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ORIGINAL ARTICLE

Bilirubin rinse of the graft ameliorates ischemia reperfusion injury in heart transplantation

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Summary

Ischemia and reperfusion contribute to substantial organ damage in transplantation. Clinically feasible measures for the prevention thereof are scarce. We tested whether rinsing rodent hearts with the antioxidant bilirubin ameliorates ischemia reperfusion injury (IRI). Left ventricular end-diastolic pressure (LVEDP), left ventricular developed pressure (LVDevP), rate per pressure product (RPP), coronary flow, maximum ($+dP/dt$) and minimum ($-dP/dt$) rate of contraction were analyzed in Lewis rat hearts rinsed with bilirubin prior to reperfusion on a Langendorff apparatus after 12 h of cold ischemia. *In vivo*, isogenic C57Bl/6 mouse hearts rinsed with bilirubin were transplanted after 12 h of cold ischemia. Cardiac function and apoptosis were assessed 24 h after reperfusion. Heart lysates recovered 15 min after reperfusion were probed for the total and the phosphorylated forms of extracellular signal-related protein kinases (ERK), JNK, p38-MAPK, and Akt. In isolated perfused hearts, bilirubin rinse resulted in significantly lower LVEDP and improved LVDevP, RPP, coronary flow, $+dP/dt$ and $-dP/dt$. *In vivo*, after reperfusion, all mitogen-activated protein kinases (MAPKs) were suppressed significantly by bilirubin pretreatment. Bilirubin rinse improved cardiac scores (3.4 ± 0.5 vs. 2.0 ± 1.0 in controls, $P < 0.05$) and significantly suppressed apoptosis. *Ex vivo* administration of bilirubin to heart grafts prior reperfusion ameliorates IRI and provides a simple and effective tool to ameliorate outcome in heart transplantation.

Introduction

Drastically increasing numbers of extended criteria deceased donors are used recently due to the lack of optimal grafts for solid organ transplantation [1,2]. To improve short- and long-term outcomes in recipients of those 'marginal' organs that are maximally prone to ischemia reperfusion injury (IRI)-mediated damage, strategies that reduce reperfusion injury have to be established. Among them, without ethical constraints for the deceased donor and without the risk of unwanted side effects–drug interactions of systemically applied drugs in the recipient, local treatment of the allograft is a promising approach.

The family of mitogen-activated protein kinases (MAPK) includes extracellular signal-related protein kinases (ERKs), p38-MAPK, and stress-activated protein kinases/c-Jun NH2-terminal protein kinases (SAPKs/JNKs). Activated MAPK pathways play an important role in cell differentiation, cell proliferation, cell death, cell homeostasis, IRI, and a variety of human diseases [3–5]. In solid organ transplantation, activation of the MAPKs occurs directly after reperfusion in the recipient due to oxidative stress [6–8]. Modulation of MAPK signaling has an

impact on organ function in various animal models of IRI [9–15].

Heme oxygenase-1 (HO-1) is an ubiquitous enzyme that has multiple beneficial effects when overexpressed [16,17] HO-1 catalyzes the rate-limiting step in the conversion of heme into carbon monoxide, free iron, and biliverdin, which subsequently is converted to bilirubin by the enzyme biliverdin reductase (BVR) [18]. Even a plethora of experimental studies has underlined the beneficial effects of HO-1; ways to clinically use the HO-1 system are still lacking. The very potent antioxidants biliverdin and bilirubin [19] account, at least in part, for many of the beneficial effects of HO-1 induction. Their use in humans presumably is safe, as both are naturally occurring in mammals and supranormal (but not pathological) serum levels of bilirubin are associated with an inverse correlation to the incidence of several diseases [20–24]. Both have been shown to ameliorate IRI in experimental models *ex vivo* and *in vivo* [23].

Based on the rationale that bilirubin has been shown to effectively ameliorate IRI and that reperfusion injury is initiated shortly after reperfusion, we hypothesized that having the potent antioxidant bilirubin 'on board' after local administration, via a simple rinse prior to reperfusion, would limit cardiac IRI after heart transplantation validated by measuring cardiac function, early MAPK activation, and apoptosis.

Methods

Reagents

Bilirubin and biliverdin (Frontier Scientific, Logan, UT, USA) were dissolved in 0.2 N NaOH, neutralized with 1 N HCl to pH 7.4, adjusted to 10 mM concentration with PBS, sterilized by filtration, and stored at −20°C and dissolved in Ringer's lactate (RL) for experimental use. Bilirubin ditaurate (Frontier Scientific) was dissolved in sterile water. All experiments were carried out avoiding direct light.

