Prolyl-hydroxylase inhibition preserves endothelial cell function in a rat model of vascular ischemia reperfusion injury

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Abstract

Storage protocols of vascular grafts need further improvement against ischemia/reperfusion (IR)-injury. Hypoxia elicits a variety of complex cellular responses by altering the activity of many signalling pathways, such as the oxygen-dependent prolyl-hydroxylase-domain containing enzyme (PHD). Reduction of PHD-activity during hypoxia leads to stabilisation and accumulation of hypoxia inducible factor (HIF) 1α. We examined the effects of PHD-inhibition by dimethyloxalylglycine on the vasomotor responses of isolated rat aorta and aortic vascular smooth muscle cells (VSMC) in a model of cold ischemia/warm reperfusion. Aortic segments underwent 24h cold ischemic preservation in saline or DMOG-supplemented saline solution. We investigated endothelium-dependent and -independent vasorelaxations. To simulate IR-injury hypochlorite (NaOCl) was added during warm reperfusion. VSMCs were incubated in NaCl or DMOG solution at 4ºC for 24h after the medium was changed for a supplied standard medium at 37ºC for 6h. Apoptosis was assessed by the TUNEL-method. Gene expression analysis was performed by quantitative real-time PCR. Cold ischemic preservation + NaOCl induced severe endothelial dysfunction, which was significantly improved by DMOG supplementation (maximal relaxation of aortic segments to acetylcholine: control 95±1 vs. NaOCl 44±4 vs. DMOG 68±5%). Number of TUNEL-positive cell nuclei was significantly higher in the NaOCl-group and DMOG-treatment significantly decreased apoptosis. Inducible heme-oxygenase 1 mRNA expressions were significantly higher in the DMOG group. Pharmacological modulation of oxygen sensing system by DMOG in an in vitro model of vascular IR effectively preserved endothelial function. Inhibition of PHDs could be therefore a new therapeutic avenue for protecting endothelium and vascular muscle cells against IR-injury.
Introduction

Endothelial and myocardial damage with temporary cardiac dysfunction is a well-described in cardiac surgery (Szabo et al., 2009). The most important limiting factor is the protection of tissue against ischemia-reperfusion injury. This step may determine the acute and chronic outcome of vessel and organ grafts. The cold storage induced ischemic injury is well-known and in the blood vessel may lead to acute endothelial dysfunction and long-term graft vasculopathy. Some clinical studies showed that hypoxia and inflammation are closely related and hypoxia promotes inflammation (Eltzschig and Carmeliet, 2011). The development and the grade of inflammation in response to hypoxia are clinically relevant. Ischemia in organ grafts increases the risk of inflammation and graft failure or rejection (Kruger et al., 2009).

The cold ischemic storage is the most extended conservation process to avoid the loss of functional integrity of tissues and organs. The main foundation of cold ischemic storage is that at lower temperature, usually at 4°C the metabolism of the tissue is suppressed. The deleterious side of this preservation method is cell swelling, acidosis and release of reactive oxygen species (ROS) (Maathuis et al., 2007).

The oxygen sensing system plays a pivotal role in the adequate reactions to ischemic adaptation. The oxygen sensing molecules are responsible for sensing oxygen tension and regulate adequately hypoxia inducible factor (HIF) activity in an oxygen dependent way (Fraisl et al., 2009). These oxygen sensing molecules (prolyl-hyroxylase-domain containing enzymes, PHDs) belong to the superfamily of non-heme iron (Fe^2+)-containing 2-oxoglutarate (2-OG)-dependent oxygenases (Fraisl et al., 2009). HIF is a heterodimeric transcription factor consisting of the constitutively expressed HIF-ß and a regulatory HIF-α subunit (Czibik, 2010). The alpha-subunit of HIF is hydroxylated by the PHDs and recognised by von Hippel-Lindau Protein which leads to ubiquitination and rapid proteosomal degradation under
normoxic conditions. However, if the cellular oxygen tension is reduced, the activity of PHD is inhibited, which causes that HIF-α and HIF-β-subunit heterodimerise and translocate to the nucleus. The HIF-complex leads to subsequent induction of gene transcription and adaptive responses to accommodate cellular function in ischemia, hypoxia and inflammation (Fraisl et al., 2009).

