Brain dead donor graft deterioration and attenuation with N-octanoyl dopamine preconditioning

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CHAPTER 2

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. Since 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, NOD or were left untreated and subsequently subjected to static cold storage (8-12 h). Dopamine and NOD treated cardiomyocytes displayed a better viability compared to untreated cells after hypothermia. In untreated cardiomyocytes, cell damage was reflected by lactate-dehydrogenase (LDH) release and a decrease in intracellular ATP. NOD was about 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 re-warming 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 β_1-adrenoceptor antagonist atenolol and D_1/D_2 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. 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 [1, 2]. Iron mediated production of reactive oxygen species (ROS), impairment of Ca^{2+} homeostasis and ATP depletion seem to be of instrumental importance in the sequel of hypothermic preservation injury [3-6]. Earlier reports have concluded that prolonged cold ischemic time is not a risk factor for decreased long-term survival in cardiac allograft recipients [7], yet more recent studies have indicated that it is a predictor of five year conditional mortality [8]. The effect of cold ischemic time on survival after heart transplantation is dependent on donor age, with greater tolerance among grafts from younger donors [9]. Cold ischemic time also shows a significant linear relationship with post transplantation length of stay in the intensive care unit [10]. Hence, there is a socio-economical demand for measures that improve cardiac allograft preservation over longer periods of time without affecting cardiac transplantation outcome [11].

Clinical studies have shown that dopamine (DA) treatment of the donor reduces the incidence of delayed graft function after renal transplantation [12, 13]. The beneficial effect of DA was more pronounced when cold ischemic time exceeded 17 hours, and this in turn, translated in a better graft survival in patients in this particular subgroup [12]. Based on a substantial amount of in vitro studies current evidence indicates that DA mitigates cell damage related to cold preservation. [4, 14, 15]. The cytoprotective properties of DA directly originate from structural entities, which enable the molecule to act as an iron chelator and to scavenge ROS [15].

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 three years. [16]. It must be mentioned however 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 biological 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 cytprotective properties compared to DA in protecting cardiomyocytes against cold preservation injury and that this does not require catecholamine receptor engagement [17].

chapter 2

Materials and Methods

Animal care

Care of the animals was taken in accordance with the Committee on Animal Research of the regional government (Regierunspräsidium Karlsruhe, Germany), who 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 (NRCM)

One to three day old Wistar rats were sacrificed by decapitation and the hearts were removed. Cardiomyocytes were isolated from hearts as described previously [18]. Briefly, 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, Germany) to release single cells. The cell suspension was placed on a Percoll™-gradient (GE-Healthcare, Freiburg, Germany) to separate cardiomyocytes from other cell types. Thereafter the myocyte fraction was seeded in 12- or 24 well plates at a density of 300,000 and 150,000 cells/cavity respectively and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (PAA), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM 5'-bromo-2'-deoxyuridine (BrdU) to prevent overgrowth of non-cardiomyocyte cell types. In all cultures cell contractions were observed after 24 h. For all experiments the cells were used 3 to 4 days after isolation.

Cold storage and re-warming

Cardiomyocytes were stimulated for 1 h with indicated substances. Hereafter, the cells were extensively washed with phosphate buffered saline and then stored at 4 °C in University of Wisconsin (UW, Southard and Belzer, 1995) solution for 8-12 h. The incubation time was based on initial experiments, showing that preservation shorter than 6 h did not significantly damage the cells. Cells were evaluated either directly after cold storage or after a re-warming period of 1 h 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 OD490 nm, corrected for the blank value. Each concentration was tested in triplicate in all experiments. The EC_{50} values were estimated from a total of 5 experiments.

