Dopamine and Lipophilic Derivates Protect Cardiomyocytes against Cold Preservation Injury

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ABSTRACT

Donor heart allografts are extremely susceptible to prolonged static cold storage. Because donor treatment with low-dose dopamine improves clinical outcome after heart transplantation, we tested the hypothesis that dopamine and its lipophilic derivate, N-octanoyl dopamine (NOD), protect cardiomyocytes from cold storage injury. Neonatal rat cardiomyocytes were treated with dopamine or NOD or left untreated and subsequently subjected to static cold storage (8–12 hours). Dopamine- and NOD-treated cardiomyocytes displayed a better viability compared with untreated cells after hypothermia. In untreated cardiomyocytes, cell damage was reflected by lactate dehydrogenase (LDH) release and a decrease in intracellular ATP. NOD was approximately 20-fold more potent than dopamine. Similarly to cardiomyocytes in vitro, rat hearts perfused with NOD before explantation showed significantly lower LDH release after static cold storage. ATP regeneration and spontaneous contractions after cold storage and rewarming only occurred in treated cardiomyocytes. Hypothermia severely attenuated isoprenaline-induced cAMP formation in control but not in dopamine- or NOD-treated cells. Esterified derivates of NOD with redox potential and lipophilic side chains reduced cell damage during cold storage similarly to NOD. In contrast to dopamine, neither NOD nor its derivates induced a significant β-adrenoceptor-mediated elevation of cellular cAMP levels. The β1-adrenoceptor antagonist atenolol and D1/D2 receptor antagonist fluphenazine had no impact on the protective effect of NOD or dopamine. We conclude that dopamine as well as NOD treatment mitigates cold preservation injury to cardiomyocytes. The beneficial effects are independent of β-adrenoceptor or dopaminergic receptor stimulation but correlate with redox potential and lipophilic properties.

Introduction

The insufficient availability of donor heart allografts, a rising waiting list mortality, and increasing demand for donor organs have substantially contributed to extension of traditionally accepted criteria for evaluation of cardiac grafts (Koerner et al., 1997; Tenderich et al., 1998). With this liberalization of donor eligibility criteria, donor hearts are being harvested from remote locations, thereby increasing cold ischemic times. Although static cold storage of solid organs is common practice in organ transplantation, it is generally acknowledged that prolonged hypothermic preservation negatively affects graft quality (Shoskes and Halloran, 1996; Taylor et al., 2009).

Iron-mediated production of reactive oxygen species, impairment of Ca2+ homeostasis, and ATP depletion seem to be of instrumental importance in the sequel of hypothermic preservation injury (Rauen et al., 2000; Brinkkoetter et al., 2006; Bartels-Stringer et al., 2007; Radovits et al., 2008). Earlier reports have concluded that prolonged cold ischemic time is not a risk factor for decreased long-term survival in cardiac allograft recipients (Morgan et al., 2003), yet more recent studies have indicated that it is a predictor of a 5-year conditional mortality (Taylor et al., 2008). The effect of cold ischemic time on survival after heart transplantation is dependent on donor age, with greater tolerance among grafts from younger donors (Russo et al., 2007). Cold ischemic time also shows a significant linear relationship with post-transplantation length of stay in the intensive care unit (Rylski et al., 2010). Hence, there is a socioeconomic demand for measures that improve cardiac allograft preservation over longer periods of time without affecting cardiac transplantation outcome (Rao et al., 2001).

Clinical studies have shown that dopamine (DA) treatment of the donor reduces the incidence of delayed graft function

ABBREVIATIONS: BB, 2,5-bisacetoxy-benzoic acid; BBNB, 2,5-bisacetoxybenzoyl-N-butylamide; BBN, 2,5-bisacetoxybenzoyl-N-octanoylamide; CS, cold storage; DA, dopamine; ISO, isoprenaline; LDH, lactate dehydrogenase; NOD, N-octanoyl dopamine; NRCM, neonatal rat cardiomyocyte; SKF-38393, 1-phenyl-2,3,4,5-tetrahydro-1H-3-benazepine-7,8-diol; UW, University of Wisconsin.
after renal transplantation (Schnuelle et al., 2004; Schnuelle et al., 2009). The beneficial effect of DA was more pronounced when cold ischemic time exceeded 17 hours, and this in turn translated into a better graft survival in patients in this particular subgroup (Schnuelle et al., 2009). Based on a substantial number of in vitro studies, current evidence indicates that DA mitigates cell damage related to cold preservation (Yard et al., 2004; Brinkkoetter et al., 2006; Rudic et al., 2010). The cytoprotective properties of DA directly originate from structural entities, which enable the molecule to act as an iron chelator and to scavenge reactive oxygen species (Yard et al., 2004).

