could only investigate the effect of MRP 4 inhibition using the leukotriene receptor inhibitor MK-571. Although MK-571 is a known leukotriene receptor inhibitor, several studies indicated that MK-571 potently inhibits MRP 4 mediated transport as well [117, 118]. MRP 4 has previously been reported as a potential target for therapeutic intervention of pulmonary arterial hypertension [119] and other cardiovascular diseases [120]. In addition the importance of cGMP export in the regulation of vascular relaxation was recently illustrated [112], suggesting that MRP 4 inhibition could be of value in corporal relaxant responses as well. However the functional effect of MRP 4 inhibition and the cGMP-dependency of this effect in corpora cavernosa was never explored. The results in our study on cGMP export, indeed showed for the first time that MK-571 potently relaxed corpora cavernosa through an increase of basal cGMP levels. This effect was less pronounced to that of sildenafil, suggesting the existence of both a degradation and a cGMP export pathway in the corpora cavernosa. Moreover, MK-571 enhanced cGMP-mediated relaxations even in diabetic mimicking conditions, suggesting that MRP 4 might be a valuable alternative target for treating (diabetic-related) ED. Although this study identified positive effects of MRP 4 inhibition on isolated cavernosal tissue, it is hard to extrapolate these in vitro findings to normal physiologic response of intact erectile tissue considering the absence of arterial flow, venous outflow, autonomic innervation and other homeostatic factors that might interfere with the cavernosal tone. As the in vivo

experiments performed in this study already revealed that intracavernosal administration of MK-571 exerts pro-erectile effects, future in vivo experiments evaluating for instance oral administration should confirm the overall in vivo relevance of MRP 4 inhibition on erectile function in normal as well as in pathological conditions.

VIII.2.4 General future considerations

Based on our in vitro work, resveratrol, quercetin and MRP 4 inhibition are suggested to be useful for improving erectile function. So what concentration should be obtained in man to benefit from these positive effects? Several aspects hamper to answer this question. First, extrapolating the in vitro concentrations of for instance resveratrol used in this study that yielded beneficial effects on the erectile tissue (ranging from 10 – 100 μ M) would mean that plasma concentration in man should reach about 2 250 – 22 500 ng/mL. As previously mentioned, due to the low bioavailability, these concentrations are however far above achievable considering that plasma levels of unchanged resveratrol only peaked to 539 ng/mL (or 2.4 μ M) after oral intake of 5 g of resveratrol. However due to possible species differences, the in vitro concentrations used in mouse tissues cannot be extrapolated as such to humans. Confirmation of the positive results of resveratrol, quercetin and MRP 4 inhibition found on mouse erectile tissues, is therefore necessary in human tissue. Second, in vitro concentrations of compounds needed to cause a direct relaxant effect in corpora cavernosa might be higher than their concentrations needed to exert an in vivo effect. For instance free plasma concentrations in man peak at 300 ng/mL, 400 ng/mL and 20 ng/mL after a single oral dose of sildenafil (100 mg), tadalafil (20 mg) and vardenafil (20 mg) respectively [121]. These concentrations are notably lower than the concentrations of these PDE-5 inhibitors causing direct relaxation of corpora cavernosa in vitro [122]. Thus, taken together although our studies could clearly indicate positive effects of resveratrol, quercetin and MRP 4 inhibition on the corpora cavernosa, in order to consider these compounds as potential therapeutic option to treat erectile dysfunction and to determine their effective dose, confirmation of these in vitro results is necessary in (i) human tissue and (ii) in vivo.

Resveratrol, quercetin and MRP4 inhibition showed positive effects on the erectile tissue, however, a major limitation of basically any compound that is under consideration as alternative for treating ED, is the unwanted systemic (side)effects. Indeed the perfect

