Effect of pre-treatment with catecholamines on cold preservation and ischemia/reperfusion-injury in rats

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Treatment of organ donors with catecholamines reduces acute rejection episodes and improves long-term graft survival after renal transplantation. The aim of this study was to investigate the effect of catecholamine pre-treatment on ischemia/reperfusion (I/R)- and cold preservation injury in rat kidneys. I/R-injury was induced by clamping the left kidney vessels for 60 min along with a contralateral nephrectomy. Cold preservation injury was induced by storage of the kidneys for 24 h at + 4°C in University of Wisconsin solution, followed by syngeneic transplantation. Rats were pre-treated with either dopamine (DA), dobutamine (DB), or norepinephrine (2, 5, and 10 μg/kg/min, each group) intravenously via an osmotic minipump for 24 h before I/R- and cold preservation injury. Pre-treatment with DA (2 or 5 μg/kg/min) and DB (5 μg/kg/min) improved recovery of renal function after I/R-injury and dose dependently reduced mononuclear and major histocompatibility complex class II-positive cells infiltrating the kidney after I/R-injury. One day after I/R-injury, upregulation of transforming growth factor (TGF)-β 1 and 2 and phosphorylation of p42/p44 mitogen-activated protein kinases was observed in kidneys of animals treated with DA or DB. DA (5 μg/kg/min) and DB (5 μg/kg/min) pre-treatment reduced endothelial cell damage after 24 h of cold preservation. Only DA pre-treatment improved renal function and reduced renal inflammation after 24 h of cold preservation and syngeneic transplantation. Our results demonstrate a protective effect of pre-treatment with catecholamines on renal inflammation and function after I/R- or cold preservation injury. This could help to explain the potent organoprotective effects of catecholamine pre-treatment observed in human kidney transplantation.

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Ischemia/reperfusion (I/R)- and cold preservation injury are major contributors to the development of primary failure and delayed graft function after renal transplantation. Despite better HLA-matching, kidneys from cadaver donors show a poorer graft survival than those from living donors.[1,2] It is widely recognized, that not only antigen-dependent factors, but also antigen-independent factors, such as donor brain death, cold preservation, and I/R during revascularization influence allograft survival.

Convincing evidence exists that prolonged cold and warm ischemia of donor kidneys increase the incidence of delayed graft function after transplantation,[3] leading to decreased graft and patient survival in the short and long term.[4] Tissue damage and inflammatory response in the kidney caused by cold preservation and Ischemia/reperfusion (I/R)-injury[5] in the early stage after transplantation may increase the immunogenicity of the graft and thus initiate allograft rejection.[6] Hence, the development of new strategies to reduce initial graft damage would reduce the need of re-transplantations and expand the pool of donor organs.

In two independent retrospective clinical studies,[7,8] administration of catecholamines to cadaveric organ donors reduced the number of acute rejection episodes in the first month after renal transplantation and markedly improved long-term graft survival. In a rat model of brain death characterized by a strong renal inflammation, treatment with dopamine (DA) reduced renal monocyte infiltration and major histocompatibility class (MHC) II and P-selectin expression in renal tissue.[9] *In vitro*, we have recently demonstrated that catecholamines protect cells against cold preservation injury either by scavenging of or inhibiting production of reactive oxygen species.[10] The effect of catecholamine pre-treatment on I/R-injury and cold preservation injury in kidneys was not studied so far. In the present study, we used a rat model of I/R-injury and a syngeneic model of renal transplantation to evaluate the effect of catecholamine pre-treatment on renal function and renal inflammation after I/R-injury and cold preservation injury.

RESULTS

I/R-injury

Recovery of I/R-injury, induced by clamping of the left kidney vessels for 60 min, was significantly improved in rats

pretreated for 24 h with DA 2 μg/kg/min ($P<0.05$), DA 5 μg/kg/min ($P<0.05$), and dobutamine (DB) 5 μg/kg/min ($P<0.01$) (Figure 1a and b). In contrast, higher dosage of DA (10 μg/kg/min) and lower dosages of DB (2 μg/kg/min) had no protective effect on kidney function, whereas higher dosage of DB (10 μg/kg/min) worsened kidney function before induction of I/R-injury, leading to progressive renal failure and death of all animals. Norepinephrine (NA) pre-treatment dose dependently delayed recovery of renal function after I/R-injury (Figure 1c). Most animals died between days 2 and 3 after I/R-injury (NA2: 1/8; NA5: 3/8; NA10: 7/8).

