Cardiomyocyte mitochondria as targets of humoral factors released by remote ischemic preconditioning

Nilguen Gedik1, Leonardo Maciel1,2, Christiane Schulte1, Andreas Skyschally1, Gerd Heusch1, Petra Kleinbongard1

1 Institute for Pathophysiology, West German Heart and Vascular Centre Essen, University of Essen, Medical School, Essen, Germany
2 Laboratory of Cardiac Electrophysiology, Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil

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Abstract

Introduction: Remote ischemic preconditioning (RIPC) reduces myocardial infarct size, and protection can be transferred with plasma to other individuals, even across species. Mitochondria are the end-effectors of cardioprotection by local ischemic conditioning maneuvers. We have now analyzed mitochondrial function in response to RIPC.

Material and methods: Plasma from pigs undergoing placebo or RIPC (infarct size reduction by 67% in RIPC pigs compared to placebo) was transferred to isolated perfused rat hearts subjected to 30 min global ischemia followed by 120 min reperfusion for infarct size measurement. Additional experiments were terminated at 10 min reperfusion to isolate mitochondria for functional measurements. Effects of RIPC pig plasma were compared to local ischemic preconditioning (IPC) or to infusion of tumor necrosis factor α (TNF-α).

Results: Ischemia/reperfusion (I/R) induced an infarct of 41 ±2% of total ventricular mass. Placebo pig plasma did not affect infarct size (38 ±1, p = 0.13). The RIPC pig plasma reduced infarct size (27 ±2, p < 0.001), as did IPC (20 ±1, p < 0.001) and TNF-α (28 ±2, p < 0.001). Associated with cardioprotection, reductions of mitochondrial adenosine diphosphate (ADP)-stimulated respiration, adenosine triphosphate (ATP) production and calcium retention capacity (CRC) by I/R and placebo pig plasma were prevented by RIPC pig plasma, as they were by IPC and TNF-α. Mitochondrial reactive oxygen species production (nmol H₂O₂/100 µg protein) induced by I/R (272 ±34) was comparable in response to placebo pig plasma (234 ±28, p = 0.37) and was reduced by RIPC pig plasma (83 ±15, p < 0.001) as well as by IPC (78 ±21, p < 0.001) and TNF-α (125 ±42, p = 0.002).

Conclusions: In rat myocardium, mitochondria are an intracellular target of protection induced by humoral factors retrieved from pigs undergoing RIPC.

Key words: cardioprotection, humoral factor, mitochondria, remote ischemic preconditioning.

Introduction

Repetitive brief episodes of myocardial ischemia/reperfusion (I/R) before sustained myocardial ischemia and subsequent reperfusion, i.e. local ischemic preconditioning (IPC), protect the myocardium from irreversible I/R injury [1]. Ischemic preconditioning in an organ remote from the heart (RIPC) confers similar cardioprotection. The protection by RIPC has been
confirmed experimentally by infarct size reduction in all animal species tested so far [2]. Likewise, RIPC by repetitive limb I/R attenuates myocardial injury in patients undergoing elective intervention [3] or surgical coronary revascularization [4–6], and also in patients with acute myocardial infarction [7–10]. The protection was confirmed by reduced cardiac biomarker release [3–6], by cardiac imaging [7, 8, 10], and by improved short- [6, 8] and long-term clinical outcome [3, 5, 11]. However, in two recent large-scale randomized trials, ERICCA and RIPCHeart, RIPC neither reduced biomarker release nor improved clinical outcome of patients undergoing cardiac surgery [12, 13]. To improve the effective use of RIPC in patients, a better understanding of how the remote stimulus is transferred from the ischemic/reperfused organ or limb to the heart, as well as identification of its intracellular target in the protected organ, is needed [14, 15].