Assessment of intracellular ATP amount

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

Determination of cAMP formation

Cardiomyocytes were subjected to cold preservation conditions for 12h. After 30 min of re-warming at cell culture conditions, 1 mM of IBMX was applied and cells were incubated for an additional 30 min. Thereafter the cardiomyocytes were stimulated for 10 min with the indicated concentrations of isoprenaline and then lysed in 0.1 M HCl. cAMP formation was assessed by using enzyme linked immunosorbant assay kits (cAMP EIA Kit, Biomol, Hamburg, Germany for concentration dependent curves and after CS, and 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 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 anaesthetised with 6 mg/body weight of xylazine (Rompun 2%®,

Bayer Vital GmbH, Leverkusen, Germany) and 100 mg/body weight of ketamine (Ketamin 10%®, Intervet GmbH, Unterschleißheim, Germany) and heparinized (100 IE, Heparin-Natrium ratiopharm®, Ratiopharm GmbH, 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 super-hepatic vena cava with 30 ml, 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 and 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 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, Mannheim, Germany). 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 ± SEM and were based on three or more separate experiments. Differences between groups were determined by Student's t-test or one-way Anova followed by Bonferroni's multiple-comparison. A *p*-value of less than 0.05 was considered statistically significant. Mathematical curve fitting and calculation of EC_{50}- or IC_{50}-values was performed with GraphPad Prism5 (GraphPad Software, San Diego).

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. While cold storage up to 4 h was not associated with profound cell damage, LDH release in the supernatants significantly increased with increasing preservation time (F*igure 1A)*. Since LDH release reached a maximum between 8 and 24 h of cold storage, most experiments were performed using a cold storage time of 8 h 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 h with increasing concentrations of DA or NOD and subsequently subjected to 8 h 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 (F*igure 1B*). In line with the observation that DA or NOD pre-treatment was protective, the cellular ATP content was significantly higher in treated than in untreated cells after cold storage (DA or NOD treated vs. untreated, $p < 0.01$, F*igure 1C*). In both settings, NOD was more potent than DA. Half-maximal inhibition of LDH release and ATP depletion occurred at about 20-fold lower NOD than DA concentrations. (LDH release IC_{50} DA vs. NOD: 50 ± 25 μM vs. 1.3 ± 0.3 μM; ATP production EC_{50} 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 re-warmed in culture medium directly after cold storage, ATP regeneration did not occur in untreated cardiomyocytes. In contrast, 1 h of re-warming was sufficient to regain similar intracellular ATP concentrations in DA or NOD pre-treated cells as in NRCM not subjected to cold storage (F*igure 1D*). NOD was also protective under conditions similar to those occurring during heart explantation 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 h, 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 (approx. 35%, F*igure 1E*). A 4 h time period of cold storage was chosen as the usually accepted maximal time for organ preservation in human heart transplantation is to 4-5 h.