The analysis of heart transplant recipients who had received a cardiac allograft from a donor enrolled in the randomized dopamine trial revealed that donor DA may also improve the clinical course after heart transplantation, ultimately resulting in superior survival after 3 years (Benck et al., 2011). However, it must be mentioned that, in approximately 12.5% of the donors that enrolled in the randomized dopamine trial, dopamine infusion had to be discontinued because of tachycardia or hypertension. This underscores the need for compounds that have superior cytoprotective properties and yet are devoid of hemodynamic actions. The present study was undertaken to provide a biologic plausibility for the clinical finding that DA improves transplantation outcome in cardiac allograft recipients. In addition, we sought to test the hypothesis that N-octanoyl dopamine (NOD), a lipophilic DA derivative devoid of hemodynamic effects, has superior cytoprotective properties compared with DA in protecting cardiomyocytes against cold preservation injury and that this does not require catecholamine receptor engagement (Lösel et al., 2010).

Materials and Methods

Animal Care. Care of the animals was taken in accordance with the Committee on Animal Research of the regional government (Regierungspräsidium Karlsruhe, Germany), which reviewed and approved all experimental protocols according to the Guide for the Care and Use of Laboratory Animals published by the Directive 2010/63/EU of the European Parliament and the corresponding German legislation.

Isolation and Culture of Neonatal Rat Cardiomyocytes. One- to 3-day-old female and male Wistar rats were killed by decapitation, and the hearts were removed. Cardiomyocytes were isolated from hearts, as described previously (Will et al., 2010). In brief, hearts were minced and subjected to serial digestion in a mixture of 0.5 mg/ml collagenase type II (Cell Systems, Troisdorf, Germany) and 0.6 mg/ml pancreatin (Sigma-Aldrich, Taufkirchen, Germany) to release single cells. The isolated cells were digested in a mixture of 0.5 mg/ml collagenase and subjected to serial digestion in a mixture of 0.5 mg/ml collagenase and 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM 2,7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4), and then stored at 4°C in University of Wisconsin (UW) (Southard and Belzer, 1995) solution for 8 to 12 hours. The incubation time was based on initial experiments, showing that preservation less than 6 hours did not significantly damage the cells. Cells were evaluated either directly after cold storage or after a rewarming period of 1 hour at 37°C.

Cold Storage and Rewarming. Cardiomyocytes were stimulated for 1 hour with indicated substances. Hereafter, the cells were extensively washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4), and then stored at 4°C in University of Wisconsin (UW) (Southard and Belzer, 1995) solution for 8 to 12 hours. The incubation time was based on initial experiments, showing that preservation less than 6 hours did not significantly damage the cells. Cells were evaluated either directly after cold storage or after a rewarming period of 1 hour at 37°C.

Lactate Dehydrogenase Assay. Lactate dehydrogenase (LDH) assays were performed as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany). A 100-μl aliquot of each supernatant was used to determine LDH release in the preservation solution. In each experiment, 100 μl of preservation solution was used as blank. The results are expressed as OD492 nm, corrected for the blank value. Each concentration was tested in triplicate in all experiments. The EC50 values were estimated from a total of five experiments.

Assessment of Intracellular ATP Amount. Intracellular ATP was extracted directly after cold preservation or after 1 hour of re-warming in cell culture medium. ATP was assessed by luciferase-driven bioluminescence using the ATP Bioluminescence Assay Kit (Roche Diagnostics), according to the manufacturer’s instructions. Absolute ATP concentrations, given in the legends of the figure, were normalized for protein concentrations in the lysates.