Isolated perfused heart

Function of the left ventricle (LV) was assessed using a standardized Langendorff apparatus. Lewis rat hearts (LEW/CrlBR; Charles River, Wilmington, MA, USA; n = 6–8/group) were rapidly excised after heparinization (500 U), perfused with and stored in 4 °C UW solution (Viaspan, Wilmington, MA, USA) for an extended period of cold ischemia of 12 h. At the end of cold ischemia time (CIT), hearts were rinsed with bilirubin (125 μM), biliverdin (125 μM), or RL via a 22-g canula for 2 min (1 ml/min) via the aorta, kept at 4 °C for additional 15 min, and then mounted and perfused with phosphate-free Krebs–Henseleit buffer on the Langendorff apparatus. Control hearts with no CIT were mounted immediately after recovery. A water-filled balloon was inserted into the LV for recording of ventricular pressure and heart rate with the use of a commercially available data acquisition system (MacLab, ADInstruments, Colorado Springs, CO, USA). Balloon volume was adjusted to achieve a left ventricular end-diastolic pressure (LVEDP) of 0 mmHg. After stabilization for 30 min, the balloon volume was increased by 50 μl every 5 min as described elsewhere (Fig. 1a) [25].

Mouse heart transplantation

Isogeneic hearts of 10-week-old male C57BL/6 (Charles River, Sulzfeld, Germany) donors were heterotopically transplanted using a standardized nonsuture cuff technique [8]. Briefly, arterial anastomosis was made between the aorta of the graft and the carotid artery of the recipient. Venous drainage was achieved by connecting the pulmonary artery to the external jugular vein. In sham-operated controls, the sternocleidomastoid muscle was resected and the jugular vein and carotid artery were ligated. After CIT (12 h), hearts were rinsed with or without bilirubin or bilirubin ditaurate (12.5 μM or 125 μM) containing RL (0.5 ml/min for 2 min) via the aorta using a 22-g canula followed by anastomosis. Control hearts without CIT were transplanted immediately after recovery. Anastomosis time was kept constantly at 30 min (Fig. 2a). For systemic treatment, bilirubin was applied i.v. as a single shot prior to reperfusion at a dose of 14 mg/kg (i.e. 0.5 ml of 125 μM bilirubin in RL). Cardiac function was assessed by palpation using a standardized score ranging from 1 to 4 [26]. Grafts were recovered at 15 min and 12 or 24 h after reperfusion and immediately snap-frozen in liquid nitrogen or fixed in buffered 4% formaldehyde. Blood samples were drawn at 12 h after reperfusion.

Measurement of CK-MB

Myocardial creatine kinase MB (CK-MB) was assessed in the serum of the mice using the Granutest 15 assay kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions (REP/EDC system, Helena Laboratories, Beaumont, TX, USA) [27].

Anesthesia/analgesia

Rats for the Langendorff preparation were anesthetized using ketamine (87 mg/kg) and xylazine (13 mg/kg). Mice were anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg). Postoperative analgesia was done with s.c. buprenorphine injections every 8 h after the operation until the end of the experiment (0.1 mg/kg). Euthanasia was done by applying a lethal dose of the anesthetic.

