DOI:10.14753/SE.2017.1905

Maintained cGMP levels improve endothelial and vascular function after oxidative stress

Ph.D. Doctoral Dissertation

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Budapest
2015

DOI:10.14753/SE.2017.1905

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DOI:10.14753/SE.2017.1905

2 List of abbreviations

ACh	acetylcholine
ADMA	asymmetric dimethylarginine
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BH2/BH4	dihydrobiopterin / tetrahydrobiopterin
BAX	Bcl-2-like protein 4; pro-apoptotic regulator factor
Bcl-2	B-cell lymphoma 2; anti-apoptotic regulator factor
cAMP	cyclic adenosine monophosphate
CAT	catalase
cGMP	cyclic guanosine monophosphate
CO_3^-	carbonate radical
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
ET-1	endothelin-1
GSH-Px	glutathione peroxidase
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxyde
HIF	hypoxia-inducible factor
HTK	histidine-triptophane-ketoglutarate
I/R	ischemia reperfusion
ICAM	intercellular cell adhesion molecules
IHD	ischemic heart disease
Il	Interleukin
iNOS	inducible nitric oxide synthase
K^+_{ATP}	ATP sensitive K^+ channel
LTB	leukotrien B
MAPK	mitogen-activated protein kinase

MDA	malondialdehyde
MMA	monomethylarginine
MMP	matrix metalloproteinase
MnSOD	manganese superoxide dismutase
mPTP	mitochondrial permeability transition pore
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NaOH	sodium hydroxide
NF-κB	nuclear factor kappa b
$NO^{\cdot}$	nitric oxide
NO_2	nitrogen dioxide
NOS	nitric oxide synthases
$O_2^{-\cdot}$	superoxide anion
OCl^-	hypochlorite anion
$^{\cdot}OH$	hydroxide anion
$ONOO^-$	peroxynitrite anion
PAF	platelet activating factor
PARP	poly(ADP-ribose) polymerase
PCR	polymerase chain reaction
PDE	phosphodiestherase
PE	phenylephrine
PKG	cGMP dependent protein kinase
R_{max}	maximal relaxation
ROS	reactive oxygen species
S.E.M.	standard error of the mean
sGC	soluble guanylate cyclase
SNP	sodium nitroprusside
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

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3 Introduction

3.1 Epidemiology of cardiovascular diseases

Cardiovascular disease (CVD) is the cause of more than 4 million deaths in Europe and over 1.9 million deaths in the European Union each year. Since decades, it is the leading cause of morbidity and mortality statistics in the developed part of the world. According to epidemiological studies, risk factors such as smoking, diabetes, hyperlipidemia, nutrition factors and age dispose changes in the cardiovascular system that in time may lead to CVD. The common in these changes are the atherosclerotic alterations in the vasculature. In most cases CVD manifests also in form of coronary artery disease, which is characterized by reduced blood supply to the heart. In Hungary, more than 10% of the population suffers in circulatory disorders of which half suffers in ischemic heart disease (IHD). Currently IHD is responsible for every fourth death and is prognosed to become the leading cause of death worldwide in ten years [1,2]. The mortality caused by the acute manifestations of IHD has been decreased by the high niveau invasive treatment of percutan coronary intervention, of which in Hungary more than 15 thousand performed annually. However, with the decrease of acute IHD mortality the prevalence of the chronic forms (heart failure) is increasing. It is estimated, that 26 million people have heart failure worldwide up to 6 million Europeans among them, with an incidence of 1 million people in the European Union and the USA alone. According to the approximations shared by the Hungarian Society of Cardiology, 3% of the adult population suffers in heart failure, 6-10% of elderly people above the age of 65. The overall CVD is estimated to cost the European Union nearly 196 billion Euro per year. The total cost of CVD in the EU consists of numerous sub-components: approximately just 54% is due to direct health care costs, 24% of it is due to productivity losses and 22% of it is known to be caused by the informal care of people with CVD [3].

The pathophysiology of cardiovascular diseases associated to hypercholesterolemia, atherosclerosis, hypertension, diabetes, cardiac insufficiency and the phenomenon of restenosis, ischemia-reperfusion injury and sepsis share many common attributes [4-8]. Growing evidence indicates, that *in vivo* formation of free radicals in the vascular wall plays a pivotal role in the development of endothelial dysfunction, that contributes to

initiation and progression of these diseases [9, 10]. The vascular system, especially the endothelium itself is very sensitive to oxidative stress. Resulting from an exceeding production of reactive oxygen species, the increased oxidative stress has a critical role in determining vascular injury.

3.2 Ischemia reperfusion injury

Historically, the first suspicion of a severe injury caused by reperfusion dates back to the second world war, when Bywaters and Beal [11] noted fatal metabolic dysfunction followed by death after the release of crushed limbs in air-raid casualties. In full extent, tissue injury induced by reperfusion was described by Parks and Granger in 1986 [12]. Reimer and his colleagues [13] introduced the term lethal reperfusion injury in 1989 as they interpreted cell necrosis resulting from reperfusion and the role of free radicals and neutrophils in reperfusion injury.

The reperfusion of an ischemic organ is fundamental for its viability and functional recovery. However, the arrival of blood oxygen leads to a series of lesions. Reperfusion injury is a complex and paradoxical phenomenon, where cellular dysfunction and cellular death exacerbates after the restoration of blood supply to the preliminary ischemic tissues. When blood flow is reestablished to an organ or tissue exposed to prolonged ischemia, renewed blood flow aggravates tissue damage by causing additional injury. The arterial clamping/unclamping in vascular surgery, cardiac surgery with or without extracorporeal circulation, transplant surgery, the use of tourniquet in orthopedic surgery, haemorrhagic shocks, septicaemia and low blood flow states are only some examples from the operating theatre which illustrate daily ischemia-reperfusion injury in clinical practice [14].

The reperfusion stage can be broken up into two phases. First, initial return of blood to ischemic tissue results in the return of aerobic respiration. Due to molecular changes and the drop in the antioxidant capacities in the cells during hypoxic stage, enzyme systems in their modified state start the rapid generation of reactive oxygen species damaging cellular functions. The second phase of reperfusion injury elicits through these biochemical and cellular changes an acute inflammatory response that is

characterized by inflammation mediated by interactions among neutrophils, platelets and endothelium, resulting in further oxidant production and endothelial barrier failure [15, 16]. While a massive ischemic injury through the deprivation of oxygen more likely manifests in cellular necrosis, reperfusion injury is rather dominated by cellular apoptosis.

3.3 Endothelium and oxidative stress

Endothelial cells have multiple vital duties: they control vascular tonicity and local blood flow, modulate coagulation and inflammation, intervene in the immune system, control the transfer of micro- and macromolecules towards the interstitial region, convert prohormones into active hormones (angiotensin II) and intervene in the formation of new blood vessels. For the understanding of how oxidative stress leads to the damage of the endothelium and how endothelial dysfunction leads to impaired vascular function, the prior discussion of endothelial physiology, the source of reactive oxygen species, and their effect on cellular function is essential.

3.3.1 Endothelial physiology

Endothelial cells show vasodilator, anti-coagulant and anti-adhesive properties thus contributing to the upkeep of the balance between the pro- and anti-inflammatory mediators, haemostatic balance, cell-proliferation and vascular permeability [17]. The vasomotor tone is regulated by the release of vasodilator agents (such as nitric oxide, prostacyclin, bradykinin, endothelium derived hyperpolarizing factor), and vasoconstrictors (endothelin-1, angiotensin II, thromboxane). The predominant nitric oxide is a free radical with short, only a few seconds long biological half-life. Its synthesis is catalyzed by a family of nitric oxide synthases (NOSs) from the aminoacid L-arginine in a reaction requiring tetrahydrobiopterin (BH4) as a cofactor, and leads to the relaxation of smooth muscle cells by increasing intracellular cyclic guanosine-monophosphate levels. The constitutive members of the NOS family (endothelial nitric oxide synthase and neuronal nitric oxide synthase) produce constantly a small amount of nitric oxide that is enough to maintain vasodilatation, inhibition of platelet

aggregation and adhesion of leukocytes. Namely, endothelin-1 could be the counterpart of NO^- by causing vasoconstriction, vascular smooth muscle cell proliferation through ET-A receptor, and release of inflammatory mediators (Il-1, 6, 8). The most important physiologic trigger of the constitutive $NO^\cdot$ synthase of endothelial cells (eNOS) is shear stress (tangential distortion of endothelial cells) generated by blood flow [18]. A constant shear stress maintains the homeostasis of vascular endothelium, while the acute loss of shear stress (e.g. in ischemia) results in the depolarization of the cell and within short to the inactivation of eNOS [17].

Under normal oxygen tension, oxidative phosphorylation is the major source of energy production by mitochondria in the cell. This results in formation of ATP, which serves as energy currency of the cell. ATP is converted to ADP, and the energy released is used to maintain intracellular ion homeostasis and to cover the energy need of metabolic reaction. The complex process of ATP synthesis is carried out by the respiratory chain (electron transport chain) in the mitochondria. The respiratory chain is a set of enzymes embedded in the mitochondrial inner membrane. Each enzyme is responsible for a specific step, which causes release of electrons that are sequentially transferred down the chain; ATP is the end product. NADH dehydrogenase and cytochrome oxidase are two of the most important enzyme complexes involved in the electron transfer chain. The superoxides generated by them are only produced in small amounts, and are quickly eliminated by the several scavenger mechanisms including enzymes such as enzyme manganese superoxide dismutase (MnSOD), catalase and glutathione peroxidase, and non enzymatic antioxidants such as vitamin E, vitamin C, beta-carotine and heme binding proteins [15, 19].

3.3.2 Changes under ischemic conditions

Hypoxia induces changes in various enzymes participating in the energy metabolism of the cells, and directly to the mitochondrial mechanisms themselves, causing uncoordinated production of ROS on resumption of oxygen supply during reperfusion. Enzyme systems affected by I/R injury include cytochrome oxidase, xanthine oxidase, reduced nicotinamide adenine dinucleotide (phosphate) oxidase and the mitochondrial electron transport chain. I/R injury causes the jumble in the function of NADH

dehydrogenase and cytochrome oxydase enzymes, leading to excess superoxides that rapidly depleting stores of MnSOD fails to neutralize.

More than hypoxia, that refers to the absolute or relative reduction of oxygen supply, the term ischemia (Greek *isch: restriction*, and *haema*: blood) translates as the absolute or relative reduction, or cessation of blood flow to and from a tissue and generally coincides with the reduced tissue oxygen delivery/demand ratio of 2:1 or below. The severity of its impact on the parenchyma depend on the intensity and duration of the ischemia, on the type and metabolic need of the affected cell. Oxygen is essential for the homeostasis of human cells. The ultimate consumer of oxygen is the oxidative phosphorylation reaction in the mitochondria, that supplies the cell with energy in the form of ATP. The large number of metabolic reactions requiring these high-energy phosphates make cells dependent of their oxygen supply. Within a few seconds after the cessation of blood flow, the oxygen content of oxyhaemoglobin, myoglobin or neuroglobin is consumed [20, 21]. Low level of ATP production is maintained by the less effective anaerobic glycolysis mainly supplied by glycogenolysis, but the demand quickly exceeds the anaerobic production. As the cytosol turns to acidic by the accumulation of lactate and protons and by the reduction in the oxidation of nicotinamide adenine nucleotide phosphate ($NADPH^{+}$) by mitochondria acidifies the cell, anaerobic glycolysis slows down [22]. The lack of ATP promotes the split of high energy phosphate from ADP and finally from AMP. In apoptotic cells pannexin hemichannels contribute to the release of ADP and AMP nucleotides out of the dieing cell, of which the 5'ecto-nucleotidase enzyme generates free adenosine in the extracellular space [23]. The generated adenosine can freely diffuse in and out of the cell, thus increasing the pool of xanthine oxidase substrates.

3.3.3 Endothelial pathophysiology in oxidative stress

Endothelial dysfunction is a corner-stone of cardiovascular diseases associated to hypercholesterolemia, hypertension, diabetes mellitus, ischemia reperfusion injury and sepsis [9, 10]. Under normal circumstances in healthy cells, a balance exists between the formation of ROS and their effective elimination by protective antioxidant mechanisms.

During oxidative stress, the disruption of this balance favours ROS production thus impairing cellular functions on multiple levels via a wide range of processes: impaired vasodilator responses, oxidizing proteins and lipids in higher concentrations, causing the oxidative damage of the DNA and leading to apoptosis, autophagy or necrosis of the cell.

Oxidative stress through the inactivation of the constitutive nitric oxide synthase results in the decreased production of $NO^{\cdot}$ and increased release of vasoconstrictors, expression of adhesion molecules (P-selectin), inflammatory mediators (PAF), leukotriene B4 (LTB4) and cytokines (Il-8) thus promoting a pro-inflammatory, pro-coagulant and proliferative state [24]. This condition is called endothelial activation.
The superoxide anion scavenges nitric oxide and forms peroxynitrite, triggering proinflammatory signals and inhibiting endothelial repair [25]. Superoxide has been demonstrated to play a key role in apoptosis through a reaction with nitric oxide to form peroxynitrite, which in turn induces tyrosine nitration and deleterious protein changes [26].

Methylarginine substratespőü, like asymmetric dimethylarginine (ADMA) and monomethylarginine (MMA) are the endogenous inhibitors of NOS, derived from the proteolysis of methylated arginine residues in various (nuclear) proteins by a group of enzymes (protein-arginine methyl transferases). These methylarginines are subsquently degraded to L-citrulline and dimethylamine by the enzyme dimethylarginine dimethylamine hydrolase (DDAH). The enzymatic activity of dimethylamine hydrolase has been shown to be decreased by oxidative stress causing the accumulation of NOS inhibiting ADMA and thus to decreased $NO^{\cdot}$ production [27].

3.3.3.1 Reactive oxygen species

Reactive oxygen species include superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hypochlorite (OCl^-), hydroxyl ions ($^{\cdot}OH$) and peroxynitrite ($ONOO^-$) which is one of the most harmful oxidant species from the reaction of superoxide and nitric oxide. They are produced by various exogenous sources such as neutrophyl granulocytes, macrophages or circulating xanthine oxidase, and by endogenous systems as well. Although NAD(P)H oxidase can be found in endothelial cells too, in neutrophil granulocytes and

platelets it is one of the most important exogenous source of ROS, especially involved in ischemia reperfusion injury [28]. The NAD(P)H oxidase-induced ROS production is more aggressive in neutrophils (oxidative burst) as compared with slower release in endothelial cells [29]. This enzyme produces superoxide that may interact with $NO^{\cdot}$ [30] thus producing reactive nitrogen species. By the oxidation of specific enzymes and co-factors, reactive oxygen species are capable to create self-perpetuating mechanisms to intensify their own production.

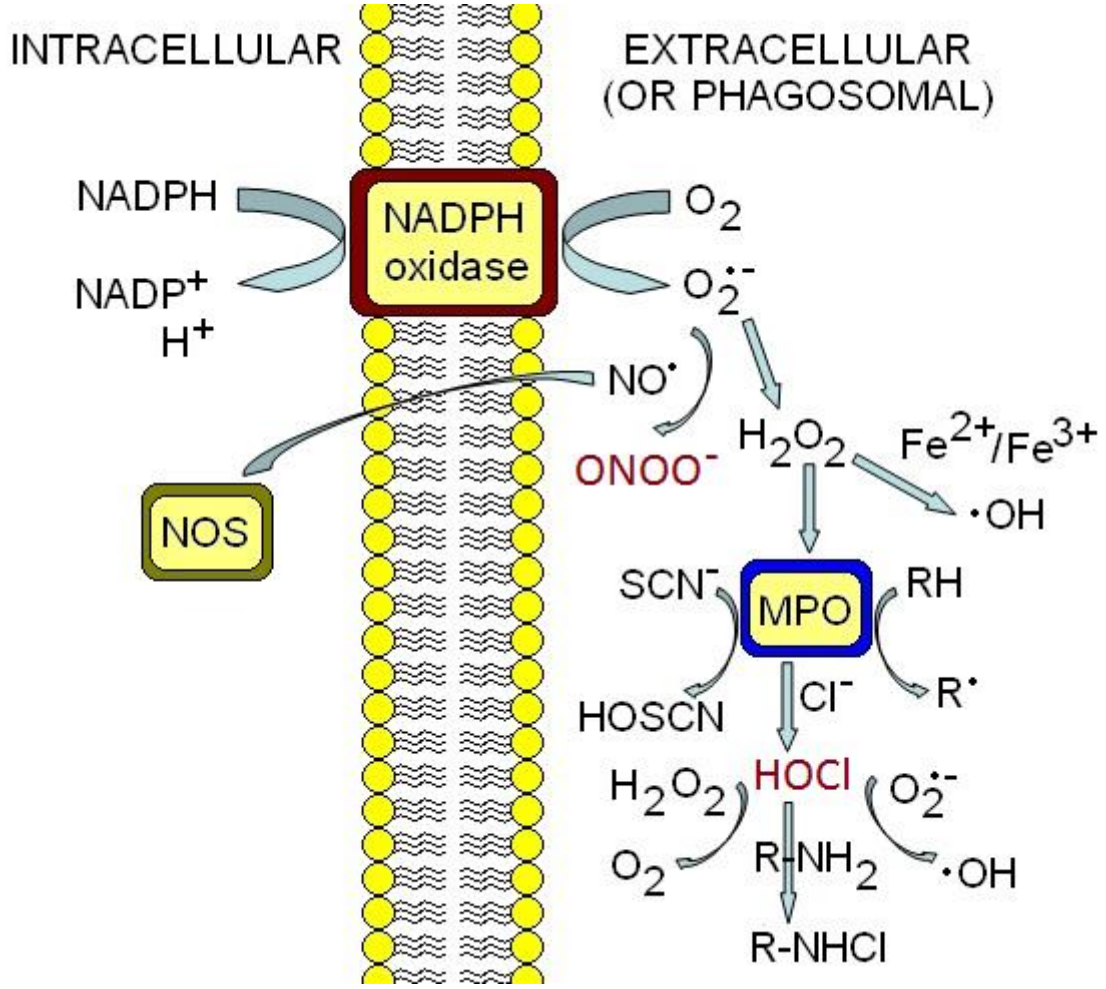

Figure 1. Generation of peroxynitrite and hypochlorite and subsequent free roots

H_2O_2: hydrogen-peroxide; MPO: myeloperoxidase; NADP(H^+): Nicotinamide adenine dinucleotide phosphate (reduced); $NO^{\cdot}$: nitric oxide; NOS: nitric oxide synthase; $O_2^{-\cdot}$: superoxide anion; $^{\cdot}OH$: hydroxide anion; R-NH_2: organic amino group; R-NHCl: organic N-chloramines; (HO)SCN^-: (hypo)thiocyanous acid;

In some cases ROS might also act as a second messenger and have significant effect on signaling pathways involving mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) Jun N-terminal kinase (JNK) as well as on regulatory proteins like NF-κB, hypoxia-inducible factor-1 (HIF-1), or activator protein-1 (AP-1) [31]. In endothelial cells NF-κB is responsible for the up-regulation of cell adhesion molecules (VCAM-1, ICAM-1), ET-1, MMPs and VEGF thus enhancing leukocyte and platelet adhesion to the endothelial surface and leukocyte excavation into the vascular wall. Recruited leukocytes serve as exogenous sources of ROS thus further accelerating the oxidative stress [32]. Endothelial cells in response to TNF-α, Il-1β, platelet derived growth factor can generate endogenous ROS by cyclo-oxygenase and NADPH oxidase.

