Dopamine induces the expression of heme oxygenase-1 by human endothelial cells in vitro

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Background. In a retrospective study of the kidney transplantations performed at our institution, we found that the administration of dopamine (DA) to the organ donors resulted in a significant improvement of long-term organ survival of the retrieved kidneys. To study the mechanisms underlying the organ protection associated with the administration of DA prior to transplantation, we questioned whether DA induces the antioxidative enzyme heme oxygenase-1 (HO-1) in cultured endothelial cells.

Methods. Human umbilical vein endothelial cells (HUVECs) in culture were incubated with varying concentrations of DA for different time periods. Cells were subsequently assessed for the expression of HO-1 by Western blot and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR).

Results. The presence of DA resulted in a dose- and time-dependent up-regulation of HO-1 both on RNA and protein level, whereas HO-1 was barely detectable under basal conditions. RT-PCR indicated the increased presence of HO-1 messenger RNA after 2 hours of incubation with DA, which peaked after 24 hours. The induction of HO-1 antigen was detectable after eight hours, as visualized by Western blot analysis. The addition of the antioxidant agents ascorbic acid and N-acetylcysteine both lead to dose-dependent inhibition of DA-mediated HO-1 induction. DA-mediated up-regulation of HO-1 was not influenced by the addition of either the D2-receptor antagonist haloperidol or the D1-receptor antagonist SCH 23390.

Conclusion. We conclude that DA induces the expression of the protective enzyme HO-1 in cultured endothelial cells by an oxidative mechanism. These findings may explain the beneficial effect of DA administration to kidney donors and indicate the potential role of DA in organ preconditioning.

METHODS

Reagents

The following reagents were used: DA (Fresenius, Bad Homburg, Germany); haloperidol (Janssen-Cilag GMBH, Neuss, Germany); ascorbic acid, KCl, ammonium sulfate (all Merck, Darmstadt Germany); hemin, SCH 23390, collagenase V, trypsin, penicillin-streptomycin, HEPES, egtazic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), Igpal, Tris HCl, and MgCl (all from Sigma, St. Louis, MO, USA); medium M199, phosphate-buffered saline (PBS), fetal calf serum (FCS), endothelial cell growth factor, TRIZOL dopamine (DA) and/or norepinephrine resulted in superior graft survival in long-term follow-up [1]. Interestingly, the majority of clinical studies examining the effect of DA administration following transplantation have failed to show a beneficial effect [2–4].

In searching for an explanation for the protective effect of DA administration prior to transplantation, we wondered how catecholamine pretreatment may protect the organs from the insult of ischemia/reperfusion injury. A possible mechanism may be via the induction of antioxidative enzymes such as heme oxygenase-1 (HO-1). HO-1 is an oxygen stress-inducible heat shock protein that catalyzes the oxidation of heme to biliverdin, iron, and carbon monoxide [5]. Via this mechanism, HO-1 removes pro-oxidant destabilized heme molecules and generates the antioxidant bilirubin [6]. The induction of HO-1 has been associated with a protective effect in models of heme-induced renal failure [7, 8], and the presence of HO-1 has been linked to the prevention of chronic allograft rejection and improved xenograft survival in animal transplant models [9, 10].

Since HO-1 is up-regulated by oxidative stress, and catecholamines (especially DA) are known to be powerful pro-oxidants [11, 12], we questioned whether DA may be capable of inducing HO-1 in endothelial cells.
after 16 hours. Cytoplasmic proteins were isolated and buffer containing 10 mmol/L HEPES, pH 7.9, 10 mmol/L of increasing doses of either haloperidol (1 to 10 μg/mL) or ascorbic acid (0.5 to 500 μg/mL) for 16 hours. Cells were harvested after 16 hours and assessed for the expression of HO-1 antigen by Western blot.

**Inhibition with antioxidants**

Human umbilical vein endothelial cells were treated with medium alone or medium containing DA (10 μg/mL) in the presence or absence of increasing doses of either NAC (N-acetyl cysteine; 0.1 to 500 μg/mL) or ascorbic acid (0.5 to 500 μg/mL) for 16 hours. Cells were harvested after 16 hours and assessed for the expression of HO-1 antigen or mRNA by Western blot and RT-PCR, respectively.

**RNA isolation and RT-PCR analysis**

Total RNA was isolated using TRIZOL reagent according to the manufacturer’s instructions. Two micrograms of total RNA was reverse transcribed into cDNA by oligo-dT and random hexamer priming. Deoxy-oligonucleotide primers were constructed from the published cDNA sequence of HO-1 [14] and GAPDH [15] and resulted in 284 and 268 bp products, respectively. GAPDH controls were performed for all PCRs. The sequences of the primers were as follows: HO-1 forward, GCT CAA CAT CCA GCT TTT GGA GG; HO-1 reverse, GAC AAA GTT CAT GGC CCT GGG A; GAPDH forward, GTC TTC ACC ACC ATG GAG AA; and GAPDH reverse, ATC CAC AGT CTG GGT GG.