therapy for treating ED is one that is specifically targeted towards the corpora cavernosa, not causing systemic (adverse) effects. At present such a compound is still an illusion. Even though the first-line therapy for ED, PDE-5 inhibitors, are widely used and have shown their efficacy in treating ED, there are several well-documented adverse effects associated with their use such as headache, facial flushing, nasal congestion, and dyspepsia [123]. These side effects are a result of their systemic dispersal following ingestion, and the target enzyme (PDE-5) being expressed in a variety of other tissues besides the corpora. Similarly the relaxant effect of resveratrol, quercetin and MK-571, that were studied in this thesis, was not restricted to the corpora cavernosa since all compounds possessed potent relaxant capacity in aorta. The results of the relaxant effect of MK-571 on mouse aorta are represented in **Figure IX.5** in the supplementary data. Therefore it is not unlikely that a systemic hypotension effect would occur in vivo which would limit the use of resveratrol, quercetin or MRP 4 inhibition as alternative strategy for treating ED. However it should be noted that several PDE-5 inhibitors like sildenafil, vardenafil and tadalafil also potently relax isolated aorta rings [124], yet their cardiovascular effects are usually minor [123]. Future research should clarify whether resveratrol, quercetin and MRP 4 inhibition cause, despite their in vitro vasorelaxant effect, similar minimal systemic effects. However, as systemic (side) effects might occur, other administration routes such as local application to the penis might represent a minimally invasive option to surpass potential systemic blood pressure lowering (side) effects. Recently, a new topical application and delivery method has been developed by which nanoparticles encapsulating erectogenic agents are applied as a gel to the glans and penile shaft [125]. This method has also been proven effective for the topical application of NO-releasing nanoparticles in a rat model of radical prostatectomy [126]. A topical application of nanoparticles containing erectogenic agents could avoid variation in absorption profiles, first-pass metabolism, and systemic effects, and might thus be an elegant and excellent alternative for oral medication in the future. Another way to overcome possible side effects might be to combine current PDE-5 inhibitors to the tested compound in this thesis. In chapter VII it was shown that MK-571 improved sildenafil-induced relaxations in corpora cavernosa, at least in vitro. Therefore coupling or combining MK-571 to sildenafil might be an option to specifically target the corpora cavernosa and thus limit systemic effects. Future research should reveal whether similar potentiating effects occur in vivo and whether these also account for resveratrol and quercetin.

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In conclusion, our studies exploring new therapeutic options for the treatment of ED, demonstrated (i) an unraveled relaxant effect of resveratrol on mouse corpora cavernosa, even in diabetic mimicking conditions; (ii) an antioxidant effect of resveratrol and quercetin that protects mouse corpora cavernosa from diabetic-induced damage and (iii) a relaxant effect, enhancement of cGMP-mediated relaxations of mouse corpora cavernosa and pro-erectile effect of MRP 4 inhibition. Taken together, these results suggest that resveratrol, quercetin and MRP 4 inhibition might improve erectile function and be of value in the treatment and/or prevention of (diabetic-induced) ED. However, the in vivo relevance of resveratrol, quercetin and cGMP export still needs to be explored.