3.3.3.1.1 Peroxynitrite

The peroxynitrite ($ONOO^-$) anion is a short-lived reactive oxidant species that is produced by the reaction of nitric oxide ($NO^·$) and superoxide ($O_2^{-·}$) radicals at diffusion controlled rates (**Figure 1**). The sites of peroxynitrite formation is associated with the sources of superoxide (such as plasma membrane NAD(P)H oxidases or mitochondrial respiratory complexes) in space and time, because though $NO^·$ is a relatively stable and highly diffusible free radical, superoxide has a much shorter lifetime and restricted diffusion across biomembranes [33]. On the other hand, peroxynitrite is able to cross cell membranes thus despite its short half-life at physiological pH (~10 ms) peroxynitrite generated from a cellular source can influence surrounding target cells within one or two cell diameters (~5-20 μm). In biological systems one fundamental reaction of $ONOO^-$ is to react with carbon dioxide (in equilibrum with physiological levels of bicarbonate anion) thus forming carbonate (CO_3^-) and nitrogen dioxide (NO_2^-) radicals [34]. These one-electron oxidants can oxidize amino acids thus creating radicals such as cysteinil from cysteine or tyrosyl from tyrosine. NO_2^- can also undergo radical-radical termination reactions with biomolecules in a diffusion-controlled manner, resulting in nitrated compounds. Another fundamental but significantly slower reaction of peroxynitrite is the homolytic fission of its protonated form (ONOOH) to generate one-electron oxidant hydroxyl (HO^-) and NO^-_2 radicals. Although this proton-catalysed

decomposition of $ONOO^-$ is a modest component of the *in vivo* reactivity of peroxynitrite, $^{\cdot}OH$ and NO^-_2 radicals gain relevance in hydrophobic phases, resulting in the initiation of lipid oxidation, nitration and protein tyrosine nitration processes. Moreover, ONOOH in the membranes may undergo direct reactions with transition metal centres such as hemin, membrane-associated thiols and lipids [25, 35]. Deoxyribose and purine nucleotides of the DNA are also vulnerable targets of peroxynitrite producing 8-oxo and 8-nitroguanine as major products, but it can also cause deoxyribose oxidation and single strand breaks [36]. Single strand DNA breakage is the obligatory inducer of the poly(ADP-ribose) polymerase (PARP) pathway.

Peroxynitrite formation results in cardiovascular dysregulation through various mechanisms; reduction of $NO^{\cdot}$ biovailability, the inhibition of prostacycline synthase, MnSOD, mitochondrial NAD dehydrogenase (complex I), sarcoplasmic reticular calcium-ATP-ase are only a few examples [37].

Due to the very short half-life the steady-state concentration of peroxynitrite is low and cannot be directly measured *in vivo*. Indirectly, protein-3-nitrotyrosine (NT) as footprints of peroxynitrite give reliable information about the oxidative load of peroxynitrite [38].

3.3.3.1.2 Hypochlorite

Hypochlorous acid is a highly reactive cytotoxic agent generated by activated polymorphonuclear leukocytes. It has major role in immune defence, however hypochlorous acid is a major contributor of endothelial oxidative damage during reperfusion injury. Activated phagocytes, neutrophils release the heme enzyme myeloperoxidase (MPO), while their membrane-bound NADPH oxidase generates superoxide radicals ($O_2^{-\cdot}$) and hence H_2O_2, via an oxidative burst (**Figure 1**). The reaction of MPO with H_2O_2 in the presence of chloride ions generates HOCl (the physiological mixture of hypochlorous acid and its anion present at pH 7.4). HOCl is reactive toward a variety of biological substances such as ascorbate, amines, thiols, sulfides and disulfides, nucleotides, DNA, proteins and unsaturated fatty acids. Exposure of amino groups to hypochlorite leads to generation of long-lived and reactive chloramines. Exposure of glycosaminoglycan chloramides to superoxide promotes the

chloramide decomposition and glycosaminoglycan fragmentation. HOCl interacts with DNA in a rather slow, but very efficient manner, assumed by the concomitant denaturation. Unlike by $^{\cdot}OH$, denaturation is not primarily due to DNA fragmentation but by chlorination of amino- and heterocyclic NH-groups of the bases, with the consequences that the double strand dissociates into single strands due to the loss of hydrogen bonds. However, even a partial chlorination of DNA bases may interfere with vital biological functions and activate PARP pathway. The presence of low-valent transition metal ions (Cu^{2+} or Fe^{2+}) facilitates the fragmentation of both the DNA and glycosaminoglycans by one-electron reduction of the chloramides [39-42].

3.3.3.2 eNOS uncoupling

Endothelial NOS is a dimeric enzyme with a reductase domain for NADPH and an oxidase domain for the co-factor oxygen and L-arginine. The co-factor BH4 is essential to catalyse the reaction of generating $NO^{\cdot}$ and L-citrulline. When BH4 levels are inadequate, "eNOS uncoupling" may occur: uncoupling of NADPH oxidation and $NO^{\cdot}$ synthesis, with oxygen instead of L-arginine as terminal electron acceptor, resulting in the formation of superoxide [43]. Moreover, the interaction between $NO^{\cdot}$ and superoxide leads to the formation of peroxynitrite that further oxidises BH4 to BH2 which competitively replace eNOS-bound BH4 thus promoting eNOS uncoupling. Hence eNOS uncoupling is greatly dependent on intracellular BH4:BH2 ratio and by the decrease of BH4 relative availability eNOS becomes the most important endogenous ROS generator [44]. The salvage re-synthesis pathway of BH4 by the dihydrofolate-reductase requires NADPH. Peroxynitrite directly oxidizes this co-factor while creating H_2O_2, thus cytoplasmic NADPH level is rapidly decreasing in oxidative stress. Not only superoxide and peroxynitrite lead to eNOS uncoupling. Through the chlorination of L-arginine OCl- may damage $NO^{\cdot}$ synthesis [45].

3.3.3.3 Other endogenous sources of ROS

Parallel to eNOS, increased oxidative stress causes the jumble in the function of cyclooxygenase, mitochondrial NADH dehydrogenase and cytochrome oxydase

enzymes, leading to excess superoxides that scavenger mechanisms are unable to neutralize.

The inducible NOS (iNOS) stimulated by different noxas affecting the endothelium (inflammation, oxidative stress) produces larger and more persistent amount of nitric oxide, thus leading to its detrimental actions: hypotension and transformation to peroxynitrite [17].

Xanthine dehydrogenase is an enzyme responsible for the safe transformation of hypoxanthine to uric acid. When oxidized by ROS, xanthine dehydrogenase is converted to xanthine oxidase and uses oxygen as a substrate for converting hypoxanthine to urate, meanwhile superoxides are produced as a by-product of the reaction [46]. This pathway gains special significance in ischemia reperfusion injury.

Reactive ferryl species have also been recognized as contributors to oxidative stress [47, 48] in trauma settings when myoglobin and haemoglobin are released into the plasma. *In vitro* experiments have shown that under conditions as they prevail in IR injury, haemoglobin is oxidized to an intermediate reactive ferryl form and causes lipid peroxidation in endothelial cells [49]. The iron-dependent formation of highly reactive species is triggered by a cold-induced increase in the cellular chelatable iron pool [50, 51]. This is particularly important in cardiac surgery when hearts are perfused and stopped with cold preservation solution. As vascular grafts are also stored in hypothermic saline or preservation solutions, they often exhibit significant loss of structurally intact endothelium following reperfusion.

3.3.3.4 Lipid peroxidation

Lipid peroxidation is the direct damage of cellular and organelle membranes resulting in structural damage and release of various autolytic enzymes. Regardless of the nature of the free radicals, they attack the phospholipids of the cell and organelle membranes culminating in irreversible structural and functional damage. Polyunsaturated fatty acids are more vulnerable to this insult than monounsaturated fatty acids [52]. Experimental evidence proves the prevalence of this mechanism in almost every organ system exposed to oxidative stress: kidney, retina, blood vessels, lungs, liver, myocardium, brain, vessels and placenta [53-58]. As a result of this

process, biologically active by-products are formed, and as mediators, contribute to the self-perpetuating nature of this injury. One such secondary by-product produced is malonaldehyde (MDA). MDA level can be used as a marker of suffered oxidative stress in various disease conditions, such as eclampsia and renal ischemia reperfusion injury [59].

3.3.4 Ischemia reperfusion injury in organ preservation

In transplantation and cardiac surgery I/R injury is one of the determining factors of acute and chronic graft failure. Organs are preferably stored in cold (4ºC) preservation solutions of which a wide scale is known in the clinical practice, such as histidine-triptophane-ketoglutarate (HTK or Custodiol), or University of Wisconsin solutions. Vascular grafts are also often stored in heparinised blood or saline. The basic concept behind the usual cold storage is to slow down the ATP consuming metabolic activities of the cells during the ischemic period. However, there are some deleterious consequences of cold [60].

Cellular swelling: the impaired activity of Na^+/K^+ ATP dependent pump leads to changes in cellular substructures, to swelling and to formation of protruding pockets [61]. Na^+ enters the cell creating a hyperosmolar enviroment and water influx.

Acidosis: Even at low temperature of preservation intracellular ATP content is depleting. In order to maintain ATP production for cellular processes under ischemic conditions the switch to the less effective anaerobic metabolism is promoted thus generating lactic acid and acidosis. Severe acidosis activates proteases and phospholipases causing lysosomal damage and cell death.

Calcium: Through the drop of cellular ATP content and the disturbances in Na^+ balance, the active transport mechanisms cannot maintain Ca^{2+} homeostasis. In the cold phase accumulation leads to activation of calcium-dependent calpain activation and protein kinase C signaling, and to loss of cell structure by breakdown of cytoskeletal

spectrin [62]. Calpain activity increases in cold-stored cells, and further increases during rewarming [63].

Enzymes: proteases and MMPs may be activated due to cold preservation, leading to detachment of endothelial cells from the underlaying matrix [64, 65]. The other relevant family of enzymes activated during cold preservation is the apoptosis-related caspases. During cold ischemia free iron is released form cytochrome P-450 [66]. In combination with hydrogen-peroxide free iron leads to severe ROS production.

3.4 NO - cGMP - PKG pathway in the vascular wall

It has been widely discussed previously, that $NO^{\cdot}$ plays a crucial role in regulation of vascular relaxation, thrombocyte activation and clotting, but it is also responsible for the intracellular homeostasis of endothelial cells. At physiologically relevant concentrations $NO^{\cdot}$ was shown to inhibit mitochondrial cytochrome oxidase [67], to inhibit activation of NF-kB [68], while enzymes such as caspase and cysteine protease, that are involved in apoptosis, were inhibited by S-nitrosylation in the presence of $NO^{\cdot}$[69].

The impairment of nitric oxide production by oxidative stress through $NO^{\cdot}$ scavenging, eNOS uncoupling, or its overproduction by iNOS leads to endothelial and vascular dysfunction. The regulation of eNOS activity is complex due to a variety of factors, including shear stress, bradykinin, histamine, VEGF, thrombin, estrogen. Acetylcholine increases eNOS activity in endothelial cells by binding to its muscarinic-3 receptor thus causing Ca^{2+} signal. eNOS activity is regulated on transcriptional level and by post-transcriptional interactions and modifications, such as binding of calcium-dependent calmodulin, phosphorylation, acylation, sub-cellular compartmentalisation [70]. It has been shown, that NOS activation can inhibit NADPH oxidase via interfering with its assembly [71, 72] and changing its expression [73-75].

Soluble guanylate cyclase (sGC) is the downstream molecule in the NO-cGMP signaling pathway, and is responsible for the conversion of GTP to the messenger molecule cGMP. The native structure of sGC is heterodimeric consisting of an A- and a

heme-containing B-subunit. In normal conditions, $NO^{\cdot}$ generated by the endothelial constitutive NOS acts as a paracrine mediator and diffuses into the neighbouring cells, such as vascular smooth muscle cells, platelets, even heart or brain cells. In these target cells $NO^{\cdot}$ activates sGC by binding to its ferrous heme iron (Fe^{2+}). By the binding of $NO^{\cdot}$ the sGC-catalyzed conversion of GTP to cGMP is activated approximately 200-fold [76]. The generated cGMP activates cGMP-dependent phosphodiesterases, protein kinases, ion channels, thus exerting its effects such as the reduction of cytosolic calcium concentration and/or calcium desensitization of the contractile apparatus, which result in smooth muscle relaxation and vascular relaxation. The activation of the cGMP dependent protein kinase G (PKG) leads to the phosphorylation of proteins at the so-called maxi-potassium channels (large conductance calcium-activated potassium channel). This results in an outflow of potassium ions into the extracellular space with subsequent hyperpolarization, with inhibition or blockade of voltage-dependent calcium channels and therefore a decrease in intracellular Ca^{2+} ion concentrations [77]. The availability of the messenger cGMP is regulated by not only its synthesis, but through its degradation by phosphodiestherases, which are thereby also cornerstone regulators of the pathway. From eleven currently known members of the phosphodiestherase family more than seven may interact with cGMP, but in the cardiovascular system (including vessel wall) the cGMP selective phosphodiestherase-5 (PDE-5) is responsible dominantly for its metabolism. cGMP facilitates its own degradation by negative feed-back through the up-regulation and marked activation of PDE-5 [78].

Though the role of $NO^{\cdot}$ in vasodilation is clearly established, the mechanism of action of cGMP and PKG still seems less defined. An early and consistent finding was that $NO^{\cdot}$ donor drugs, cGMP analogues, and PKG all lower intracellular Ca^{2+} levels, especially when elevated with a Ca^{2+}-mobilizing agonist [79]. Ca^{2+} is the major activation signal of the myosin light chain (MLC) kinase and cross-bridge cycling in SMCs.

Smooth muscle contraction and relaxation are tightly coupled to the phosphorylation and dephosphorylation, respectively, of the regulatory myosin light chain. Myosin chain phosphorylation state is determined by the relative activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). MLCK phosphorylates

MLC leading to contraction, and MLCP dephosphorylates MLC, leading to relaxation. Both MLCK and MLCP are highly regulated, MLCK is activated by the binding of calcium/calmodulin and thus is the primary mechanism linking intracellular calcium concentration to smooth muscle contractility. MLCP is regulated by both vasodilator and vasoconstrictor stimuli, and is therefore responsible for much of the calcium-independent regulation of contractility [80]. PKG interacts directly with the regulatory myosin binding subunit of MLCP, which is critical for the PKG dependent activation of MLCP.

There are at least three major known mechanisms by which PKG appears to lower intracellular Ca^{2+} levels in VSMCs. First, PKG activates Ca^{2+} sensitive potassium (K^{+}_{Ca}) channels, leading to hyperpolarization and to an inhibition of voltage-dependent Ca^{2+} channels. The second well described mechanism is that PKG increases the uptake of Ca^{2+} into intracellular stores such as sarcoplasmatic and endoplasmatic reticulum through the phosphorylation of phospholamban and activation of Ca^{2+} ATP-ases [81, 82]. The importance of phospholamban phosphorylation *in vivo* VSMC relaxation has been questioned using studies on phospholamban-deficient mice, where VSMCs demonstrated predictable enhanced sensitivity to agonist-evoked contraction. On the other hand, the animals do not display impaired vascular relaxation in response to $NO^{\cdot}$ donor drugs [83]. However, the critical role of phospholamban phosphorylation in the K^{+}_{Ca} regulation has also been evidenced [84]. Thirdly, through the inhibition of PIP-IP3-Ca^{2+} signaling pathway. It has been shown, that PKG catalyzes phosphorylation of the type I inositol 1,4,5- trisphosphate receptor in the SR *in vitro* in rat aorta [85-87] which decreases Ca^{2+} release from the intracellular compartment. Many of the PKG substrate proteins are yet uncharacterized. Some of these are relatively small molecular weight entities. One such protein is the heat-shock protein (HSP) -20 [88]. HSP-20 has been described to play a crucial role in the ischemic preconditioning in rat and dog hearts, and in the NO-cGMP dependent relaxation as a regulator of myosine phosphorylation and thin filament function [89].

3.4.1 The therapeutic perspective of the NO - sGC - cGMP pathway

Impaired endothelial function is correlated with cardiovascular diseases, therefore therapeutic strategies aimed at limiting vascular oxidative stress and improving endothelial function may have clinical benefits. Organic nitrates act as a source of $NO^{\cdot}$, but drug tolerance develops when used as sustained therapy [90]. Their efficacy is limited by the absence of clinically relevant anti-platelet activity [91] and the inability to activate NO-insensitive sGC. Oxidative stress affects the heme-containing $NO^{\cdot}$ receptor of sGC by both decreasing its expression and potentially impairing NO-induced activation [92]. Oxidative stress leads to the oxidation of sGC and to the dissociation of the heme thereby inactivating the enzyme and destines to degradation [93]. Moreover, in cardiovascular diseases due to oxidative stress significantly increased PDE-5 expression was detected to accelerate cGMP degradation [78]. As a result, $NO^{\cdot}$ donors and other pharmacological agents that protect vascular function through NO-dependent activation of sGC may not be as beneficial in the setting of oxidative stress or I/R injury. As an alternative therapeutic approach, a novel class of drugs is aimed to modulate the NO-sGC-cGMP signal transduction pathway (**Figure 2A**).

3.4.1.1 Cinaciguat

Cinaciguat (BAY 58-2667, molecular mass: 565.697 g/mol) is a compound that activates NO- and heme-independent sGC but devoid tolerance and potential cytotoxic actions of $NO^{\cdot}$ (**Figure 2B**). It triggers selectively a state of sGC that is indistinguishable from the NO-insensitive oxidized/heme-free state of the enzyme [94]. In preclinical studies, the sGC activator cinaciguat has been shown to bypass the impaired NO-sGC-cGMP pathway by activation of the oxidized (Fe^{3+})/heme-free forms of sGC and to preferentially dilate the diseased versus non-diseased vasculature [76, 95-97]. In a phase I clinical trial in healthy human participants, intravenously administered cinaciguat had a favourable safety profile and was well tolerated [98]. Moreover the phase IIa clinical study proved its effectivity in patients with acute decompensated heart failure [99].

3.4.1.2 Vardenafil

Vardenafil (molecular mass: 488.604 g/mol) is a selective PDE-5 inhibitor, an exciting small molecule, that hinders the degradation of cGMP by inhibiting its predominant regulator enzyme (**Figure 2C**).