The cardioprotective signal from the ischemic/reperfused organ or limb to the heart is transferable with plasma [16–18] or a plasma-derived dialysate/filtrate [19, 20] from one individual to another, even across species. Investigations deciphering possible myocardial intracellular targets of RIPC were inspired by studies on local IPC or postconditioning. Mitochondria are viewed as end-effectors of these cardioprotective strategies. The preservation of mitochondrial function after I/R is decisive for survival of cardiomyocytes and thus salvage of the myocardium [21, 22]. Whether mitochondria are also the target of cardioprotection by RIPC is still under investigation. In rats, myocardial infarct size reduction by RIPC is associated with preserved mitochondrial morphology [23], maintained mitochondrial membrane potential and increased mitochondrial manganese superoxide dismutase (MnSOD) content [24]. Several studies have also reported changes in mitochondrial function by RIPC: mitochondrial respiration was improved in rat hearts [25] and in right atrial appendages in situ of patients undergoing cardiac surgery [26, 27]. Transfer of rabbit RIPC plasma dialysate to isolated perfused neonatal rabbit hearts also improved mitochondrial respiration but had no effect on mitochondrial permeability transition pore (mPTP) opening [28]. However, none of these latter studies demonstrated an association of improved mitochondrial function with infarct size reduction.

The available data can therefore not clarify a relationship between improved mitochondrial function and cardioprotection, i.e. infarct size reduction by RIPC. Whether or not mPTP opening, which is decisive for cardiomyocyte survival or death at early reperfusion [29–31], is associated with infarct size reduction by RIPC is also not clear at this point. Therefore, in this study we used an established set-up with humoral across-species transfer of RIPC’s cardioprotection from an anesthetized pig model in situ to an in vitro isolated perfused rat heart model subjected to global I/R [18]. Mitochondria were isolated from the isolated perfused rat hearts at early reperfusion. Mitochondrial function, i.e. mitochondrial adenosine diphosphate (ADP)-stimulated respiration, calcium retention capacity (CRC) to estimate mPTP opening, adenosine triphosphate (ATP) production and reactive oxygen species (ROS) production, was measured at physiological temperature of 37°C. For comparison of humorally transferred RIPC cardioprotection, isolated perfused rat hearts were also subjected to local IPC and to infusion of tumor necrosis factor α (TNF-α), which is known to be an endogenous humoral factor of cardioprotection [32–34].

Material and methods

Materials

Chemicals were of the highest quality available, and all solutions were freshly prepared using Milli-Q water or high quality analytical grade organic solvents and, where appropriate, sterilized prior to use. Materials were obtained from Sigma-Aldrich (Deisenhofen, Germany) or purchased as indicated.

Plasma separation after RIPC or placebo maneuver in pigs

Anesthetized pigs were subjected to RIPC (4 × 5 min/5 min hindlimb I/R, n = 10) or placebo (n = 10) before 60 min/180 min coronary occlusion/reperfusion. One hour after the RIPC or placebo maneuver, 100 to 120 ml of arterial blood was withdrawn and sampled in vials containing lithium-heparin and immediately centrifuged at 800 g and 4°C for 10 min. Separated plasma was centrifuged at 4500 g and 4°C for an additional 10 min. The separated plasma was stored at −80°C for later use and again centrifuged for 10 min at 4500 g and filtered (0.2 µm pore size) before use. For details see Skyschally et al. [18].

Experiments in isolated rat hearts

The experimental protocols were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany (B1322/12), and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by NIH Publication 85–23, revised 1996 [35].

Male Lewis rats (age: 10–14 weeks, weight: 250–400 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (400 mg/kg; Narcoren, Merial, Hallbergmoos, Germany). The injection was supplemented with 1000 IU of heparin to attenuate coagulation. The hearts were rapidly excised and placed in ice-cold saline before they were mounted.
on a Langendorff apparatus. Modified Krebs-Henseleit buffer (KHB; in mmol/l: NaCl 118.0, KCl 4.7, MgSO\textsubscript{4} 1.6, KH\textsubscript{2}PO\textsubscript{4} 1.2, glucose 5.6, NaHCO\textsubscript{3} 24.9, sodium pyruvate 2.0, CaCl\textsubscript{2} 2.0; gassed with 95% O\textsubscript{2} and 5% CO\textsubscript{2} in a 37°C pre-warmed reservoir) was used for retrograde perfusion at constant pressure (65–68 mm Hg). The perfusate temperature was held constant by a heat exchanger located next to the aortic cannula. A fluid-filled latex balloon was inserted through the left atrium into the left ventricle and connected to a pressure transducer (CODAN pvb Medical, Lensahn, Germany). Minimal left ventricular pressure (LVP\textsubscript{min}) was set to 5–15 mm Hg by balloon inflation to control ventricular preload, and maximal systolic left ventricular pressure (LVP\textsubscript{max}) was measured. Heart rate was set to 400 bpm by atrial pacing. Coronary flow (CF) was measured by an inline ultrasonic transit time flow probe (ME2-PXN Transonic, Maastricht, NL) connected to the aortic cannula. During the whole experiment, the hearts were continuously immersed in 37°C warm buffer to avoid hypothermia. Hearts were allowed to stabilize for 20 min before a protocol was started (the last 30 s were defined as baseline).