Determination of cAMP Formation. Cardiomyocytes were subjected to cold preservation conditions for 12 hours. After 30 minutes of re-warming at cell culture conditions, 1 mM 3-isobutyl-1-methylxanthine was added and cells were incubated for an additional 30 minutes. Thereafter, the cardiomyocytes were stimulated for 10 minutes with the indicated concentrations of isoprenaline and then lysed in 0.1 M HCl. cAMP formation was assessed by using enzyme-linked immunosorbent assay kits (cAMP EIA Kit, Biomol, Hamburg, Germany, for concentration-dependent curves and after cold storage (CS); EIA Kit, Biotrend, Koeln, Germany, for β-adrenergic receptor activation).

Determination of Cardiomyocyte Contractility. Cardiomyocyte contractions were determined optically using a phase-contrast microscope. The results are expressed as the percentage of cavities of 24-well plates containing contracting neonatal rat cardiomyocytes (NRCM), irrespective of the number of contracting cells.

Cold Preservation of and LDH Measurement in Explanted Rat Hearts. Male Lewis rats, weighing 250–300 g, were used. Animals were kept under standard conditions and fed standard rodent chow and water ad libitum. All procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences and approved by the local authorities.
Animals were anesthetized with 6 mg/body weight of xylazine (Rompun 2%; Bayer Vital, Leverkusen, Germany) and 100 mg/body weight of ketamine (Ketamin 10%; Intervet, Unterschleißheim, Germany) and heparinized (100 IE, Heparin-Natrium ratiopharm; Ratiopharm, Ulm, Germany). Long midline incision was used to enter the abdominal cavity. The abdominal aorta and inferior vena cava were exposed, and aorta and inferior vena cava were cut to drain the blood. An incision through the thoracic wall was applied to expose the chest organs. The heart was perfused through the superhepatic vena cava with 30 ml of 4°C cold UW solution with and without 50 μM NOD to cool and arrest its beating. The ascending aorta, pulmonary artery, and pulmonary veins were transacted. By cutting distal from the ligature, the heart can be harvested. Subsequently, the explanted hearts were preserved at 4°C in cold 10 ml of UW solution with and without NOD over 4 hours. Thereafter, preservation solution was collected from the heart ventricles to measure LDH release of cardiac cells. LDH was assessed according to the manufacturer’s instructions (Roche Diagnostics). The following groups were investigated: vehicle, hearts in UW solution, n = 4; NOD, 50 μM in UW solution, n = 4.

Statistical Analysis. Data are presented as mean ± S.E.M. and were based on three or more separate experiments. Differences between groups were determined by Student’s t test or one-way analysis of variance, followed by Bonferroni’s multiple comparison. A P value of less than 0.05 was considered statistically significant. Mathematical curve fitting and calculation of EC50 or IC50 values were performed with GraphPad Prism5 (GraphPad Software, Inc., San Diego, CA).

Results

Dopamine and N-Octanoyl Dopamine Protect Cardiomyocytes from Cold-Inflicted Cell Damage and ATP Depletion. We first assessed the susceptibility of cultured cardiomyocytes for damage to hypothermia. To this end, cardiomyocytes were subjected to cold storage for various time intervals. Thereafter, the supernatants were immediately analyzed for LDH activity. Although cold storage up to 4 hours was not associated with profound cell damage, LDH release in the supernatants significantly increased with increasing preservation time (Fig. 1A). Because LDH release reached a maximum between 8 and 24 hours of cold storage, most experiments were performed using a cold storage time of 8 hours, unless otherwise stated.