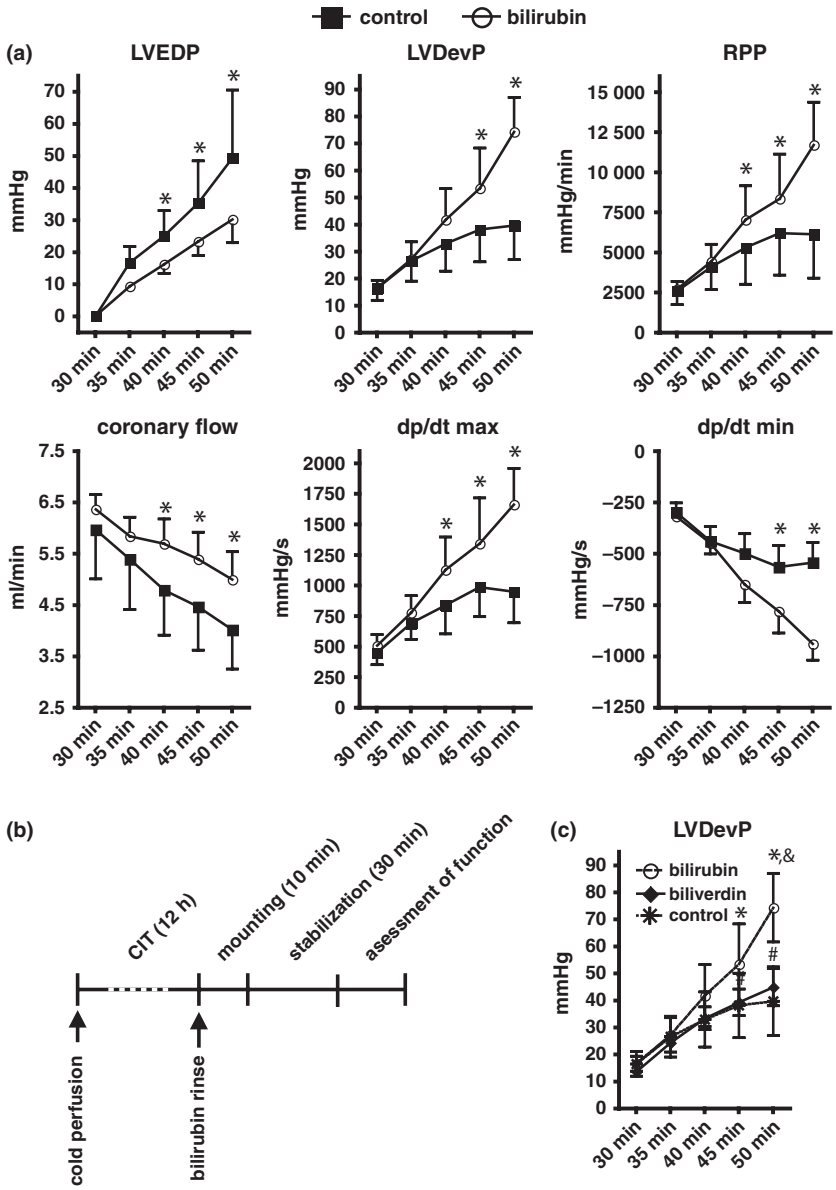

Figure 1 (a) Bilirubin rinse prior to reperfusion improves cardiac performance in *ex vivo* perfused rat hearts. Lower LVEDP and improved LVDevP, RPP, coronary flow, +d*P*/d*t,* and −d*P*/d*t* were observed ($P < 0.05$ for all parameters vs. control). (b) Graph depicting ischemia time, treatment, and assessment of function on the Langendorff apparatus. (c) Biliverdin does not improve cardiac performance in *ex vivo* perfused rat hearts (*$P < 0.05$ vs. control; &$P < 0.05$ vs. biliverdin; #P = NS vs. control).

All experiments were carried out respecting the Directive 2010/63/EU of the European Parliament. Approval for the animal experiments was given by the Austrian 'Bundesministerium für Wissenschaft und Forschung' (BMWF-66011-67-2007).

Western blotting

Protein separation, immunoblotting, and chemoluminescence were performed as previously described [28]. Primary antibodies directed against the total and the phosphorylated forms of ERK, JNK, p38, and Akt were used. Blots were quantified measuring optical density (OD) using IMAGEJ software (http://imagej.nih.gov/ij/).

TUNEL staining

Five serial sections (every 200 μm) of each heart graft were cut from formalin-fixed, paraffin-embedded blocks. Multiple sections were stained using the *In Situ* Cell