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Chapter VIII

Discussion and future perspectives

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Chapter IX

Supplementary data

IX.1 Supplementary data of the effect of PVAT in mesenteric arteries

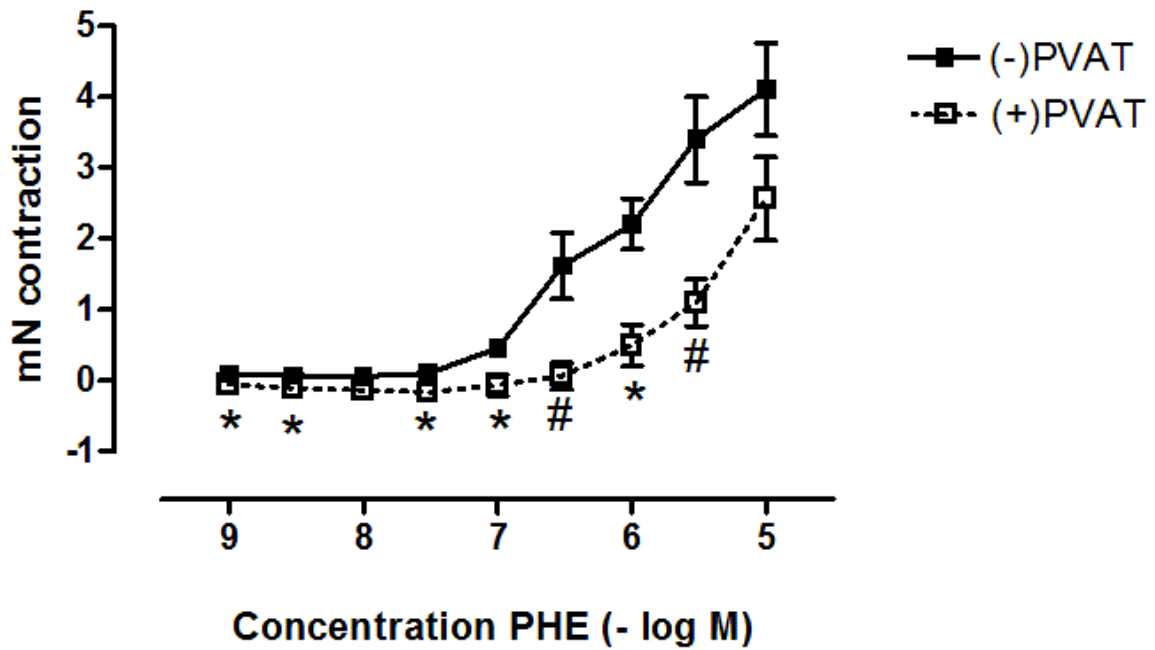


Figure IX.1 shows that phenylephrine (PHE, 1 nM – 10 μ M) elicited concentration-dependent contractions in mesenteric arteries without ((-)PVAT) and with ((+)PVAT) adherent adipose tissue. The presence of PVAT significantly attenuated contraction responses to PHE. Data are expressed as mN contraction; Mann-Whitney U test; * $p < 0.05$; # $p < 0.01$; $n = 6-7$.

IX.2 Supplementary data of the effect of resveratrol on corpora cavernosa from sGC β_1 knock-in mice

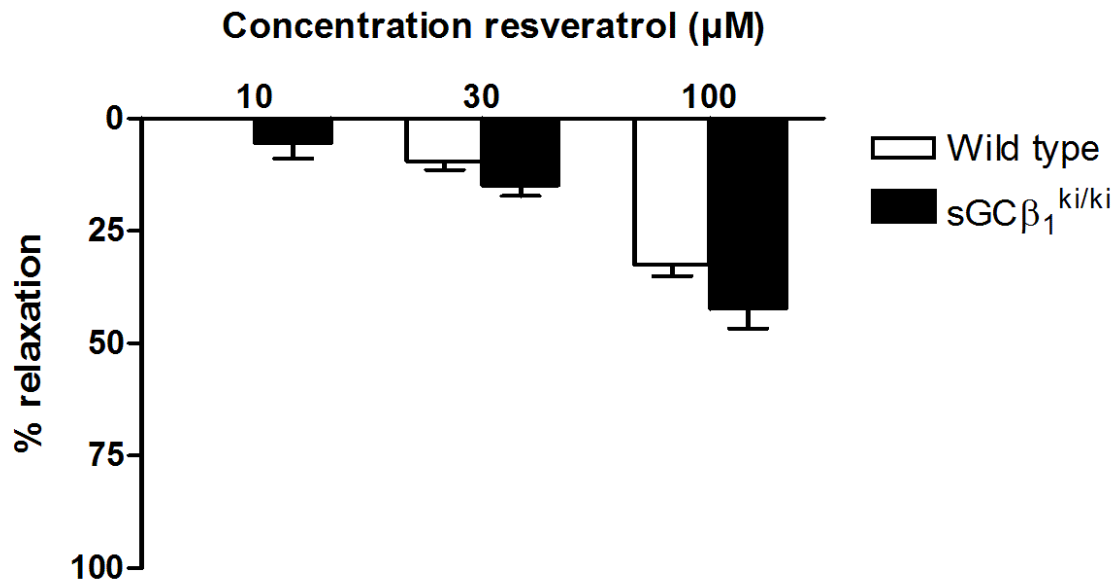


Figure IX.2 indicates the effect of resveratrol (10-100 μM , 15 min) and corpora cavernosa from sGC β_1 ^{ki/ki} mice after precontraction with norepinephrine 5 μM . Resveratrol-induced corporal relaxations in sGC β_1 ^{ki/ki} mice did not differ from those obtained in wild type controls. Data are expressed as % relaxation of the NOR-induced tone; Mann-Whitney U test; n=8.