The biological effects of HIF target genes are diverse and include determining steps in cell metabolism and survival. Plenty of the HIF-target genes constitute a reasonable adaptation to low oxygen tension in mammalians, such as angiogenesis (Bateman et al.), vasodilatation (Loor and Schumacker), scavenging of free radicals (heme-oxygenase-1(HO-1)), erythropoiesis (EPO), increased glucose uptake (glucose transporter-1), switch of metabolism to glycolysis (several key enzymes of glycolysis) and increased lactate utilisation (lactate dehydrogenase) (Heyman et al., 2011). With pharmacological inhibiton of the PHDs, HIF can be activated under normoxic conditions, which allows the initiation of an inflammatory response before tissues become hypoxic (Eltzschig and Carmeliet, 2011).

The role of oxygen sensing pathway was already discussed in different hypoxia related disease models (myocardial-, cerebral infarction (Fraisl et al., 2009; Czibik, 2010), solid tumours (Giaccia et al., 2003)). N-oxalylglycine, the product of dimethyloxalilglycine (DMOG) is able to mimic 2-OG to the catalytic domain and inhibits PHDs by blocking the entry of cosubstrate (Fraisl et al., 2009). It has been published by Czibik et al. that in murine hearts gene therapy with HIF1α was associated with higher serum bilirubin levels and resulted in cardioprotective effect. This cardioprotective effect was mimicked by remote HMOX-1 (HO-1) treatment (Czibik et al., 2009).

In our study we investigated the role of oxygen sensing system on cold ischemia-warm reperfusion injury on isolated rat aortic segments. Our experiments are based on
pharmacological treatment with a non-selective inhibition of PHDs under normoxic conditions resulting in HIF stabilisation and gene expression modulations by DMOG in an in vitro experimental model of vascular cold ischemic-warm reperfusion injury.

To further explore the role of HIF-complex induced adaptive gene-expression, the regulation of HO-1 in acute vessel dysfunction has also been examined.
Methods

Animals

Male Sprague–Dawley rats (250–350 g; Charles River, Sulzfeld, Germany) were used in the experiments. The animals were housed in a room at a constant temperature of 22±2°C with 12h light/dark cycles and were fed a standard laboratory rat diet and water ad libitum. The rats were randomly assigned to different groups. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures and handling of animals during the investigations were reviewed and approved by the Ethical Committee of the Land Baden-Württemberg for Animal Experimentation.

Preparation of Aortic rings

Rats were anesthetized with an intraperitoneal pentobarbital (60 mg/kg) injection. After lateral thoracotomy thoracic aorta was removed and immediately placed in cold (4°C) Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1.77 mM CaCl₂, 25 mM NaHCO₃, 11.4 mM glucose; pH=7.4). After dissection of adhering fat and connective tissue, 4 mm length segments were placed in a testing tube in different solutions (NaCl or DMOG supplemeted NaCl solution, see experimental groups). The not for organbath used, cleaned aortic segments were used for smooth muscle cell isolation.

Experimental groups

Aortic segments of organ bath experiments were randomized into 3 groups: 1) the aortic rings in the control group were not treated, were immediately mounted in the organ bath, 2) in the NaOCl group aortic rings were preserved in saline at 4°C for 24 hours after explantation 3) in
the DMOG group rings were stored at 4°C for 24h in saline or in 10⁻⁴ M DMOG-supplemented saline.

Vascular smooth muscle cells (VSMC) were divided into 3 groups: 1) control group without cold ischemia and warm reperfusion, 2) NaCl group: cells were stored at 4°C in saline for 24h followed by 6h warm reperfusion in normal medium at 37°C, 3) DMOG (10⁻³ M) group: cells were stored at 4°C in DMOG supplemented saline for 24 h followed by 6h warm reperfusion in normal medium at 37°C.

The used DMOG concentrations were based on previous literature data and our pilot studies on aortic rings and cell culture (primary cardiomiocytes and vascular smooth muscle cells) (Takeda et al., 2009; Perman et al., 2011).