Pre-treatment with catecholamines significantly reduced renal inflammation 5 days after I/R-injury in a dose-dependent manner. The amount of ED1- (Figure 2a) and MHC class II-positive cells (Figure 2b) was significantly reduced in DA pre-treated rats compared to vehicle pre-treated rats. Similarly, DB pre-treatment significantly reduced the number of ED1- (Figure 3a) and MHC class II-expressing cells (Figure 3b). There was no effect of NA on ED1- and MHC class II-positive infiltrating cells in kidneys from surviving animals (data not shown).

On day 1 after I/R-injury, pre-treatment with DA (5 μg/kg/min) or DB (5 μg/kg/min) significantly reduced infiltration of MHC II-positive cells (Figure 4) and showed a tendency to a reduced number of ED1-positive cells in renal tissue. Furthermore, there were significantly less TdT-mediated dUTP nick-end labeling (TUNEL)-positive cells in renal tissue of animals pre-treated with DA or DB compared to vehicle-treated rats (Figure 5a–c). Ribonuclease protection assay of renal tissue harvested 1 day after I/R-injury (Figure 6) showed a significant upregulation of transforming growth factor (TGF)-β 1 and TGF-β 2 in animals pre-treated with DA (TGF-β 1: 0.55 ± 0.02; TGF-β 2: 0.21 ± 0.02) or DB (0.53 ± 0.04; 0.22 ± 0.03), but not in animals pre-treated with vehicle (0.41 ± 0.02; 0.11 ± 0.02) when scored by densitometry ($P<0.05$ DA or DB vs vehicle, analysis of variance (ANOVA)). No changes in tumor necrosis factor-α, interleukin-1, or interferon-γ could be detected by ribonuclease protection assay. Additionally, renal tissue was investigated for activation (i.e. phosphorylation) of the extracellular signal-regulated kinase (ERK) p42/p44. Animals pre-treated with DA or DB (5 μg/kg/min) showed an increase in p42/p44 phosphorylation compared to vehicle-treated rats 1 day after I/R-injury as detected by densitometry ($P<0.05$, ANOVA) (Figure 7). Measurement of malondialdehyde before induction of I/R-injury and on day 1 after I/R-injury in peripheral blood as a marker of oxidative stress revealed no differences between the groups (data not shown).

Cold preservation injury

In kidneys subjected to cold storage in University of Wisconsin (UW) solution for 24 h, cold preservation injury was demonstrated by the presence of TUNEL-positive cells. Serial cryostat sections that were stained either with an anti-rat endothelial cell monoclonal antibody or by TUNEL

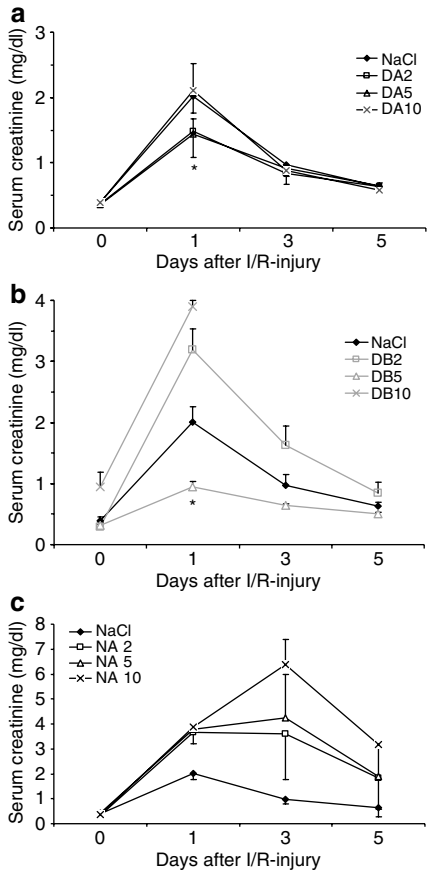

Figure 1 | Serum creatinine (mg/dl) after I/R-injury in uni-nephrectomized rats pre-treated with catecholamines. (**a**) Pre-treatment with DA at different doses (DA 2, 5, or 10 μg/kg/min). DA pre-treatment with 2 or 5 μg/kg/min significantly reduces the rise in serum creatinine on day 1 after I/R-injury (*$P<0.05$). (**b**) DB pre-treatment with 5 μg/kg/min significantly reduces the rise in serum creatinine on day 1 after I/R-injury (*$P<0.01$) (ANOVA). Higher dosage of DB (10 μg/kg/min) worsened renal function before induction of I/R-injury, leading to progressive renal failure and death of all animals. (**c**) NA pre-treatment dose dependently delayed recovery of renal function after I/R-injury. Most animals died between days 2 and 3 after I/R-injury (NA2: 1/8; NA5: 3/8; NA10: 7/8).

revealed that TUNEL-positive cells were predominantly found in peritubular capillaries and larger vessels. Although TUNEL positivity was also detected in tubular cells, the fluorescence intensity was markedly lower in these cells compared to endothelial cells (Figure 8). We observed significant less TUNEL-positive cells after 24 h of cold preservation in rats pre-treated with DA (5 μg/kg/min) or DB (5 μg/kg/min) compared with saline pre-treated rats. Noradrenaline pre-treatment did not influence cold preservation injury, as quantified by TUNEL-positive cells (Figure 9). The addition of DB to the preservation solution without systemic pre-treatment over 24 h significantly reduced TUNEL-positive cells after 24 h of cold preservation (Figure 9).