IX.3 Supplementary data of the involvement of adenosine monophosphate-activated protein kinase in the relaxant effect of resveratrol on mouse corpora cavernosa

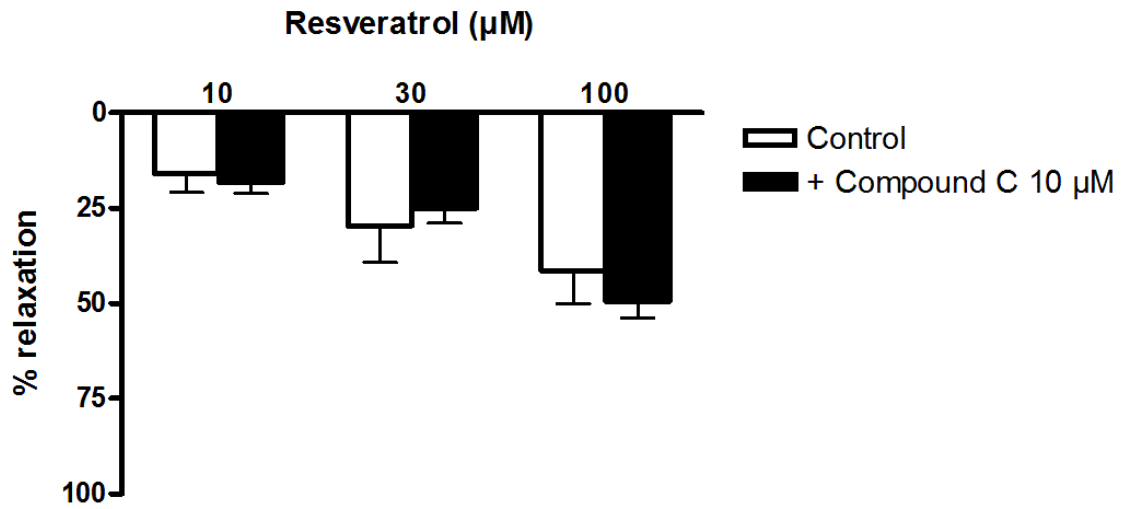


Figure IX.3 shows the relaxant effect of resveratrol on precontracted (5 μM norepinephrine (NOR)) corpora cavernosa from Swiss mice in absence and presence of the adenosine monophosphate-activated protein kinase inhibitor, compound C (10 μM). Data are expressed as % decrease of NOR-induced tone; Mann-Whitney U test; (n=9).