Vardenafil is a drug approved by the US Food and Drug Administration (FDA) with the primary indication of the treatment of erectile dysfunction in men, and its effectivity is supported by clinical studies [100]. However, its cardiovascular effects has already been widely discussed. As previously mentioned, oxidative stress leads to increased PDE-5 activity. Intracellular cGMP accumulation is proven to reduce tissue injury in conditions associated with increased free radical release and oxidative stress [101, 102]. Vardenafil was shown to have beneficial effects against myocardial I/R injury after preconditioning-like treatment in rabbits [103] and to have advantageous protective effect on vascular endothelium [104]. The protective effect of PDE-5 inhibition on endothelial dysfunction following I/R injury was also demonstrated in a human study, nevertheless sildenafil was used [105].

DOI:10.14753/SE.2017.1905

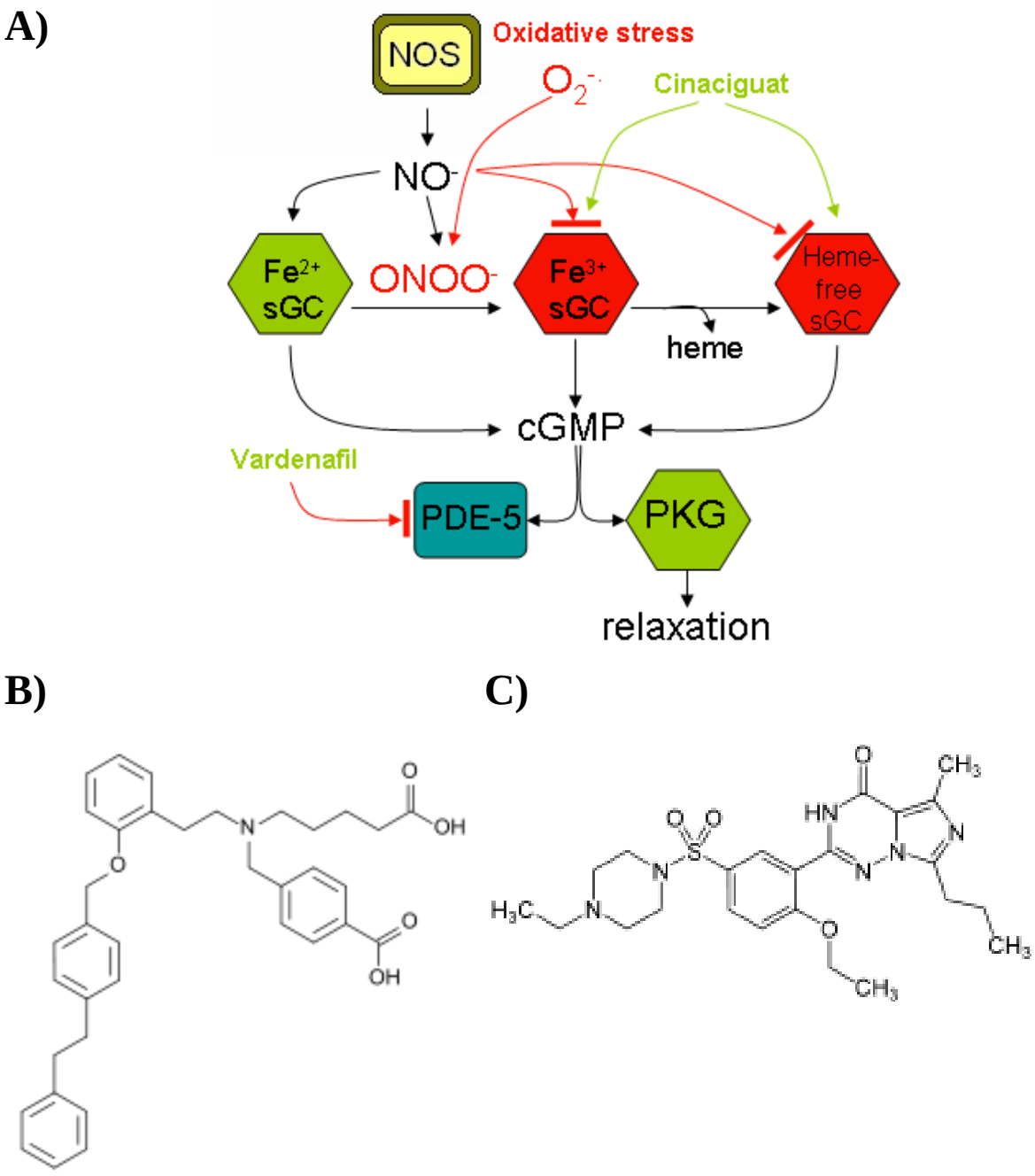

Figure 2. (A) The schematic figure of the eNOS-sGC-PKG pathway with the pharmacologic targets of cinaciguat and vardenafil; molecular structure of (B) Cinaciguat ($C_{36}H_{39}NO_5$) and (C) Vardenafil ($C_{23}H_{32}N_6O_4S$);

$NO^{\cdot}$: nitric oxide; NOS: nitric oxide synthase; $O_2^{\cdot-}$: superoxide anion; $ONOO^-$: peroxynitrite; PKG: cGMP dependent protein kinase; PDE-5: phosphodiestherase-5; sGC: soluble guanylate cyclase;

DOI:10.14753/SE.2017.1905

4 Objectives

Based upon the described mechanisms how oxidative stress leads to endothelial and vascular dysfunction, the present studies investigate whether increased cGMP levels contribute to the protection of vascular function and structure against acute oxidative stress.

1. The aims of the first *in vitro* model of vascular oxidative stress induced by peroxynitrite incubation was:
    - the investigation of vascular dysfunction and the contribution of decreased cGMP level to it after an acute oxidative stress;
    - testing the effect of the soluble guanylate cyclase activator cinaciguat on vascular dysfunction induced by peroxynitrite and underlying cellular and molecular changes in the vessel wall;

2. The aim of the second model of vascular oxidative stress induced by the model of *in vitro* ischemia and reperfusion was:
    - the investigation of I/R injury on vascular function, structure and cGMP levels;
    - testing the effect of the selective phosphodiesterase -5 inhibitor vardenafil-maintained cGMP levels on vascular dysfunction induced by *in vitro* I/R injury;

As a summary, the main goal of the studies was to establish novel potent therapeutic strategies facilitating the NO-sGC-cGMP pathway for ameliorating the endothelial and vascular dysfunction associated with acute oxidative stress.

DOI:10.14753/SE.2017.1905

5 Methods

5.1 Experimental models

5.1.1 *In vitro* model of vascular dysfunction induced by peroxynitrite exposure

Thoracic aortic rings were isolated from rats. In organ bath experiments for isometric tension the effect of *in vitro* peroxynitrite exposure on vasoconstriction, endothelium-dependent and independent vasorelaxation was measured as described detailed below. Endothelial injury was induced by incubating the isolated aortic rings in peroxynitrite (200 μmol/L) for 30 minutes.

5.1.2 *In vitro* model of vascular dysfunction induced by long term cold preservation, reoxygenation and hypochlorite exposure

Thoracic aortic rings were isolated from rats and incubated in cold hypoxic solution for 24 hours. In organ bath experiments for isometric tension the effect of *in vitro* hypochlorite exposure on vasoconstriction, endothelium-dependent and independent vasorelaxation was measured as described below. Endothelial injury was induced by reoxygenation and incubating the isolated aortic rings in hypochlorite (200 μmol/L) for 30 minutes.

5.2 Animals

Male Sprague-Dawley rats (250-330 g, Charles River, Sulzfeld, Germany) were housed in a room at constant temperature of 22 ± 2 °C with 12-hour light/dark cycles and were fed a standard laboratory rat diet and water ad libitum. All procedures concerning animals were conformed to the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86–23, revised 1996). The

investigations were reviewed and approved by the local Ethical Committee for Animal Experimentation.

5.3 Experimental groups and treatment protocols

5.3.1 Experimental groups and treatment for cinaciguat experiments

Rats were treated orally 2 times at an interval of 17 hours with vehicle (1% methylcellulose solution) or with the sGC activator cinaciguat (10 mg/kg). One hour after the last treatment, animals were exsanguinized. After excision and preparation of the descending thoracic aorta (as described below), aortic rings were placed in Krebs-Henseleit solution (118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 1.77 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$, and 11.4 mmol/L glucose; pH=7.4) at 37 °C, aerated with 95% O_2 and 5% CO_2 and divided into 4 groups (n= 12-15 rings in each group from N= 4-5 animals) as follows: Control group (rats pretreated orally with methylcellulose vehicle, then aortic rings were incubated for 30 minutes in 4,7% NaOH vehicle), Peroxynitrite group (pretreatment of rats with methylcellulose, aortic rings were exposed to 200 μmol/L peroxynitrite), Cinaciguat + peroxynitrite group (pretreatment of rats with cinaciguat, then aortic rings exposed to peroxynitrite), and Cinaciguat group (pretreatment of rats with cinaciguat, exposure of aortic rings to NaOH). The concentration of peroxynitrite was chosen on the basis of previous studies [106].

5.3.2 Experimental groups and treatment for vardenafil experiments

Rats were euthanized by an overdose of sodium pentobarbital before exsanguination. After the excision and preparation of the descending thoracic aorta (as described below), aortic rings were divided into 5 groups (n= 15-20 in each group form N= 4-5 animals): Saline group (aortic rings were incubated in physiological saline for 24 hours at 4 ºC), Vardenafil groups (aortic rings were divided and incubated in 4 different concentration (10^{-12} mol/L, 10^{-11} mol/L, 10^{-10} mol/L, 10^{-9} mol/L) of PDE-5 inhibitor vardenafil supplied saline for 24 hours at 4 ºC) and Control group (no cold

ischemic storage, and received no hypochlorite exposition). The solutions were previously equilibrated for 15 minutes with nitrous oxide to extrude oxygen from the solution.

5.4 Preparation of isolated aortic rings

Rats were euthanized with sodium pentobarbital (60 mg/kg intraperitoneally) before exsanguination. The left chamber was pierced with a 4G needle and 20 ml Krebs–Henseleit solution was infused at a speed of 1ml/sec transcardially to wash out any blood from the aorta. Then, thoracic aorta was carefully excised, cleaned from connective tissue and cut transversely into 4 mm wide rings (n=4 from each animal with a mean diameter of 1.7-1.8 mm) under an operation microscope. Special attention was paid to avoid damaging the endothelium.

5.5 In vitro organ bath experiments

Isolated aortic rings were mounted on stainless steel hooks in individual organ baths (Radnoti Glass Technology, Monrovia, CA, USA), containing 25 ml of Krebs–Henseleit solution at 37 °C and aerated with 95% O_2 and 5% CO_2. Isometric contractions were recorded using isometric force transducers of a myograph (159901A, Radnoti Glass Technology, Monrovia, CA, USA), digitalized, stored and displayed with the IOX Software System (EMKA Technologies, Paris, France). The aortic rings were placed under a resting tension of 2g (found optimal in preliminary experiments [106, 107]) and equilibrated for 60 minutes. Tension was periodically adjusted to the desired level during this period and the Krebs–Henseleit solution was changed in every 30 minutes. At the beginning of each experiment, the maximal contraction forces in response to potassium chloride (KCl, 80 mmol/L) were determined and then aortic rings were washed until the resting tension was obtained again. Afterwards, to simulate free radical burst which occurs usually *in vivo* during reperfusion [108], determined by the experimental setup, 200 μmol/L hypochlorite or 200 μmol/L peroxynitrite was added to the baths for 30 minutes, then washed out. Aortic preparations were preconstricted with α-adrenergic receptor agonist phenylephrine (10^{-6} mol/L) until stable plateau was

reached, and relaxation responses were examined by adding cumulative concentrations of endothelium-dependent dilator acetylcholine (10^{-9}–10^{-4} mol/L). For testing relaxing response of smooth muscle cells, a direct nitric oxide donor, sodium nitroprusside (10^{-10}–10^{-5} mol/L) was used. Half-maximal response (EC_{50}) values were obtained from individual concentration–response curves by fitting experimental data to a sigmoidal equation using Origin 7.0 (Microcal Software, Northampton, USA). Contractile responses to phenylephrine are expressed as percent of the maximal contraction induced by KCl. The sensitivity to vasorelaxants was assessed by pD_2=−log EC_{50} (mol/L), vasorelaxation (and its maximum [R_{max}]) is expressed as percent of the contraction induced by phenylephrine (10^{-6} mol/L).

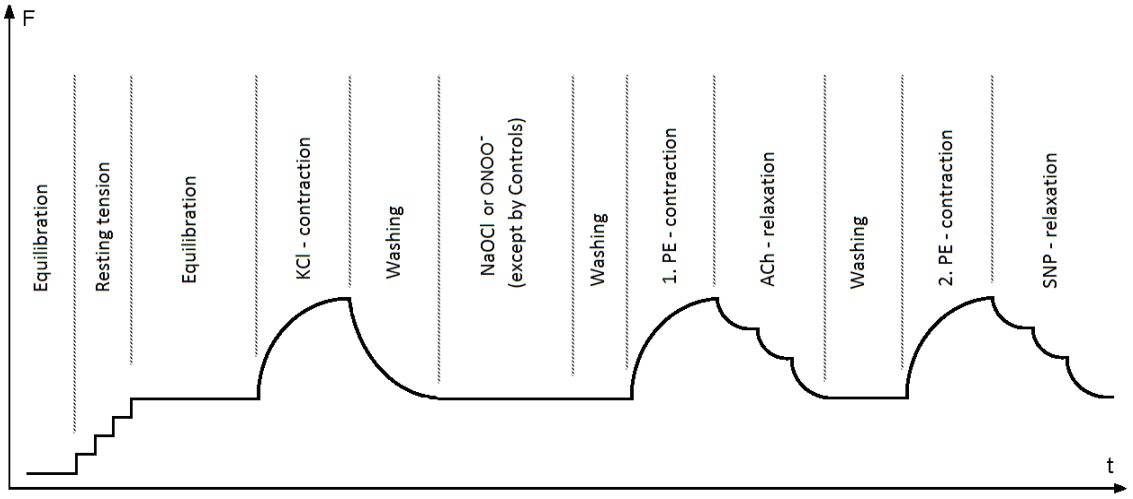

Figure 3: Organ bath protocol

The phases of the organ bath experiments on time (t) - force (F) scale.

ACh: acetylcholine; KCl: potassium chloride; NaOCl: sodium hypochloride; PE: phenylephrine; SNP: sodium nitroprusside;

5.6 Histopathological processing

Aortic segments from each experimental group were fixed in paraformaldehyde solution (4%) and embedded in paraffin. 3-µm-thick sections cut by microtome were placed on adhesive slides.

DOI:10.14753/SE.2017.1905

5.6.1 Immunohistochemical staining

5.6.1.1 Nitrotyrosin immunohistochemical staining

According to previously described methods [109], we performed immonohistochemical staining on aortic rings for nitrotyrosine, a marker of peroxynitrite-mediated damage. Peroxynitrite has very short lifetime, therefore it's generation is usually characterized by the "footprint" of peroxynitrite generation nitrotyrosine, a product of the reaction of peroxynitrite and tyrosine. Immunohistochemical staining for nitrotyrosine was performed by using polyclonal sheep anti-nitrotyrosine antibody (OXIS, Portland, OR, USA) incubation was performed (1:80) for 2 hours at room temperature. Negative controls were performed by omitting the primary antibody. Sections were counterstained with Gill's hematoxylin, mounted with Permount, and coverslips were placed on the section.

5.6.1.2 Cyclic GMP immunohistochemical staining

Cyclic GMP immunohistochemical staining was performed for the identification of intracellular cGMP content. Rehydrated sections were blocked (3% goat serum), rabbit polyclonal anti- cGMP primary antibody (Abcam plc, Cambridge, UK) incubation was performed (1:1000) for 2 hours at room temperature. Tissue sections were overnight incubated at 4ºC [110], then secondary biotinylated anti-rabbit immunglobuline E (BioGenex, CA, USA) incubation followed which allowed reacting with alcaline phosphatase-conjugated streptavidin (BioGenex, CA, USA). A red reaction product at the site of the target antigen was formed by use of Fast Red substrate (DakoCytomation, Hamburg, Germany). Negative controls were performed by omitting the primary antibody. Sections were counterstained with Gill's hematoxylin, mounted with Permount, and coverslips were placed on the section.

5.6.2 Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

TUNEL assay was performed for detection of DNA strand breaks (free 3'-OH DNA ends). The detection was performed using a commercial kit according to the protocol provided by the manufacturer (Chemicon International, Temecula, CA, USA). Rehydrated sections were treated with 20μg/ml DNAse-free Proteinase K (Sigma-Aldrich, Taufkirchen, Germany) to retrieve antigenic epitopes, followed by 3% hydrogen-peroxide to quench endogenous peroxidase activity. Free 3′-OH termini were labeled with a reaction mixture of terminal deoxynucleotidyl transferase and digoxigenin-deoxynucleotidyl (dUTP) for 1 hour at 37°C. Incorporated digoxigenin-conjugated nucleotides were detected using a horseradish peroxidase–conjugated anti-digoxigenin antibody and 3,3′-diaminobenzidine. Sections were counterstained with methylgreen. Dehydrated sections were cleared in xylene and mounted with Permount (Fischer Scientific, Germany), and coverslips were applied.

5.6.3 Quantification of immunostainings and TUNEL assay

Semiquantitative histomorphological assessment was performed on all of the stained specimens of cinaciguat and vardenafil projects in a blinded fashion using conventional microscopy. The results were expressed with a scoring system. Section with the most intense labeling signals was used as reference for maximum labeling intensity. All other tissue sections were comparatively evaluated. Colours were measured using densitometry and sorted in four colour classes: one class for background staining and three classes for positively stained areas. On the basis of the measured intensity, the colour classes were coupled with score values 0-4 where 0 meant no positive staining and 4 meant extensive staining. Using the Cell-A software (Olympus Soft Imaging Solutions GmbH, Germany) we measured the area of the objects in each class in each field, and assigned an area score (0 = 0%, 1 = up to 30% positive cells, 2 = 31–60% positive cells, 3 = 61–90% positive cells, 4 = >91% positive cells), and calculated an average score for the whole picture (intensity score multiplied by area score, 0–16). Finally, each specimen was characterized by 4 adjacent fields.

For the assessment of TUNEL-labeled cells four different fields were pictured with digital camera from each section at a magnification of x200. TUNEL positive and negative cell nuclei were counted and the TUNEL positive cell nuclei were calculated as percentage of total cell number.