Model of in vitro cold ischemic storage/ warm reperfusion induced vascular injury

After 24 hours cold storage in different solution (NaCl or DMOG supplemented NaCl) we investigated in vitro vascular function in an organ bath experiment. As the major source of free radicals and oxidants produced during ischemia–reperfusion, are activated leukocytes in vivo, which are absent in the present in vitro model, it was necessary to add an external oxidant source to the aortic rings to better simulate the clinical situation. Thus the aortic rings were investigated in a similar manner, with additional exposure to hypochlorite (200 µM) for 30 minutes and rinsing before phenylephrine pre-contraction (Radovits et al., 2008). Special attention was paid during the preparation to avoid damaging the endothelium. The different preservation solutions were aerated with nitrous oxide to reduce oxygen concentration to simulate hypoxic conditions.
In vitro assessment of vascular function on aortic rings:

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% O₂ and 5% CO₂. Isometric contractions were recorded using isometric force transducers of a myograph (159901A, Radnoti Glass Technology, Monrovia, CA, USA), digitized, stored and displayed with the IOX Software System (EMKA Technologies, Paris, France). The aortic rings (n=16-28 in each group) were placed under a resting tension of 2 g and equilibrated for 60 min. During this period, tension was periodically adjusted to the desired level and the Krebs–Henseleit solution was changed every 30 min. At the beginning of each experiment, maximal contraction forces to potassium chloride (KCl, 80 mM) were determined and aortic rings were washed until the resting tension was again obtained. Aortic preparations were preconstricted with an α-adrenergic receptor agonist, phenylephrine (10⁻⁶ M) until a stable plateau was reached, and relaxation responses were examined by adding cumulative concentrations of endothelium-dependent dilator acetylcholine (10⁻⁹–10⁻⁴ M). For testing relaxing responses of smooth muscle cells, a direct nitric oxide-donor, sodium nitroprusside (SNP, 10⁻¹⁰–10⁻⁵ M) was used. Half-maximal response (EC₅₀) values were obtained from individual concentration–response 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 were assessed by pD₂=−log EC₅₀ (M), vasorelaxation (and its maximum (Rₘₐₓ)) is expressed as percent of the contraction induced by phenylephrine (10⁻⁶ M).
E. Investigation of cold ischemic storage warm reperfusion injury on aortic smooth muscle cells in cell culture:

Vascular smooth muscle cells (VSMC) were isolated with Liberase®, resuspended in base medium plated and incubated on 6-well plates. The cells were grown over 70% of the plate. To verify the quality of the cells $\alpha$-smooth muscle immunostaining was performed. We performed the experiments with the 3-5 passages of the cells. Medium was changed for saline or DMOG supplemented saline solution, incubated for 24 hours and stored for hypothermic ischemia at 4ºC. After the cold storage, complete cell culture medium was added and reperfusion was simulated by further incubation at 37 ºC for 6 hours. Samples were harvested in RLT lysis buffer and stored at -80°C for later quantitative real-time PCR measurement of mRNA expression.

F. Aortic and VSMC mRNA expression by quantitative real time polymerase chain reaction (qRT-PCR):

Aortic rings and smooth muscle cells used for RT-qPCR were snap-frozen in liquid nitrogen after harvesting and were homogenized. Total RNA was extracted by using RNeasy Fibrous Tissue Mini Kit (Quiagen, Hilden, Germany) $\beta$-mercaptoethanol completed Buffer RLT. RNA concentration and purity were determined photometrically (at 260, 280 and 230 nm). RNA (1$\mu$g from each group) was reverse transcribed with QuantiTect Reverse Transcription Kit (Quiagen, Hilden, Germany). Real-time PCR reactions were performed on the Light Cycler 480 Real-time PCR detection system by using the LightCycler 480 Probes Master and Universal Probe Library (UPL) probes (Roche, Mannheim, Germany). Expression of HO-1 of aortic rings and VSMCs was determined. From aortic rings mRNA was isolated at 0, 2, 4, 6 h of warm reperfusion.
**G. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction**

Aortic segments were fixed in 4% buffered formalin, dehydrated and embedded in paraffin. Then, 3 µm thick sections were placed on adhesive slides. TUNEL assay was performed for detection of DNA strand breaks. The detection was carried out using a commercial kit following the protocol provided by the manufacturer (Chemicon International, Temecula, CA, USA). Rehydrated sections were treated with 20 μg/ml DNAse-free Proteinase K (Sigma-Aldrich, Germany) to retrieve antigenic epitopes, followed by 3% hydrogen peroxide to quench endogenous peroxidase activity. Free 3′-OH termini were labeled with digoxigenin-dUTP for 1h at 37°C utilizing a terminal deoxynucleotidyl transferase reaction mixture (Chemicon International, Temecula, CA, USA). 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, mounted with Permount (Fischer Scientific, Germany) and coverslips were applied. Four representative pictures were made with 200x magnification from each aortic ring. TUNEL positive and negative cell nuclei were counted and the TUNEL positive cell nuclei were calculated as percentage of total cell number.