IX.4 Supplementary data of the effect of MK-571 on mouse aorta

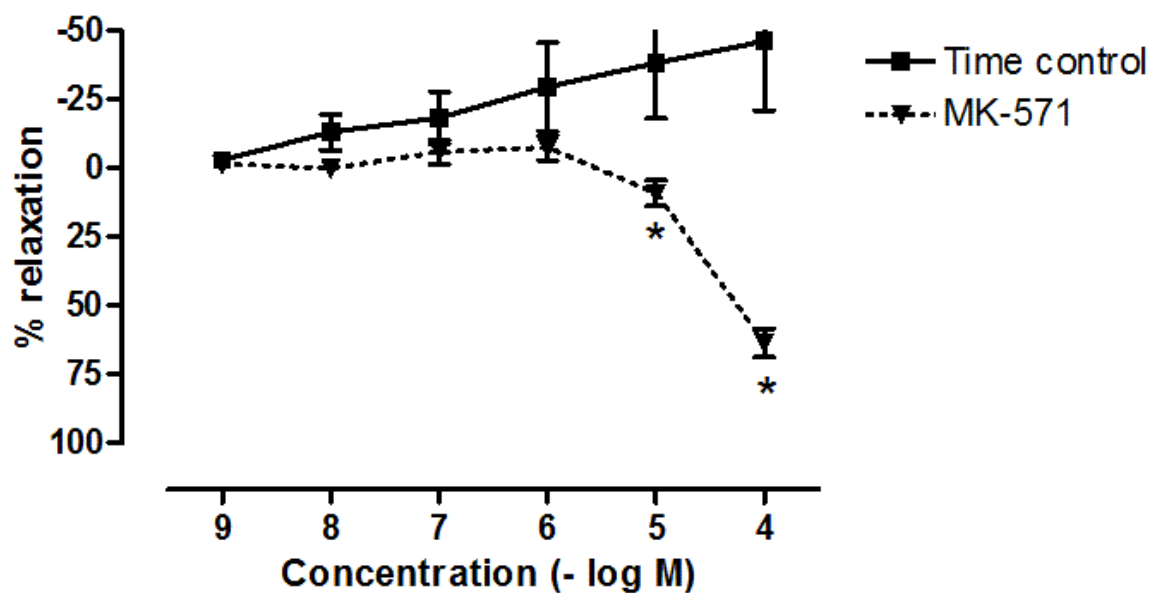


Figure IX.4 shows the relaxant effect of MK-571 on precontracted (5 μ M Phe) aorta from Swiss mice versus time controls. Data are expressed as % decrease of Phe-induced tone; Mann-Whitney U test; # $p < 0.01$ and * $p < 0.05$; (n=4).

Chapter X

Summary

Summary

Blood flow to the organs and tissues is determined by a balance between relaxation and contraction of the smooth muscle cells in the walls of the blood vessels. Any disturbance of this balance, will result in impaired blood flow regulation which can lead to pathological conditions, such as hypertension and erectile dysfunction. Vasoactive factors released from neighboring cells (i.e. paracrine modulators) including the perivascular adipose tissue play a substantial role in the regulation of blood flow. Under physiological conditions the adipose tissue secretes vasodilatory and vasoconstrictory adipokines, which are described in **chapter III**, and elicit a net beneficial relaxing effect.

In **chapter IV**, possible alterations in the vasorelaxing effect of perivascular adipose tissue (PVAT) were investigated using an in vitro set-up based on isometric tension measurements in arteries mounted in a wire myograph. Resveratrol, a polyphenol present in the skin of grape and highly abundant in red wine, is a compound with known relaxant, antioxidant and anti-inflammatory properties and exerts beneficial effects on adipogenesis as well as on adipose tissue function. We demonstrated that resveratrol decreases the relaxing influence of PVAT surrounding mouse thoracic aorta and that in presence of PVAT the positive effects of resveratrol are neutralized. This study could not provide evidence for resveratrol as potential candidate to alleviate complications associated with adipose tissue dysfunction. In addition testosterone depletion, known to affect adipose tissue distribution and functioning, did not alter the vasorelaxing effect of PVAT.

Like vascular function, erectile function depends on a delicate balance between smooth muscle contraction and relaxation. Nitric oxide (NO) with subsequent intracellular accumulation of cGMP, is generally accepted to be the main activator of arterial and corporal smooth muscle relaxation. The efficacy of phosphodiesterase-5 (PDE-5) inhibitors for the treatment of erectile dysfunction clearly demonstrate the importance of the NO/cGMP pathway for normal penile erection. However, some limitations and drawbacks in the use of PDE-5 inhibitors, necessitate research on other potential strategies and/or targets. Since the red wine polyphenols, resveratrol and quercetin have shown their beneficial effects in the cardiovascular system, the potential benefits for erectile function were explored using in vitro tension measurements. In **chapter V** it was demonstrated that

resveratrol, but not quercetin, relaxed mouse corpora cavernosa, however the underlying mechanism(s) remain(s) elusive. Furthermore, resveratrol and to a lower extent quercetin, prevented obesity-like induced damage to neurogenic-mediated relaxant responses of the corpora cavernosa. These data suggest a beneficial effect of resveratrol on erectile (dys)function.

To gain further insight whether resveratrol and quercetin could be beneficial in the treatment of diabetic-associated erectile dysfunction, an in vitro model was created to mimick the diabetic circumstances which lead to vascular and erectile dysfunction. In **chapter VI** it was found that treatment of mesenteric arteries and corpora cavernosa with high concentrations of glucose in combination with methylglyoxal, a reactive glucose metabolite, induced damage to endothelium- and neurogenic-dependent relaxations. Oxidative stress was suggested as underlying mechanism for these diabetic deficits. In the corpora cavernosa these defects were prevented by both resveratrol and quercetin. The results suggest a protective effect of both polyphenols on mouse corpora cavernosa against diabetic-induced damage.