Amplification of cDNA by PCR was performed by a modification of the procedure described by Saiki et al [16] One microliter of cDNA, 20 pmol of each primer, and 0.5 pmol Taq-DNA-polymerase were added to a final volume of 50 μL (for HO-1, 75 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.8, 1.5 mmol/L MgCl₂, 0.25 mmol/L of each dNTP; for GAPDH, 15 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.9, 1.5 mmol/L MgCl₂, 75 mmol/L ammonium sulfate, 0.25 mmol/L of each dNTP). The mixture was heated at 95°C for three minutes followed by 27 (HO-1) or 30 (GAPDH) cycles, each consisting of incubation for one minute at 95°C, one minute at 62°C (HO-1) or 59°C (GAPDH), and two minutes at 72°C. After termination of the last cycle, the samples were chilled at 4°C.

Polymerase chain reaction products were separated by electrophoresis on a 1% agarose etidium bromide gel. Identity of the bands was confirmed by sequencing on an ABI310 automatic sequencer. The semiquantitative character of the PCR was ascertained by twofold serial dilutions of cDNA and controlled for equal expression of the GAPDH PCR product.

**Western blot analysis**

Human umbilical vein endothelial cell monolayers were harvested with trypsin EDTA, and subsequently, the cell pellet was washed twice in ice-cold PBS. Cytoplasmic proteins were isolated by adding 100 μL of lysis buffer containing 10 mmol/L HEPES, pH 7.9, 10 mmol/L

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**Cell culture**

Human umbilical vein endothelial cells (HUVECs) were prepared from fresh umbilical cords according to the method described by Jaffe et al [13]. Briefly, endothelial cells were isolated from umbilical veins by digestion with collagenase V for 20 minutes at 37°C. Thereafter, the vein was flushed with sterile medium to collect endothelial cells. The culture medium consisted of medium 199 supplemented with 10% FCS, endothelial cell growth factor, and penicillin-streptomycin. The cells were cultured in 25 or 75 cm² flasks coated with 1% gelatin. Confluent cells were harvested with 0.02% EDTA/0.05% trypsin and were subcultured in T25 flasks.

All experiments were performed with cells from passages two to six. HUVECs were characterized on the basis of positive staining for factor VIII-related antigen and the endothelial marker EN4 (CD31).

**Dopamine and norepinephrine stimulation**

All experiments were performed with confluent monolayers of HUVECs, seeded in T25 culture flasks. HUVECs were treated with concentrations of 0.1, 1, 10, 50, and 100 μg/mL of DA or norepinephrine in complete culture medium. Cells were harvested after 16 hours and were assessed for the induction of HO-1 by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot. Cells were incubated with hemin at 5 μmol/L as a positive control for HO-1 induction.

The kinetics of DA-mediated HO-1 induction were studied by incubation of HUVECs with medium or medium containing DA (10 μg/mL). Cells were harvested at various time points, and HO-1 induction was assessed by Western blot and RT-PCR.

**Inhibition with DA receptor antagonists**

To study the mechanism of DA-induced HO-1 expression, HUVECs were treated with medium alone or medium containing DA (10 μg/mL) in the presence or absence of increasing doses of either haloperidol (1 to 10 μg/mL) or SCH 23390 (0.05 to 50 μg/mL). Cells were harvested after 16 hours. Cytoplasmic proteins were isolated and assessed for the expression of HO-1 antigen by Western blot.
Fig. 1. Dopamine (DA)-mediated up-regulation of heme oxygenase-1 (HO-1) mRNA in human umbilical vein endothelial cells (HUVECs). Cells were incubated with medium alone (lane 1), Hemin 5 μmol/L (lane 2), DA 0.1 μg/mL (lane 3), DA 1 μg/mL (lane 4), DA 10 μg/mL (lane 5), DA 50 μg/mL (lane 6), DA 100 μg/mL (lane 7). After 16 hours, mRNA was isolated and assessed by RT-PCR for the expression of HO-1 and GAPDH. Data are representative of three independently performed experiments.