After 24 h of cold preservation and syngeneic transplantation, only donor pre-treatment with DA (5 μg/kg/min) significantly improved renal function on the first day after

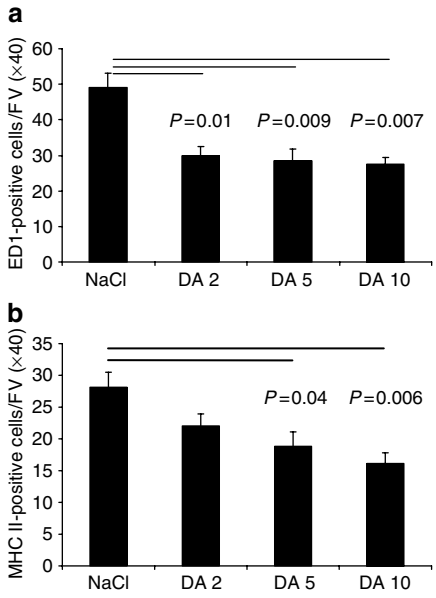

Figure 2 | ED1- and MHC II-positive cells in renal tissue after DA pre-treatment and I/R-injury. Number of (**a**) ED1- and (**b**) MHC II-positive cells per field of view (original magnification $\times 40$) in renal tissue 5 days after I/R-injury. Pre-treatment with DA dose dependently reduced infiltration of ED1- and MHC II-positive cells (ANOVA).

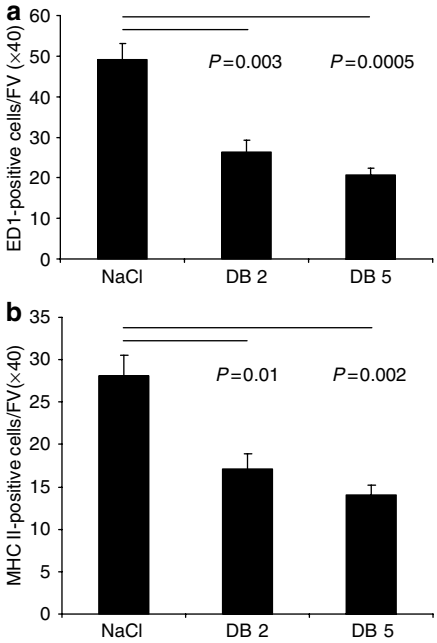

Figure 3 | ED1- and MHC II-positive cells in renal tissue after DB pre-treatment and I/R-injury. Number of (**a**) ED1- and (**b**) MHC II-positive cells per field of view (original magnification, $\times 40$) in renal tissue 5 days after I/R-injury. Pre-treatment with DB or 5 μg/kg/min dose dependently reduced infiltration of ED1- and MHC II-positive cells (ANOVA). All animals pre-treated with DB 10 μg/kg/min developed progressive renal failure and died before day 5.

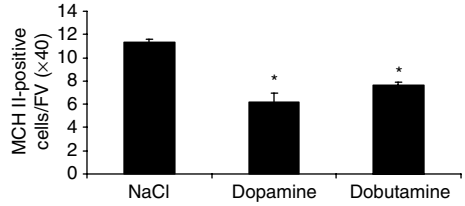

Figure 4 | Number of MHC II-positive cells per field of view (original magnification $\times 40$) in renal tissue 1 day after I/R-injury. Pre-treatment with dobutamine 5 μg/kg/min or DA 5 μg/kg/min reduced infiltration of MHC II-positive cells (*$P<0.05$ isotonic saline (NaCl) vs dopamine or dobutamine ANOVA).

Figure 5 | TUNEL-positive cells in renal tissue 1 day after I/R-injury. Immunohistochemistry for TUNEL-positive cells in renal tissue harvested 1 day after I/R-injury of animals pre-treated with (**a**) vehicle (isotonic saline) , (**b**) DA (5 μg/kg/min), or (**c**) DB (5 μg/kg/min).