Besides PDE's also multidrug resistance protein (MRP) 4 and MRP 5, that export cGMP out of the smooth muscle cells, determine intracellular cGMP levels. **Chapter VII** explored the functional effect of inhibiting MRP 4 in mouse corpora cavernosa in vitro to evaluate it as potential alternative target for the treatment of erectile dysfunction. Inhibition of MRP 4 relaxed mouse corpora cavernosa by increasing cGMP levels and exerted pro-erectile effects in vivo. In addition, MRP 4 inhibition enhanced cGMP-dependent relaxations, suggesting that inhibition of cGMP export might be a valid alternative strategy to treat erectile dysfunction.

In conclusion, this work demonstrates that the vasorelaxing influence of adipose tissue is not altered by testosterone depletion, while it was decreased by resveratrol administration. With respect to the erectile function studies, resveratrol, quercetin and MRP 4 inhibition exerted positive effects on the contractility of the mouse corpora cavernosa including a relaxant and/or antioxidant effect. Therefore it is suggested that resveratrol, quercetin and MRP 4 inhibition might improve erectile function and be of value in the treatment and/or prevention of (diabetic-induced) ED, alone or in combination with current therapies.

Chapter XI

Samenvatting

Samenvatting

De doorbloeding van de organen en weefsels wordt in belangrijke mate bepaald door de balans tussen relaxatie en contractie van de gladde spiercellen in de wand van de bloedvaten. Een verstoring van deze balans leidt tot een onregelde doorbloeding, wat op zich de ontwikkeling van verscheidene pathologische aandoeningen zoals hypertensie en erectiestoornissen in de hand kan werken. Vasoactieve substanties vrijgesteld uit naburige weefsels, ook wel paracrine modulators genoemd, zoals uit het vetweefsel dat de arteriën omringt (het perivasculair vetweefsel), spelen hierbij een belangrijke rol. Onder fysiologische omstandigheden stelt het vetweefsel vasodilerende en vasoconstraherende adipokines vrij (beschreven in **hoofdstuk III**) en oefent het vetweefsel hierdoor een netto positief relaxerend effect uit op de bloedvaten.

In **hoofdstuk IV** werd onderzocht welke omstandigheden het vasorelaxerend effect van het perivasculair vetweefsel kan versterken of aantasten. Hiervoor werd gebruik gemaakt van een in vitro techniek die gebaseerd is op het meten van isometrische spanningsverschillen in arteriën opgespannen in een draadmyograaf. Resveratrol, een polyfenol die voorkomt in de schil van druiven en dus teruggevonden wordt in rode wijn, is een component met gekende relaxerende, antioxidante en anti-inflammatoire eigenschappen, alsook met mogelijke gunstige effecten op de vorming en de functie van het vetweefsel. We toonden aan dat resveratrol het relaxerend effect van het vetweefsel rond de thoracale muis aorta verzwakt. Verder zorgde de aanwezigheid van het perivasculair vetweefsel ervoor dat de positieve effecten van resveratrol niet tot uiting kwamen. Deze studie kon niet bewijzen dat resveratrol complicaties die te wijten zijn aan stoornissen in de secretiefunctie van het vetweefsel zou kunnen verbeteren. Daarnaast had een tekort aan testosteron, wat de ophoping en de functie van het vetweefsel negatief te beïnvloedt, geen effect op het vasorelaxerend vermogen van het perivasculair vetweefsel.

Net zoals bij het functioneren van de arteriën, is het goed functioneren van het erectiele weefsel afhankelijk van een delicate balans tussen gladde spiercel contractie en relaxatie. Algemeen wordt aanvaard dat stikstofmonoxide (NO) met erop volgend een intracellulaire ophoping van het cGMP, de belangrijkste activator is van arteriële en corporale gladde spiercel relaxatie. De doeltreffendheid en het succes van fosfodiësterase-5 (PDE-5) inhibitoren bij de behandeling van erectiestoornissen illustreren duidelijk het belang van de