KCl, 0.1 mmol/L EGTA, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, and 12.5 μL of 10% Igpal, followed by centrifugation (12,000 g) to remove undissolved debris. Protein concentrations of the supernatants were determined by the Coomassie Blue assay. Twenty-five micrograms of total protein was then separated on 10% SDS-PAGE according to Laemmli [17] and semidry blotted on a PVDF membrane. The membrane was incubated overnight in 5% milk powder in TBS (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl) solution. Thereafter, the blot was incubated for one hour with polyclonal rabbit-anti-HO-1 antibodies. After washing, the blot was incubated with HRP conjugated goat-anti-rabbit-IgG antibodies for 30 minutes. Antibody binding was visualized by chemiluminescence on a BIO-MAX film.

RESULTS

To examine the effect of DA on the expression of HO-1 by endothelial cells, HUVECs were exposed to increasing doses of DA. Hemin at a dose of 5 μmol/L served as a positive control. mRNA was isolated after 16 hours and analyzed for HO-1 expression by semiquantitative RT-PCR. While HO-1 mRNA was barely detectable under basal conditions, the addition of DA resulted in a dose-dependent increase in the expression of HO-1 clearly detectable at a DA dose of 1 μg/mL and with a maximum at a DA concentration of 50 μg/mL (Fig. 1). DA concentrations above 100 μg/mL resulted in detachment of the cells. No induction of HO-1 was detected when HUVECs were treated with norepinephrine in a range of 0.1 to 200 μg/mL (data not shown). A twofold serial dilution of cDNA obtained from HUVECs treated with DA (10 μg/mL) for 16 hours demonstrated a fourfold induction of HO-1 mRNA when compared with cDNA obtained from cells treated with medium alone (data not shown). The up-regulation of HO-1 on protein level was examined by Western blot (Fig. 2) and showed a corresponding induction of HO-1 antigen.

The kinetics of HO-1 up-regulation was determined using DA 10 μg/mL and incubation of endothelial cells for up to 72 hours. Cells were harvested and assessed for HO-1 mRNA by RT-PCR and for HO-1 antigen by Western blot. As shown in Figure 3A, an increase in HO-1 mRNA was detectable after 2 hours and peaked after 6 to 12 hours. HO-1 antigen was detectable after 8 hours of incubation with DA and increased for up to 48 hours as demonstrated by Western blot (Fig. 3B).

To assess the mechanism of DA-mediated HO-1 up-regulation, HUVECs were treated with DA in the presence of either the D<sub>1</sub>-receptor antagonist SCH 23390 or the D<sub>2</sub>-receptor antagonist haloperidol. As shown in Figure 4, neither increasing doses of SCH 23390 (0.1 to 100 μg/mL) or haloperidol (0.1 to 10 μg/mL) influenced
Fig. 4. Addition of DA-receptor antagonists haloperidol and SCH 23390. HUVEC were incubated with medium alone, DA (10 μg/mL), or DA (10 μg/mL) in the presence of increasing doses of either haloperidol (A) or SCH 23390 (B). Cells were harvested after 16 hours and were assessed for the expression of HO-1 by Western blot. Data are representative of three independently performed experiments.

Fig. 5. Inhibition of DA-mediated HO-1 induction by ascorbic acid. HUVECs were incubated with medium alone, DA (10 μg/mL), or DA (10 μg/mL) in the presence of increasing doses ascorbic acid. After 16 hours, mRNA was isolated and assessed by RT-PCR for the expression of HO-1 and GAPDH (A). Western blot analysis of HUVECs treated as described (B). Data are representative of three independent experiments.

In Figure 6, the addition of NAC to DA stimulated HUVECs leads to a complete abrogation of HO-1 induction on both protein and mRNA levels.

**DISCUSSION**

This article demonstrates the induction of the endothelial synthesis of HO-1 by DA in vitro. The DA dose of 1 μg/mL, which leads to a powerful induction of both HO-1 RNA and protein in the dose response experiments, is within the upper range of therapeutic steady-state DA levels measured in patients receiving intermediate dose DA treatment [18, 19]. Our data demonstrate that DA exerts its effect via an oxidative mechanism. While the DA-mediated induction of HO-1 was not influenced by the addition of the D₂-receptor antagonist haloperidol or the specific D₁-receptor antagonist SCH 23390, the induction of both HO-1 mRNA and antigen was inhibited by the addition of the antioxidant vitamin C and completely blocked in the presence of NAC.

Possible oxidative effects of DA have generated a strong interest in the context of Parkinson’s disease and schizophrenia [11, 20]. The auto-oxidation of DA in the presence of transition metals, for example, iron or copper, leads to the generation of quinones/semiquinones and reactive oxygen species, including the superoxide...
radical (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) [12]. DA has been shown to induce apoptosis in neuronal cell lines via oxidative mechanisms. Treatment of mesencephalic cell cultures with nontoxic levels of the auto-oxidizable compound L-DOPA leads to resistance to the toxic effects of the strong oxidant tert-butyl hydroperoxide [21].