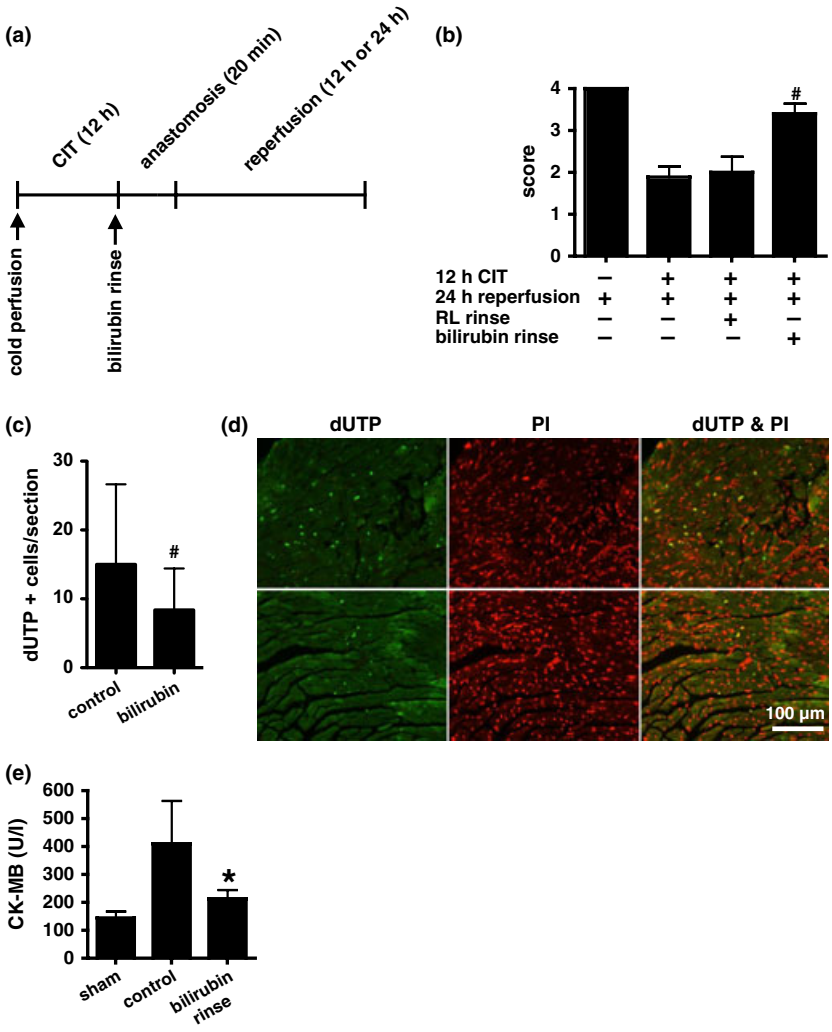

Figure 2 (a) Mouse heart grafts were rinsed with bilirubin at 125 µM prior to anastomosis. (b) Bilirubin rinsed grafts showed significantly better cardiac scores when compared with RL-rinsed controls (#$P < 0.05$). (c, d) dUTP and propidium iodide (PI) staining of heart grafts 24 h after reperfusion. Bilirubin rinsed hearts showed significantly less apoptotic cells when compared with controls (#$P < 0.05$). (e) CK-MB levels of heart grafts. Bilirubin rinsed grafts had significantly lower serum levels of CK-MB (*$P < 0.001$).

Death Detection Kit, Fluorescin (Roche Diagnostics GmbH, Vienna, Austria). TUNEL positive nuclei per section were counted at a magnification of 10-fold in a blinded fashion.

Assessment of serum bilirubin concentrations

Blood samples for measuring serum bilirubin were acquired at 5 min after reperfusion in mice that were untreated, treated with a bilirubin rinse (0.5 ml, 125 µM) or treated systemically i.v. (0.5 ml, 125 µM; i.e. 14 mg/kg). Bilirubin levels were measured with the total bilirubin assay kit (Sigma Aldrich, Hamburg, Germany) in duplicates.

Statistical analysis

Statistical analysis was performed with the GRAPHPAD PRISM 4 (Graphpad Software, La Jolla, CA, USA) for Mac using ANOVA. Results are expressed as mean ± standard deviation (SD).

Results

Bilirubin rinse ameliorates left ventricular function during reperfusion in isolated perfused hearts

Rat hearts that underwent 12 h of CIT showed a significantly higher LVEDP (57.6 ± 16.5 mmHg vs. 10.9 ± 2.3 mmHg, $P < 0.01$), a decrease in left ventricular developed pressure (LVDevP, 43.7 ± 12.6 mmHg vs.