5.7 Quantitative Real-Time Polymerase Chain Reaction (PCR)

After homogenization of the chosen aortic rings total RNA was isolated from the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). RNA concentration and purity were determined at 260, 280, and 230 nm wave-lengths with spectrophotometer. Reverse transcription was performed with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) using 400 μg RNA in a volume of 20 μL. Quantitative real-time PCR was performed on a LightCycler 480 system with the Universal ProbeLibrary probes (Roche, Mannheim, Germany). Primers were obtained from TIB Molbiol (Berlin, Germany), their sequences and UPL probes used are represented on **Table I**. Evaluation was performed with LightCycler 480 SW1.5 software (Roche, Mannheim, Germany).

Efficiency of the PCR reaction was confirmed with standard curve analysis. Every sample was quantified in duplicate, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

5.8 Western-blot analysis

Proteins were extracted from the tissue homogenisate, and concentration was determined. Homogenates (15 μg) were denatured, separated on sodium dodecyl sulfate polyacrylamide gel, transferred to polyvinylidene fluoride membrane. Membranes were blocked, incubated overnight with primary antibodies specific to p17 caspase-3 fragment (1:1000, Abcam, Cambridge, UK), Bax (1:200, Abcam, Cambridge, UK), Bcl-2 (1:100, Abcam, Cambridge, UK). Blots were incubated for 1 hour with horseradish peroxydase conjugated secondary antibody (1:5000, Santa Cruz Biothechnology, Heidelberg, Germany). Immunoreactive protein bands were developed using Enhanced Chemiluminescence system (PerkinElmer, Rodgau-Juegesheim, Germany). Band

intensities were detected with Hyperfilm ECL System. For GAPDH housekeeping (1:500, Santa Cruz Biothechnology, Heidelberg, Germany) incubation and detection was repeated after stripping the blots form primary antibodies (Restore Plus Western Blot Stripping Buffer, Thermo Scientific, Rockford, USA). Target protein densities were normalized to housekeeping densities of the same samples, respectively.

5.9 Statistical Analysis

Data were tested for normal distribution (Shapiro-Wilk) and where met the requirements for parametric analysis, means were tested by one-way ANOVA followed by Student's unpaired t-test with Bonferoni's correction test. For the analysis of PCR results Kruskal-Wallis one-way analysis of variance with Dunn's post hoc test was used. A p value <0.05 was considered statistically significant.

5.10 Preparation and application of chemical reagents

Cinaciguat (BAY 58-2667), an amino dicarboxylic acid, was kindly provided by Bayer HealthCare (Wuppertal, Germany). It was dissolved in 1% methylcellulose solution vehicle and administered orally at a dose of 10 mg/kg at a volume of 10 ml/kg. The application and dosage of cinaciguat have been determined according to the pharmacokinetic and dynamic properties [98] as well as to the results of previous rodent experiments [111]. Vardenafil was provided by Bayer HealthCare (Wuppertal, Germany) and was diluted in physiologic saline to 10^{-12} mol/L, 10^{-11} mol/L, 10^{-10} mol/L and 10^{-9} mol/L. Peroxynitrite (Calbiochem, San Diego, CA, USA) was diluted with 4.7% NaOH. Sodium-hypochlorite solution (AppliChem, Darmstadt, Germany) was diluted with distilled water. Phenylephrine, acetylcholine and sodium nitroprusside (Sigma-Aldrich, Germany) were dissolved in normal saline.

DOI:10.14753/SE.2017.1905

Table I

The sequences for the forward (F) and reverse (R) primers (from 5’ to 3’) and Universal Probe Library (UPL) probes

Assay	**Sequence**	**UPL probes**
BAX	F: 5’-TAGCAAACTGGTGCTCAAGG R: 5’-GCCACCCTGGTCTTGGAT	69
Bcl-2	F: 5’-GTACCTGAACCGGCATCTG R: 5’-GGGGCCATATAGTTCCACAA	75
Caspase-3	F: 5’-AAACCTCCGTGGATTCAAAA R: 5’-AGCCCATTTCAGGGTAATCC	56
Endothelin-1	F: 5’-TGTCTACTTCTGCCACCTGGA R: 5’-CCTAGTCCATACGGGACGAC	115
eNOS	F: 5’-TGACCCTCACCGATACAACA R: 5’-CGGGTGTCTAGATCCATGC	5
GAPDH	F: 5’-CTACCCACGGCAAGTTCAAT R: 5’-ATTTGATGTTAGCGGGATCG	111
iNOS	F: 5’-CAGCGGCTCCATGACTCT-3’ R: 5’-ATCTCCTGCATTTCTTCCTGAT-3’	82

BAX: Bcl-2-like protein 4, Bcl-2: B-cell lymphoma, eNOS: endothelial nitric oxide synthase, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, iNOS: inducible nitric oxide synthase

DOI:10.14753/SE.2017.1905

6 Results

6.1 Vascular dysfunction induced by peroxynitrite in vitro - effects of cinaciguat

6.1.1 Contractile responses of aortic rings

The contractile responses of aortic segments to phenylephrine (10^{-6} mol/L), an α_1-adrenergic agonist are shown in Table II. Incubation of aortic rings with peroxynitrite significantly increased the phenylephrine-induced maximum contraction compared to the control rings. However, treatment of rats with cinaciguat did not significantly reduce increased contractile responses to phenylephrine. In the absence of peroxynitrite, cinaciguat treatment did not have any effect (**Table II**).

Table II.

Values of maximal relaxation (R_{max}, %) and pD_2 (affinity) to the vasorelaxant actions of acetylcholine (ACh) and sodium nitroprusside (SNP), and contraction values induced by phenylephrine (PE % of KCl) in percentage of the contraction induced by 0.1 mol/L potassium-chloride caused depolarization in rat thoracic aortic rings.

	Control	Peroxynitrite	Cinaciguat + Peroxynitrite	Cinaciguat
R_{max} to ACh (%)	93.2 ± 2.0	**44.5 ± 5.9***	**67.1 ± 3.5*,#**	**93.9 ± 1.1#**
pD_2 to ACh	7.6 ± 0.1	**6.6 ± 0.2***	7.0 ± 0.1	**7.9 ± 0.1#**
P_{max} to SNP (%)	100.1 ± 0.2	100.2 ± 0.3	100.2 ± 0.2	101.6 ± 0.2
pD_2 to SNP	8.8 ± 0.2	**8.2 ± 0.1***	**8.2 ± 0.1***	**9.1 ± 0.3#**
Phenylephrine (% of KCl)	73 ± 5	**114 ± 3***	**108 ± 5***	**78 ± 5#**

Values represent mean ± S.E.M. of 12-15 experiments.

Statistical relevance is highlighted with **bold**.

*$p < 0.05$ versus control;

# $p < 0.05$ versus peroxynitrite group;

6.1.2 Endothelium-dependent vasorelaxation of aortic rings

In aortic rings precontracted with 10^{-6} mol/L phenylephrine, 10^{-9} to 10^{-4} mol/L acetylcholine induced a concentration-dependent relaxation. In contrast, exposure of aortic rings to the reactive oxidant peroxynitrite (200 μmol/L) for 30 minutes significantly attenuated the maximal relaxation to acetylcholine and decreased pD2 values for the concentration-response curves as compared to control (NaOH only) segments (**Table II, Figure 4A**). Treatment of rats with cinaciguat significantly improved the acetylcholine-induced, endothelium-dependent, NO-mediated vasorelaxation after exposure of aortic rings to peroxynitrite. In the absence of peroxynitrite, cinaciguat treatment did not alter maximal relaxation and the sensitivity to acetylcholine compared with the control group (**Table II, Figure 4A**).

6.1.3 Endothelium-independent vasorelaxation of aortic rings

Figure 4B shows concentration-dependent relaxations induced by 10^{-10} to 10^{-5} mol/L sodium nitroprusside, an endothelium-independent vasodilator. In contrast to acetylcholine, maximal relaxation did not differ significantly among the different experimental groups (**Table II, Figure 4B**). However, incubation of aortic rings with peroxynitrite caused a significant shift of the sodium nitroprusside concentration-response curves to the right. Cinaciguat has no effect on this level of damage (**Table II, Figure 4B**). In the absence of peroxynitrite, treatment of rats with cinaciguat did not alter maximal relaxation and the sensitivity to acetylcholine compared with the control group (**Table II, Figure 4B**).

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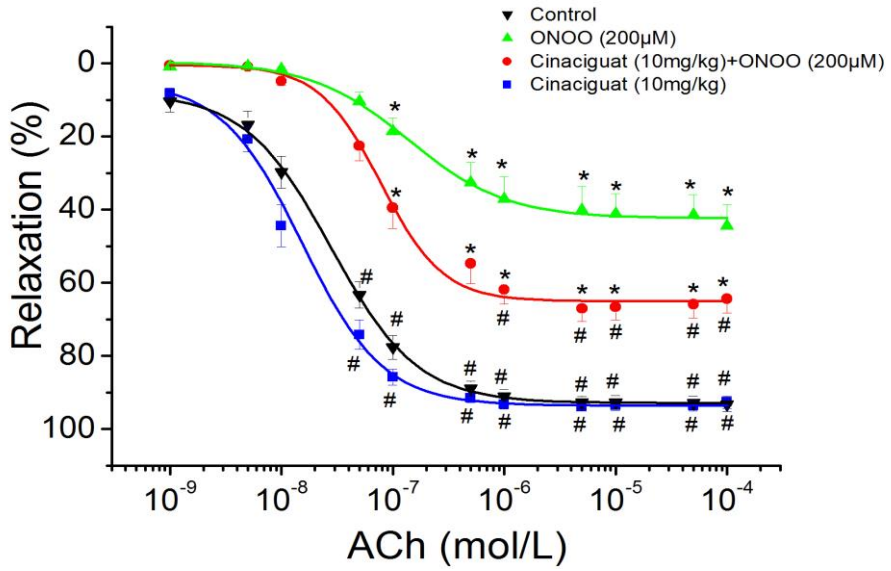
A)



B)

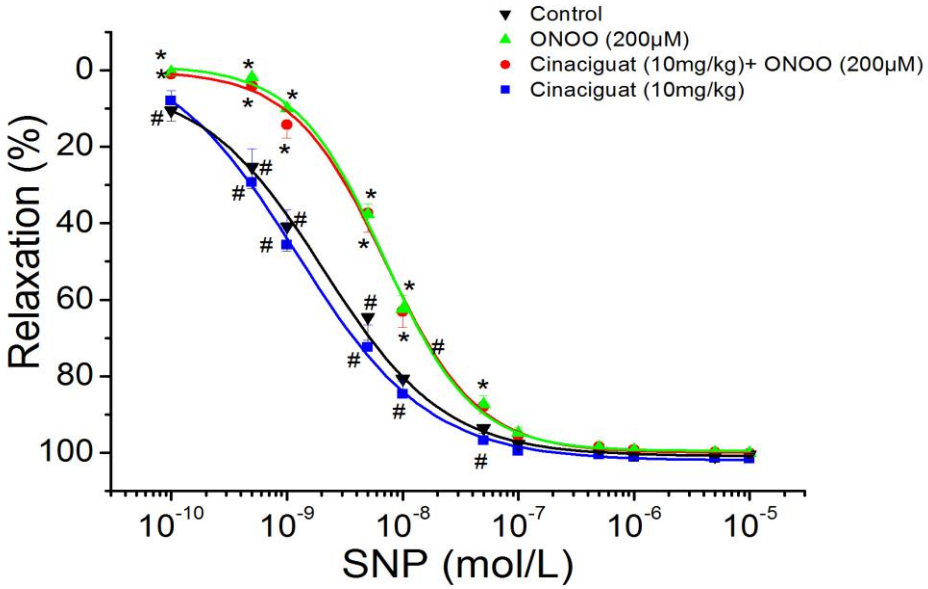


Figure 4. Effect of cinaciguat on vascular relaxation in rat thoracic aortic ring after peroxynitrite exposure

(A) Acetylcholine (ACh)-induced endothelium-dependent vasorelaxation; (B) sodium nitroprusside (SNP)-induced endothelium-independent vasorelaxation; Each point of the curves represents mean ± S.E.M. of 12-15 experiments in thoracic aortic rings of the different groups. $p < 0.05$: * vs. Control; # vs Peroxynitrite group.

6.1.4 Cinaciguat decreases nitro-oxidative stress and DNA strand breaks in aortic rings exposed to peroxynitrite

To evaluate the levels of oxidative and nitrosative stress in the aortic rings after peroxynitrite exposure, we assessed nitrotyrosine immunoreactivity. There was a large increase in the intensity of nitrotyrosine staining in the peroxynitrite-incubated rings compared to the control segments which was reduced after cinaciguat pretreatment, as evidenced by decreased brown staining (Control: 3.8 ± 0.4 vs. $ONOO^-$: 6.4 ± 0.4; $p<0.05$; $ONOO^-$ vs. Cinaciguat+$ONOO^-$: 4.4 ± 0.3; $p<0.05$; Cinaciguat: 4.4 ±0.4) (**Figures 5A and 6A**).

Increased density of TUNEL-positive nuclei was observed in the wall of peroxynitrite-exposed aortic rings indicating DNA-fragmentation (**Figure 5B and 6B**). Pretreatment of rats with cinaciguat significantly decreased peroxynitrite-induced DNA strand breaks (Control: 35 ± 2 vs. $ONOO^-$: 49 ± 3; $p<0.05$; $ONOO^-$ vs. Cinaciguat+$ONOO^-$: 34 ± 3 and Cinaciguat: 29 ± 5; $p<0.05$) (**Figure 5B and 6B**).

6.1.5 Cinaciguat increases cGMP levels in aortic rings exposed to peroxynitrite

In peroxynitrite-exposed rings, we detected a tendency toward lower cGMP immunoreactivity compared with control (without reaching the level of statistical significance). However, after treatment of rats with cinaciguat, in the media of peroxynitrite-incubated rings a significantly higher score of cGMP staining was observed when compared with the peroxynitrite-incubated segments, as evidenced by increased red staining ($ONOO^-$: 5.9 ± 0.7; vs. Cinaciguatt+$ONOO^-$: 7.9 ± 0.6; $p<0.05$; Control: 7.2 ± 0.6; Cinaciguat: 7.7 ± 0.8) (**Figures 5C and 6C**).

DOI:10.14753/SE.2017.1905

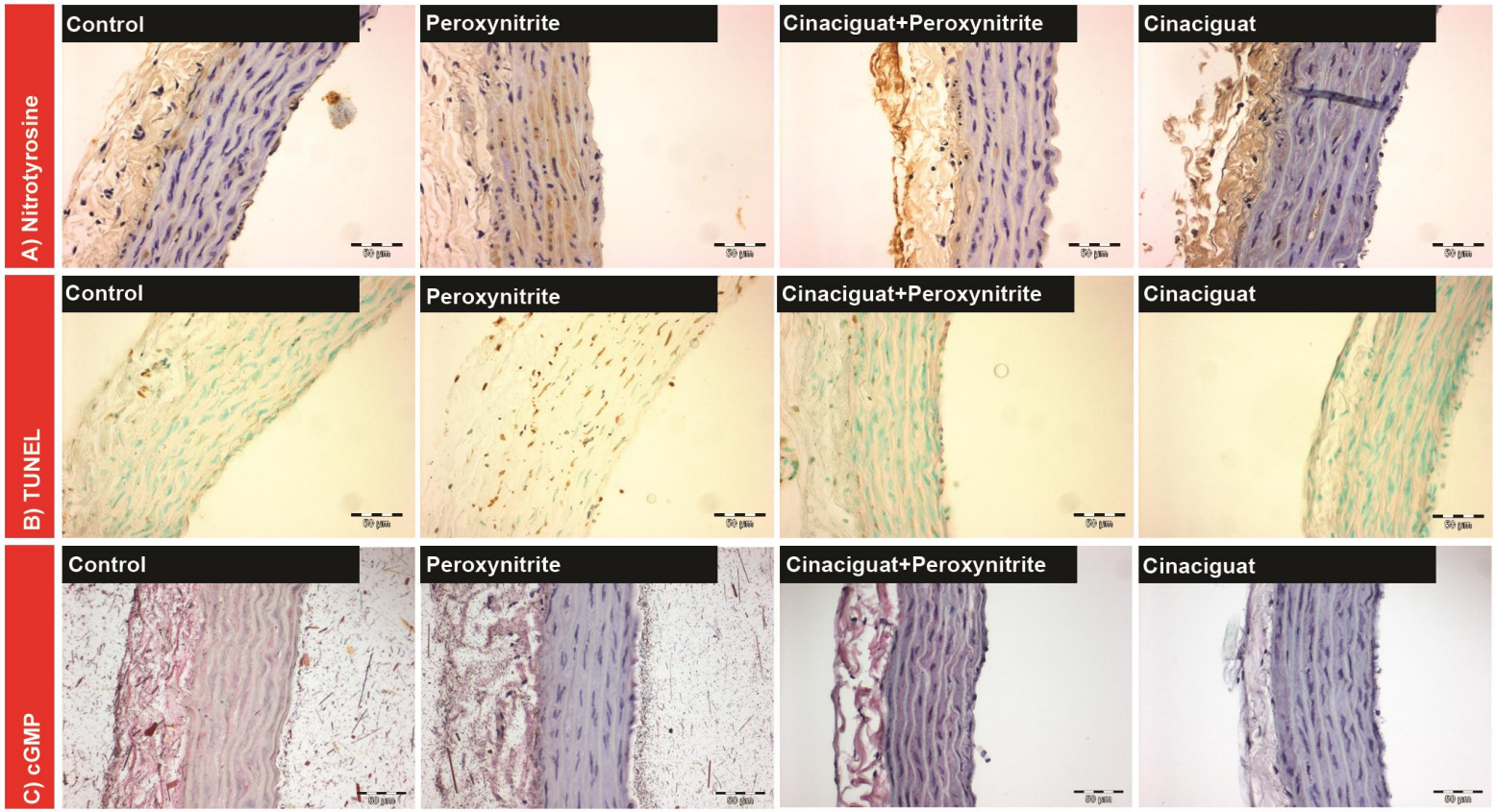


Figure 5. Effects of cinaciguat on nitro-oxidative stress, DNA strand breaks, and cyclic GMP levels in aortic rings exposed to peroxynitrite.

Representative photomicrographs of (A) nitrotyrosine immunohistochemistry staining (brown staining), (B) TUNEL assay in the cell nuclei (brown staining), and (C) cyclic GMP immunohistochemistry (red staining) in the aortic vascular wall (magnification x200, scale bar: 50µm). GMP indicates guanosine monophosphate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUDP nick end labeling.