**Experimental protocols**

Sham: Hearts were perfused with KHB solution for 60 or 170 min.

I/R: Global ischemia was induced for 30 min by full stop of retrograde perfusion followed by 10 or 120 min reperfusion.

Placebo/RIPC pig plasma: Infarct size of pig hearts was reduced with RIPC (12 ±2% of area at risk) in comparison to placebo (36 ±6%; \( p < 0.05 \)) and was comparable with the size measured in our prior study \([18]\). Pig plasma was infused for 8 min via a syringe pump into the KHB solution (1:10 volume ratio) before passing the heat exchanger. After 2 min washout of the plasma with KHB solution to avoid adherence of proteins from stagnant plasma to the Langendorff apparatus, global ischemia was induced as in the I/R protocol, and plasma infusion was continued during the initial 5 min of reperfusion.

Local IPC: Three cycles of 3 min/3 min of I/R were induced immediately before induction of the I/R protocol.

TNF-\(\alpha\) infusion: TNF-\(\alpha\) (0.5 ng/ml) was perfused for 7 min via a syringe pump into the KHB solution before passing the heat exchanger. A washout with KHB solution for 10 min was performed before induction of the I/R protocol to activate the cardioprotection by TNF-\(\alpha\) \([36]\).

For infarct size measurement experimental protocols were terminated after 120 min reperfusion. For measurement of mitochondrial function, experimental protocols were terminated after 10 min reperfusion to isolate mitochondria from the rat hearts.

**Mitochondria isolation and measurement of mitochondrial function**

All procedures were performed on ice or at 4°C. After 10 min reperfusion isolated rat hearts were rapidly removed from the Langendorff apparatus, placed in ice-cold isolation buffer (pH 7.4) containing (in mmol/l) 250 sucrose, 10 HEPES, 1 ethylene glycol tetraacetic acid (EGTA), with 0.5% w/v bovine serum albumin (BSA), minced thoroughly using scissors, and then homogenized with a tissue homogenizer (Ultra-Turrax, IKA, Staufen, Germany) using two 10 s treatments at a shaft rotation rate of 6,500 rpm to release the subsarcolemmal mitochondria. Further homogenization with a Teflon pestle in the presence of proteinase type XXIV (8 IU/mg tissue weight) then released interfibrillar mitochondria. The homogenate containing subsarcolemmal and interfibrillar mitochondria was centrifuged at 700 g for 10 min. The supernatant was collected and centrifuged at 14,000 g for 10 min. The resulting pellet was resuspended in isolation buffer without BSA and centrifuged at 10,000 g for 5 min. The latter procedure was repeated, and the pellet was resuspended in isolation buffer \([37]\). The protein concentration of the resuspended pellet was determined using a protein assay (Lowry method, Bio-Rad, Hercules, CA, USA) with BSA as standard (Thermo Scientific, Waltham, MA, USA).

**Mitochondrial respiration**

Mitochondrial respiration was measured with a Clark-type electrode (Strathkelvin, Glasgow, UK) at 37°C during magnetic stirring in incubation.
buffer containing in mmol/l: 125 KCl, 10 MOPS, 2 MgCl₂, 5 KH₂PO₄, 0.2 EGTA with 5 glutamate and 5 malate as substrates for complex I. The oxygen electrode was calibrated using a solubility coefficient of 217 nmol O₂/ml at 37°C.

For the measurement of complex I respiration, suspended mitochondria (corresponding to a protein amount of 50 µg) were added to 0.5 ml of incubation buffer. After 2 min, 1 mmol/l ADP was added and ADP-stimulated respiration was measured over 2–3 min. Hereafter, mitochondria were used to either measure complex IV respiration and maximal uncoupled oxygen uptake in the respiration chamber, or incubation buffer containing mitochondria was taken from the respiration chamber to measure ATP production or ROS production, respectively. Complex IV respiration was stimulated by adding N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD, 300 µmol/l) and ascorbate (3 mmol/l), which donates electrons to cytochrome oxidase via the reduction of cytochrome c. Maximal uncoupled oxygen uptake was measured in the presence of 30 nmol/l carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone (FCCP) [37].