64.8 ± 11.8 mmHg, $P < 0.05$), rate per pressure product RPP (7111 ± 2402 vs. 13 462 ± 3497, $P < 0.05$), coronary flow (3.5 ± 1.2 ml/min vs. 6.2 ± 2.5 ml/min, $P < 0.05$), maximum rate of contraction (+d*P*/d*t*; 865.0 ± 230.2 mmHG/s vs. 1898.0 ± 417.5 mmHG/s, $P < 0.05$), and relaxation (−d*P*/d*t*; −491.7 ± 205.0 mmHg/s vs. −956.6 ± 178.9 mmHg/s, $P < 0.05$) when compared with hearts without CIT measured 50 min after reperfusion (data not shown). When hearts were rinsed with bilirubin at a concentration of 125 μM 15 min prior to reperfusion, a significantly lower LVEDP (30.3 ± 7.2 mmHg) and improved LVDevP (74.4 ± 12.7 mmHg), RPP (11 735 ± 2647), coronary flow (5.0 ± 1.1 ml/min), +d*P*/d*t* (1665.5 ± 295.5 mmHg/s), and −d*P*/d*t* (937.7± 159.3 mmHg/s, $P < 0.05$ for all parameters vs. control) were observed (Fig. 1b). In contrast, a rinse with biliverdin at a concentration of 125 μM prior to reperfusion showed no beneficial effects on left ventricular developed pressure (LVDevP 45.0 ± 11.9, $P = 0.6$ vs. control and $P = 0.026$ vs. bilirubin; Fig 1c).

Bilirubin rinse improves functional scores in a mouse model of heart transplantation

Mouse hearts transplanted after 12 h of cold ischemia showed significantly worse functional scores (2.0 ± 1.1) when compared with control hearts that were transplanted immediately after recovery (3.8 ± 0.4, $P = 0.0019$) as assessed 24 h after reperfusion. When hearts were rinsed with bilirubin dissolved in RL at a concentration of 125 μM prior to anastomosis (Fig. 2b), a significant improvement of cardiac grafts was seen (score 3.4 ± 0.5) when compared with the 12-h cold ischemia controls that were rinsed with RL alone (score 2.0 ± 1.0, $P = 0.0212$; Fig. 2b). This effect was not seen when bilirubin was used at a concentration of 12.5 μM (data not shown).

Bilirubin rinse reduces the number of apoptotic myocardial cells and CK-MB levels

At 24 h after reperfusion, a significant number of TUNEL positive nuclei was being observed in the 12-h cold ischemia control hearts ($n = 15.0 \pm 8$/section). Bilirubin-rinse-treated hearts showed significantly less TUNEL positive cells when compared with the controls ($n = 8.3 \pm 0.6$/section, $P < 0.0001$, Fig 2c and d). As a marker of myocardial injury, serum CK-MB was being assessed in hearts that underwent 12 h of cold ischemia. CK-MB dramatically increased when compared with sham-operated controls (410 U/l vs. 144 U/l, respectively; $P = 0.0051$). When hearts were treated with a single bilirubin rinse prior to reperfusion, CK-MB levels were significantly lower after 12 h of reperfusion (213 U/l, $P < 0.02$ vs. controls; Fig. 2e).

Bilirubin rinse decreases activation of MAPKs and PI3K/Akt

We analyzed activation of MAPKs that are involved in the mediation of cell injury, death, and inflammation related to IRI [8]. Grafts that were kept in UW solution and rinsed with RL with or without bilirubin were probed for the phosphorylated forms of p38 MAPK, JNK, ERK, and Akt. In control hearts, a dramatic increase in MAPK and Akt activation was observed at 15 min after reperfusion (ERK 12.9-fold; JNK 22.4-fold; p38 6.2 fold, Akt 2.3 fold; Fig. 3a–e). When hearts were rinsed with bilirubin prior to reperfusion, activation of MAPKs and Akt was clearly suppressed (ERK 6.7-fold, $P = 0.0001$; JNK 9.6-fold, $P = 0.0178$, p38 3.5 fold, $P = 0.041$; Akt 0.8 fold, $P = 0.0119$ vs. vehicle-treated control, Fig. 3a–e).

Beneficial effects were not seen when the grafts were rinsed with bilirubin ditaurate or after systemic treatment

We analyzed whether hydrophilic bilirubin ditaurate applied locally to the grafts improves outcomes after reperfusion in the recipient such as the lipophilic counterpart bilirubin. However, no improvement in cardiac scores (2.0 ± 0.6 vs. 1.8 ± 0.4 in the controls, $P = 0.56$) nor suppression of ERK activation was seen when the heart grafts were rinsed with bilirubin ditaurate prior to reperfusion (Fig. 4a and b).

