Maintained cGMP levels improve endothelial and vascular function after oxidative stress

Ph.D. Theses

Péter Hegedűs, M.D.

Doctoral School of Basic Medicine
Semmelweis University

Supervisor: Prof. Gábor Szabó, M.D., med. habil.

Official reviewers:
Mária Szekeres, M.D., Ph.D.
István Szokodi, M.D., D.Sc.

Head of the Final Examination Committee:
Prof. Péter Sándor, M.D., D.Sc.

Members of the Final Examination Committee:
Tamás Ivanics, M.D., med. habil.
Gábor Jancsó, M.D., med. habil.

Budapest

2016

Introduction

Cardiovascular diseases (CVD) 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. Endothelial dysfunction is a corner-stone of cardiovascular diseases associated to hypercholesterolemia, hypertension, diabetes mellitus, ischemia reperfusion injury and sepsis. 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. The vascular system, especially the endothelium itself is very sensitive to oxidative stress. Under normal circumstances in healthy cells, a balance exists between the formation of reactive oxygen species (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: oxidizing proteins and lipids, the oxidative damage of the DNA may lead to apoptosis, autophagy or in higher concentrations causing necrosis of the cell. 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. The superoxide anion scavenges nitric oxide ($NO^{\cdot}$) and forms peroxynitrite, which in turn induces tyrosine nitration and deleterious protein changes, triggering proinflammatory signals and inhibiting endothelial repair.

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. It has been widely discussed, that NO - cyclic guanosine monophosphate (cGMP) – protein kinase G pathway regulates vascular tone, platelet aggregation, cellular growth and proliferation, and extracellular matrix deposition. Their dysfunction is therefore the target of many therapeutic drugs derived from NO/cGMP stimulators and blockers. Intracellular cGMP accumulation has been shown to reduce tissue injury in conditions associated with increased free radical release and oxidative stress. As novel therapeutic approach, new classes of drugs are aimed to modulate the $NO^{\cdot}$- sGC- cGMP pathway.

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. ROS affect the heme-containing $NO^{\cdot}$ receptor of sGC by both decreasing its expression and by causing the dissociation of the heme. Oxidation thus potentially impairs NO-induced activation and destines the enzyme to degradation. 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. In preclinical studies the novel heme-independent 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. In a phase I clinical trial in healthy human participants, intravenously administered cinaciguat had a favourable safety profile and was well tolerated.

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. In cardiovascular diseases due to oxidative stress significantly increased PDE-5 expression was detected to accelerate cGMP degradation. Vardenafil is a well-known selective PDE-5 inhibitor that was recently shown to have beneficial effects against myocardial I/R injury after preconditioning-like treatment in rabbits and to have advantageous protective effect on vascular endothelium.

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.

Methods

I. Experimental models

1. In vitro model of vascular dysfunction induced by peroxynitrite exposure

Thoracic aortic rings were isolated from male Sprague-Dawley 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. Rats of the treated group received orally two times sGC activator cinaciguat (10 mg/kg).

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 saline 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. Cold hypoxic solution of the treated group was enriched with different concentrations the of PDE-5 inhibitor vardenafil (10^{-12} mol/L, 10^{-11} mol/L, 10^{-10} mol/L, 10^{-9} mol/L).

II. In vitro organ bath experiments

Thoracic aorta was carefully excised, cleaned from connective tissue and cut transversely into 4 mm wide rings. Isolated aortic rings were mounted on stainless steel hooks in individual organ baths, 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. The aortic rings were placed under a resting tension of 2g and equilibrated for 60 minutes. 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, 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

(PE, 10^{-6} mol/L) until stable plateau was reached, and relaxation responses were examined by adding cumulative concentrations of endothelium-dependent dilator acetylcholine (ACh, 10^{-9}–10^{-4} mol/L). For testing relaxing response of smooth muscle cells, a direct nitric oxide donor, sodium nitroprusside (SNP, 10^{-10}–10^{-5} mol/L) was used. Contraction responses are expressed in gramm, relaxational responses are expressed in percentage of phenylephrine contractions.

III. Histopathological processing

1. Immunohistochemical and TUNEL (terminal deoxynucleotidyl transferase-mediated dUDP nick end-labeling) stainings

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. According to previously described methods, we performed immonohistochemical staining for the detection of nitrotyrosine (marker of peroxynitrite-mediated damage) and for the detection of cyclic GMP content. TUNEL assay was performed for detection of DNA strand breaks (free 3'-OH DNA ends).

2. 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. The results were expressed with a scoring system in regard to the intensity and the ratio of the stained cells. For the assessment of TUNEL-labeled cells TUNEL positive and negative cell nuclei were counted and the TUNEL positive cell nuclei were calculated as percentage of total cell number.

IV. Quantitative Real-Time Polymerase Chain Reaction (PCR)

From the aortic rings total RNA was isolated, transcribed, and quantitative real-time PCR was preformed for the detection of mRNA expression of ET-1, Caspase-3, BAX, Bcl-2, eNOS, iNOS. 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.