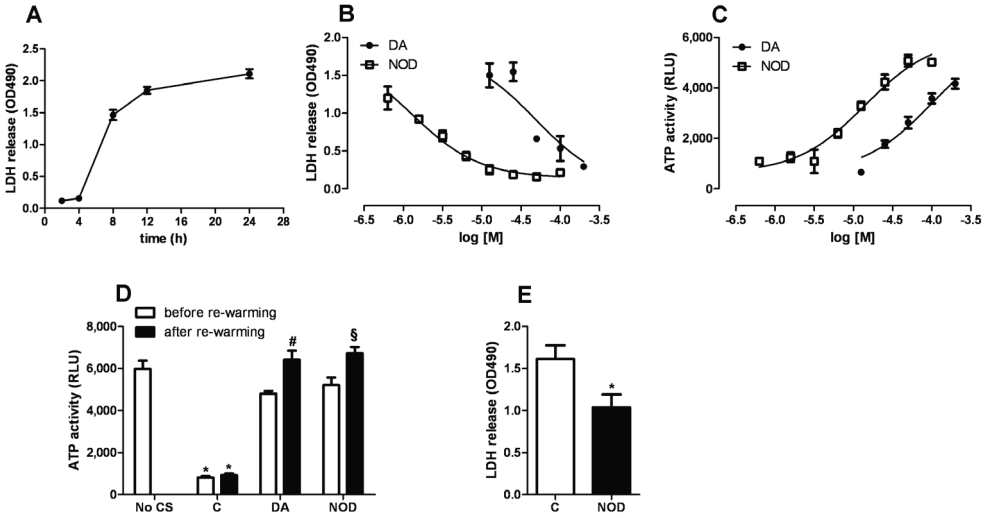

Figure 1. Influence of DA and NOD on cold preservation injury of cultured neonatal rat cardiomyocytes (NRCM) and perfused rat hearts. A. NRCM were subjected to various time intervals of cold storage. B. + C. NRCM were treated for 1 h with increasing concentrations of DA or NOD as indicated. Thereafter the cells were extensively washed with phosphate buffered saline and stored for 8 h at 4 ˚C in UW solution. LDH release in the preservation solution was assessed directly after cold preservation as described in the method section. The results are expressed as mean OD490 values ± SEM (B). Intracellular ATP was assessed directly after cold preservation by luciferase driven bioluminescence. The results are expressed as mean relative light units (RLU) ± SEM. Absolute ATP concentration in [nM/ mg protein] ranged from 13.2 ± 2.1 to 2.2 ± 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. D. NRCM were stimulated for 1 h with DA (100 μM) or NOD (50 μM). Hereafter the cells were extensively washed with phosphate buffered saline and stored for 8 h at 4 ˚C in UW solution. Intracellular ATP was measured either directly before (open bars) or after (filled bars) 1 h of re-warming 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) ± SEM. Absolute ATP concentration in [nM/ mg protein] were 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), 13.6 ± 2.2 and 17 ± 2.1 (NOD treated cells before and after rewarming respectively). A total of 6 independent experiments were performed. For each experiment all conditions were tested in triplicates (* $p \leq 0.05$ vs. No CS, $^{\#}p \leq 0.05$ vs. DA before re-warming, $^{\S}p \leq 0.05$ vs. NOD before re-warming). E. Rat hearts were perfused with UW or UW containing 50 μM NOD prior to explantation. After 4 h at 4 °C LDH-release was determined as described in the method section. Results are expressed as mean values ± SEM, n = 4 for each group (* $p \leq 0.05$ vs. control).

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 re-warming. To address this issue, we stuied 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 pre-treatment, spontaneous cardiomyocyte contractions were not detectable immediately after cold storage. However, when the preservation solution was replaced by culture medium and cells were re-warmed to 37 °C for 1 h, in the majority of cavities of a 24 well plate DA or NOD pre-treated NRCM regained their ability to contract spontaneously-(DA 89%, NOD 94%, F*igure 2A*). In untreated NRCM spontaneous contraction after cold storage and rewarming was only observed in 16% of the cavities. When untreated cardiomyocytes were maintained in culture for another 24 h after cold preservation their ability to contract ceased while the beating capacity remained constant in DA and NOD treated cells. A video of such a comparison is presented in the supplement (S1 no treatment; S2 NOD treatment). To further demonstrate that protected NRCM regain functionality after rewarming the ability to respond to positive inotropic stimuli, was assessed by measuring by β-adrenoceptor induced cAMP formation after 12 h of cold storage and 1 h of re-warming. The β-adrenoceptor agonist isoprenaline (ISO) concentration-dependently increased cAMP production in DA or NOD pre-treated cardiomyocytes whereas the efficacy of ISO to induce cAMP formation was severely impaired in untreated cells that were subjected to cold preservation and re-warming (F*igure 2B*). As shown in *figure 2C*, the maximal extent of ISO-stimulated cAMP formation in DA- or NOD-pretreated NRCM was not significantly different to cardiomyocytes that were not subjected to cold preservation.