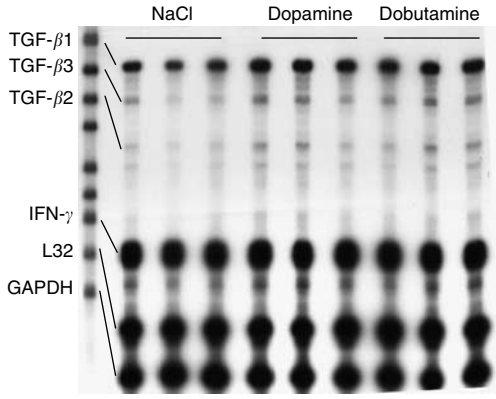

Figure 6 | RNA protection assay of renal tissue harvested 1 day after I/R-injury of animals pre-treated with isotonic saline, DA (5 μg/kg/min), or DB (5 μg/kg/min). Treatment with DA or DB led to an increase in TGF-β 1 and TGF-β 2 expression ($n=5$ each group, three animals of each group are shown exemplary). No changes in interferon-γ and tumor necrosis factor-α were detected.

transplantation ($P=0.02$) (Figure 10). Pre-treatment with DA significantly reduced renal infiltration with MHC class II- and ED1-positive cells 5 days after transplantation (Figures 11 and 12). DB or noradrenaline pre-treatment did not reduce renal inflammation (data not shown).

DISCUSSION

In the present study, we demonstrate that pre-treatment with catecholamines reduces I/R-injury and cold preservation injury in rat kidneys in a substance- and dose-dependent manner. Renoprotection was observed in organs treated with low to medium doses of DA or DB, whereas higher doses and the use of NA had no or even deleterious effects on renal function and morphology.

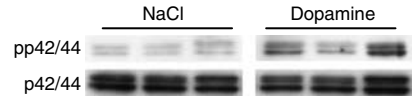

Figure 7 | Western blot of renal tissue harvested day 1 after I/R-injury for the MAPKs pp42/44 and p42/44. Treatment with DA or DB (only DA is shown) led to a significant increase of pp42/44, that is, phosphorylation (i.e. activation) of p42/44. Unphosphorylated p42/44 is not changed in renal tissue ($P<0.05$ isotonic saline vs DA or DB when scored by densitometry, ANOVA).

Low-dose DA administration has been advocated for many years in oliguric and critically ill patients on the basis of its action on dopaminergic renal receptors. However, a meta-analysis of DA on prevention, development, and course of acute renal failure and mortality in patients treated by intensive care did not show any beneficial effect.[11] Concordantly, authors concluded that 'dopamine should be eliminated from routine clinical use for this indication'. A similar conclusion was drawn by Holmes and Walley,[12] who argued that, in addition to the lack of renal efficacy, DA may be harmful in patients with critical illnesses. Likewise, the use of NA in critically ill patients is often fraught with controversy, as renal dysfunction can occur as a consequence of its vasoconstrictor effect on renal vascular bed.[13]

However, there may be clinical situations in which catecholamines can be safely used to protect kidneys from injury in severely ill patients. Analysis of two independent registries, one based on results from cadaveric renal transplantations at our center and the other on data from the Eurotransplant International Foundation, revealed that administration of catecholamines to brain-dead organ donors reduced the number of acute rejection episodes and markedly prolonged long-term graft survival without adverse side effects for the recipients.[7,8] Similar to clinical transplantation, we found *in vitro* that pre-treatment with catecholamines also protects cells very effectively against preservation injury.[10] Protection was not receptor-mediated and did not require *de novo* protein synthesis. The study demonstrated that catecholamines prevent cold preservation injury either by scavenging of reactive oxygen species or by inhibition of reactive oxygen species generation. Previous studies have reported catecholamines to possess antioxidative properties in addition to their pro-oxidative effects.[14–18] In a direct comparison, Miura *et al.* showed that DB was a more powerful inhibitor of iron-catalyzed lipid peroxidation than DA or isoproterenol. This effect was largely achieved by scavenging of reactive oxygen species. In our study, we were not able to demonstrate protective effects of DA or DB treatment regarding a reduction of oxidative stress in the kidney as measured by malondialdehyde content in peripheral blood, but we can not exclude an effect of scavenging of reactive oxygen species by catecholamines in this model. In addition, catecholamines also exert indirect antioxidative properties by inducing expression of enzymes such as heme oxygenase-1.[19]