V. Western-blot analysis

Proteins expression of p17 caspase-3 fragment, Bax and Bcl-2 was determinated by conventional Western-blot method. Target protein densities were normalized to housekeeping GAPDH densities of the same samples, respectively.

VI. 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.

VII. Preparation of chemical reagents

Cinaciguat (BAY 58-2667) was dissolved in 1% methylcellulose solution vehicle, while peroxynitrite was diluted with 4.7% NaOH. Vardenafil, phenylephrine, acetylcholine and sodium nitroprusside were diluted in physiologic salin. Sodium-hypochlorite solution was diluted with distilled water.

Results

I. Vascular injury induced by peroxynitrite in vitro - effects of cinaciguat

1. Vascular dysfunction of aortic rings

Exposure of aortic rings to the reactive oxidant peroxynitrite significantly attenuated the maximal relaxation response to the acetylcholine-induced, endothelium-dependent, NO-mediated vasorelaxation. In harmony with our expectations, pre-treatment of rats with cinaciguat significantly improved the endothelium-dependent relaxation after exposure of aortic rings to peroxynitrite. Maximal relaxations to endothelium-independent vasodilator sodium nitroprusside did not differ significantly among the different experimental groups (Table I).

	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#
PE (% of KCl)	73 ± 5	114 ± 3*	108 ± 5*	78 ± 5#

Table I: 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.
Values represent mean ± S.E.M. of 12-15 experiments.
*$p < 0.05$ versus control; # $p < 0.05$ versus peroxynitrite group;

2. Immunohistochemistry and TUNEL

A large increase in the intensity of nitrotyrosine staining was detected 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; $p<0.05$).

Though we detected no significant change after peroxynitrite exposure in the aortic rings, pre-treatment of rats with cinaciguat, resulted in a significantly higher score of cGMP compared to the peroxynitrite-incubated segments ($ONOO^-$: 5.9±0.7; vs. Cinaciguatt+$ONOO^-$: 7.9±0.6; $p<0.05$; Control: 7.2±0.6; Cinaciguat: 7.7±0.8).

Increased density of TUNEL-positive nuclei was observed in the wall of peroxynitrite-exposed aortic rings indicating DNA-fragmentation. 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; $p<0.05$).

3. Cinaciguat regulates gene expression

Exposure of aortic rings to peroxynitrite significantly up-regulated the expression of ET-1, BAX and Caspase-3 mRNA, compared to native control rings. Cinaciguat pre-treatment significantly moderated this up-regulation caused by peroxynitrite compared to the $ONOO^-$ group. The significant reduction of Bcl-2 mRNA expression in the peroxynitrite exposed rings was totally overturned by cinaciguat treatment. 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. In the expression of inducible $NO^\cdot$ synthase mRNA no significant difference was detected among the groups.

4. 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. 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.

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

1. Vascular dysfunction of aortic rings

Aortic segments exposed to 24 hours long cold ischemic storage followed by hypochlorite (200 µmol/l) incubation showed significantly attenuated maximal relaxation to the acetylcholine induced endothelium-dependent, $NO^\cdot$ mediated vasorelaxation, as compared to control group. Supplementation of the conservation solution with 10^{-11} mol/L Vardenafil significantly improved the acetylcholine induced vasorelaxation after the exposure of rings to

hypochlorite, compared to saline group. There was no statistical difference among the vardenafil treated groups. In contrast to acetylcholine, endothelium-independent vasorelaxation of the aortic rings to sodium-nitroprusside showed no significant difference in maximal relaxation among the experimental groups (Table II).

	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*

Table II: 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.Values represent mean ± S.E.M. of 15-20 experiments.
*$p < 0.05$ versus Control; $^{\#}$ $p < 0.05$ versus Saline group;

2. Immunohistochemistry and TUNEL

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 control group. Vardenafil-supplementation led to significantly higher score of cGMP staining, compared with the saline group.

Cold ischemic conservation, reoxygenation and 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 oxidative stress caused DNA-fragmentation in the aortic wall. Vardenafil-supplementation significantly decreased DNA strand breaks (saline vs. vardenafil 10^{-11}: 14±5; $p<0.05$).

3. Vardenafil regulates aortic gene expression

Exposure of aortic rings to 24 hours cold ischemic conservation followed by 30 minutes hypochlorite incubation significantly up-regulated the expression of ET-1, BAX and Caspase-

3 mRNA, compared to native control rings. These changes were significantly moderated by vardenafil supplementation of preservation solution. Long ischemic storage and hypochlorite exposure caused a significant down-regulation of Bcl-2 mRNA expression, which was improved by vardenafil. Vardenafil supplementation of the storage solution did not influence the mRNA level of endothelial and inducible nitric oxide synthases.

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

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. Expression of the anti-apoptotic Bcl-2 protein was significantly decreased in saline group compared to control, while the supplementation of vardenafil maintained Bcl-2 levels on the level of controls.

Conclusions

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

- Acute oxidative stress such as preoxynitrite load or 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.

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