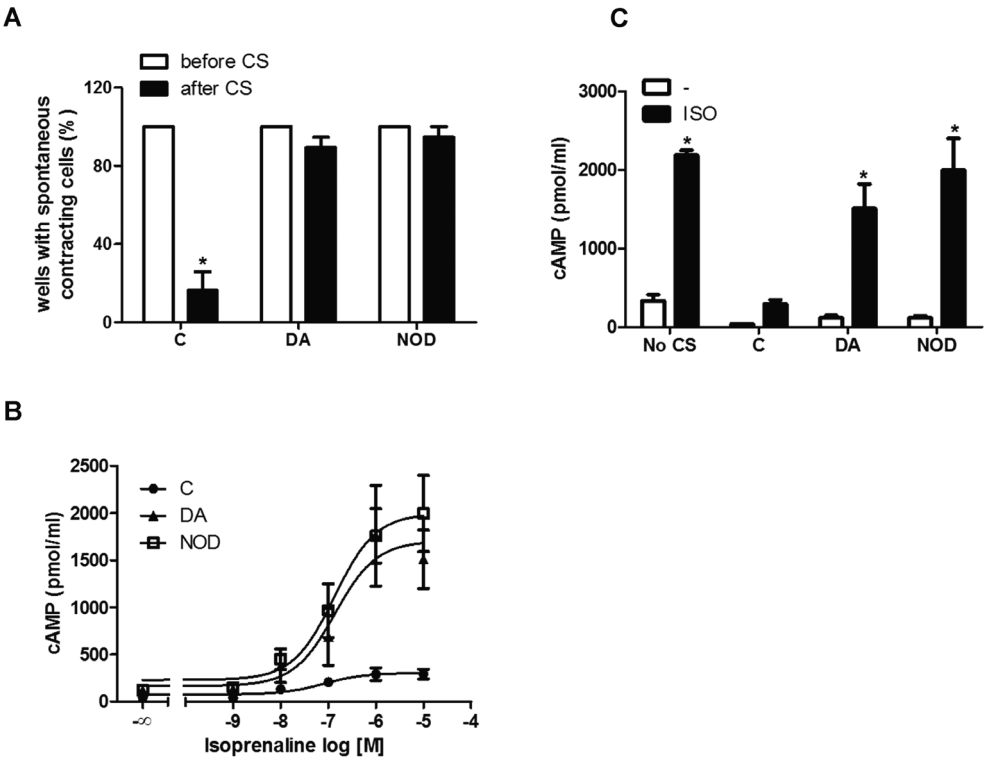

Figure 2. Influence of DA and NOD treatment on cardiomyocyte function after cold preservation and re-warming. Cardiomyocyte contractions were determined microscopically using a phase-contrast microscope. A. NRCM were treated with DA (100 μM) or NOD (50 μM) for 1 h or were left untreated. The percentage of wells containing contracting cardiomyocytes was assessed before cold storage (open bars) or after cold preservation followed by 1 h of re-warming (filled bars). The results are expressed as the mean percentage of wells that contain contracting cardiomyocytes ± SEM, irrespective of the number of contracting cells (* $p \leq 0.05$ vs. control before CS). B. Isoprenaline oncentration-response curve in DA or NOD-treated and untreated cells after cold preservation and re-warming. Cardiomyocytes were treated with DA (100 μM), NOD (50 μM) for 1 h prior to cold storage or left untreated. After re-warming, NRCM were stimulated with the indicated concentrations of the β_1/β_2-adrenoceptor agonist isoprenaline (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 ± SEM (* $p \leq 0.05$ vs. not treated control). In B and C a total of 3 independent experiments were performed. For each experiment all conditions were tested in duplicates.

Similar to NOD, lipophilic, esterified derivates of gentisic acid protect cardiomyocytes from cold storage injury

To explore the structure-activity relationship of DA and NOD, we additionally 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 f*igure 3A*, all three substances are derivates of gentisic

acid (2,5-hydroxybenzoic acid), a naturally occurring compound with known radical scavenging properties [19]. The para position and esterification of the hydroxy groups of these compounds makes them however unlikely candidates for any activation of β-adrenoceptors. Yet, similar toDA 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 case of BB (F*igure 3A*). To compare the cytoprotective effects of these genistic acid derivates cardiomyocytes were treated with 50 μM BB, BBNB and BBNO 1 h prior to cold storage. A comparable protection from cold-induced cell damage by NOD (F*igure 1)* was only observed with BBNB and BBNO in which a hydrophobic side chain was added to the aromatic core (F*igure 3B*).