To study whether treatment of cardiomyocytes with DA or its lipophilic derivative NOD is protective against cold preservation injury, cultured cardiomyocytes were treated for 1 hour with increasing concentrations of DA or NOD and subsequently subjected to 8 hours of cold storage in UW solution. In untreated cardiomyocytes, cold storage resulted in profound cell damage, as demonstrated by a profound release of LDH into the preservation solution. The release of LDH was inhibited in a concentration-dependent manner by prior treatment with either DA or NOD before the start of cold storage (Fig. 1B). In line with the observation that DA or NOD pretreatment was protective, the cellular ATP content was significantly higher in treated than in untreated cells after cold storage (DA or NOD treated vs. untreated, P < 0.01; Fig. 1C). In both settings, NOD was more potent than DA. Half-maximal inhibition of LDH release and ATP depletion occurred at approximately 20-fold lower NOD than DA concentrations (LDH release IC50 DA vs. NOD, 50 ± 25 μM vs. 1.3 ± 0.3 μM; ATP production EC50 DA vs. NOD, 100 ± 25 μM vs. 13 ± 3 μM, P < 0.05). No significant difference in the maximally achievable protection was observed between DA and NOD treatment. When the cells were rewarmed in culture medium directly after cold storage, ATP regeneration did not occur in untreated cardiomyocytes. In contrast, 1 hour of rewarining was sufficient to regain similar intracellular ATP concentrations in DA- or NOD-pretreated cells as in NRCM not subjected to cold storage (Fig. 1D). NOD was also protective under conditions similar to those occurring during heart transplantation and cold storage of donor organs. When rat hearts were perfused with UW solution containing 50 μM NOD prior to explanation and kept at 4°C for 4 hours, the LDH content in the preservation solution taken from the heart ventricles of treated and untreated hearts was significantly lower in the NOD-treated group (approximately 35%; Fig. 1E). A 4-hour time period of cold storage was chosen, as the usually accepted maximal time for organ preservation in human heart transplantation is up to 4 to 5 hours. DA could not be tested in this setting, as the presence of 50 μM DA in the preservation solution gave a strong positive LDH reading, although no heart allografts were stored herein. The false-positive LDH reading was only observed in the heart preservation study for DA but not when cultured cardiomyocytes were pretreated with DA, extensively washed, and subsequently stored at 4°C.

NRCM Treated with Dopamine or N-Octanoyl Dopamine Regain Positive Inotropic Capacity after Cold Storage and Rewarming. Even though both DA and NOD are able to protect NRCM against cold storage injury, this does not necessarily imply that protected cells regain full functionality upon rewarming. To address this issue, we studied two aspects of NRCM functionality, i.e., their ability to regain spontaneous contractions after cold storage and to respond to β-adrenoceptor stimulation. Irrespective of the pretreatment, spontaneous cardiomyocyte contractions were not detectable immediately after cold storage. However, when the preservation solution was replaced by culture medium and cells were rewarmed to 37°C for 1 hour, DA and NOD treated cells regained their ability to contract spontaneously (% of wells with spontaneous contractions: 89% for DA and 94% for NOD treated cells vs. 15% for control cells) (Fig. 2A). In untreated NRCM, spontaneous contraction after cold storage and rewarining was only observed in 16% of the cultures. When untreated cardiomyocytes were maintained in culture for another 24 hours after cold preservation, their ability to contract ceased, whereas the beating capacity remained constant in DA- and NOD-treated cells. A video of such a comparison is presented in the Supplemental Data (Supplemental Video 1, no treatment; Supplemental Video 2, NOD treatment). To further demonstrate that protected NRCM regain functionality after reawarming, the ability to respond to positive inotropic stimuli was assessed by measuring by β-adrenoceptor–induced cAMP formation after 12 hours of cold storage and 1 hour of rewarining. The β-adrenoceptor agonist isoprenaline (ISO) concentration-dependently increased cAMP production in DA- or NOD-pretreated cardiomyocytes, whereas the efficacy of ISO to induce cAMP formation was severely impaired in untreated cells that were subjected to cold preservation and rewarining (Fig. 2B). As shown in Fig. 2C, the maximal extent of ISO-stimulated cAMP formation in DA- or NOD-pretreated NRCM was not significantly different from cardiomyocytes that were not subjected to cold preservation.

Similar to NOD, Lipophilic, Esterified Derivates of Gentisic Acid Protect Cardiomyocytes from Cold Storage Injury. Because NOD contains a redox active catechol structure, we assessed whether the catechol structure is strictly
required for protection. Similar to catechols (ortho-dihydroxy-
yquinones), para-dihydroxyquinones are redox active and
ehref{https://jpet.aspetjournals.org/doi/suppl/10.1124/jpet.104.066879}{} hence may have the propensity to act protective in our assays.