DOI:10.14753/SE.2017.1905

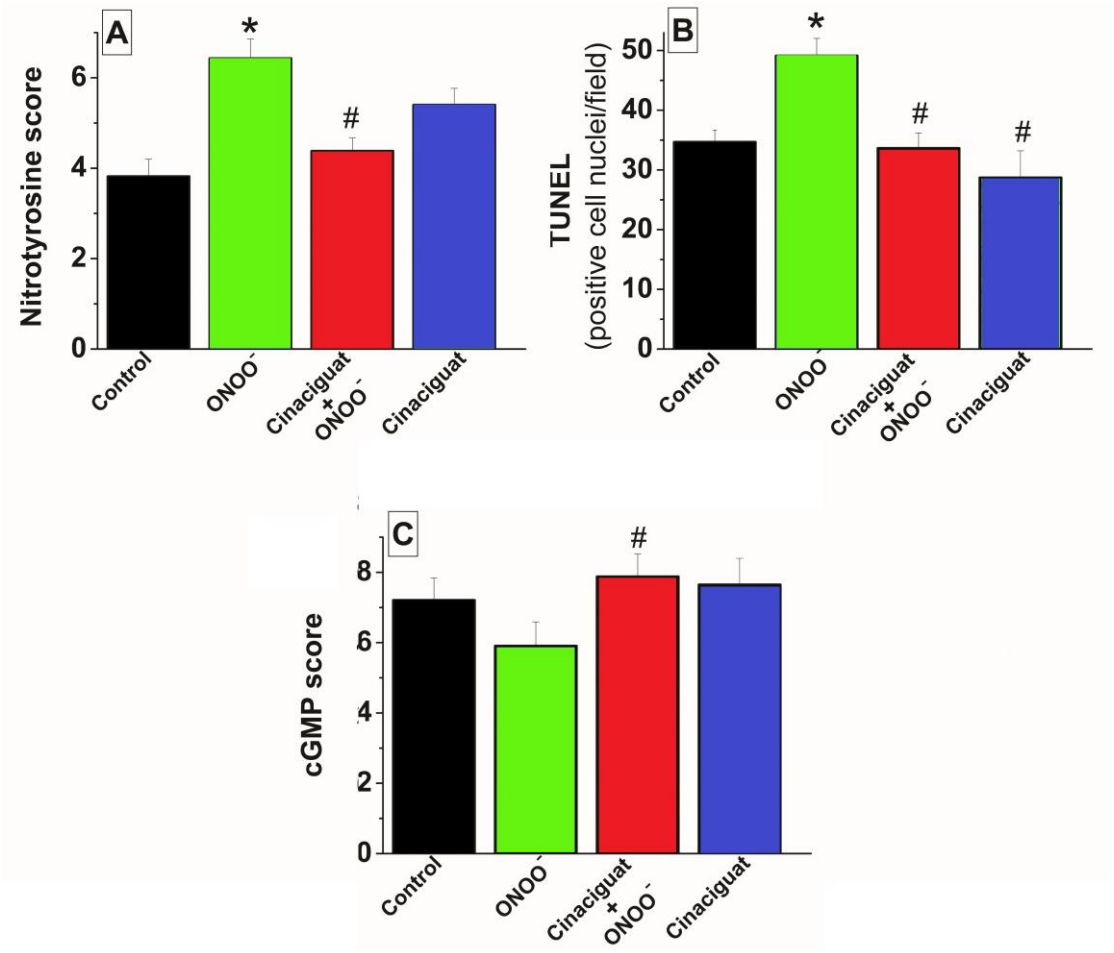


Figure 6. Scoring of nitrotyrosine and cGMP immunohistochemistry and TUNEL assay.

Immunohistochemical scores for (A) nitrotyrosine, (B) average number of TUNEL-positive cell nuclei in a microscopic field, and (C) cGMP in the vessel wall of aortic rings. Values represent mean ± S.E.M. (n=7 in each group).

$p < 0.05$: * vs. Control; # vs. Peroxynitrite ($ONOO^-$) group. GMP indicates guanosine monophosphate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUDP nick end labeling.

6.1.6 Cinaciguat regulates gene expression

The expression of endothelin-1 mRNA in aortic rings exposed to peroxynitrite was significantly up-regulated compared to native control rings. The previous

cinaciguat treatment significantly decreased this up-regulation caused by peroxynitrite compared to the $ONOO^-$ group however, ET-1 mRNA level remained significantly higher than in control. Cinaciguat treatment itself did not influence the ET-1 expression in native rings (**Figure 7A**).

Peroxynitrite exposure caused a significant up-regulation in the mRNA expression of the pro-apoptotic Caspase-3 and BAX genes, compared to control rings, which up-regulation was significantly decreased by previous cinaciguat treatment. Cinaciguat treatment itself did not influence either Caspase-3 or BAX mRNA expressions in native rings (**Figure 7B, C**).

A significant reduction in the mRNA expression of Bcl-2 anti-apoptotic gene was detected in the peroxynitrite exposed rings compared to controls, which change was totally overturned by cinaciguat treatment (**Figure 7D**).

The mRNA expression of eNOS was significantly suppressed by peroxynitrite exposure. Peroxynitrite exposed rings after cinaciguat treatment did not show significant difference compared to control group. Cinaciguat treatment itself did not affect the expression of eNOS in aortic rings compared to control (**Figure 7E**).

In the expression of inducible $NO^\cdot$ synthase mRNA no significant difference was detected among the groups (**Figure 7F**).

6.1.7 Effect of cinaciguat on cleaved caspase-3 level, Bax and Bcl-2 protein expression

Densitometric analysis of the bands revealed a 2-fold increase of caspase-3 p17 cleavage protein presence and a 5-fold increase of BAX protein presence in peroxynitrite-exposed rings. Cincaiguat treatment significantly decreased the presence of these pro-apoptotic proteins in rings exposed to peroxynitrite. Cinaciguat treatment alone caused no significant changes in protein levels **(Figure 8A, B).**

Expression of the anti-apoptotic Bcl-2 protein was significantly decreased in the peroxynitrite exposed group compared to control, while the cinaciguat resulted in maintained Bcl-2 levels in both cinaciguat treated groups (**Figure 8C**).

DOI:10.14753/SE.2017.1905

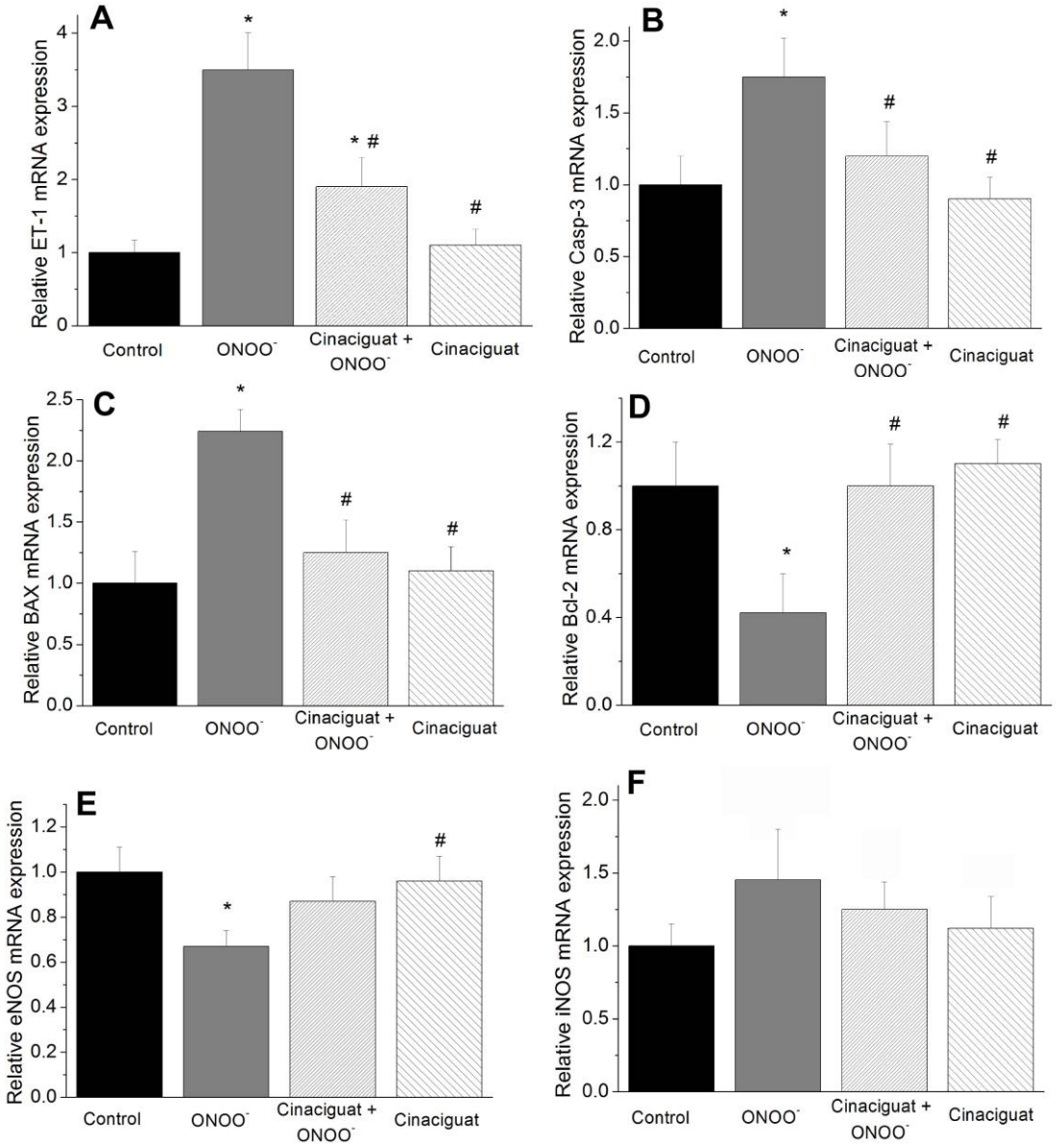


Figure 7. Effects of *in vivo* cinaciguat pretreatment and *in vitro* peroxynitrite exposure on the gene expressions of isolated aortic rings

(A) endothelin-1 (ET-1), (B) caspase-3 (casp-3), (C) Bax, (D) Bcl-2, (E) endothelial nitric oxide synthase (eNOS), and (F) inducible nitric oxide synthase (iNOS)

Controls were given the arbitrary value of 1 (n=6 in each group).

Values represent median ± quartiles; $p<0.05$: * vs. control; # vs. $ONOO^-$

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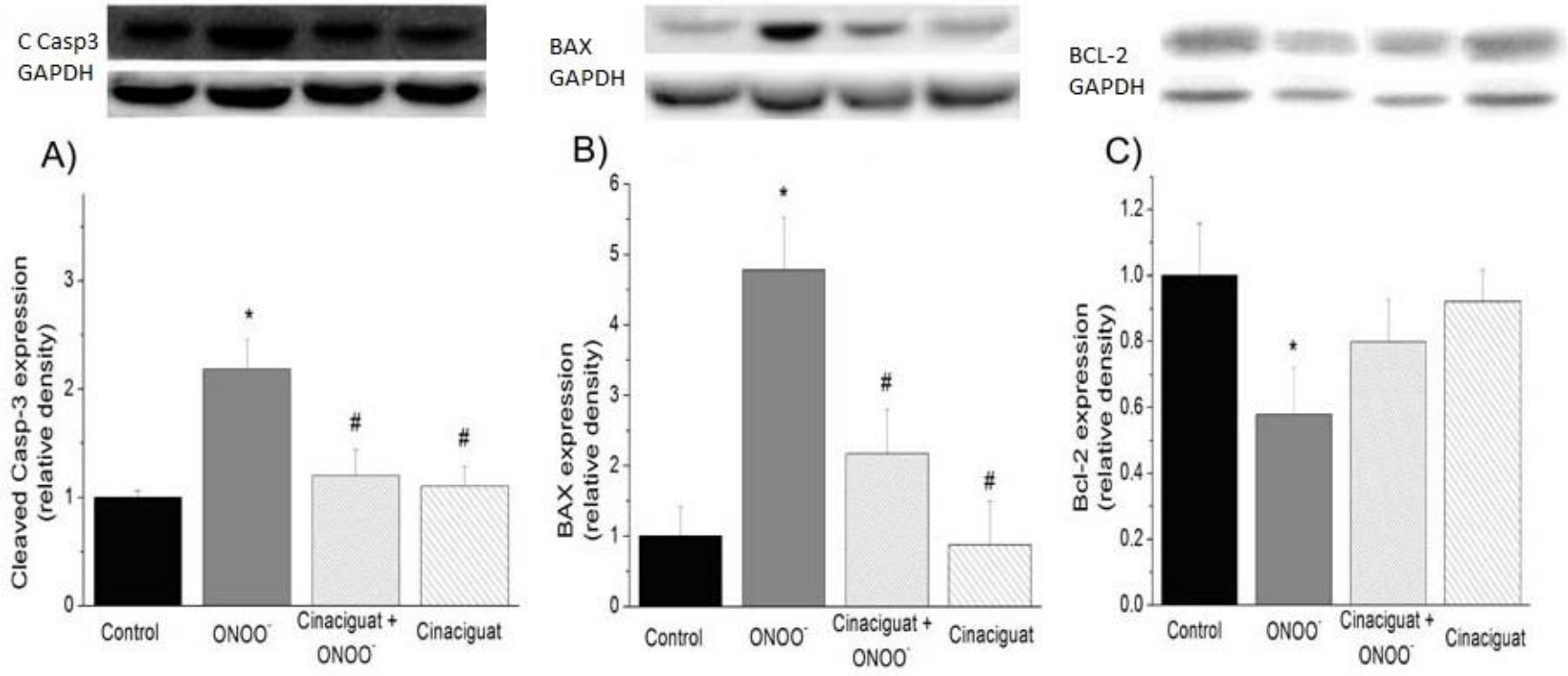

Figure 8. Effects of *in vivo* cinaciguat pretreatment and *in vitro* peroxynitrite exposure on the protein expressions of isolated aortic rings

(A) cleaved caspase-3 (casp-3), (B) Bax and (C) Bcl-2 (n=6 in each group).

Values represent mean ± S.E.M.; *p<.05 versus control; #p<.05 versus $ONOO^-$

DOI:10.14753/SE.2017.1905

6.2 Vascular dysfunction induced by cold preservation, in vitro reoxygenation and hypochlorite - effects of vardenafil

6.2.1 Contractile responses of aortic rings

Contractile responses of aortic rings to KCl are shown in Figure 9. Hypochlorite exposure of aortic segments after 24 hours long cold ischemia did not alter contractile responses to KCl. However, vardenafil supplementation of the storage solution resulted in the significant decrease of contractile responses to KCl caused depolarisation, when compared to control. Vardenafil supplementation in 10^{-11} and 10^{-9} mol/L concentration resulted in a significant decrease compared to the Saline group (**Figure 9A**).

Compared to the control group, exposure of aortic segments to hypochlorite after 24 hours long cold ischemic preservation significantly increased phenylephrine-induced maximum contraction in the saline treated and in the 10^{-12} mol/L vardenafil treated rings. However, contractile responses to PE did not significantly differ in the groups treated with 10^{-11} mol/L or higher concentration of vardenafil, when compared to control (**Figure 9B).**

6.2.2 Endothelium-dependent vasorelaxation of aortic rings

In aortic rings precontracted with 10^{-6} mol/L phenylephrine, 10^{-9} to 10^{-4} mol/L acetylcholine induced a concentration-dependent relaxation. Aortic segments exposed to 24 hours long cold ischemic storage followed by hypochlorite (200 μmol/l) incubation showed significantly attenuated R_{max} to acetylcholine and a significant increase of pD_2 that implies to a shift of the acetylcholine concentration-response curves to the right, as compared to control group (**Table III, Figure 10**). Though conservation of aortic segments in vardenafil supplemented saline solution improved the acetylcholine induced endothelium-dependent, $NO^{\cdot}$ mediated vasorelaxation after the exposure of rings to hypochlorite, the improvement of R_{max} have reached the level of statistical significance only at the concentration of 10^{-11} mol/L vardenafil supplementation. There was no statistical difference among the vardenafil treated groups. (**Table III, Figure 10**).

DOI:10.14753/SE.2017.1905

6.2.3 Endothelium-independent vasorelaxation of aortic rings

Figure 10. and 11. shows concentration-dependent relaxations induced by 10^{-10} to 10^{-5} mol/L sodium nitroprusside, an endothelium-independent vasodilator. In contrast to acetylcholine, endothelium-independent vasorelaxation of the aortic rings to sodium-nitroprusside showed no significant difference in R_{max} among the experimental groups. However, long, cold ischemic preservation and incubation of aortic rings with hypochlorite caused a significant shift of the sodium nitroprusside concentration-response curves to the right. Only vardenafil at the concentration of 10^{-11} mol/L have caused significant increase of the pD_2 value (**Table III, Figure 11**).

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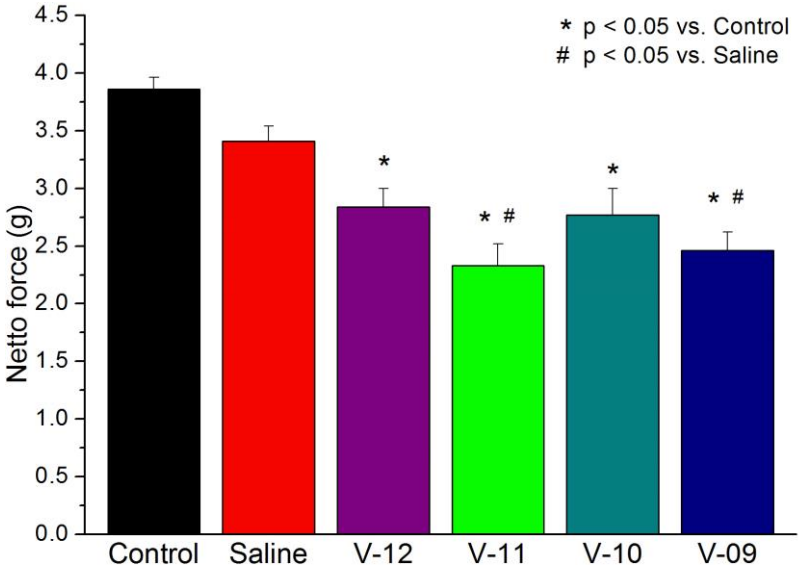
A)



B)

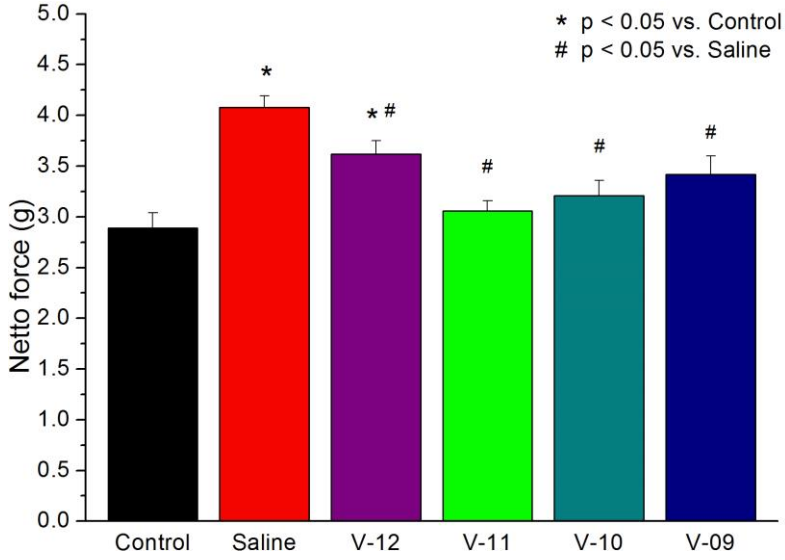


Figure 9. Effect of cold ischemic storage, reoxygenation and hypochlorite incubation on the vascular contractility

Netto contraction forces induced by (A) KCl (0.1mol/L) caused depolarisation and (B) phenylephrine (10^{-6} mol/L) in the groups of control, saline, and vardenafil [V] (10^{-12} mol/L; 10^{-11} mol/L; 10^{-10} mol/L; 10^{-9} mol/L). Values represent mean ± S.E.M. of 15-20 experiments in thoracic aortic rings of the different groups.