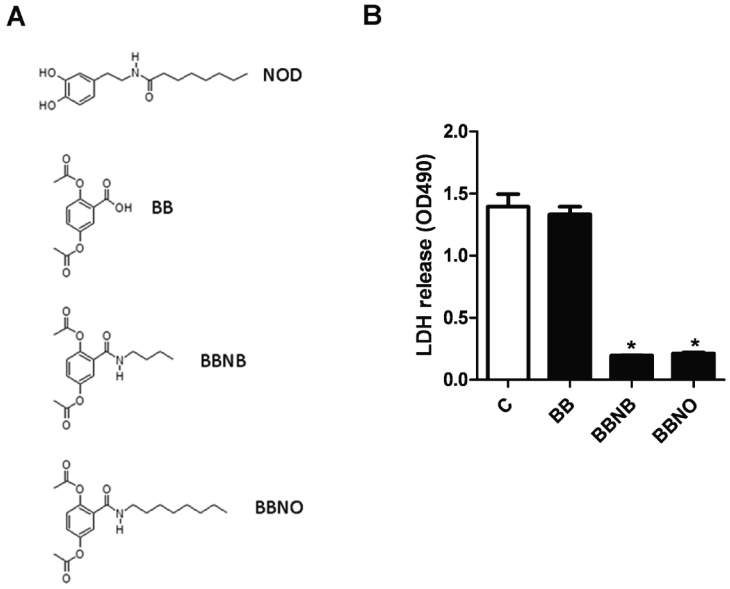

Figure 3. The cytoprotective effect requires a lipophilic structure with radical scavenging redox potential. A. Chemical structure of N-octanoyl dopamine (NOD), 2,5-bisacetoxy-benzoic acid (BB), 2,5-bisacetoxybenzoyl-N-butylamide (BBNB) and 2,5-bisacetoxybenzoyl-N-octanoylamide (BBNO). B. NRCM were treated with the gentisic acid derivates BBNO, BBNB, or BB (50 μM). Thereafter cells were extensively washed with phosphate buffered saline and stored for 8 h at 4 ˚C in UW solution. LDH release in the preservation solution was assessed directly after cold preservation as described in the method section. The results are expressed as mean OD490 values ± SEM. A total of six independent experiments were performed. For each experiment all conditions were tested in triplicates (* $p \leq 0.05$ vs. not treated control (C)).

Neither β-adrenoceptor nor D_1/D_2 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 if 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 to isoprenaline, which increases intracellular cAMP formation approximately by 10-fold, only DA led to a significant increase in intracellular cAMP, while no significant rise in intracellular cAMP concentration was observed after stimulation with 50 μM of NOD, BB, BBNB or BBNO (F*igure 4A*). The DA-induced increase in intracellular cAMP levels could be completely blocked by addition of the β_1-adrenoceptor specific antagonist atenolol (F*igure 4B*). Although we have previously demonstrated that the cytoprotective effect of dopamine could not be overcome by β-adrenoceptor blockade [15], this has not been tested thus far for NOD. To exclude any receptor engagement in the cytoprotective properties of NOD cardiomyocytes were treated for 1 h with NOD alone or with NOD in the presence of 10 μM atenolol prior to 8 h of cold storage. As shown (F*igure 4C*), pre-treated cardiomyocytes were equally protected against cold-induced damage, independently of β_1-adrenergic activation. Similarly, the D_1/D_2-receptor antagonist fluphenazine neither abrogated the protective effect of dopamine nor that of NOD over large range of fluphenazine concentrations (F*igure 4D)*, which data exclude the involvement of dopaminergic receptors on the protection of cardiomyocytes to cold inflicted damage.