In our experiments, no up-regulation of HO-1 was observed when HUVECs were treated with norepinephrine. This is in keeping with a weaker toxic effect of norepinephrine compared with DA [22].

A recent retrospective study of the cadaveric kidney transplants performed at our center showed that the administration of DA to the organ donors prior to transplantation leads to a significant reduction of acute graft rejection episodes and improvement of long-term survival [1]. Studies on the administration of DA after renal transplantation have either failed to demonstrate an effect [2–4] or report an adverse association between donor catecholamine use and immediate graft function [23, 24].

It has been demonstrated that E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 are up-regulated in models of brain injury and cerebral ischemia [25, 26]. The pretreatment of HUVECs with the inotropes amrinone and DA leads to an inhibition of IL-1β–mediated up-regulation of adhesion molecules [27]. Thus, possibly the down-regulation of elevated adhesion molecules in the transplanted grafts may contribute to the beneficial effect of donor treatment with DA.

However, an inhibition of adhesion molecules by catecholamines would not explain why the administration of DA after the transplantation procedure has not proven to be beneficial. The timely induction of protective genes prior to the actual ischemia/reperfusion insult may be an alternative explanation. Of these genes, HO-1 is a possible candidate. HO-1 is a 32 kD microsomal enzyme that has been linked to a protective effect in a broad variety of disease models. It catalyzes the conversion of heme to biliverdin, iron, and carbon monoxide (CO) [5]. Via this mechanism, HO-1 degrades destabilized potentially toxic heme molecules and generates biliverdin, which is subsequently converted to the antioxidant bilirubin [6]. The iron released during this process is sequestered by ferritin, which is induced together with HO-1 [28]. An additional beneficial effect may arise from the synthesis of CO, which similar to NO is a potent vasodilator [5].

In vivo models of renal disease have underscored the potential protective role of HO-1. The induction of HO-1 confers resistance to the development of renal failure in glycerol-induced rhabdomyolysis in the rat [7, 8]. Recent studies have demonstrated that the expression of HO-1 is linked to an improved outcome both in models of xenotransplantation and chronic rejection [9, 10]. A number of recent studies indicate an important protective of HO-1 in the setting of ischemia/reperfusion. Both the induction of HO-1 with either cobalt protoporphyrin or gene therapy prevent ischemia/reperfusion injury in livers of genetically fat Zucker rats [29]. The inhibition of HO-1 with tin mesoporphyrin markedly worsens renal failure in a model of ischemic renal failure [30]. The protective effects of HO-1 have been linked to the action of bilirubin [31, 32].

Thus, the induction of protective genes such as HO-1 prior to transplantation may provide the organ with a resistance to the insult of ischemia/reperfusion and inflammation that results in the improved outcome observed after donor DA administration. Since ischemia/reperfusion is thought to have a strong impact on the development of acute and chronic rejection [33], the reduction of reperfusion injury is a major task in transplant medicine. A possible approach is the emerging concept of organ preconditioning [34]. Short periods of ischemic stress render the organ (for example, the heart) resistant to a subsequent episode of prolonged ischemia. Two distinct phases of protection have been identified. The early phase develops within minutes and has been linked to the release of adenosine [35]. The second phase is referred to as late preconditioning and takes hours to become apparent. The generation of reactive oxygen species is an essential component [36], and the induction of nuclear factor-κB and nitric oxide synthase are both necessary for the development of organ protection [37, 38].

Our results indicate that DA may provide its beneficial effect in renal transplantation by pharmacological preconditioning with the induction of antioxidative and possibly anti-inflammatory genes. The powerful pro-oxidative effects may also explain why the administration of DA after transplantation is of no benefit or may even be harmful. DA given during the phase of reperfusion may lead to additional oxidative stress with glutathione depletion, resulting in increased damage and possibly immunogenicity of the transplanted organ.

Although of obvious importance, the induction of HO-1 of course may not be the only beneficial gene induced in response to DA-mediated oxidative stress. Further studies are required to determine whether other candidate genes such as superoxide dismutase, glutathione, nitric oxide synthetase, or catalase are induced by DA. Furthermore, although ample evidence of oxidative metabolism of catecholamines in the brain exists [11], we do not know whether DA administered in vivo is oxidized sufficiently to induce the stress necessary for the HO-1 induction observed in our in vitro system. Studies to examine the induction of HO-1 by DA and its effect on ischemia/reperfusion injury in the presence of the antioxidative armamentarium of the living organism are underway.

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