Mitochondrial ATP production

After measurement of ADP-stimulated respiration, the incubation buffer containing mitochondria was taken from the respiration chamber and immediately supplemented with ATP assay mix (diluted 1 : 5). Mitochondrial ATP production after measurement of respiration was determined immediately and compared with ATP standards using a 96-well white plate and a Cary Eclipse spectrophotometer (Varian, Mulgrave, Victoria, Australia) at 560 nm emission wavelength [37].

Calcium retention capacity

Calcium retention capacity was determined using suspended mitochondria (corresponding to a protein amount of 100 µg) in 1 ml of CRC buffer containing (in mmol/l) 125 KCl, 10 MOPS, 2 MgCl₂, 5 KH₂PO₄ with 5 glutamate and 5 malate as substrates in the presence of ADP (0.4 mmol/l), at 37°C. Calcium green-5N (0.5 µmol/l, Life Technologies, Carlsbad, CA, USA) was used to measure the extramitochondrial calcium concentration in a spectrophotometer (Varian, Mulgrave, Victoria, Australia) at 500 nm emission and 530 nm extinction wavelengths. Pulses of 5 nmol CaCl₂ were added every minute until a rapid increase in calcium green fluorescence indicated mPTP opening [38]. The immunosuppressant cyclosporine A delays mPTP opening by interaction with cyclophilin D to keep the pore closed [39]. Therefore, additional measurements were performed with cyclosporine A (10 µmol/l) as a positive control.

ROS production

The Amplex Red Hydrogen Peroxide Assay (Life Technologies, Carlsbad, CA, USA) was used to determine the ROS concentration in the extramitochondrial space. Amplex Red reacts in a 1 : 1 stoichiometry with peroxides under catalysis by horseradish peroxidase (HRP) and produces highly fluorescent resorufin. The incubation buffer containing mitochondria was removed from the respiration chamber and immediately supplemented with 50 µmol/l Amplex UltraRed and 2 U/ml HRP. The supernatant was collected after 120 min of incubation in the dark. The ROS concentration was determined and compared with H₂O₂ standards using a 96-well black plate and a Cary Eclipse fluorescence spectrophotometer (Varian, Mulgrave, Victoria, Australia) at 540 nm emission and 580 nm extinction wavelengths [37].

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Coronary flow and LVDP were analyzed by two-way ANOVA for repeated measures. Infarct size, mitochondrial respiration, ATP production, CRC and ROS production were analyzed by one-way ANOVA. When a significant difference was detected, one-way and two-way ANOVA were followed by Fisher's LSD post-hoc tests (SigmaStat 2.03, SPSS Inc., Chicago, IL, USA). Differences were considered significant at the level of p < 0.05.

Results

Coronary flow and left ventricular developed pressure in isolated perfused rat hearts

Coronary flow and LVDP were not different between groups at baseline and did not change during the experiments in the rat hearts with sham. After plasma infusion, during ischemia, and during reperfusion, CF and LVDP were comparable between I/R, placebo and RIPC pig plasma, respectively. CF and LVDP of rat hearts with IPC and TNF-α infusion were comparable with I/R during ischemia, but rat hearts recovered better with IPC at reperfusion (Table I).

Infarct size of isolated perfused rat hearts

Almost no infarction was detectable with sham. Compared to I/R, infarct size was similar with placebo pig plasma, whereas RIPC pig plasma reduced infarct size. Comparable infarct size reduction was observed with local IPC and TNF-α infusion (Figure 1).
Table I. Coronary flow and left ventricular developed pressure of isolated perfused rat hearts

