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 signaling pathways, such as the oxygen-dependent prolyl-hydroxylase domain-containing enzyme (PHD). Reduction of PHD activity during hypoxia leads to stabilization 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 (VSMCs) in a model of cold ischemia/warm reperfusion. Aortic segments underwent 24 hours of cold ischemic preservation in saline or DMOG (dimethyloxalylglycine)-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 4°C for 24 hours after the medium was changed for a supplied standard medium at 37°C for 6 hours. Apoptosis was assessed using the TUNEL method. Gene expression analysis was performed using quantitative real-time polymerase chain reaction. Cold ischemic preservation and NaOCl induced severe endothelial dysfunction, which was significantly improved by DMOG supplementation (maximal relaxation of aortic segments to acetylcholine: control 95% ± 1% versus NaOCl 44% ± 4% versus 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 therefore be a new therapeutic avenue for protecting endothelium and vascular muscle cells against IR injury.

Introduction

Endothelial and myocardial damage with temporary cardiac dysfunction is well described in cardiac surgery (Szabo et al., 2009). The most important limiting factor is the protection of tissue against ischemia-reperfusion (IR) 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 (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 to regulate adequately hypoxia inducible factor (HIF) activity in an oxygen-dependent way (Fraisl et al., 2009). These oxygen sensing molecules [prolyl-hydroxylase domain–containing enzymes (PHDs)] belong to the superfamily of non-heme iron (Fe²⁺)-containing 2-oxoglutarate–dependent oxygenases (Fraisl et al., 2009). HIF is a heterodimeric transcription factor consisting of the constitutively expressed HIF-β and a regulatory HIF-α subunit (Czibik, 2010). The alpha-subunit of HIF is hydroxylated by the PHDs and recognized by von Hippel-Lindau Protein, which leads to ubiquitination and rapid proteosomal degradation under normoxic conditions. However, if the cellular oxygen tension is

ABBREVIATIONS: DMOG, dimethyloxalylglycine; HIF, hypoxia inducible factor; HO-1, heme-oxygenase-1; IR, ischemia-reperfusion; PHD, prolyl-hydroxylase domain–containing enzyme; qRT-PCR, quantitative real-time polymerase chain reaction; VSMC, vascular smooth muscle cell.
reduced, the activity of PHD is inhibited, which causes the HIF-α and HIF-β-subunit to heterodimerize 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 biologic 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., 2007), vasodilatation (Loor and Schumacker, 2008), scavenging of free radicals (heme-oxygenase–1 (HO-1)), erythropoiesis, increased glucose uptake (glucose transporter–1), switch of metabolism to glycolysis (several key enzymes of glycolysis), and increased lactate use (lactate dehydrogenase) (Heyman et al., 2011). With pharmacological inhibition 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 tumors (Giaccia et al., 2003)]. N-oxalylglycine, the product of dimethylxalilglycine (DMOG), is able to mimic 2-oxoglutarate 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 HIF-1α was associated with higher serum bilirubin levels and resulted in cardioprotective effect. This cardioprotective effect was mimicked by remote 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 nonselective inhibition of PHDs under normoxic conditions, resulting in HIF stabilization 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.

**Materials and 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 12-hour 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 (National Institutes of Health 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 i.p. 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-supplemented NaCl solution; see experimental groups). The residual, not for organ bath used, particles of 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 and 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; and (3) in the DMOG group, rings were stored at 4°C for 24 hours in saline or in 1 × 10−4 M DMOG-supplemented saline.