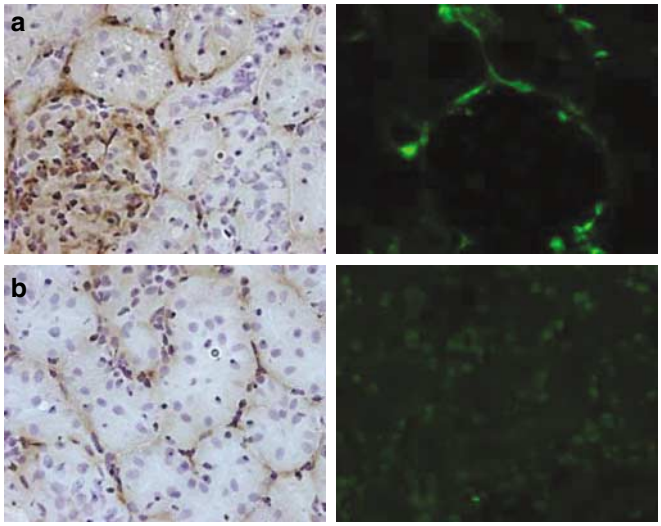

Figure 8 | Influence of cold storage on cell damage in the kidney. Kidneys from Lewis rats pre-treated with (**a**) isotonic saline and rats pre-treated with (**b**) DB were stored for 24 h in UW solution at 4°C. Serial cryostat sections were stained with (panels to the left) an anti-rat endothelial cell monoclonal antibody or (panels to the right) stained by TUNEL. DB pre-treatment (**b**) reduced the extent of cold-induced endothelial cell damage. Original magnification, ×40.

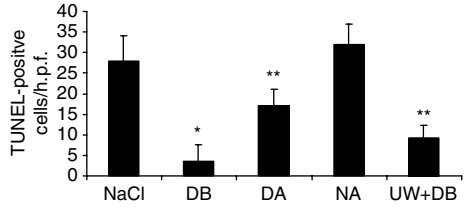

Figure 9 | Kidneys from Lewis rats pre-treated with either DB, DA, or NA in a dose of 5 μg/kg/min for 24 h until explantation were stored at 4°C in UW solution for 24 h. Rats pre-treated with isotonic saline served as controls. Pre-treatment with DB or DA significantly protected against cold preservation injury, as quantified by the presence of TUNEL-positive cells in cold preserved kidneys. Addition of DB to the preservation solution without systemic pre-treatment over 24 h significantly reduced TUNEL-positive cells in cold preserved kidneys. *$P<0.01$, **$P<0.05$ (ANOVA).

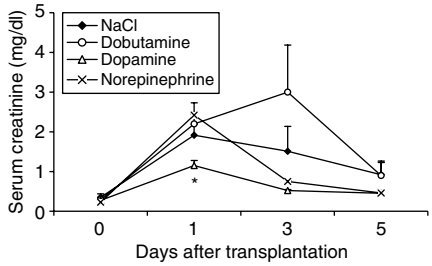

Figure 10 | Renal function, as measured by serum creatinine concentration, after 24 h of cold preservation and syngeneic transplantation of kidneys from DA (5 μg/kg/min), DB (5 μg/kg/min), NA (5 μg/kg/min), or vehicle-treated donor rats. Only DA pre-treatment improved renal function on the first day after transplantation. (*$P=0.03$ DA vs isotonic saline, ANOVA).

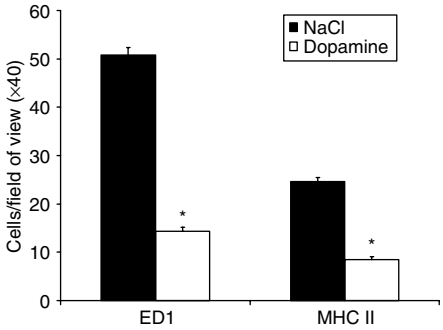

Figure 11 | Donor DA pre-treatment significantly reduced MHC class II- and ED1-positive cells in syngenically transplanted kidneys 5 days after transplantation (*$P < 0.0001$ DA vs isotonic saline, unpaired *t*-test).