Meta-Diarylquinones were not tested because these structures have extremely low redox activities. We analyzed the cytoprotective properties of 2,5-acetoxybenzoic acid (BB), 2,5-acetoxybenzoyl-N-butylamide (BBNB), and 2,5-acetoxybenzoyl-N-octanoylamide (BBNO). As shown in Fig. 3A, all three substances are derivatives of gentisic acid (2,5-hydroxybenzoic acid), a naturally occurring compound with known radical scavenging properties (Chen et al., 2002). The para position and esterification of the hydroxy groups of these compounds make them, however, unlikely candidates for any activation of β-adrenoceptors. Yet, similar to DA and NOD, they have a redox active moiety at the aromatic ring, provided that the acetylated hydroxy groups in the aromatic ring are hydrolyzed by intracellular esterases. BB, BBNB, and BBNO vary in their hydrophobic side chains, which range from eight C-atoms (the same length as NOD) to four and none in the case of BB (Fig. 3A). To compare the cytoprotective effects of these genistic acid derivatives, cardiomyocytes were treated with 50 μM BB, BBNB, and BBNO 1 hour prior to cold storage. A comparable protection from cold-induced cell damage by NOD (Fig. 1) was only observed with BBNB and BBNO in which a hydrophobic side chain was added to the aromatic core (Fig. 3B).

**Neither β-Adrenoceptor nor D1/D2 Receptor Agonism Is Involved in Protection of NRCM from Cold Storage Injury.** To investigate a putative relation between β-adrenergic or dopaminergic agonism and cytoprotection, we first tested whether the different compounds were able to increase intracellular cAMP concentration upon treatment and, if so, to what extent the increased cAMP was inhibited by the β-receptor blocker atenolol. Compared with isoprenaline, which increases intracellular cAMP formation ~10-fold, only DA led to a significant increase in intracellular cAMP, whereas no significant rise in intracellular cAMP concentration was observed after stimulation with 50 μM NOD, BB, BBNB, or BBNO (Fig. 4A). The DA-induced increase in intracellular cAMP levels could be completely blocked by the addition of the β1-adrenoceptor–specific antagonist atenolol (Fig. 4B). Although we have previously demonstrated that the cytoprotective effect of dopamine could not be overcome by β-adrenoceptor blockade (Yard et al., 2004), this has not been tested to date for NOD. To exclude any receptor engagement in the cytoprotective properties of NOD, cardiomyocytes were treated for 1 hour with NOD alone or with NOD in the presence of 10 μM atenolol before 8 hours of cold storage. As shown in Fig. 4C, pretreated cardiomyocytes were

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**Fig. 1.** Influence of DA and NOD on cold preservation injury of cultured NRCM and perfused rat hearts. (A) NRCM were subjected to various time intervals of cold storage, and LDH release in the supernatants was assessed hereafter. *P ≤ 0.01 vs. time point 2 hours. (B and C) NRCM were treated for 1 hour with increasing concentrations of DA or NOD, as indicated. Thereafter, the cells were extensively washed with phosphate-buffered saline and stored for 8 hours at 4°C in UW solution. LDH release in the preservation solution was assessed directly after cold preservation, as described in [Materials and Methods](#). The results are expressed as mean OD<sub>490</sub> values ± S.E.M. (B) Intrasacellular ATP was determined directly after cold preservation by luciferase-driven bioluminescence. The results are expressed as mean relative light units (RLU) ± S.E.M. Absolute ATP concentration in nanograms per milligram protein ranged from 13.2 ± 2.1 to 22 ± 0.5 for both DA and NOD (C). A total of five independent experiments were performed. For each experiment, all conditions were tested in triplicates. *P ≤ 0.01, NOD vs. DA. (D) NRCM were stimulated for 1 hour with DA (100 μM) or NOD (50 μM). Hereafter, the cells were extensively washed with phosphate-buffered saline and stored for 8 hours at 4°C in UW solution. Intracellular ATP was measured directly before (open bars) or after (filled bars) 1 hour of rewarming in culture medium. NRCM that were not subjected to cold storage (No CS) were included in each experiment. The results are expressed as mean relative light units (RLU) ± S.E.M. Absolute ATP concentration in nanograms per milligram protein was as follows: 15.4 ± 2.2 (No CS), 2.1 ± 0.6 before and after rewarming of untreated cells (C), 12.9 ± 0.8 and 16.8 ± 2.1 (DA-treated cells before and after rewarming, respectively), and 13.6 ± 2.2 and 17 ± 2.1 (NOD-treated cells before and after rewarming, respectively). A total of six independent experiments were performed. For each experiment, all conditions were tested in triplicates. *P ≤ 0.05 vs. No CS; #P ≤ 0.05 vs. DA before rewarming; $P ≤ 0.05 vs. NOD before rewarming. (E) Rat hearts were perfused with UW or UW containing 50 μM NOD prior to explantation. After 4 hours at 4°C, LDH release was determined, as described in [Materials and Methods](#). Results are expressed as mean values ± S.E.M., n = 4 for each group, *P ≤ 0.05 vs. control.
equally protected against cold-induced damage, independent of β₁-adrenergic activation. Likewise, the D₁/D₂-receptor antagonist fluphenazine abrogated neither the protective effect of dopamine nor that of NOD over large range of fluphenazine concentrations (Fig. 4D), the data of which exclude the involvement of dopaminergic receptors on the protection of cardiomyocytes to cold-inflicted damage.