Serum bilirubin levels in recipients after rinsing of the grafts with bilirubin did not show significant changes when compared with RL-rinsed controls (0.25 ± 0.10 mg/dl vs. 0.19 ± 0.09 mg/dl, $P = 0.47$, Fig 4c). We further analyzed whether the same amount of bilirubin applied locally to the grafts administered systemically would similarly improve outcomes after reperfusion in the recipient. However, no improvement in cardiac scores (2.5 ± 0.5 vs. 2.5 ± 0.5 in the controls, $P = 1.0$) nor suppression of ERK activation was seen under systemic treatment at this dose and time point when compared with the controls (Fig. 4d and e).

Discussion

Our study provides evidence that pretreatment of heart grafts with bilirubin prior to reperfusion ameliorates IRI *ex vivo* and *In vivo*. We initiated our studies based on the observation that induction of HO-1 in the organ donor (alone) ameliorates IRI [29]. As induction of HO-1 has not yet found its way into clinical application because specific and nontoxic promotors of HO-1 activity are lacking, research has focused on the end products of heme catabolism, namely CO, Fe++, and biliverdin/bilirubin that, at least in part, account for the beneficial effects seen under HO-1 induction [23,30]. Bilirubin is among the most

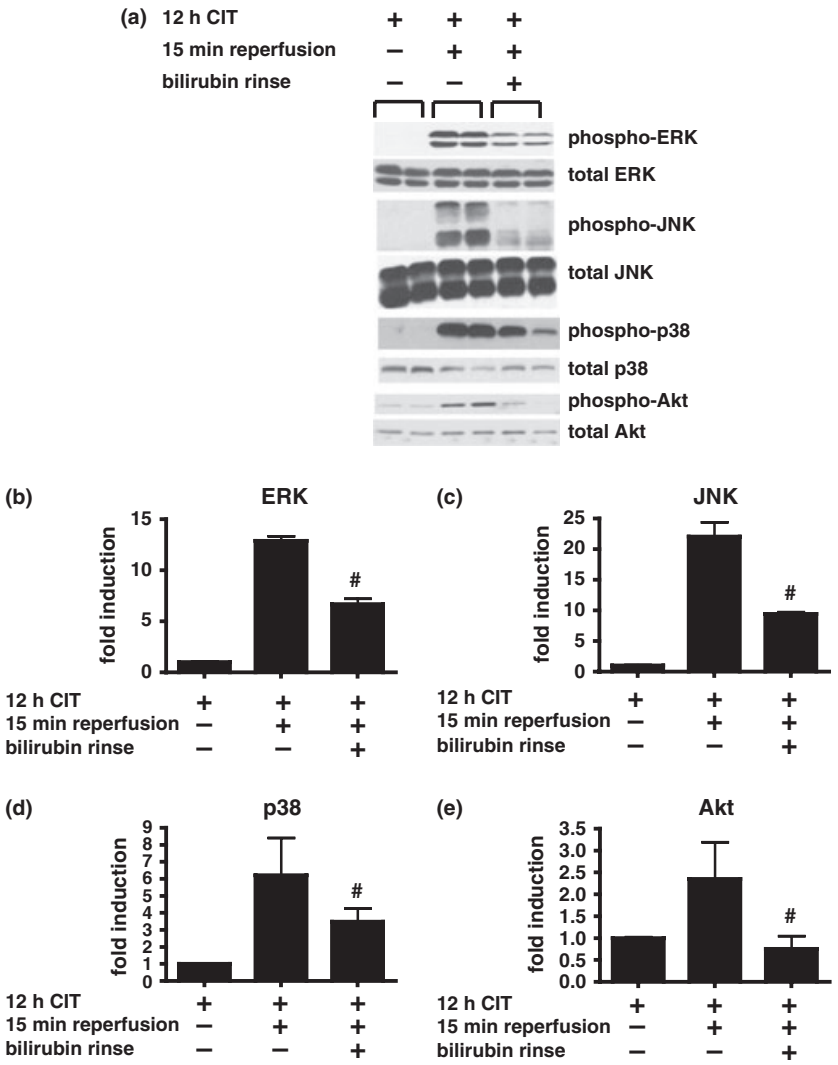

Figure 3 (a) Representative Western blots for the total and the phosphorylated forms of mitogen-activated protein kinases (MAPKs). Bilirubin rinse suppresses activation of extracellular signal-related protein kinases (ERK), p38, JNK, and PI3K/Akt. (b–e) Quantification of MAPK phosphorylation (#$P < 0.05$).

potent endogenous antioxidants [19], and its use has been tested in various animal models successfully [23]. Aim of our study was to show that bilirubin prevents IRI, what has been demonstrated by various groups previously [20,21,31–37]. We focused on the use of a potent naturally occurring thus presumably nontoxic (in the concentration applied) substance we have considerable experience with, namely bilirubin, in an ambitious and easily applicable way to administer it in an animal model of heart transplantation.