$p<0.05$: * vs. Control; # vs. Saline

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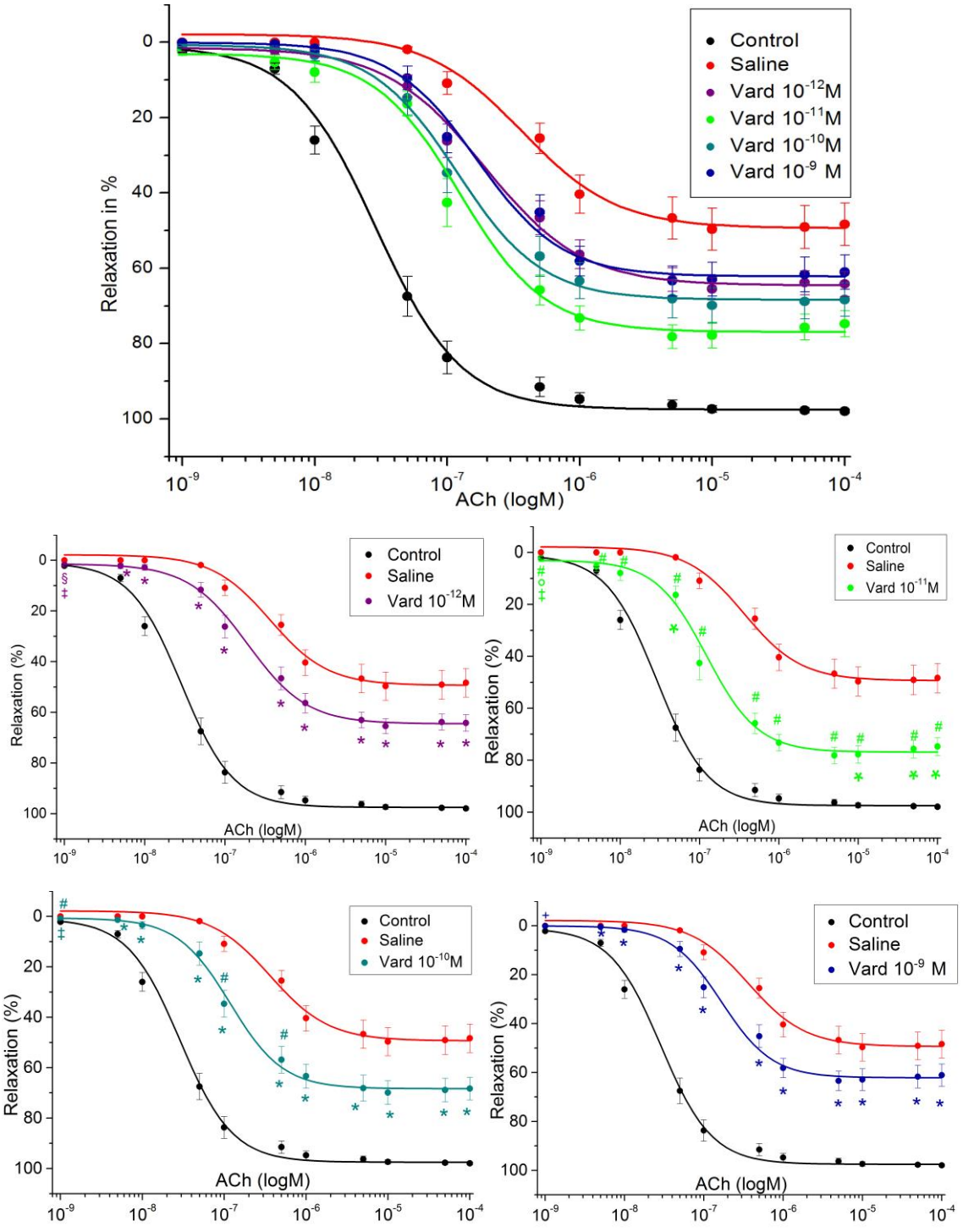

Figure 10. Effect of cold ischemic storage, reoxygenation and hypochlorite incubation on the acetylcholine (ACh)-induced endothelium-dependent vasorelaxation

Each point of the curves represents mean ± S.E.M. of 15-20 experiments in thoracic aortic rings of the different groups.

$p < 0.05$: * vs. Control; # vs. Saline; ‡ vs. V^{-12}; † vs. V^{-11}; § vs. V^{-10}; ° vs. V^{-9}

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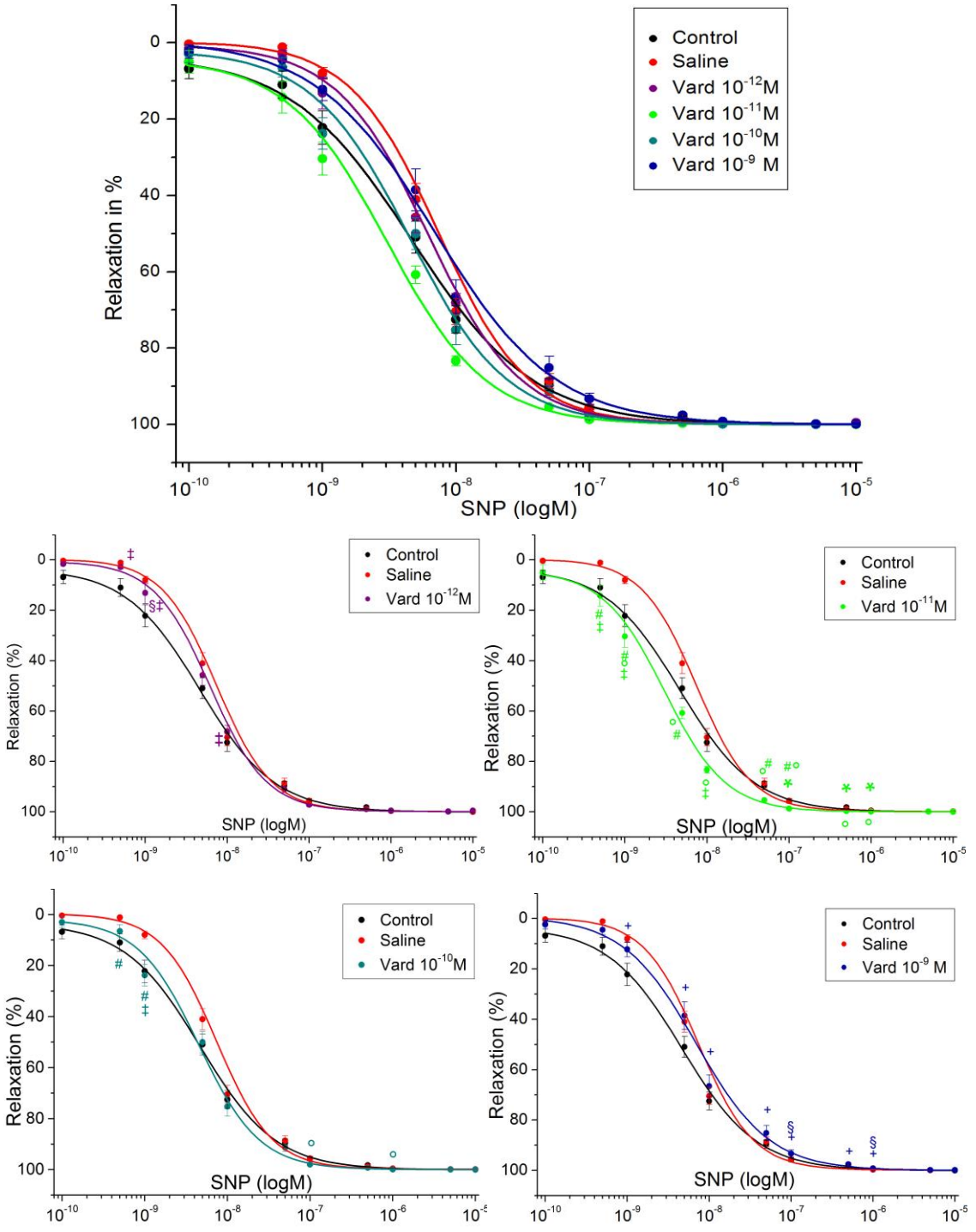

Figure 11. Effect of cold ischemic storage, reoxygenation and hypochlorite incubation on the sodium nitroprusside (SNP)-induced endothelium-independent vasorelaxation

Each point of the curves represents mean ± S.E.M. of 15-20 experiments in thoracic aortic rings of the different groups.

$p < 0.05$: * vs. Control; # vs. Saline; ‡ vs. V^{-12}; † vs. V^{-11}; § vs. V^{-10}; ° vs. V^{-9}

DOI:10.14753/SE.2017.1905

Table III.

Values of maximal relaxation (R_{max}, %) and pD_2 (affinity) to the endothelium-dependent vasorelaxant action of acetylcholine (ACh) and sodium nitroprusside (SNP), and contraction induced by phenylephrine (PE % of KCl) in percentage of the contraction induced by 0.1 mol/L potassium-chloride caused depolarization in rat thoracic aortic rings.

	Control	Saline	Vardenafil (10^{-12} M)	Vardenafil (10^{-11} M)	Vardenafil (10^{-10} M)	Vardenafil (10^{-9} M)
R_{max} to ACh (%)	97.9 ± 0.56	**48.3 ± 5.6***	**64.2 ± 3.3***	***74.8 ± 3.5**#***	**68.3 ± 4.5***	**61.0 ± 4.5***
pD_2 to ACh	7.6 ± 0.09	**6.4 ± 0.1***	**6.7 ± 0.1***	***6.9 ± 0.1**#***	**6.83 ± 0.1***	**6.7 ± 0.1***
R_{max} to SNP (%)	99.9 ± 0.02	99.8 ± 0.1	99.5 ± 0.4	99.9 ± 0.1	99.9 ± 0.1	99.8 ± 0.1
pD_2 to SNP	8.3 ± 0.07	8.2 ± 0.1	8.3 ± 0.1	**8.8 ± 0.2***	8.4 ± 0.1	8.2 ± 0.1
PE (% of KCl)	75.5 ± 2,75	**121.2 ± 1.9***	**117.3 ± 5.8***	**122.1 ± 4.3***	**110.4 ± 4.9***	**124.9 ± 7.9***

Values represent mean ± S.E.M. of 15-20 experiments.

Statistical relevance is highlighted with **bold**.

*$p < 0.05$ versus Control;

$^{\#}$ $p < 0.05$ versus Saline group;

6.2.4 Vardenafil decreases DNA strand breaks in aortic rings

Cold ischemic conservation for 24 hours followed by reoxygenation and 30 minutes hypochlorite incubation led to significantly increased density of TUNEL-positive nuclei in the aortic segments, compared to control rings (control: 10 ± 6 vs. saline: 72 ± 4; $p>0.05$). This indicates DNA-fragmentation caused by oxidative stress in the wall (intima and media) of the aortic rings. Preservation of aortic segments with vardenafil-supplemented saline solution significantly decreased DNA strand breaks (saline: 72 ± 4 vs. vardenafil (V) 10^{-12} : 31 ± 6; 10^{-11}: 14 ± 5; 10^{-10}: 22 ± 4; 10^{-9}: 43 ± 11) (**Figure 12A, 13A**).

6.2.5 Vardenafil increases cGMP levels in aortic rings

Cold ischemic conservation for 24 hours followed by reoxygenation and 30 minutes hypochlorite incubation led to significantly lower cGMP immunreactivity in the saline group compared to the aortic segments of the control group. In the vardenafil-supplemented group a significantly higher score of cGMP staining was observed when compared with the saline group (**Figure 12B, 13B**).

6.2.6 Vardenafil regulates aortic gene expression

In the aspect of protection of vascular function, cGMP homeostasis and DNA injury the vardenafil concentration of 10^{-11} mol/L proved to be the most effective. Therefore, from the four different vardenafil concentrations tested, this group was chosen for further PCR analysis.

The expression of endothelin-1 mRNA in aortic rings exposed to 24 hours cold ischemic conservation followed by 30 minutes hypochlorite incubation was significantly up-regulated compared to native control rings, and was significantly decreased by vardenafil supplementation. However, ET-1 mRNA level remained significantly higher than in control (**Figure 14A**).

Long ischemic storage and hypochlorite exposure caused a significant up-regulation in the mRNA expression of the pro-apoptotic Caspase-3 and BAX genes compared to

control rings, which up-regulation was significantly decreased by vardenafil supplementation in the storage solution (**Figure 14B, C**).

A significant reduction in the mRNA expression of Bcl-2 anti-apoptotic gene was detected in the saline group compared to control, which change was totally overturned by vardenafil supplementation (**Figure 14D**).

Vardenafil supplementation of the storage solution did not influence the mRNA level of endothelial and inducible nitric oxide synthases (eNOS and iNOS) (**Figure 14E, F**).

6.2.7 Effect of vardenafil on cleaved caspase-3 level, Bax and Bcl-2 protein expression

In the aspect of protection of vascular function, cGMP homeostasis and DNA injury the vardenafil concentration of 10^{-11} mol/L proved to be the most effective. Therefore, from the four different vardenafil concentrations tested, this group was chosen for further Western-blot analysis.

Densitometric analysis of caspase-3 p17 cleavage and Bax bands after 24 hours of cold storage and hypochlorite exposure showed a significant increase in saline group compared to control group. This up-regulation of protein level was significantly moderated by vardenafil supplementation (**Figure 15A, B**). Expression of the anti-apoptotic Bcl-2 protein (**Figure 15C**) was significantly decreased in saline group compared to control, while the supplementation of vardenafil maintained Bcl-2 levels on the level of controls.

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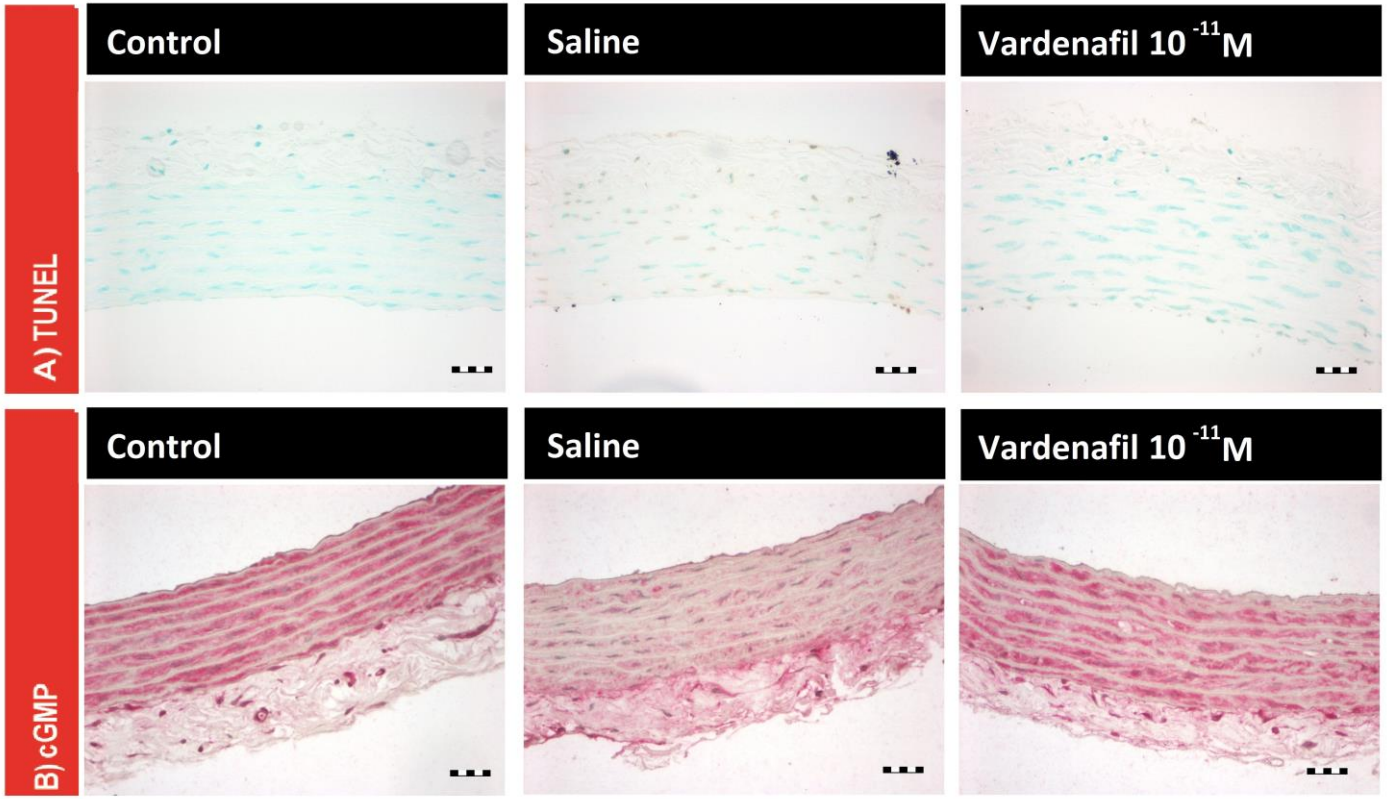
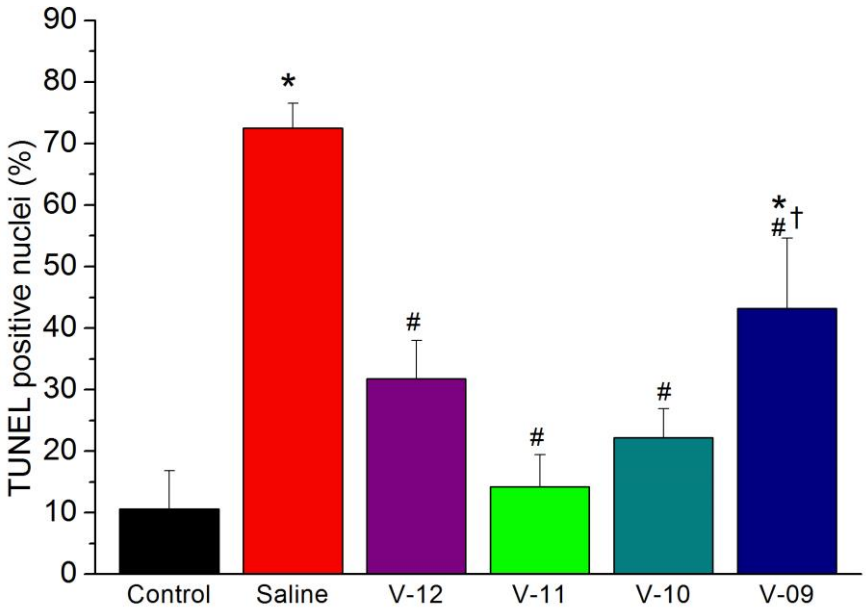


Figure 12. Photomicrographs of TUNEL staining and cGMP staining

Respective photomicrographs of the (A) TUNEL reaction (brown staining), and the (B) immunohistochemical staining for cGMP (red staining) in the vessel wall of control and in saline or in vardenafil (10^{-11}M) conserved and hypochlorite exposed thoracic aortic rings (magnification: x200; scale bar: 50µm).