NO/cGMP signaaltransductie cascade voor zowel inductie als aanhouden van een erectie. Omwille van sommige beperkingen of neveneffecten, is een behandeling met PDE-5 inhibitoren niet altijd aangewezen, wat verder wetenschappelijk onderzoek naar nieuwe potentiële strategieën en/of doelwitten noodzakelijk maakt. Aangezien de rode wijn polyfenolen resveratrol en quercetin verscheidene positieve effecten uitoefenen in het cardiovasculair systeem, werden hun mogelijke gunstige effecten voor de erectiele functie nagegaan gebruikmakend van in vitro spanningsmetingen. In **hoofdstuk V**, werd aangetoond dat resveratrol, maar niet quercetin, relaxatie veroorzaakte van corpora cavernosa van muizen. Het onderliggende mechanisme van dit relaxerend effect blijft echter ongekend. Daarenboven, kon resveratrol, en in mindere mate quercetin, de op obesitas-lijkende schade aan zenuw-afhankelijke relaxaties van de muis corpora cavernosa voorkomen. Deze resultaten suggereren dat resveratrol een positief effect kan uitoefenen op erectiele (dys)functie.

Om verder inzicht te verwerven of resveratrol en quercetin nuttig kunnen zijn bij de behandeling van diabetes-geassocieerde erectiestoornissen, werd een in vitro model gecreëerd waarbij de diabetes omstandigheden, die leiden tot vasculaire en erectiestoornissen, nagebootst werden. In **hoofdstuk VI** zagen we dat de behandeling van muis mesenterische arteriën en corpora cavernosa met hoge concentraties aan glucose in combinatie met methyglyoxal, een reactief glucose metabooliet, schade aan endotheel- en zenuw-afhankelijke relaxaties teweegbracht. Oxidatieve stress werd gevonden als onderliggend mechanisme van deze diabetische defecten. In de corpora cavernosa konden zowel resveratrol als quercetin deze schade voorkomen. Uit deze data blijkt dat beide polyfenolen de corpora cavernosa kunnen beschermen tegen schade veroorzaakt door diabetes.

Naast PDE's bepalen ook multidrug resistente eiwitten (MRP) 4 en 5 de intracellulaire hoeveelheid van cGMP, aangezien deze het intracellulair cGMP uit de gladde spiercellen kunnen transporteren. **Hoofdstuk VII** ging in vitro na wat het functioneel effect was van de belemmering van MRP 4 in de muis corpora cavernosa en evalueerde of MRP 4 een mogelijk alternatief doelwit zou kunnen zijn voor de behandeling van erectiestoornissen. Inhibitie van MRP 4 relaxeerde de muis corpora cavernosa door een stijging in cGMP te veroorzaken en lokte in vivo erectie uit. Daarenboven werden cGMP-afhankelijke relaxaties versterkt door

remming van MRP 4, hetgeen doet vermoeden dat het verhinderen van cGMP export inderdaad een mogelijk alternatieve strategie kan zijn bij de behandeling van erectiestoornissen.

Samenvattend, toont dit werk aan dat de relaxerende invloed van vetweefsel onveranderd blijft door een tekort aan testosteron, terwijl resveratrol het relaxerend effect van vetweefsel afremt. Voor wat betreft de studies over de erectiele functie werd aangetoond dat resveratrol, quercetin en inhibitie van MRP 4 positieve effecten hebben op de contractiliteit van de muis corpora cavernosa via een relaxerend en/of antioxidant effect. Bijgevolg wijst dit erop dat resveratrol, quercetin en MRP 4 inhibitie de erectiele functie mogelijks zouden kunnen verbeteren en dat ze waardevol zouden kunnen zijn in de behandeling en/of preventie van (diabetes-geassocieerde) erectiestoornissen, in monotherapie of in combinatie met huidige therapieën.

Curriculum vitae

Curriculum vitae

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IV. Scientific publications in peer-reviewed journals

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Pauwels B, **Boydens C**, Vanden Daele L, Van de Voorde J. Ruthenium-based nitric oxide-donating and carbon monoxide-donating molecules. *Journal of Pharmacy and Pharmacology*. 2016;68(3):293-304

V. Abstracts and presentations

Decaluwé K, Pauwels B, **Boydens C**, Van de Voorde J. Mechanisms involved in the vasorelaxing influence of CO-releasing molecule-2 (CORM-2) on mice aorta. *Belgian Society of Physiology and Pharmacology*, Autumn meeting (2013)

Boydens C, Pauwels B, Decaluwé K, Van de Voorde J. Mechanisms involved in resveratrol-induced relaxation of isolated mice corpora cavernosa. Bullet presentation at the *Science Day Ghent University*, Gent, Belgium (2014)