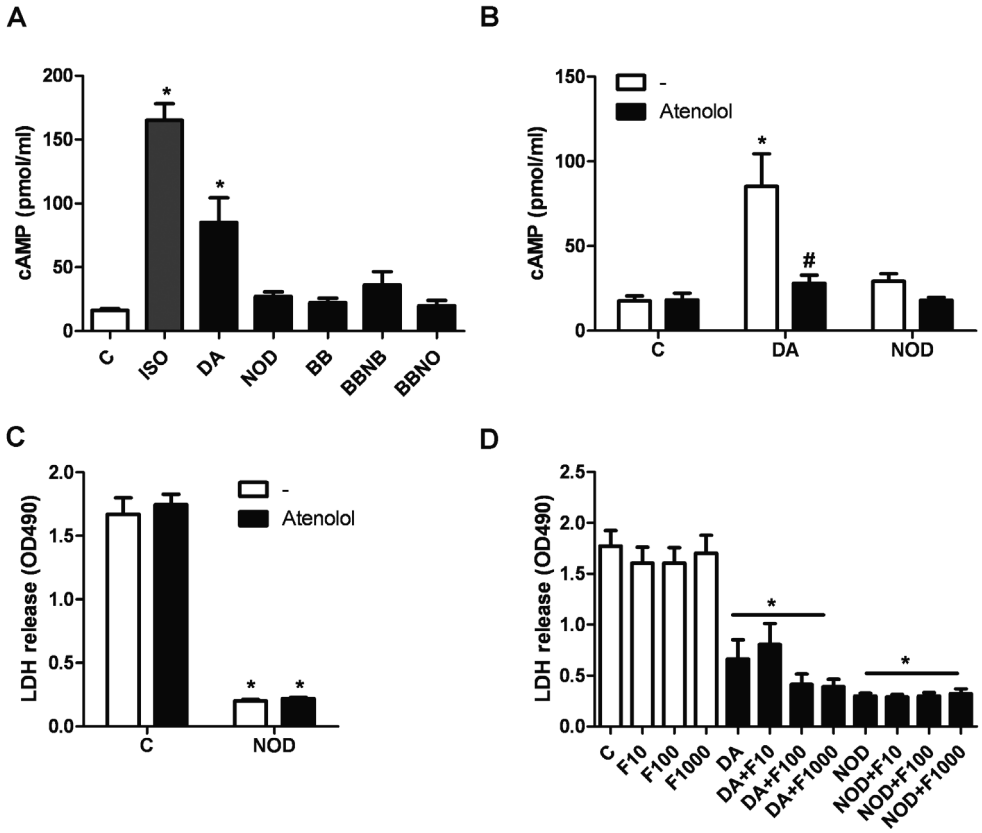

Figure 4. The cytoprotective effect does not require β-adrenoceptor or D_1/D_2 receptor engagement. A. β-adrenoceptor–induced cAMP formation by isoprenaline (ISO, 10 μM), DA (100 μM), NOD (50 μM), and the gentisic acid derivates BB, BBNB and BBNO (50 μM each). B. cAMP formation in DA treated NRCM was inhibited by 10 μM atenolol. In A and B, cAMP formation was quantified after 10 min of stimulation in the presence of 1 mM IBMX. All samples were tested in duplicate. A total of 4 experiments were performed, the results are expressed as mean cAMP concentration [pmol/ ml] ± SEM (* $p \leq 0.05$ vs. control (C), # $p \leq 0.05$. DA vs. DA plus atenolol). C. NRCM were treated for 1 h with NOD (50 μM) or left untreated. To each of these conditions 10 μM of atenolol was added. Hereafter the cells were subjected to 8 h of cold storage and supernatants were collected to assess the LDH release. The results are expressed as mean OD490 values ± SEM. A total of three independent experiments were performed. For each experiment all conditions were tested in triplicates (* $p \leq 0.05$ vs. untreated control). D. NRCM were treated for 1 h with DA (100 μM), NOD (50 μM) (filled bars) or normal culture medium (open bars) in the presence of different fluphenazine concentrations (F; 10, 100, 1000 nM). In all experiments DA, NOD or culture medium alone (C) without addition of fluphenazine was included. Hereafter the cells were subjected to 24 h of cold storage and supernatants were collected to assess the LDH release. The results are expressed as mean OD490 values ± SEM. A total of three independent experiments were performed. For each experiment all conditions were tested in triplicates (* $p \leq 0.05$ vs. medium controls (open bars)).

Discussion

Dopamine and its lipophilic derivates provide cytoprotection from cold preservation injury based on their redox potential and cellular uptake

In the present study we sought to explore the biological 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 [16]. We hypothesized that dopamine pre-treatment increases the viability of cardiomyocytes during cold preservation and that NOD is superior in this regard. Our data clearly substantiate this hypothesis as pre-treatment 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 prevented, and as a consequence spontaneous contractility as well as responsiveness to adrenergic stimuli is preserved upon re-warming.