DOI:10.14753/SE.2017.1905

A)



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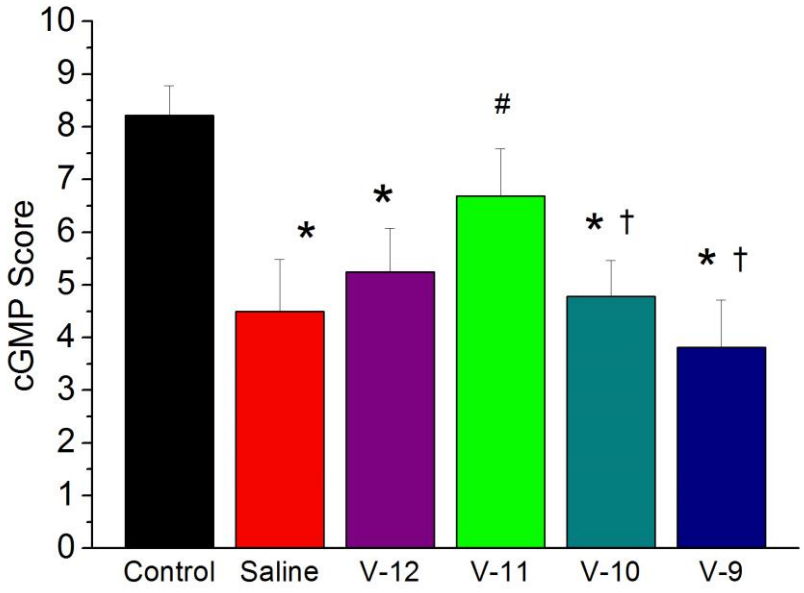

Figure 13. Effects of vardenafil on DNA strand breaks and cyclic GMP levels in aortic rings

Scoring of (A) TUNEL staining (in percentage of total cell number) and (B) semiquantitative scoring of cGMP immunohistochemistry. Values represent mean ± S.E.M. of 7-10 experiments in thoracic aortic rings of the different groups.

$p < 0.05$: * vs. Control; # vs. Saline; ‡ vs. V^{-12}; † vs. V^{-11}; § vs. V^{-10}; ° vs. V^{-9}

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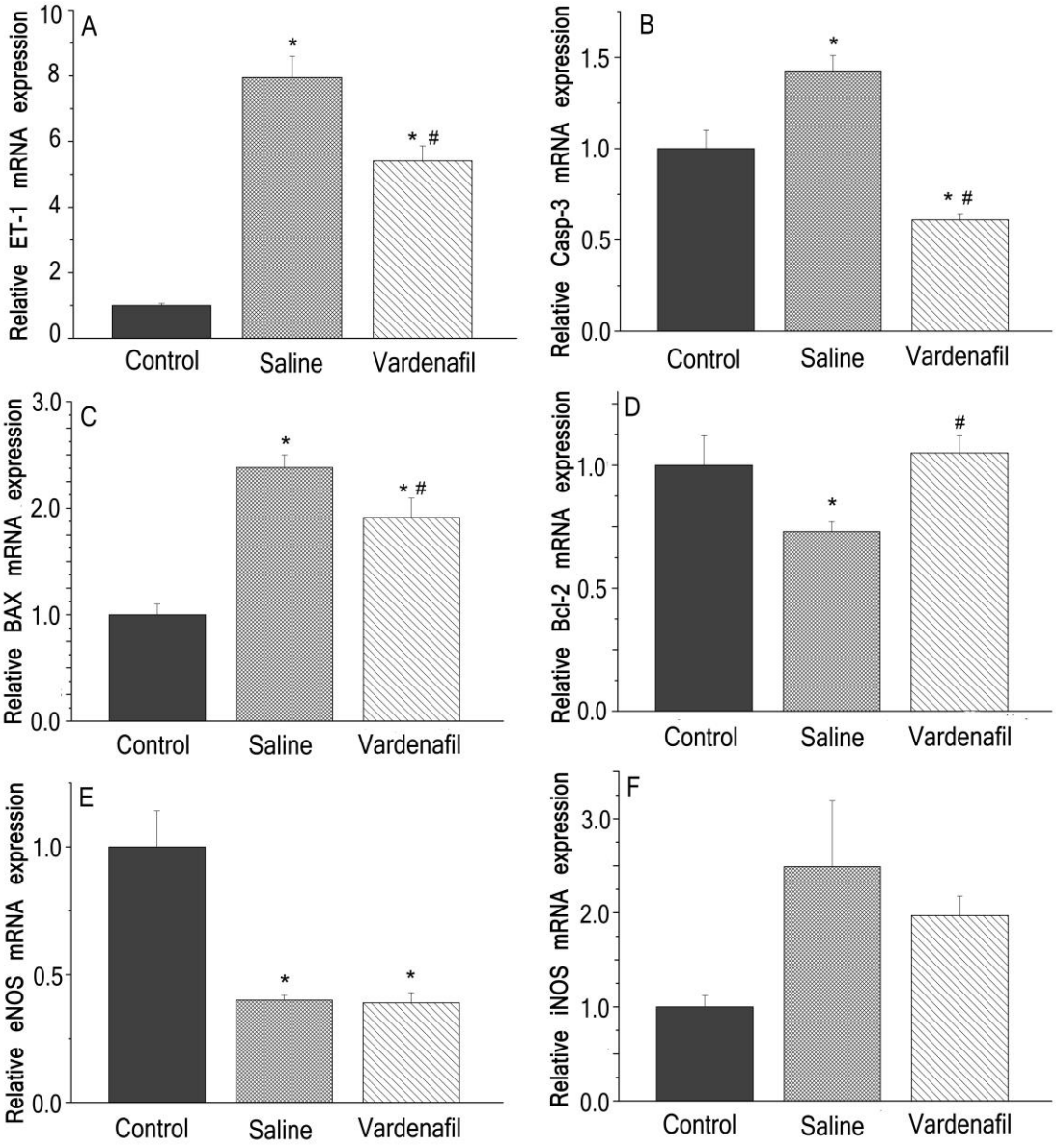

Figure 14. Effects of vardenafil in isolated aortic rings on the gene expressions

(A) endothelin-1 (ET-1), (B) caspase-3 (casp-3), (C) Bax, (D) Bcl-2, (E) endothelial nitric oxide synthase (eNOS) and (F) inducible nitric oxide synthase (iNOS) after long term cold preservation, reoxygenation and sodium-hypochlorite exposition (n=6 per group). Controls were given the arbitrary value of 1.

Values represent median ± quartiles; $p<0.05$: * vs. control; # vs. saline.

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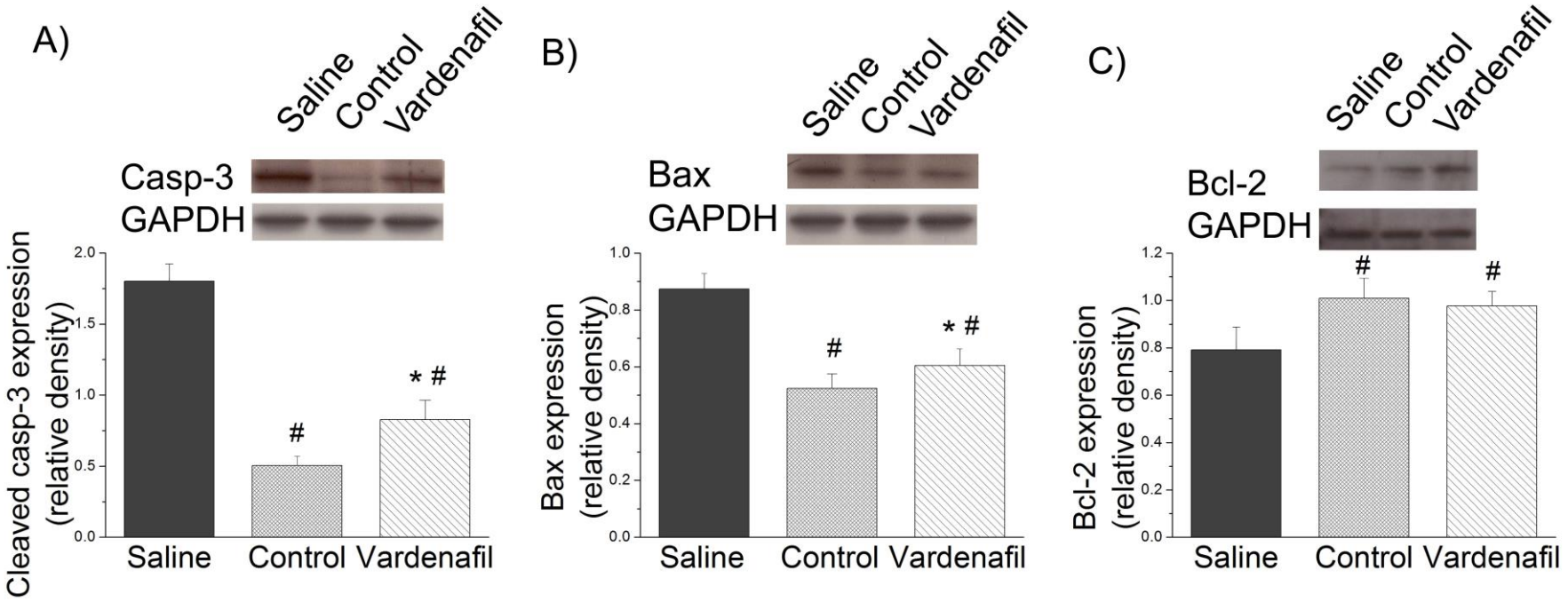

Figure 15. Effects of vardenafil in isolated aortic rings on the protein expressions

(A) cleaved caspase-3 (casp-3), (B) Bax and (C) Bcl-2 in isolated aortic rings after long term cold preservation, reoxygenation and sodium-hypochlorite exposition (n=6 per group). Values represent mean ± S.E.M.; *P<.05 versus control; #P<.05 versus saline

DOI:10.14753/SE.2017.1905

7 Discussion

Experimental evidence demonstrates the advantageous effect of maintained cGMP levels against oxidative stress and I/R injury in heart [112], in lungs [113] and in liver [114]. The main findings of the presented *in vitro* studies are the evidence that also vascular dysfunction caused by ROS can be improved by the maintenance of intracellular cGMP levels.

In order to test the possible favourable effects of increased cGMP levels - caused either by the sGC activator cinaciguat or by the PDE-5 inhibitor vardenafil - on the oxidative stress associated vascular dysfunction, two approved *in vitro* models of vascular oxidative damage were used. The first model is a relevant model of an acute, intensive oxidative stress with one of the most reactive oxygen species, peroxynitrite. The second model claims to mimic the oxidative injury that is occurring during reperfusion after cold storage by reoxygenation and neutrophil activation after prolonged ischemic period.

It must be noted, that in both presented studies, molecular biological analysis of the aortic rings were performed. Due to the technical limitations, immunohistochemical, qRT-PCR, and Western-blot analyses could not be executed on the vascular endothelium separately, but were performed on the vessel segment including endothelium and vascular smooth muscle cells of the intimal and medial layers. The adventitia was already removed during the preparation of the vessels for the *in vitro* incubations.

7.1 In vitro model of vascular dysfunction induced by peroxynitrite

In the first study vascular rings were exposed to peroxynitrite at a concentration of 200 μmol/L. The contribution of the elevated production of reactive oxygen and nitrogen species to cellular dysfunction via induction of oxidative damage to cell macromolecules, such as lipids, DNA, and proteins is widely described in the literature. The DNA damage induced by peroxynitrite is a known phenomenon and has been reviewed by Szabó and Ohshima [115].

Under pathophysiologic conditions *in vivo* formation of peroxynitrite has been demonstrated as the highly reactive coupling product of $NO^{\cdot}$ and superoxide [116], which is an important mediator of tissue injury in various forms of inflammation, shock, and ischemia / reperfusion injury [117]. Whereas apoptosis is a typical consequence of low to moderate concentrations of peroxynitrite, exposure of cells to higher concentrations of this oxidant has been associated with necrosis [118]. In moderate concentrations it is cytotoxic and causes vascular dysfunction in several ways, such as causing tyrosine nitration of the prostacyclin synthase thereby shutting down prostacycline (PGI_2) production (also considered as the reserve pathway of relaxation) within the endothelium. $ONOO^-$ is also a strong inhibitor of the sGC thereby making sGC nonresponsive to $NO^{\cdot}$ [90], therefore under all conditions where $NO^{\cdot}$ deficit is encountered, the process of vascular dysfunction is initiated or accelerated. Several studies have indicated that exposure of vessels to $ONOO^-$ or to other reactive oxygen species leads to impairment of endothelium dependent relaxation [106, 108, 119, 120].

7.2 Effect of cinaciguat on peroxynitrite induced vascular dysfunction

Vascular rings exposed to peroxynitrite at a concentration of 200 μmol/L exhibited reduced endothelium-dependent vasorelaxations to acetylcholine (Table II, Figure 4A). Activation of the muscarinic M3 receptor with acetylcholine stimulates the generation of $NO^{\cdot}$ from the endothelial cells. The released $NO^{\cdot}$ then diffuses into the smooth muscle cells where it binds to and activates sGC. This enzyme catalyzes the conversion of GTP to cGMP. The elevation of cGMP ultimately initiates vascular smooth muscle relaxation [121]. In line with our results Szabo et al. showed, that exposure of peroxynitrite caused a marked impairment of the endothelium-dependent relaxation [119]. In our model cinaciguat pretreatment significantly improved relaxation responses to acetylcholine after $ONOO^-$ exposure. The endothelial dysfunction observed after peroxynitrite incubation can be associated with the accumulation of oxidized and heme-free sGC that cannot be activated by $NO^{\cdot}$. It has been shown that the oxidation of sGC from Fe^{2+} to Fe^{3+} state by peroxynitrite leads to the desensitization of the enzyme to endogenous $NO^{\cdot}$ and NO-releasing drugs, thereby inhibiting $NO^{\cdot}$ signaling [90]. Moreover, peroxynitrite also causes tyrosine nitration of the prostacyclin synthase,

thereby inhibiting prostacyclin formation within the endothelium, and in higher concentration peroxynitrite-mediated oxidation of tetrahydrobiopterin, an essential $NO^{\cdot}$ synthase cofactor leads to the dysfunction of NOS, thereby causing endothelial NOS uncoupling [122]. Pharmacological activation of sGC by cinaciguat can potently bind and activate the oxidized and / or heme-free sGC (as introduced didactically by Figure 2C), producing selective sGC activation and vasodilation of diseased blood vessels [95]. In this work, oral pretreatment of rats with cinaciguat significantly ameliorated the peroxynitrite-induced endothelial dysfunction, which indicates, that the sGC stabilizing features of cinaciguat might help to overcome this imbalance by preventing sGC from degradation [123].

Aortic rings incubated with peroxynitrite exhibited decreased sensitivity and normal maximal relaxation responses to the endothelium-independent vasodilator sodium nitroprusside (Figure 4B). These results show a slight damage of the relaxation apparatus in the smooth muscle cells. Under normal conditions, sodium nitroprusside breaks down spontaneously to yield $NO^{\cdot}$, thus causing endothelium-independent vasodilation by the same effector mechanism as $NO^{\cdot}$ released from endothelium, that is, activation of sGC [121]. In synchrony with the decreased sensitivity to sodium nitroprusside we found an increase in the contractile responses of the smooth muscle cells to the α_1-adrenergic agonist phenylephrine after peroxynitrite-induced impairment of cGMP levels. The increase in intracellular cGMP production stimulates cGMP-dependent protein kinases, leading to the inhibition of calcium entry into the cell, thereby decreasing cytoplasmatic Ca^{2+} concentrations and decreased vasoconstriction [121]. However, in the presented study cinaciguat had no effect on the phenylephrine-induced increased maximal contraction. The enhanced contractile response to phenylephrine after peroxynitrite exposure, which occurs despite the elevated levels of cGMP by cinaciguat may suggest the involvement of vascular structural and functional components that are not cGMP-mediated. One such component could be the loss of basal prostacycline and prostaglandin dilator agents through the nitration and inhibition of cyclooxygenases [124]. The decrease of these prostanoids leads to the reduction of intracellular cAMP levels in the VSMCs and ultimately to increased contractile responsiveness. ROS may also contribute to decreased cAMP levels through the

activation of PDE-1, which is activated by the increased Ca^{2+} influx caused by the ROS load.

It shall be noted, that in this experimental setup after oral treatment of rats, aortic segments are mounted in organ bath. According to the protocols, the tissues are subjected to repeated washings (aortic rings for histological purposes were also washed during the incubation time). Even though cinaciguat is washed out in this *in vitro* model, the intracellular cGMP levels remained elevated. These observations suggest that cinaciguat continues to exert its pharmacological effect in the aortic preparations previously washed.

As previously written, the nitration of tyrosine residues to produce nitrotyrosine is a sensitive marker elicited by peroxynitrite. In the aortic segments subjected to peroxynitrite exposure immunohistochemical reaction revealed a marked nitrotyrosine presence (Figure 5A). Consistently, Mihm et al. have demonstrated that the preincubation of rat thoracic aorta segments with clinically relevant concentrations of 3-nitrotyrosine, a biomarker of peroxynitrite formation observed in various pathophysiological states, resulted in concentration-dependent impairment of endothelium-dependent vascular relaxation and induced DNA damage in vascular endothelial cells [125]. In accordance with other biomolecular targets, DNA is damaged by peroxynitrite through nitration and oxidation. Li et al showed that direct exposure of primary rat aortic smooth muscle cells to peroxynitrite induces apoptosis in a concentration-dependent manner, as confirmed by means of quantitative fluorescence staining and TUNEL assay [126]. In accordance with these results only 30 minutes of peroxynitrite exposure significantly increased the TUNEL positive staining compared to the control (Figure 5B). Marked up-regulation of the mitochondrial pro-apoptotic factor BAX and apoptotic cascade executor caspase-3 was also confirmed on transcriptional and translational levels in these samples along with the significant down-regulation of the anti-apoptotic Bcl-2.