**H. Statistic**

Statistical analysis was performed by using the Origin 7.0. Data distribution was tested by the Shapiro-Wilk’s test. Normally distributed data are expressed as mean±standard deviation. Two groups were compared with Student’s t test, more than 2 groups were compared by one-way ANOVA and Bonferroni’s test (e.g. PCR, immunohistochemical scores in the media, VSMC assay). Values of P<0.05 were considered as statistically significant.
H. Reagents

DMOG was provided by Cayman Chemical (Ann Arbor, Michigan, USA) and diluted in saline to concentration of $10^{-3}$ and $10^{-4}$ M. For anesthetic sodium-phenobarbital (MerialGmbH, Hallbergmoos, Germany) was used. Phenylephrin, acetylcholine and sodium nitroprusside were obtained from Sigma-Aldrich (Taufkirchen, Germany). Sodium-hypochlorite solution was produced by Grüssing, Filsum, Germany.
Results

1) Effects of prolyl-hydroxylase inhibition on vascular function:

Endothelium-dependent vasorelaxation of aortic rings: Endothelial dysfunction induced by cold ischemic storage followed by warm reperfusion and with additional hypochlorite was indicated by reduced \( R_{\text{max}} \) and right shift of the concentration-response curves of aortic segments to acetylcholine when compared with the control group (Figure 1.). Treatment of aortic rings with DMOG \( 10^{-4}\text{M} \) significantly improved the acetylcholine-induced, endothelium-dependent, NO-mediated vasorelaxation after cold ischemic storage and warm reperfusion (Figure 1., Table 1.).

Endothelium-independent vasorelaxation of aortic rings: Indicated by the vasorelaxation of aortic rings to SNP the endothelium-independent vascular smooth muscle function was not significantly altered by cold storage followed warm reperfusion injury as compared with the control group (Table 1).

Contractile responses of aortic rings: Table 1. shows the effects of hypochlorite on the contraction forces induced by KCl (80 mM) and phenylephrine (\( 10^{-6}\text{M} \)). Contractile response to high \( K^+ \)-induced depolarization was significantly reduced compared to the control group at high concentration of DMOG (\( 10^{-4}\text{M} \)). The contraction induced by \( \alpha_1 \)-adrenergic receptor agonist phenylephrine was not different among the groups.

2) Results of histopathological stainings

We found pronounced DNA damage in the wall of aortic segments in the hypochlorite treated group, as reflected by the quantitative assessment of TUNEL-staining. DMOG treatment compared to NaOCl significantly decreased the cold ischemic storage and warm reperfusion induced DNA strand breaks, which we measured as indicator of apoptosis (Figure 2.A, B.).
3) Effects of prolyl-hydroxylase inhibition on HO-1 gene expression:

Effects of DMOG 10^{-4}M on relative heme-oxygenase-1 mRNA-expression of isolated aortic rings:

We observed at the beginning of the warm reperfusion (t0) that expression of HO-1 in the NaOCl group was significantly reduced compared to control. From the second hour of “reperfusion” the aortic segments, treated with prolyl-hydroxylase inhibitor showed a significantly increased level of inducible form of HO compared to NaCl stored aortic rings (Figure 3.).

The impact of prolyl-hydroxylase inhibition on aortic smooth cell culture: After 24 hours cold storage followed by 6 hours warm reperfusion relative mRNA-expression of HO-1 was significantly higher in the DMOG group when compared to that of the NaCl group (Figure 4.).
Discussion

In our in vitro experiments we tested the role of the oxygen sensing system on cold hypoxic storage and warm reperfusion induced vascular dysfunction. Activation of the oxygen sensing pathway during normoxia with a non-specific inhibitor of prolyl-hydroxylases was found to induce HO-1 mRNA expression. We showed in vascular smooth muscle cells and aortic segments that HO-expression was significantly higher in the DMOG treated groups.

Nowadays, the potency of oxygen sensing system became in focus in several ischemia related diseases. The role of PHD-HIF system in IR injury and other hypoxia related disorders was already proven in different animal models, such as myocardial, cerebral ischemia, liver ischemia-reperfusion and cancer (Loor and Schumacker, 2008; Zhang et al., 2008; Zhong et al., 2008; Nagel et al., 2011; Kiss et al., 2012).