Our data further indicate that the beneficial effects on NRCM are independent of β_1-adrenoceptor or D_1/D_2 dopaminergic receptor engagement. At the concentrations used, only DA stimulated cAMP formation in NRCM by activation of β_1-adrenoceptors. It is well documented that dopamine displays β-adrenoceptor agonism at higher concentrations (>3 μM), whereas cardiac effects at lower concentrations are attributed to DA-induced release of noradrenaline in vivo [20]. The DA derivate NOD on the other hand has no hemodynamic effects when applied *in vivo*, [17] but is more potent with regard to cytoprotection. Like the derivates of gentisic acid, NOD did not induce a significant elevation of intracellular cAMP and its protective effect was unaffected by β_1-adrenoceptor antagonists.

It has recently been described that various dopamine D_1 receptor agonists exert cytoprotective effects on oxidative injury in cultured cardiomyocytes which raises the question of dopamine receptor involvement [21]. Gerö et al. however propose that some of the positively tested D_1 receptor agonists confer cytoprotection through indirect inhibition of the poly(ADP-ribose) polymerase, without receptor engagement [21]. Another study on ischemia/reperfusion injury in neonate rat cardiomyocytes supports that cardiac D_1 receptors are not involved in cytoprotection, since selective activation of D_1 receptors with the agonist SKF-38393 induces apoptotic cell death [22]. As NOD has been shown not to act on dopamine receptors, [17] our data clearly support that DA's cytoprotective action does not require dopamine receptor engagement but depends on the molecule's redox activity and its relative hydrophobicity [4, 16, 22]. 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_1/D_2 receptor antagonist fluphenazine. While the redox activity of DA and NOD is similar, NOD displays a higher degree of hydrophobicity compared to DA, which allows improved cellular uptake [17]. Subsequently higher intracellular concentrations can be achieved with lower dosages and thus reaching cytoprotective intracellular levels at much lower concentrations [17]. This likely explains the higher potency of NOD compared to DA in protection from cold storage injury. The data obtained with gentisic acid derivates clearly support the hypothesis that an intracellular redox potential and sufficient cellular uptake are required for cytoprotective activity. Gentisic acid is a naturally occurring compound in mould fungi and plants [19]. It also is a naturally occurring metabolite of acetylsalicylic acid [23]. While its high redox potential has been clearly demonstrated *in vitro*, both *in vitro* and *in vivo* findings suggest that this property may convey protection towards geno- and hepatotoxicity of cyclophosphamide and may lower LDL oxidation [19, 24, 25]. We have modified gentisic acid in two ways. The free phenolic hydroxyl groups have been esterified a) to protect the compound from oxidation outside of the cell where they cannot be hydrolized by esterases and b) to increase hydrophobicity and thus increase cellular uptake. In analogy to the structure of NOD, longer lipophilic side chains have been applied as amides of gentisic acids. Our data clearly show that like in NOD, the lipophilic side chain enhances the ability of the compounds to provide cytoprotection already at low concentrations.

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, since 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. Due to the enhanced susceptibility of the cardiac allograft, it is of utmost importance to keep cold ischemic time below 4 h in heart transplantation [2, 26]. A considerable number of heart transplants are still performed after prolonged cold ischemia, which promotes early graft dysfunction and increases mortality post transplantation [2]. 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 centres [16]. 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 (MAO) and catechol-O-methyl transferase (COMT). 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, wwe reported that DA treatment was prematurely discontinued in 12.5% of donors due to circulatory side effects, such as tachycardia and/or hypertension [12]. Such a discontinuation of DA infusion will however result in rapid loss of protection due to MAO and COMT 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, pre-clinical data on mitigation of ischemia induced acute kidney injury in rats are promising and suggest superior protection than DA [27]. 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 biological plausibility for our observation that donor preconditioning with DA may improve the outcome after heart transplantation. Since 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 proof that the beneficial effect of donor dopamine treatment on cardiac transplantation outcome is due to the protective properties of dopamine on hypothermic preservation injury. Nonetheless, this study provides a biological 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 pre-treatment 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.

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