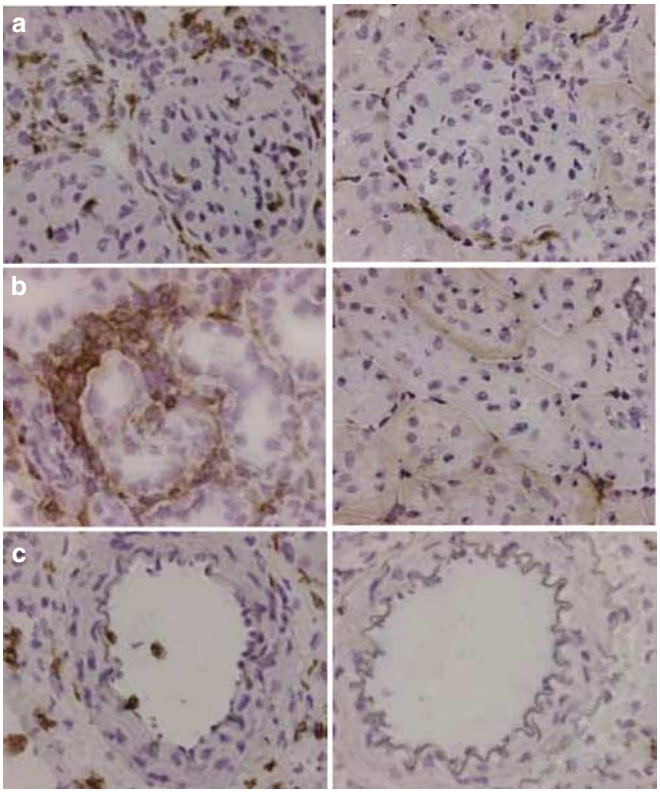

Figure 12 | Immunohistology of donor kidneys pre-treated with (panel to the right) DA or (panel to the left) isotonic saline 5 days after isogeneic transplantation. Note the strong infiltrates found in rats pre-treated with isotonic saline in (**a**) glomerular and (**b**) tubular areas, and in (**c**) larger vessels (**c**). Original magnification $\times 40$.

It was hypothesized that stimulation of adrenergic and/or dopaminergic receptors by catecholamines may contribute to prevention of ischemia-related injury through an alteration of systemic or renal hemodynamics. However, several lines of evidence argue against a receptor-mediated mechanism. First, Schaub *et al.*[9] found that intravenous infusion of DA in a dose of 10 μg/kg/min over 6 h had no significant effect on arterial blood pressure and renal blood flow in anesthetized rats. Second, Schnuelle *et al.*[8] could also show that the beneficial effect of DA on immediate graft function after transplantation is independent of systolic and diastolic blood pressure. Finally, the addition of DB to the preservation solution for 24 h without previous intravenous administration to the donor markedly reduced the number of TUNEL-positive cells in the cold-stored kidney. Thus, it is very unlikely that adrenergic and/or dopaminergic receptor stimulation plays a major role in protecting kidneys from ischemia-related damage.

Recently, the role of mitogen-activated protein kinases (MAPKs) in I/R-injury was explored by several groups.[20–23] MAPKs are a class of protein kinases that include ERK, which play an important role in the determination of cell survival. The ERK MAPK family consists of p42 and p44 isoforms, which are activated by various stimuli including oxidative stress and several G protein-coupled receptor agonists. It has recently been shown that DA induces ERK activation and mitogenesis in proximal tubular cells. The mechanism was not receptor mediated but required DA uptake into the cells, its metabolism by monoamine oxidase, and generation of H_2O_2, indicating that other catecholamines may also induce some of its effects through ERK activation.[24] The DA d2-like receptor agonist bromocriptine stimulates p42/44 MAPKs in proximal tubular epithelial cells, causes mitogenesis via p42/44 MAPKs in kidney cells, and protects the kidney against I/R injury in a rat model.[25,26] The mitogenic activity of DA could play a key role in normal cell growth and repair processes. This might be of importance in our model as proliferation of tubular cells is essential for recovery from acute renal failure.[27] In myocardial[28–30] and renal[20,23,31–33] cell damage, activation of ERKs is considered to be a sign of tissue regeneration and protection. In agreement with these results, we found an increase in ERK activation in animals pre-treated with DA and DB, which were protected against I/R-injury.

DA- and DB-treated animals showed an increased expression of TGF-β 1 day after I/R-injury. A recent study has shown an increase in TGF-β secretion in pituitary cells after stimulation with DA or bromocriptine.[34] TGF-β is a polypeptide growth factor playing an important role in wound healing and tissue regeneration.[35] TGF-β inhibits proliferation of renal proximal tubule cells *in vitro* and stimulates extracellular matrix synthesis, cell clustering, tubulogenesis, and apoptosis.[36,37] Previous studies have shown rapid and prolonged upregulation of TGF-β primarily in regenerating proximal tubules after renal ischemia, indicating a potential role for TGF-β in the regenerating kidney.[38,39] Basile *et al.*[40] demonstrated a spatial and temporal relationship with TGF-β and several extracellular matrix-related genes post-ischemia, which was partially inhibited by the administration of a TGF-β-neutralizing antibody *in vivo*, showing that TGF-β may participate in renal regenerating by promoting extracellular matrix synthesis and providing newly formed proximal tubule cells a solid

substrate for adhesion and migration. Furthermore, genotype analysis of donor and recipients of kidney transplants revealed an association of low TGF-β production with an elevated risk for early rejection and worse graft function at 4 years.[41] However, other studies failed to identify a prominent role for TGF-β in renal repair after I/R-injury.[42]