We investigated the role of oxygen sensing system under pathophysiological conditions in an in vitro model of cold ischemia warm reperfusion. We used the pan prolyl-hydroxylase inhibitor, DMOG in order to modulate the oxygen sensing system. DMOG stabilize HIF under normoxic conditions. The transcription factor HIF-1 plays a central role in regulation of gene expression (inducing and suppressing) in response to hypoxia and ischemia. In our model, the inhibition of prolyl-hydroxylases reflected in significantly ameliorated acute vessel dysfunction, suggesting opportunities to improve vascular functions after vascular and organ transplantations. In clinical settings, the ischemic grafts become inflamed, due to hypoxia-mediated inflammation. The exploration of hypoxia and hypoxia-induced genes could imply an efficient therapeutic avenue in the treatment and pretreatment of hypoxia–related pathophysiological conditions (transplant vasculopathy, graft failure).

Cold ischemic storage is a common way to preserve tissues and organs against the loss of functional integrity during ischemic period. In the vascular grafts and in the transplanted...
organs the acute cold ischemia and warm reperfusion injury leads to loss of the functional integrity of the vessels, which manifests as an acute endothelial dysfunction and later on as transplant vasculopathy. Vascular integrity of the graft is critically dependent on nitric oxide production by intact endothelial cells (Zebger-Gong et al., 2010).

Vascular endothelium has a multiple role in the adequate vascular tone, prevents platelet aggregation and smooth muscle proliferation, therefore the protection of endothelial integrity is crucial in the protection of vascular grafts. Previous experimental studies proved that short-term storage of vessels stored in saline characterized marked loss of endothelial functions (Garbe et al., 2011). Intact endothelial and vascular smooth muscle function is especially important for prevention of postoperative graft thrombosis and stenosis (He, 2005). Work by other groups determined that cold storage in the often used saline was unable to prevent the loss of functional integrity of the vasculature. This is reflected in the reduced ability for endothelium-dependent relaxation, and also, decreased development of smooth muscle tone to a high potassium concentration (Garbe et al., 2011). Previous published data from our research group that short-term storage was not able to induce a marked deficit of functional integrity (Radovits et al., 2008). Therefore an in vitro model of cold ischemic storage is not suited for reliable investigation of pharmacological attempts (Radovits et al., 2009). It has been shown, in different models of vascular diseases (e.g. diabetes, atherosclerosis, ischemia-reperfusion injury) that leukocyte-derived myeloperoxidase (MPO) plays an important role (due to formation of ROS) in the formation of vascular injury (Sand et al., 2003; Stocker et al., 2004; Zhang et al., 2004; Radovits et al., 2007). Hydrogene peroxide (H$_2$O$_2$) is a substrate of MPO, oxidize chloride, resulting in generation of hypochlorous acid (Zhang et al., 2004). In order to simulate warm reperfusion injury in an in vitro condition we used also sodium-hypochlorite mimic leukocyte activity.
We demonstrated in this study, that exposure of aortic vascular segments to cold ischemic storage followed by warm reperfusion resulted in formation of DNA strand breaks in the vessel walls as evidenced by our TUNEL staining. We investigated for the first time the vascular effects of DMOG. We clearly demonstrated that pharmacological inhibition of PHDs by DMOG is resulted in a significantly ameliorated vasorelaxation after 24 hours cold ischemia and hypochlorite induced warm reperfusion injury (Figure 1.). However, in the NaOCl group we showed a severe impaired endothelial function. Time-course of HO-1 mRNA expression was significantly higher in the DMOG group. Ameliorated endothelial function was probably caused by HO-1 mediated carbon monoxide (CO) release. CO has a physiological role in the regulation of vascular tone similar that nitric oxide (Philip et al.), one mechanism for which may be increased intracellular cyclic guanine monophosphate (cGMP). The in vitro measurements identified the role of oxygen sensing system in endothelium-dependent vasorelaxation, however not in the smooth muscle dependent relaxation. We hypothesize that, either the smooth muscle layer is not as sensitive for changes of oxygen tension as the endothelial layer or the hypochlorite induced in vitro injury could induce the damage of endothelial cells but could not penetrate to the deeper tissue layers. We also investigated the role of modulation of oxygen sensing system on isolated rat smooth muscle cell culture. In the NaCl group of vascular smooth muscle cells we could detect a significantly decreased level of HO-1 in contrast to the DMOG group (Figure 4).