Vascular smooth muscle cells (VSMCs) were divided into 3 groups: (1) control group without cold ischemia and warm reperfusion; (2) NaCl group, in which cells were stored at 4°C in saline for 24 hours followed by 6 hours of warm reperfusion in normal medium at 37°C; and (3) DMOG (1 × 10−3 M) group, in which cells were stored at 4°C in DMOG-supplemented saline for 24 hours followed by 6 hours of 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 VSMCs) (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. Because the major source of free radicals and oxidants produced during IR 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 precontraction (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 (Radiotti Glass Technology, Monrovia, CA) 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; Radiotti Glass Technology), digitized, stored, and displayed with the IOX Software System (EMKA Technologies, Paris, France). The aortic rings (16–28 in each group) were placed under a resting tension of 2 g and equilibrated for 60 minutes. During this period, tension was periodically adjusted to the desired level and the Krebs-Henseleit solution was changed every 30 minutes. 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 (1 × 10−6 M), until a stable plateau was reached, and relaxation responses were examined by adding cumulative concentrations of endothelium-dependent dilator acetylcholine (1 × 10−6–1 × 10−4 M). For testing relaxing responses of smooth muscle cells, a direct nitric oxide donor, sodium nitroprusside (1 × 10−3–1 × 10−6 M) was used. Half-maximal response (EC50) values were obtained from individual concentration response by fitting experimental data to a sigmoidal equation with use of Origin 7.0 (Microcal Software, Northampton, MA). Contractile responses to phenylephrine are expressed as percentage of the maximal contraction induced by KCl. The sensitivity to vasorelaxants was assessed by pD2 = log EC50 (M), vasorelaxation and its maximum (Rmax) is expressed as percentage of the contraction induced by phenylephrine (1 × 10−6 M).

**Investigation of Cold Ischemic Storage Warm Reperfusion Injury on Aortic Smooth Muscle Cells in Cell Culture.** VSMCs were isolated using 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, α-smooth muscle immunostaining was performed. We performed the experiments with 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 4°C. After the cold storage, complete cell culture medium was added and reperfusion was simulated by further incubation at 37°C for 6 hours. Samples were harvested in RLT lysis buffer and stored at −80°C for later quantitative real-time polymerase chain reaction (qRT-PCR) measurement of mRNA expression.

Aortic and VSMC mRNA Expression by qRT-PCR. Aortic rings and smooth muscle cells used for qRT-PCR were snap-frozen in liquid nitrogen after harvesting and were homogenized. Total RNA was extracted by using RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) β-mercaptoethanol completed Buffer RLT. RNA concentration and purity were determined photometrically (at 260, 280, and 230 nm). RNA (1 μg from each group) was reverse-transcribed with QuantiTect Reverse Transcription Kit (Qiagen). RT-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 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, and 6 hours of warm reperfusion.

Terminal Deoxynucleotidyl 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 performed using a commercial kit according to the protocol provided by the manufacturer (Chemicon International, Temecula, CA). Rehydrated sections were treated with 20 μg/ml DNAse-free Proteinase K (Sigma-Aldrich, Taukirchen, 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 1 hour at 37°C with use of a terminal deoxynucleotidyl transferase reaction mixture (Chemicon International). 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 (Fisher Scientific, Germany), and coverslips were applied. Four representative pictures were made with 200× 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.

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

Reagents. DMOG was provided by Cayman Chemical (Ann Arbor, MI) and diluted in saline to concentration of 1 × 10⁻³ and 1 × 10⁻⁴ M. For anesthetic, sodium-phenobarbital (Merial GmbH, Hallbergmoos, Germany) was used. Phenylephrin, acetylcholine, and sodium nitroprusside were obtained from Sigma-Aldrich. Sodium-hypochlorite solution was produced by Grüssing (Filsum, Germany).

Results

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 Rmax and right shift of the concentration-response curves of aortic segments to acetylcholine when compared with the control group (Fig. 1.). Treatment of aortic rings with DMOG 1 × 10⁻⁴ M significantly improved the acetylcholine-induced, endothelium-dependent, NO-mediated vasorelaxation after cold ischemic storage and warm reperfusion (Fig. 1; Table 1).

Endothelium-Independent Vasorelaxation of Aortic Rings. Indicated by the vasorelaxation of aortic rings to sodium nitroprusside, the endothelium-independent vascular smooth muscle function was not significantly altered by cold storage followed by warm reperfusion 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 phenylephrin (1 × 10⁻⁶ M). Contractile response to high K⁺–induced depolarization was significantly reduced, compared with the control group, at high concentration of DMOG (1 × 10⁻³ M). The contraction induced by α₁-adrenergic receptor agonist phenylephrin was not different among the groups.

Effects of Prolyl-Hydroxylase Inhibition on HO-1 Gene Expression

Effects of DMOG 1 × 10⁻⁴ M on Relative HO-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 with 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 with NaCl-stored aortic rings (Fig. 3).

The Impact of Prolyl-Hydroxylase Inhibition on HO-1 in VSMCs and Aortic Segments. After 24 hours cold storage followed by 6 hours warm reperfusion, relative mRNA-expression of HO-1 was significantly higher in the DMOG group, compared with that of the NaCl group (Fig. 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 nonspecific inhibitor of prolyl-hydroxylases was found to induce HO-1 mRNA expression. We showed in VSMCs and aortic segments that HO expression was significantly higher in the DMOG-treated groups.