In contrast to post-ischemic acute renal failure, only DA, but not DB and NA treatment of donor rats improved immediate graft function after 24 h of cold preservation and syngeneic transplantation. These data strikingly correlate to those of Schnuelle *et al.*,[43] who found that DA was the only catecholamine that improved initial graft function after kidney transplantation. All other adrenergic derivatives were ineffective with respect to normalizing serum creatinine after controlling for confounding factors by multivariate Cox regression. The observed differences in efficacy of catecholamines both *in vitro* and *in vivo* cannot be explained on the basis of our results and need to be investigated in future studies.

Systemic pre-treatment of organ donors or pre-treatment of donor organs by improved preservation solutions is gaining more and more interest. Preventing the initial tissue damage, originating from donor brain death, cold preservation, or I/R-injury, by pharmacological pre-treatment, may reduce immunogenicity of the graft[6] and thus improve initial function and prolong long-term survival.[44–49] Indeed, donor DA treatment has been shown, in a retrospective analyses, to increase long-term graft survival in humans[7,8] and in an experimental rat model, to improve short- and long-term outcome after prolonged cold storage and subsequent allogeneic kidney transplantation.[50] The attractiveness of the concept of pharmacological pre-treatment of organ donors is further underlined by the fact that it is not associated with any costs and side effects for the recipients. A randomized prospective clinical trial, investigating the effect of DA pre-treatment of brain-dead organ donors on short- and long-term renal graft function is currently under progress.

In summary, we have shown for the first time that pre-treatment with catecholamines reduces both I/R and cold preservation injury in rat kidneys. The use of catecholamines, when administered in higher doses, is limited by severe hemodynamic side effects. Newly synthesized chemically modified derivatives of catecholamines lacking sympathoadrenergic properties are currently under investigation and may prove beneficial for pre-treatment of organ donors.

MATERIALS AND METHODS

Animals

Inbred male Lewis (LEW, RT1^1) rats weighing 220–250 g were obtained from Harlan-Winkelmann (Borchen, Germany). Animals were kept under standard conditions and fed standard rodent chow and water *ad libitum*. All procedures were performed according to the guidelines of the American Physiological Society and were approved by the local authorities (Regierungspräsidium Karlsruhe AZ: 35-9185.81/166/01).

Experimental protocol

All surgical procedures were performed under general anesthesia with ketamine (100 mg/kg body weight intraperitoneal; CP-Pharma, Burgdorf, Germany) and xylazine (5 mg/kg body weight intraperitoneal; Rompun® Bayer, Leverkusen, Germany). For pre-treatment, an osmotic minipump (Alzet 2ML1) was implanted subcutaneously in the animals back. A catheter attached to the pump was placed in the left femoral vein and the pre-treatment solution was administered for 24 h (mean pumping rate 10 μl/h).

In the model of I/R-injury, Lewis rats received pre-treatment with either DA (2, 5, or 10 μg/kg/min), DB (2, 5, or 10 μg/kg/min), NA (2, 5, or 10 μg/kg/min), or isotonic saline (control) ($n = 8$ each dosage) for 24 h. After the pre-treatment period, the pumps were removed and the rats underwent right nephrectomy. These kidneys were snap frozen in liquid nitrogen and stored at -80°C for further analysis. Before induction of ischemia, animals received 100 IU heparin/kg intravenously to prevent renal arterial thrombosis. The vessels of the remaining left kidney were clamped for 60 min. After removal of the clamps, the abdominal wall was closed with continuous sutures, and the animals were housed as described above.

To assess cold preservation injury, Lewis donor rats were pre-treated intravenously with either DA (5 μg/kg/min), DB (5 μg/kg/min), NA (5 μg/kg/min), or isotonic saline ($n = 5$ each group) as described above. After the pre-treatment period, both donor kidneys were explanted, flushed with 1 ml UW solution each, and stored for 24 h at $+4^\circ$C in UW solution. After cold preservation, one kidney of each group was snap frozen in liquid nitrogen for further TUNEL staining to show apoptotic cells. Next, kidneys were transplanted syngeneically in bilaterally nephrectomized Lewis rats using standard microsurgical techniques. Renal perfusion was restored in all cases after 25 min.

In all animals, blood samples for measurement of serum creatinine (Dimension®, Dade-Behring, Frankfurt, Germany) were obtained on days 0, 1, 3, and 5 after induction of I/R-injury or after isogeneic transplantation. On day 5, all animals were killed, and kidneys were harvested and frozen in liquid nitrogen. Additionally, kidneys of animals pre-treated with DA 5 μg/kg/min, DB (5 μ/kg/min), or isotonic saline were harvested on day 1 after I/R-injury ($n = 5$ each group).