Vascular effects of DMOG were tested on veins by Lim et al (Lim et al., 2011). They examined prolonged mechanical stretch of inferior vena cava segments in organ bath and concluded that stabilisation of HIF by DMOG resulted in enhanced reduction of venous contractility associated with prolonged vein stretch (Lim et al., 2011). We investigated for first time the functional vascular effects of DMOG in cold ischemia warm reperfusion rat in vitro model.
Bateman et al. found that during hypoxic conditions HIF-1α on mRNA level as well as in the protein level was significantly elevated very rapidly in the first 2 hours, which was followed by the increased levels of the target genes (VEGF, GLUT-1) (Bateman et al., 2007). The kinetics of expression of HO-1 of aortic rings in the NaOCl group was significantly lower compared to by DMOG group (Figure 3). We observed the same tendency on vascular smooth muscle cells. We suppose that this phenomenon is caused by the early protective effects of HIF-stabilization caused by prolyl-hydroxylase inhibition, which could protect the endothelium against IR injury. Presumably, the aortic rings without preconditioning borne a stronger, delayed ischemia-reperfusion injury.

Czibik et al. published that in murine model after gene therapy with HIF 1α the cardioprotective effect was associated with elevated bilirubin levels in serum. This effect was mimicked by remote HO-1 treatment (Czibik et al., 2009).

We investigated for the first time the role of oxygen sensing system on cold hypoxic storage and warm reperfusion induced vascular dysfunction. We proved that the pharmacological modulation of PHD-HIF pathway improved endothelium-dependent vasorelaxation through HIF stabilization induced HO-1 upregulation after short-term storage. Based on our results, we conclude, that the usage of prolyl-hydroxylase inhibitors will be useful, by targeting the prevention of vascular dysfunction of the grafts and by adaptation of the hypoxic conditions, which can induce different adaptive genes.
Authorship Contributions

Participated in research design: Enikő Barnucz, Gábor Veres, Tamás Radovits, Gábor Szabó

Conducted experiments: Enikő Barnucz, Gábor Veres, Péter Hegedűs, Raphael Zöller, Stephanie Klein

Contributed new reagents or analytic tool: Gábor Szabó, Péter Hegedűs, Enikő Barnucz

Performed data analysis: Péter Hegedűs, Raphael Zöller, Stephanie Klein

Wrote or contributed the writing of the manuscript: Enikő Barnucz, Gábor Veres, Tamás Radovits, Sevil Korkmaz, Ferenc Horkay, Béla Merkely, Matthias Karck, Gábor Szabó
References


Footnotes:

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Legends for Figures

Figure 1.

Vascular function after 24 h cold storage, concentration-response curves of acetylcholine. Each point of the curves and each column represent the mean ±SEM. #: vs. control, *: vs. NaOCl. P<0.05

Figure 2 (A, B).

Histological analysis. TUNEL staining after 24 hours cold ischemic storage and 6 h warm reperfusion. Values represent mean ±SEM. #: vs. control, *: vs. NaOCl. P<0.05

Figure 3.

Relative expression of heme oxygenase-1 in aortic segments compared to expression of glucose-3-phosphate-dehydrogenase (GAPDH) after 24 hours cold ischemic storage followed by 0, 2, 4, 6 hours warm reperfusion in control, NaOCl and DMOG groups. #: vs. control, *: vs. NaOCl. P<0.05

Figure 4.

Relative expression of heme oxygenase-1 in vascular smooth cells compared to expression of glucose-3-phosphate-dehydrogenase (GAPDH) after 24 hours cold ischemic storage followed by 0, 2, 4, 6 hours warm reperfusion in control, NaCl and DMOG groups. Relative expression of heme oxygenase-1 compared to expression of glucose-3-phosphate-dehydrogenase (GAPDH) after 24 hours cold ischemic storage followed by 0, 2, 4, 6 hours warm reperfusion in control, NaCl and DMOG groups. #: vs. control, *: vs. NaCl. P<0.05
Table 1.

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Values of maximal relaxation ($R_{\text{max}}$) and $pD_2$ to acetylcholine and sodium-nitroprusside in control, NaOCl exposed and DMOG treated aortic rings. Values represent mean ±SEM. #: vs. control, *:vs. NaOCl. P<0.05
Figure 2.

A

% of TUNEL positive cell nuclei

Control  NaOCl  DMOG $10^{-4}$M

B

Control  NaOCl  DMOG $10^{-4}$M
Figure 3.

Relative HO-1 mRNA expression over reperfusion time (h).

- Control
- NaCl
- DMOG

* indicates statistical significance compared to control.
# indicates statistical significance compared to NaCl.
## indicates statistical significance compared to DMOG.

Reperfusion time (h): Control, t0, t2, t4, t6.
Figure 4.