At present, the potency of oxygen sensing system came into 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 to modulate the oxygen sensing system. DMOG stabilizes 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, because of 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 lead to loss of the functional integrity of the vessels, which manifests as an acute endothelial dysfunction and, later, as transplant vasculopathy. Vascular integrity of the graft is critically dependent on NO production by intact endothelial cells (Zebger-Gong et al., 2010).

Vascular endothelium has a multiple role in the adequate vascular tone and 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 decreased development of smooth muscle tone to a high potassium concentration (Garbe et al., 2011). Previous published data from our research group showed 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, IR injury), that leukocyte-derived myeloperoxidase plays an important role (because of formation of reactive oxygen species) in the formation of vascular injury (Sand et al., 2003; Stocker et al., 2004; Zhang et al., 2004; Radovits et al., 2007). Hydrogene peroxide is a substrate of myeloperoxidase, oxidize chloride, resulting in generation of hypochlorous acid (Zhang et al., 2004). To simulate warm

<table>
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<tr>
<th>Variable</th>
<th>Control</th>
<th>NaOCl</th>
<th>DMOG 10^{-8}M</th>
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<tbody>
<tr>
<td>$R_{max}$ to Ach (%)</td>
<td>95 ± 1</td>
<td>44 ± 4 *</td>
<td>68 ± 5*</td>
</tr>
<tr>
<td>pD2 to Ach</td>
<td>7.23 ± 0.1</td>
<td>6.46 ± 0.11</td>
<td>6.20 ± 0.44 *</td>
</tr>
<tr>
<td>$R_{max}$ to SNP (%)</td>
<td>101 ± 1</td>
<td>101 ± 1</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>pD2 to SNP</td>
<td>8.26 ± 0.06</td>
<td>8.11 ± 0.06</td>
<td>8.33 ± 0.10</td>
</tr>
<tr>
<td>KCl (g)</td>
<td>3.83 ± 0.15</td>
<td>2.52 ± 0.20 *</td>
<td>2.74 ± 0.20 *</td>
</tr>
<tr>
<td>Phenylephrine (g)</td>
<td>3.25 ± 0.15</td>
<td>3.45 ± 0.15</td>
<td>3.10 ± 0.16</td>
</tr>
</tbody>
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SNP, sodium nitroprusside
* versus NaOCl; $P < 0.05$
* versus control.
reperfusion injury in an in vitro condition, we used sodium-hypochlorite mimic leukocyte activity.

We revealed 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 to our knowledge, the vascular effects of DMOG. We clearly showed that pharmacological inhibition of PHDs by DMOG results in a significantly ameliorated vasorelaxation after 24 hours cold ischemia and hypochlorite-induced warm reperfusion injury (Fig. 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 release. Carbon monoxide has a physiologic role in the regulation of vascular tone similar to that of nitric oxide (Philip et al., 1999), one mechanism for which may be increased intracellular cyclic guanine monophosphate. 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 (Fig. 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 stabilization of HIF by DMOG resulted in enhanced reduction of venous contractility associated with prolonged vein stretch (Lim et al., 2011). We investigated, for the first time to our knowledge, 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 and in the protein level was significantly

![Figure 2](image-url)

**Fig. 2.** (A and B) Histologic analysis. TUNEL staining after 24 hours cold ischemic storage and 6 hours warm reperfusion. Values represent mean ± S.E.M. #versus control, *versus NaClO; P < 0.05.

![Figure 3](image-url)

**Fig. 3.** Relative expression of HO-1 in aortic segments, compared with expression of glucose-3-phosphate-dehydrogenase (GAPDH) after 24 hours cold ischemic storage, followed by 0, 2, 4, and 6 hours warm reperfusion in control, NaClO, and DMOG groups. #versus control, *versus NaClO; P < 0.05.
Fig. 4. Relative expression of HO-1 in vascular smooth cells, compared with expression of glucose-3-phosphate-dehydrogenase (GAPDH) after 24 hours cold ischemic storage, followed by 0, 2, 4, and 6 hours warm reperfusion in control, NaCl, and DMOG groups. *versus control, †versus NaCl; P < 0.05.


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