Boydens C, Pauwels B, Decaluwé K, Van de Voorde J. Mechanisms involved in resveratrol-induced relaxation of isolated mice corpora cavernosa. Oral presentation at the *2nd Benelux congress on Physiology and Pharmacology (Physphar)*, Maastricht, The Netherlands (2014)

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

Boydens C, Pauwels B, Decaluwé K, Van de Voorde J. Mechanisms involved in resveratrol-induced relaxation of isolated mice corpora cavernosa. *Acta Physiologica*. Joint meeting of the Federation of European Physiological Societies (FEPS) and the Hungarian Physiological Society, Budapest, Hungary (2014)

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

Boydens C, Pauwels B, Decaluwé K, Brouckaert P, Van de Voorde J. Relaxant and antioxidant capacity of red wine polyphenols on isolated mice corpora cavernosa. Poster presentation at the *European Council for Cardiovascular Research (ECCR)*, 18th Annual meeting, Garda, Italy (2014)

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

Boydens C, Pauwels B, Van de Voorde J. Effect of resveratrol and orchidectomy on the vasorelaxing influence of perivascular adipose tissue. *Malta polyphenols*. International Society of Antioxidant in Nutrition and Health (ISAHN) (2015)

Boydens C, Pauwels B, Van de Voorde J. Effect of resveratrol and orchidectomy on the vasorelaxing influence of perivascular adipose tissue. Poster presentation at the 1st Joint meeting of the European society for Microcirculation (ESM) and European Vascular Biology Organization (EVBO), Pisa, Italy (2015)

Vanden Daele L, **Boydens C**, Pauwels B, Van de Voorde J. Vasorelaxing effect of resveratrol on bovine retinal arteries. *Belgian Society of Physiology and Pharmacology*, Autumn meeting (2015)

Boydens C, Pauwels B, Vanden Daele L, Van de Voorde J. Protective effect of resveratrol and quercetin on in vitro-induced diabetic mouse corpus cavernosum. Oral presentation at the *Belgian Society of Physiology and Pharmacology*, Spring meeting (2016)

Boydens C, Pauwels B, Vanden Daele L, Van de Voorde J. Effect of resveratrol and quercetin on in vitro-induced diabetic mouse corpus cavernosum. Poster presentation at *Knowledge for Growth* (2016)

Vanden Daele L, **Boydens C**, Pauwels B, Van de Voorde J. Vasorelaxing effect of resveratrol on bovine retinal arteries. *Knowledge for Growth*, Ghent, Belgium (2016)

Boydens C, Pauwels B, Vanden Daele L, Van de Voorde J. Protective effect of resveratrol and quercetin on in vitro-induced diabetic mouse corpus cavernosum. 7th *European congress of Pharmacology (Ephar)*, Istanbul, Turkey (2016)

Vanden Daele L, **Boydens C**, Pauwels B, Van de Voorde J. Vasorelaxing effect of resveratrol on bovine retinal arteries. *7th European congress of Pharmacology (Ephar), Istanbul, Turkey (2016)*

VI. Teaching and supervision

- Practical courses:
 - Spirometry (2nd Bachelor Pharmaceutical Sciences; 2nd Bachelor Dentistry; 2nd Bachelor Biomedical Sciences; 1st Bachelor Logopedics and Audiology)
 - Blood pressure (2nd Bachelor Pharmaceutical Sciences; 1st Bachelor Logopedics and Audiology; 2nd Bachelor Biomedical Sciences)
 - Reflexes (1st Bachelor Biomedical Sciences; 2nd Bachelor Medicine)
 - Oxygen consumption (1st Bachelor Logopedics and Audiology, 2nd Bachelor Dentistry, 2nd Bachelor Biomedical Sciences)
 - Eye (2nd Bachelor Medicine)
 - Neuro-muscular communication (2nd Bachelor Pharmaceutical Sciences)
- ‘Is rode wijn drinken goed voor ons cardiovasculair system? Betekenis van resveratrol’
Master thesis Pieter De Mulder (student Medicine 2013-2015)
- ‘Effect van polyfenolen op glucose- en methylglyoxal-geïnhibeerde endotheel-en EVS – gemedieerde relaxaties’
Master thesis John Tuypens (student Pharmaceutical Sciences 2014-2015)