The complexity of organ transplantation would allow to interfere with the pathological processes responsible for graft dysfunction at three stages: treatment of the organ donor, the graft when recovered but not reperfused or treating the recipient [21,25,38,39]. Treating the donor implies that an effect is assumed to be beneficial for all organs being recovered what might not be the case under certain circumstances, that is, a donor treatment beneficial for the heart might be detrimental for the kidneys and *vice versa*. In addition to that, in many countries, treatment of the donor for the purpose of improving the allografts integrity is not allowed for ethical reasons. Otherwise, treating the recipient is certainly possible, and many attempts have been made to reduce IRI after solid organ transplantation. Experimentally a plethora of substances is effective in reducing IRI (e.g. antioxidants, immunosuppressants), however, no single specific treatment to reduce IRI has proven efficacy in the clinical situation by the means of improving organ function after reperfusion in the recipient. Additionally, side effects of 'IRI reducing drugs' may compromise the recipient who is in a critical condition in the

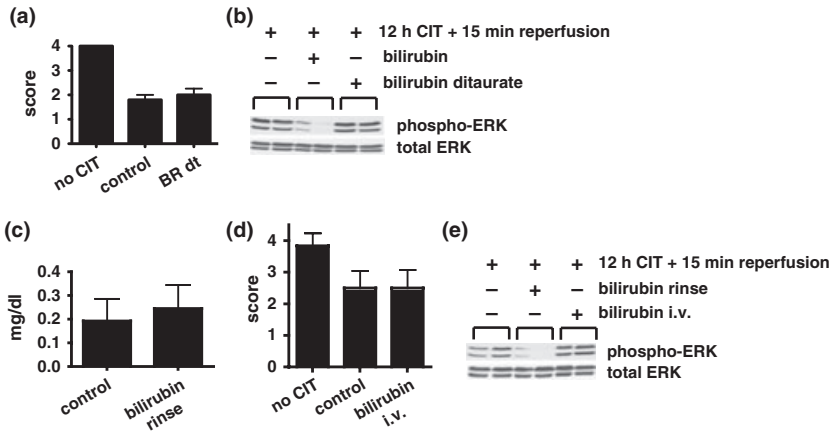

Figure 4 (a, b) Locally applied hydrophilic bilirubin ditaurate does not improve cardiac function nor suppress extracellular signal-related protein kinases (ERK) activation *in vivo*. (c) Serum bilirubin levels measured in mice after bilirubin rinse of heart grafts. (d, e) Systemic application of bilirubin at the amount that has been administered locally does not improve cardiac score nor suppress ERK activation *in vivo*.

early phase after solid organ transplantation. Thus, the concept of treating the graft only avoiding systemic treatment of the donor/recipient was tested because it is simple and has the potential of local 'protection' of the graft initiated prior to reperfusion. Years ago, it has been shown that bilirubin administered to rat hearts on a Langendorff apparatus together with the perfusate improves cardiac function [20].

We administered bilirubin briefly before reperfusion on the Langendorff apparatus after 12 h of cold ischemia by rinsing the hearts, avoiding treatment of the donor and/or the recipient. Twelve hours of cold ischemia were chosen in order to have serious damage to the grafts what was not seen with (clinically more common) 3 h or 6 h of cold ischemia, that is, no significant decrease in cardiac scores in the heart transplant model (data not shown). The decision to use bilirubin at a concentration of 125 μM was based on previous studies mimicking supernormal serum levels that have been found/achieved [22,24,28]. Bilirubin was very efficient in ameliorating cardiac function. This is exciting, especially as the period covered by the treatment is considerably short but goes along with our hypothesis that if at the moment of reperfusion a potent antioxidant in a proper concentration is present, reperfusion injury is reduced.