Discussion

Dopamine and Its Lipophilic Derivates Provide Cytoprotection from Cold Preservation Injury Based on Their Redox Potential and Cellular Uptake. In this study, we sought to explore the biologic plausibility of our clinical observation that treatment of the brain-dead cardiac donor with low-dose DA is associated with an improved clinical outcome after heart transplantation (Benck et al., 2011). We hypothesized that dopamine pretreatment increases the viability of cardiomyocytes during cold preservation and that NOD is superior in this regard. Our data clearly substantiate this hypothesis as pretreatment with DA or NOD concentration-dependently reduces cell damage and enhances tolerance of cardiomyocytes to withstand cold preservation in culture. A similar loss of damage was seen if NOD was applied to rat hearts before explantation. In cultured cardiomyocytes, ATP depletion was preserved, and, as a consequence, spontaneous contractility as well as responsiveness to adrenergic stimuli is preserved upon rewarming.

Our data further indicate that the beneficial effects on NRCM are independent of β₁-adrenoceptor or D₁/D₂ dopaminergic receptor engagement. At the concentrations used, only DA stimulated cAMP formation in NRCM undergoing cold preservation after DA or NOD treatment or no treatment (C) with cardiomyocytes not being subjected to cold preservation (No CS). Where indicated (filled bars), cells were stimulated with 10 μM ISO. The results are expressed as means ± S.E.M. *P ≤ 0.05 vs. not treated control. In (B and C), a total of three independent experiments were performed. For each experiment, all conditions were tested in duplicates.

Fig. 2. Influence of DA and NOD treatment on cardiomyocyte function after cold preservation and rewarming. Cardiomyocyte contractions were determined microscopically using a phase-contrast microscope. (A) NRCM were treated with DA (100 μM) or NOD (50 μM) for 1 hour or were left untreated. The percentage of wells containing contracting cardiomyocytes was assessed before cold storage (open bars) or after cold preservation, followed by 1 hour of rewarming (filled bars). The results are expressed as the mean percentage of wells that contain contracting cardiomyocytes ± S.E.M. irrespective of the number of contracting cells. *P ≤ 0.05 vs. control before CS. (B) Isoprenaline concentration-response curve in DA- or NOD-treated and untreated cells after cold preservation and rewarming. Cardiomyocytes were treated with DA (100 μM) and NOD (50 μM) for 1 hour before cold storage or left untreated. After rewarming, NRCM were stimulated with the indicated concentrations of the β₁/β₂-adrenoceptor agonist ISO, and intracellular cAMP was quantified. (C) Comparison of cAMP formation in NRCM undergoing cold preservation after DA or NOD treatment or no treatment (C) with cardiomyocytes not being subjected to cold preservation (No CS). Where indicated (filled bars), cells were stimulated with 10 μM ISO. The results are expressed as means ± S.E.M. *P ≤ 0.05 vs. not treated control. In (B and C), a total of three independent experiments were performed. For each experiment, all conditions were tested in duplicates.
D<sub>1</sub> receptor agonists confer cytoprotection through indirect inhibition of the poly(ADP-ribose) polymerase without receptor engagement (Gerö et al., 2007). Another study on ischemia/reperfusion injury in neonate rat cardiomyocytes supports that cardiac D<sub>1</sub> receptors are not involved in cytoprotection, because selective activation of D<sub>1</sub> receptors with the agonist SKF-38393 (1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol) induces apoptotic cell death (Li et al., 2008). As NOD has been shown not to act on dopamine receptors (Lösel et al., 2010), our data clearly support that DA’s cytoprotective action does not require dopamine receptor engagement (Brinkkoeetter et al., 2006, 2008; Benck et al., 2011). Direct evidence to underpin this assumption is provided by the data demonstrating that the protective effects of DA and NOD are not abrogated by the D<sub>1</sub>/D<sub>2</sub> receptor antagonist fluphenazine.