Immunohistochemistry

Serial cryostat sections (3–5 μm) were either fixed in acetone/methanol (70/30%) or in paraformaldehyde (2.5%) for immunohistochemical or TUNEL staining. For immunohistology, sections were incubated with specific mouse-anti-rat antibodies. Then, sections were stained with biotinylated horse-anti-mouse antibody using the avidin-biotinylated-peroxidase immunolabeling method (Vector Laboratories Inc., Burlingame, CA, USA) and counter-stained with hematoxylin. Cell populations and cell surface marker were assessed using monoclonal antibodies to macrophages and monocytes (ED1), MHC class II (F17-23-2), and endothelial cells (JG12, 1:10). Anti-endothelial antibodies were kindly provided by Professor D Kerjaschki (Vienna, Austria), and all other antibodies were purchased from Serotec Ltd (Kidlington, UK). Negative controls included omission of the first antibody or murine control immunoglobulin. Positive cells are expressed as mean $\pm$ s.e.m. of cells/field of view (c/FV). More than 20 fields of view/section were evaluated at $\times 400$.

TUNEL staining to show apoptotic cells was performed according to the manufacturer's instructions (Roche diagnostics, Mannheim, Germany). In brief, tissue sections were washed twice with phosphate-buffered saline after fixation and incubated for 15 min in permeabilization solution (0.1 mM sodium citrate) at 70°C. Hereafter, the sections were extensively washed and incubated for 30 min at 37°C with 50 μl of reaction mixture in humid chambers. In each experiment and for each staining, a negative control was included, by omitting dUTP transferase from the reaction mixture. TUNEL-positive cells were detected by immune-fluorescence microscopy using a fluorescein isothiocyanate filter. In the same experiment, TUNEL staining on paraffin-embedded renal tissue was performed using an anti-fluorescein antibody conjugated to peroxidase. TUNEL-positive cells were visualized by addition of diaminobenzidine tetrahydrochloride substrate (Vector Laboratories Inc., Burlingame, CA, USA).

RNase protection assay

Total RNA was isolated from whole kidneys harvested 24 h after using TRIzol Reagent (Invitrogen, karlsruhe, Germany). RNA was precipitated with isopropanol, washed with 70% ethanol, and subjected to RNase protection assays using BD RiboQuant™ multi-probe sets (RCK-1 and RCK-3; BD Biosciences, San Jose, CA, USA; Instruction manual is available at bdbiosciences.com, BD Biosciences, 2002. BD *RiboQuant™ Multi-Probe RNase Protection Assay System* (Instruction Manual)) following the supplier's instructions. The gels were dried and subjected to autoradiography. The RNA transcripts were identified by appropriate length of the protective fragments. In each of the template sets, the housekeeping genes L32 and glyceraldehyde-3-phosphate dehydrogenase were included to control for equal loading.

Western blot analysis

Tissue was homogenized and lysed in buffer (10 mM Tris, 2% sodium dodecyl sulfate, 0.5% β-mercaptoethanol, all from Sigma-Aldrich (St Louis, MO, USA). Protein concentrations were measured using Coomassie-Reagent (Pierce, Rockford, IL, USA). The samples were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and semidry blotted on a polyvinylidine difluoride membrane (Roche Diagnostics, Mannheim, Germany). The membrane was incubated overnight in 5% milk powder in Tris-buffered saline (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) solution. Thereafter, the blot was incubated for 1 h with a polyclonal antibody against phospho-p42/p44 (Santa Cruz, Heidelberg. Germany), followed by incubation with an appropriate horseradish peroxidase secondary antibody (Santa Cruz, Heidelberg, Germany). Proteins were visualized using enhanced chemoluminescence technology according to the manufacturer's instruction (Pierce, Rockford, IL, USA). To confirm equal protein loading, membranes were stripped with 62.5 mM Tris-HCl, 2% sodium dodecyl sulfate, and 100 mM β-mercaptoethanol, and incubated with a polyclonal antibody against p42/p44 (Santa Cruz, Heidelberg, Germany). Where indicated, the Western blots were subjected to densitometric analysis using Scion Image software.

Malondialdehyde

Plasma malondialdehyde was measured by enzymatic detection, according to the method described by Esterbauer and Cheeseman.[51]

Statistical analysis

Data are shown as means ± s.e.m. For comparison of means between different groups, ANOVA with Tukey's option for multiple comparison was applied (StatsDirect version 2.2.2). Statistical significance was defined as a P-value <0.05.

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