No 'protective' effect was seen under biliverdin treatment that experimentally has a similar antioxidant potential [19]. So far we have no explanation for this difference, one might assume that biliverdin, due to its more hydrophilic properties at physiological pH [40], does not enter the target cells (endothelial cells, myocardium). Further, that the enzyme BVR that may play an important role in biliverdin-mediated effects as suggested recently [41] is not being functional at 4 °C, and thus, biliverdin is not being converted to bilirubin, as at 4 °C all metabolic reactions are coming to a complete stop. Based on these results, we suggest that the antioxidant present in the perfusate alone is not effective and has to be incorporated into the 'effector' cells. However, this issue needs to be further explored and will be addressed in future studies. Lipophilic bilirubin was used primarily because of its beneficial effects in the isolated perfused heart in the mouse heart transplant model; however, because of the more feasible use of water-soluble bilirubin ditaurate, the latter was also tested. No effect of a bilirubin ditaurate rinse was observed with respect to functional scores nor suppression of MAPK activation was seen, confirming what has been assumed for biliverdin, that under conditions of local administration, the hydrophilic compound cannot effectively protect the cells from damage. These objective findings were accompanied by the subjective visual perception that the myocardium of the heart grafts rinsed with the lipophilic bilirubin colored orange. This was not seen when water-soluble bilirubin ditaurate was used to rinse the grafts.

Based on the ex *vivo studies,* we assumed that local administration of bilirubin to syngeneic heart grafts would ameliorate IRI *In vivo*. The heterotopic cervical mouse heart transplant model certainly bares several disadvantages: As the hearts are not transplanted orthotopically, no physiological blood flow can be achieved. Nevertheless, we chose the mouse heart model to confirm the data obtained in the isolated perfused heart as it is vascularized and thus much more relevant with respect to reperfusion damage what, at least in part, is mediated by the immune system and certainly is not seen in an isolated perfused heart.

We are aware that palpation of the grafts bares the risk of under- or overscoring; thus, the investigator was blinded to the treatment; further the heart transplantation method with the cervical cuff technique maximally facilitates palpation. No better methods so far are available in mice to assess cardiac function reliably; even echocardiography has

been described. With echocardiography, we were not able to generate reliable readings of contractions neither in mice nor in rats (data not shown). Thus, we focused on other methods to quantify damage in the heart transplant model: Apoptosis is the consequence of IRI-mediated via several pro-apoptotic pathways as well as the innate immune system that determines the fate of an organ transplanted, especially the heart. Apoptosis was not completely prevented by the bilirubin rinse, but significantly reduced. To confound these results, we measured CK-MB with a standardized assay. Again, a dramatic increase in serum CK-MB was seen what was nearly completely suppressed in mice receiving a bilirubin rinsed heart.

Recently we have described very early activation of MAPKs in a mouse model of heart transplantation [8]. Bilirubin administered locally to heart isografts suppressed MAPK and PI3K activation that is seen after reperfusion. MAPK activation in this setting may be beneficial as well as detrimental, depending on which MAPK being is activated. ERK has been described as antiapoptotic; similar observations have been made for PI3K/Akt [42,43]. This activation may be a natural defense mechanism of the cells exposed to cold ischemia and warm reperfusion and rescue them from irreversible damage. In contrast, experimental suppression of p38 and/or JNK activation is beneficial in IRI; thus, the suppression thereof under local bilirubin treatment may minimize pro-apoptotic events [44,45]. Herein, we did not analyze whether suppression of MAPK activation is the mechanism of action of local bilirubin administration but merely to have a reliable (in our hands) well-established parameter to assess early reperfusion injury.

Certainly the assumption arises that the bilirubin administered to the grafts becomes systemic upon reperfusion and then exerts its beneficial effects. The serum bilirubin levels measured in recipients after rinsing of the grafts with bilirubin did not significantly increase when compared with RL-rinsed controls. We tested whether the same amount that was rinsed into the grafts applied systemically would ameliorate IRI. Intravenous injection of bilirubin at 0.5 mg to the recipient did not affect cardiac scores nor ERK activation; we thus conclude that the effects seen are not due to systemic 'wash out' of the grafts.

In summary, our data suggest that local administration of lipophilic (unconjugated) bilirubin ameliorates transplant-related IRI, already measured in the very early phase after reperfusion. Based on our results, we conclude that bilirubin applied locally to allografts is a promising, novel strategy to ameliorate IRI.

Authorship

FB, MT, PK, and RÖ: experimental work; RO, RS, FA, SS, and DW: contributed important reagents; KY and RÖ: designed the study; KK, JT, and JP: supervised the project; FB and RÖ: wrote the paper.

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