The relative hydrophobicity of dopamine derivatives correlates with the efficacy of these compounds to protect against cold-inflicted injury (Lösel et al., 2010) and thus explains the differences in potency between DA and NOD to limit LDH release and the loss of ATP activity following cold storage. Whereas the redox activity of DA and NOD is similar, NOD displays a higher degree of hydrophobicity compared with DA, which allows improved cellular uptake (Lösel et al., 2010). Subsequently, higher intracellular concentrations can be achieved with lower dosages, thus reaching cytoprotective intracellular levels at much lower concentrations (Lösel et al., 2010).

Gentisic acid is a naturally occurring compound in mold fungi and plants (Chen et al., 2002). It also is a naturally occurring metabolite of acetylsalicylic acid (Cham et al., 1980). Its high redox potential has been clearly demonstrated in vitro; moreover, both in vitro and in vivo findings suggest that this property may convey protection toward geno- and hepatotoxicity of cyclophosphamide and may lower low-density lipoprotein oxidation (Chen et al., 2002; Ashidate et al., 2005; Nafees et al., 2012). We have modified gentisic acid in two ways. The free phenolic hydroxy groups have been esterified to protect the compound from oxidation outside of the cell where they cannot be hydrolyzed by esterases and to increase hydrophobicity and thus increase cellular uptake. It should be underscored, however, that cellular uptake of the gentisic acid derivatives was not assessed. Nonetheless, it is conceivable that, similarly as shown for cellular uptake of DA and NOD, increased hydrophobicity of the gentisic acid derivatives might increase cellular uptake.

This study did not identify a putative cellular target by which DA or NOD conveys protection. Yet it suggests that if such a target exists, the redox activity and hydrophobicity of the protective compound seem to have a strong influence on the activity of this target. NOD, BBNB, and BBNO all have the propensity to inhibit nuclear factor κB upon stimulation with tumor necrosis factor α (Hottenrott et al., 2013). Therefore, even though NOD, BBNB, and BBNO are distinct in their chemical structure, their common chemical characteristics, that...
is, redox activity and hydrophobicity, appear to be sufficient to inhibit the same cellular target. Nakao et al. (2008) have suggested that cold preservation injury occurs as a consequence of destabilization of heme-containing proteins, for example, cytochrome P450. This results in the release of heme and subsequently oxidative cell damage. The requirement for redox activity for cellular protection against cold-inflicted damage fits with the findings of Nakao et al. (2008). All protective compounds that were tested in this study are able to participate in a redox reaction and therefore have the propensity to reduce reactive oxygen intermediates. Although the less hydrophobic BB compound is also able to participate in such an oxidation reaction, it was not protective in this study. This further supports the notion that the relative hydrophobicity of the protective compound is likely to play part in the protective mechanism.