7.3 In vitro model of I/R injury caused vascular dysfunction

In the second experimental setup to simulate the whole reperfusion process in the *in vitro* model, the well established method of reoxygenation, rewarming with additional hypochlorite incubation was applied [120, 127, 128] where $HOCl^-$ was used to simulate *in vivo* reperfusion. Radovits et al have confirmed that the complex phenomenon of *in vivo* ischemia/reperfusion injury cannot be modelled in blood-free *in vitro* system as a simple restoration of oxygen supply and rewarming, as no difference was found in the maximal relaxation to acetylcholine after a maximal cold ischemic time of 120 minutes and 30 minutes reoxygenation in rat aortic rings [129]. Our preliminary experiments did not show significant impairment of maximal relaxation even after 24 hours of cold ischemic storage; although the relaxational-curve to endothelium-independent SNP showed a significant left-shift. 24 hours storage was chosen in order to maximize the effect of ischemia on the aortic rings. Endothelium seems to show good tolerance against ischemia however, it is highly vulnerable to the highly toxic oxygen and nitrogen species formed during reperfusion.

Hypochlorite is released from activated leukocytes among other less toxic oxygen species such as superoxide, hydrogen-peroxide, peroxynitrite [130] inducing endothelial dysfunction, impaired $NO^{\cdot}$ production, apoptosis in endothelial cells and in the vessel wall through oxidative stress [131, 132]. Former studies reported that $HOCl^-$ at low concentrations (<50 μmol/L) evoke mostly base modifications on DNA without inducing DNA strand breaks [133, 134], while in higher concentrations (100-500 μmol/L leads to the breakage and degradation of DNA in superoxide-dependent [135], hydroxyradical-dependent [136], and chloramine-dependent [137] fashion. At concentrations above 500 μmol/L along the increased apoptosis rate necrosis becomes dominant [131]. Radovits et al. reported that $HOCl^-$ impaired endothelium-dependent vasorelaxation in a concentration-dependent manner without affecting the endothelium-independent relaxation (at the magnitude of 100-500 μmol/L hypochlorite) [108], while other studies also encountered similar results after $HOCl^-$ exposure in rabbit arteries, rat aorta, and guinea pig coronary arteries [132, 135, 138-140]. Addition of hypochlorite is therefore a valid model to mimic the effect of activated white blood cells during reperfusion.

7.4 Effect of vardenafil on I/R induced vascular dysfunction

In order to determinate the optimal vardenafil concentration in the preservation solution, four different concentrations were tested. The functional measurements of endothelial dysfunction showed that only 10^{-11} mol/L vardenafil could provide significant improvement of R_{max} to acetylcholine, compared to saline group. Also, the highest cGMP concentration along with the lowest rate of DNA double-strand breaks were detected at this concentration, when vardenafil supplemented groups were compared to each other.

Vascular rings were stored in cold ischemic solutions for 24 hours then were rewarmed, reoxygenated and exposed to 200 μmol/L hypochlorite. Previous studies have reported potent myocardial protection by pharmacological PDE-5 inhibition [104, 107], by sGC activation [112], and by supplementation of the $NO^{\cdot}$ donor L-arginine [141]. Intracellular cGMP accumulation was shown to reduce oxidative tissue injury in conditions associated with increased free radical release and oxidative stress [112]. In this case the beneficial effect of vardenafil was demonstrated by improved vasodilator capacity, while improvement of $HOCl^{-}$ -induced endothelial dysfunction was far more pronounced compared to the slight sensitization of vascular smooth muscle to $NO^{\cdot}$ as reflected by SNP induced relaxations (Figure 11).

Different studies aimed to test individual methods to preserve vascular function during storage. In the very same experimental setup Barnucz at al. used dimethyloxalylglycine in the preservation solution in order to activate the hypoxia inducible factor -1 induced pathways to successfully protect the endothelium [128]. Another *in vitro* study demonstrated decreased rat coronary vasodilator capacity after only 8 hours of cold storage in HTK solution [142]. Zatschler at al. compared an N-acetyl-hisitdine buffered, potassium enriched and amino acid-fortified storage solution with HTK and physiologic saline solutions in a model of 4 days storage of arterial grafts. They found, that 2 hours of cold storage in HTK resulted in a significant loss of structurally intact endothelium, while their new solution maintained full endothelial function and its coupling to smooth muscle after 4 days of cold storage [143]. Although after 24 hours storage we have observed only 74% relaxation response to endothelium-dependent dilator, their model did not include ROS exposure but ischemia only.

Supplementation of preservation solutions with iron chelators is already in focus of researchers, however deferoxamine and similar macromolecules hardly prevent ROS formation in the free-iron pool due to Fenton's reaction as they barely cross cellular membranes. Low molecular weight chelators, such as LK 614 can penetrate cell membranes and have shown promising results during 24 hours of ischemia [144].
Ingemansson et al showed weak contractive ability after administration of U-46619 TxA_2 agonist after 24 hours of ischemic storage using University of Wisconsin solution [145], but the model did not include oxidative load for reperfusion, which is a key factor of cellular damage.

In accordance with the literature, impaired vasodilator capacity of aortic rings was detected after prolonged cold storage followed by rewarming and $HOCl^-$ -induced reperfusion injury (Table III, Figure 10). The preserved contraction and endothelium-independent relaxation indicated that the impaired vascular response was not a functional deficit of vascular smooth muscle. Vardenafil supplementation of preservation solution during cold ischemic storage provided protection to rat aortic segments against I/R injury, with respect to function and structure. In sight with the PCR results it must be noted, the improved dilatory response of the vardenafil treated rings after $HOCl^-$ exposure was not a consequence of an increased NOS expression of by any ways, but was rather the consequence of a maintained endothelial function. However, the downstream activity of PKG has been described to be involved in the endogenous regulation of the basal eNOS activity as PKG 1β phosphorilates eNOS on serine 116 thereby increasing its activity [146].

7.5 The possible protective down-stream mechanisms behind cGMP accumulation

The presented results are in line with the literature and confirm that increased cGMP levels provide protection against the deleterious effects of peroxynitrite, as well as against ischemia / reperfusion injury that we mimicked in our *in vitro* model. Intracellular cGMP accumulation was shown to reduce oxidative tissue injury in conditions associated with increased free radical release and oxidative stress [112].

Parallel to cellular dysfunction, the end-effector of oxidative stress leading to apoptosis is considered to be the opening of mitochondrial permeability transition pore (mPTP) [147]. The closure of ATP sensitive K^+ (K^+_{ATP}) channels, the activation of NADPH oxidase, ROS production, activation of NF-κB and activator protein-1, increased intracellular Ca^{2+} lead to the dysfunction and in time to the apoptosis of the cell [148]. Through increased Ca^{2+} or lasting depolarization mPTP opening depletes electric gradient of the mitochondria, releases deadly compounds such as cytochrome C and Bax. The main effector of the apoptotic cascade is the split of procaspase-3 to active cleavage caspase-3. We detected decreased caspase-3 cleavage presence and also decreased expression associated with maintained cGMP levels in the aortic rings.
The supposed cytoprotective mechanisms that stand behind the beneficial effects of both cinaciguat and vardenafil are those PKG-dependent mechanisms that may prevent the initiation of the apoptotic cascade. Maintained cGMP levels prevent $HOCl^-$ caused activation of the $ONOO^-$ -PARP pathway and thus protects the structural and functional integrity of the endothelium. The prevention of ATP consumption and energy depletion contributes to the prevention of apoptosis inducing factor (AIF) translocation [149] and mPTP opening. PKG is responsible for the phosphoryaltion of Ca^{2+}-ATP-ase thereby increasing Ca^{2+} efflux from the cytoplasm [105, 150].

The protective mechanisms may involve the phosphorylation of ERK, induction of Bcl-2, and opening of mitochondrial K^+_{ATP} channels [103, 151]. More recently the observed cardioprotection with pharmacologically increased cGMP levels has been reported to be mediated by hydrogen sulphide signaling in a PKG-dependent manner [94]. The significant down-regulation of the anti-apoptotic factor Bcl-2 was observed after 24 hours cold storage and $HOCl^-$ exposure, as well as after peroxynitrite exposure. However, the described changes of BAX, caspase-3 and Bcl-2 were totally reversed by previous of cinaciguat treatment and was fully reversed by vardenafil supplementation of the storage solution. The changes in the expression of the pro-apoptotic factors Bax and Caspase-3 are in synchrony with the described changes of Bcl-2.

DOI:10.14753/SE.2017.1905

7.6 Therapeutic perspectives of maintained cGMP levels against oxidative stress

It has been widely discussed, that NO- cGMP- PKG pathway regulates vascular tone, platelet aggregation, cellular growth and proliferation, and extracellular matrix deposition, therefore its dysfunction is the target of many therapeutic drugs derived from $NO^{\cdot}$/cGMP stimulators and blockers [152]. NO-independent but heme-dependent sGC stimulators have been promising in clinical trials for primary and pulmonary hypertension and have been specifically effective in treating chronic thromboembolic pulmonary hypertension [153]. After successful phase III clinical studies (CHEST and PATENT) Riociguat constitutes the first drug of this novel class of sGC stimulators with the indication of pulmonary hypertension that has been recently registered (commercial name: Adempas) [154, 155].

The most common PDE-5 inhibitor sildenafil is also used for the treatment of pulmonary hypertension, and erectile dysfunction. Solloum et al. have shown the advantageous effect of vardenafil pretreatment in rabbits in a myocardial I/R injury model [103], while *ex vivo* vascular studies have demonstrated its beneficial endothelium-protective effect [104, 156]. Nevertheless a human study of endothelial I/R injury also demonstrates the protective effect of PDE-5 inhibition in which case sildenafil was used [105].

Damaged endothelial cells are responsible for impaired vasodilatory graft-function and for the developing vasculopathy [157, 158]. Vascular tone (endothelium-dependent vasorelaxation) is particularly important in cardiac as well as in vascular surgery, as it determines postoperative coronary blood flow and it is responsible for the early and late graft thrombosis and stenosis [159]. Furthermore, the long-term benefit of revascularization surgery depends heavily on the long-term patency of bypass grafts, which are determined by several factors: the progress of heart/vascular disease, the biological properties of the implanted graft and last but not the least the degree of I/R injury. In line with the literature, this current work supports the concept that through maintained cGMP levels they could also provide new therapeutic approach for the protection of vascular function against reactive oxygen species.

However, aortic rings in this investigation were harvested and tested *ex vivo* without the involvement of nonaortic tissue, blood flow, and in the absence of

DOI:10.14753/SE.2017.1905

leukocytes. Therefore, confirmation of these observations *in vivo* is essential. Prudent interpretation of the findings obtained from this animal model is required for the extrapolation to humans.

DOI:10.14753/SE.2017.1905

8 Conclusion

The main results of this dissertation shall be resumed in 2 main theses:

- Acute oxidative stress such as reperfusion injury leads to decreased intracellular cGMP bioavailability in the vascular wall and consequently to vascular dysfunction. This phenomenon is accompanied by further molecular changes thus increasing the tendency of the cells to undergo apoptosis.
- The pharmacological maintenance of intracellular cGMP levels does not only contribute to preserved vascular function but does also prevent the otherwise unfolding pathologic subcellular changes caused by oxidative damage. Both the facilitation of cGMP synthesis by cinaciguat, and the inhibition of cGMP degradation by vardenafil efficiently improved the vascular function and hindered the development of intracellular pathologic molecular changes.

In the first study we investigated the oxidative injury and impairment of vascular responsiveness induced by peroxynitrite in the isolated rat aorta. The second study examined the effect of an *in vitro* ischemia reperfusion injury on the vascular function. In both cases the oxidative stress caused vascular dysfunction was associated with decreased cGMP levels along with increased apoptosis ratio in the vessel wall. The maintenance of cGMP levels through the activation of soluble guanylate cyclase by cinaciguat or through the inhibition of phosphodiesterase -5 by vardenafil respectively, led to improved endothelial function and decreased DNA damage. These results were coherently supported by the beneficial changes in the ratios of pro- and anti-apoptotic factors associated with increased cGMP levels. This work includes the study that provided for the first time evidence of the beneficial effect of PDE-5 inhibition on endothelial protection during cold ischemic storage and reperfusion.

Taken together, the current work supports the concept, that pharmacological activation of cGMP synthesis and/or inhibition of cGMP decomposition may represent novel potential therapy approaches to improve vascular dysfunction associated with oxidative stress.

DOI:10.14753/SE.2017.1905

9 Summary

This dissertation serves to analyze the mechanisms by which the disintegration of the nitric oxide / soluble guanylate cyclase / cyclic guanosine monophosphate signaling pathway through oxidative stress such as ischemia/reperfusion (I/R) leads to vascular dysfunction, and provides evidence that the maintenance of this pathway contributes to the attenuation vascular injury.

First, in a simple *in vitro* model of vascular oxidative stress we tested how cinaciguat pretreatment of rats affects endothelial dysfunction induced by peroxynitrite.

In the second study we investigated on an approved *in vitro* I/R model how endothelial dysfunction induced by long term cold ischemic storage followed by hypochlorite exposure improves if the storage solution is supplemented with PDE-5 inhibitor vardenafil.

In these vascular reactivity measurements on isolated rat aortic rings phenylephrine-induced contraction, endothelium-dependent and -independent vasorelaxation were registered by using acetylcholine and sodium nitroprusside.
Immunohistochemical analysis of the vessel walls was performed for nitrotyrosine and cGMP, DNA strand breaks were assessed by TUNEL method. Protein and mRNA expression of apoptotic factors were detected by Western-blot and RT-PCR.

In both *in vitro* models ROS exposure resulted in impaired endothelial function which was significantly improved by maintained cGMP levels. Maximal relaxations to the endothelium-independent dilator agent sodium nitroprusside did not differ in any groups studied, respectively to the experimental setup. Improvement of endothelial function was associated with decreased TUNEL and increased cGMP staining.

Our results demonstrate the importance of the NO- sGC- cGMP pathway in the maintenance of vascular function and structure. Oxidative stress leads to the disintegration of this pathway, to the depletion of cGMP, and to the activity loss of down-stream mediators. Pharmacologic activation of sGC or inhibition of PDE-5 represent possible therapeutic utilities to improve vascular dysfunction associated with I/R injury.

DOI:10.14753/SE.2017.1905

10 Összefoglalás

Disszertációm célja annak bemutatása, hogy milyen szerepet játszik a nitrogén-monoxid / szolubilis guanilát cikláz / ciklikus guanozin monofoszfát jelátviteli út károsodása az oxidatív stress (pl. iszkémia / reperfúzió) következtében kialakuló funkcionális és strukturális érkárosodásban. Célom továbbá annak igazolása, hogy az intracelluláris cGMP depléció megakadályozása csökkenti az oxidatív stressz következtében létrejövő károsodást.

A bemutatott első kísérletben azt vizsgáltuk, hogy a vaszkuláris stressz egyszerű *in vitro* modelljében a szolubilis guanilát cikláz aktivátor cinaciguát képes-e javítani a peroxinitrit által károsított endotélfunkciót.

A második vizsgálatban az iszkémia / reperfúzió elfogadott *in vitro* modelljén érfunkcionális mérésekkel igazoltuk, hogy a tartós hideg iszkémiát követő hipoklorit indukálta oxidatív károsodás csökkenthető, ha az érszegmensek prezervációs oldatát PDE-5 inhibitor vardenafillal dúsítjuk.

Az érfunkciós méréseket izolált patkányaorta-gyűrűkön fenilefrinnel előidézett kontrakciókkal, majd endotél-függő (acetilkolinnal) és független (nitroprussziddal) relaxációk előidézésével végeztük. Immunhisztokémiai festést végeztünk továbbá a nitrotirozin és cGMP meghatározására, valamint TUNEL reakciót a DNS lánctörések kimutatására. Az apoptózisban szerepet játszó faktorok expressziós vizsgálatát qRT-PCR és Western-blot segítségével végeztük el.

Mindkét *in vitro* modellben a reaktív oxigén szabadgyökök súlyosan károsították az endotélium működését, azonban megtartott cGMP szint mellett az endotélfunkció szignifikánsan javult. A maximális endotélium-független relaxációkban nem mutatkozott különbség az egyes csoportok között. A TUNEL reakció eredménye alapján a magasabb cGMP szintet csökkent DNS károsodás kíséri.

Jelen eredményeink jól mutatják az NO- sGC- cGMP jelpálya jelentőségét a normális érműködés megtartásában. Oxidatív stressz során a csökkent cGMP szint gátolja a jelpálya működését, azonban ez hatékonyan megelőzhető illetve gátolható sGC aktivátor cinaciguáttal, valamint a PDE-5 gátló vardenafillal, melyek új terápiás lehetőséget jelenthetnek az iszkémia / reperfúzió okozta érkárosodással szemben.

DOI:10.14753/SE.2017.1905

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12 Bibliography of the candidate's publications

List of publications related to the dissertation

1. Hegedűs P, Veres G, Radovits T, Barnucz E, Korkmaz S, Székely N, Kolonics F, Merkely B, Szabó G. (2014) A foszfodiészteráz-5 inhibitor vardenafil hatása a hipoxia-reoxigenáció okozta endotél-diszfunkcióra patkány aortában Card. Hung; 44: 224-30.

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1. Hegedűs P, Li S, Korkmaz-Icöz S, Radovits T, Mayer T, Al Said S, Brlecic P, Karck M, Merkely B, Szabó G. (2015) Dimethyloxalylglycine treatment of brain-dead donor rats improves both donor and graft left ventricular function after heart transplantation. J Heart Lung Transplant. doi: 10.1016/j.healun.2015.06.016. [Epub ahead of print]

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DOI:10.14753/SE.2017.1905

Acknowledgements

First of all, I would like to express my gratitude to my advisor and mentor Prof. Dr. Gábor Szabó for offering me the chance to take part in an international scientific cooperation between the University of Heidelberg and Semmelweis University, for giving me the possibility to perform this research work in his scientific team in Laboratory of Cardiac Surgery at the University of Heidelberg, and for his continuous guidance and personal support in my work.

I am especially grateful to Prof. Dr. Béla Merkely and Dr. Violetta Kékesi for their recommendations and encouragements during the years of my scientific work.

I would like to express my special thanks to my scientific tutors Dr. Tamás Radovits and Dr. Sevil Korkmaz for their patience, and helpful advices whenever I needed. Without their support I could not have accomplished this work.

Special thanks goes to my colleagues in the laboratory in Heidelberg: for Karin Sonnenberg, Patricia Kraft, Tobias Mayer, Lutz Hoffman for their helpful organisational support and for the valuable skills I have attained during my entire research work.