**Dopamine and Its Lipophilic Derivates as Potential Drugs to Prevent Graft Dysfunction in Heart Transplantation.** There is ample evidence that prolonged hypothermic preservation has detrimental effects on organ quality after transplantation. Unlike in renal transplantation where delayed graft function can be bridged by hemodialysis, graft dysfunction after heart transplantation has severe consequences, because post-transplant use of cardiac assist devices to aid circulatory demands goes along with high morbidity and mortality. Ischemic tolerance substantially varies between different organs. Because of the enhanced susceptibility of the cardiac allograft, it is of utmost importance to keep cold ischemic time below 4 hours in heart transplantation (Taylor et al., 2009; Jacobs et al., 2010). A considerable number of heart transplants are still performed after prolonged cold ischemia, which promotes early graft dysfunction and increases mortality post-transplantation (Taylor et al., 2009). These sequelae urgently demand a pharmacologic intervention to limit cell damage during cold preservation. DA's clinical benefit in heart transplantation has recently been shown in a cohort study with 93 patients from 21 centers (Benck et al., 2011). Although these implications have not yet been confirmed by a randomized controlled trial, treatment of the donor with DA is thus a potential option to increase the probability of graft survival after transplantation. Nevertheless, in the clinical setting, intracellular DA accumulation critically depends on the activity of its degrading enzymes monoamine oxidase and catechol-O-methyl transferase. Therefore, a pharmacokinetic
steady state needs to be maintained until cross-clamping. In a prospective randomized clinical trial on low-dose donor DA treatment and transplantation outcome in renal transplant recipients, we reported that DA treatment was prematurely discontinued in 12.5% of donors due to circulatory side effects, such as tachycardia and/or hypertension (Schnuelle et al., 2009). Such a discontinuation of DA infusion will, however, result in rapid loss of protection because of monoamine oxidase and catechol-O-methyl transferase activity. Hence, the efficacy of donor preconditioning could be further improved if a DA-like compound would be available, which at cytoprotective concentrations is devoid of dopaminergic and adrenergic action. Although NOD is not yet approved for clinical application, preclinical data on mitigation of ischemia-induced acute kidney injury in rats are promising and suggest superior protection than DA (Tsagogiorgas et al., 2012). Further biological evaluation of NOD, BBNB, and BBNO is ongoing and will clarify whether these compounds can also be considered for clinical testing.

In summary, this study provides biologic plausibility for our observation that donor preconditioning with DA may improve the outcome after heart transplantation. Because protection is mediated by antioxidant properties of the tested molecules, this study also provides a rationale for further development and evaluation of DA derivatives devoid of receptor-driven dopaminergic and adrenergic action for clinical use in organ transplantation.

Limitations of the Study. We are aware that the data presented in this study do not prove that the beneficial effect of donor dopamine preconditioning on cardiac transplantation outcome is attributed to the protective properties of dopamine on hypothermic preservation injury. Nonetheless, this study provides a biologic plausibility that urges further research in this context. A prospective trial of donor dopamine administration on outcome of cardiac transplants seems to be justified and should ideally also look at the outcome of all organs transplanted from those donors, to assess the efficacy of donor dopamine pretreatment in general. The in vitro finding that NOD might be superior to dopamine is intriguing but requires further in vivo exploration in relevant transplantation models. Moreover, because the hydrophobicity of drug compounds makes intravenous application complicated, an extensive structure activity study may also further help understanding how structural alterations of NOD affect efficacy of protection and may provide new protective compounds with lower hydrophobicity. Attempts should also be made to provide putative targets by which NOD is mediating its protective effect and how redox activity and hydrophobicity fit in this model.

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Authorship Contributions

Participated in research design: Vettel, Hottenrott, Hoeger, Wieland, Yard.

Conducted experiments: Vettel, Hottenrott, Spindler, Hoeger.

Contributed new reagents or analytic tools: Tsagogiorgas, Spindler.

Performed data analysis: Tsagogiorgas, Hoeger, Bench, Schnuelle.

Wrote or contributed to the writing of the manuscript: Vettel, Hottenrott, Bench, Krämer, Schnuelle, El-Armouche, Yard, Wieland.

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Legends for supplemental videos

**Video 1:** Cultured NRCM were subjected to 24 h of cold preservation in UW solution. Hereafter the preservation solution was exchanged for normal NRCM culture medium and the cells were rewarmed for 2 h. Spontaneous contractions were studied by phase-contrast microscopy equipped with video options. Note that no spontaneous contractions of cardiomyocytes were observed.

**Video 2:** Cultured NRCM pre-treated for 2 h with 50µM of NOD and subsequently subjected to 24 h of cold preservation in UW solution. Hereafter the preservation solution was exchanged for normal NRCM culture medium and the cells were rewarmed for 2 h. Spontaneous contractions were studied by phase-contrast microscopy equipped with video options. Note that in NOD pre-treated NRCM spontaneous contractions of cardiomyocytes were observed.