| Variable   | Time               | Rat hearts for infarct size measurement | Rat hearts for mitochondria preparation |
|------------|--------------------|----------------------------------------|-----------------------------------------|
|            | CF<sub>mean</sub> [ml/min] | LVDP<sub>max</sub> [mm Hg]             | CF<sub>mean</sub> [ml/min] | LVDP<sub>max</sub> [mm Hg] |
| I/R (n = 8/8) |                   |                                        |                                        |
| Baseline   | 13.0 ±0.6          | 74.5 ±6.7                              | 12.6 ±0.9                              | 91.3 ±6.0 |
| After intervention | 12.4 ±0.6          | 71.6 ±7.9                              | 12.6 ±0.9                              | 91.9 ±6.2 |
| Ischemia 5 min | 0.0 ±0.0*          | 0.4 ±0.2*                              | 0.0 ±0.0*                              | 0.6 ±0.3* |
| Ischemia 25 min | 0.0 ±0.0*          | 0.3 ±0.1*                              | 0.0 ±0.0*                              | 3.8 ±3.6* |
| Reperfusion 10 min | 8.5 ±0.8*          | 22.9 ±7.8*                             | 7.9 ±1.2*                              | 25.4 ±6.6* |
| Reperfusion 30 min | 7.7 ±0.9*          | 31.0 ±7.6*                             | –                                      | –         |
| pPlacebo (n = 10/10) |                   |                                        |                                        |
| Baseline   | 12.3 ±0.4          | 87.9 ±5.7                              | 13.1 ±0.4                              | 81.0 ±5.6 |
| After intervention | 12.7 ±0.4          | 80.3 ±6.9                              | 13.8 ±0.7                              | 71.5 ±7.1 |
| Ischemia 5 min | 0.0 ±0.0*          | 0.4 ±0.1*                              | 0.0 ±0.0*                              | 0.4 ±0.1* |
| Ischemia 25 min | 0.0 ±0.0*          | 0.3 ±0.1*                              | 0.0 ±0.0*                              | 0.2 ±0.1* |
| Reperfusion 10 min | 8.0 ±0.7*          | 30.1 ±5.7*                             | 8.8 ±0.6*                              | 34.6 ±5.2* |
| Reperfusion 30 min | 7.3 ±0.7*          | 34.1 ±5.8*                             | –                                      | –         |
| pRIPC (n = 10/10) |                   |                                        |                                        |
| Baseline   | 12.6 ±0.5          | 88.7 ±8.2                              | 12.1 ±0.4                              | 91.8 ±3.3 |
| After intervention | 12.8 ±0.9          | 80.5 ±9.9                              | 13.3 ±0.4                              | 87.8 ±3.1 |
| Ischemia 5 min | 0.0 ±0.0*          | 0.3 ±0.1*                              | 0.0 ±0.0*                              | 0.5 ±0.3* |
| Ischemia 25 min | 0.0 ±0.0*          | 0.5 ±0.4*                              | 0.0 ±0.0*                              | 0.2 ±0.1* |
| Reperfusion 10 min | 9.0 ±0.8*          | 30.4 ±8.4*                             | 8.9 ±0.8*                              | 21.7 ±9.0* |
| Reperfusion 30 min | 8.1 ±0.9*          | 28.8 ±8.4*                             | –                                      | –         |
| IPC (n = 8/11) |                   |                                        |                                        |
| Baseline   | 12.0 ±0.6          | 79.8 ±7.8                              | 12.1 ±0.6                              | 95.0 ±5.4 |
| After intervention | 12.2 ±0.5          | 70.4 ±7.5                              | 12.4 ±0.6                              | 85.1 ±5.1 |
| Ischemia 5 min | 0.0 ±0.0*          | 1.5 ±1.0*                              | 0.0 ±0.0*                              | 0.6 ±0.2* |
| Ischemia 25 min | 0.0 ±0.0*          | 2.0 ±1.7*                              | 0.0 ±0.0*                              | 4.8 ±4.2* |
| Reperfusion 10 min | 12.3 ±0.7*         | 44.7 ±11.6*                            | 11.8 ±1.0*                             | 69.6 ±10.4* |
| Reperfusion 30 min | 10.9 ±0.6*         | 51.0 ±13.5*                            | –                                      | –         |
| TNF-α infusion (n = 7/8) |               |                                        |                                        |
| Baseline   | 12.8 ±0.7          | 75.3 ±7.6                              | 13.6 ±0.6                              | 93.9 ±5.8 |
| After intervention | 12.0 ±0.9          | 70.5 ±9.0                              | 13.6 ±0.5                              | 96.6 ±5.8 |
| Ischemia 5 min | 0.0 ±0.0*          | 0.6 ±0.1*                              | 0.0 ±0.0*                              | 0.6 ±0.1* |
| Ischemia 25 min | 0.0 ±0.0*          | 0.1 ±0.0*                              | 0.0 ±0.0*                              | 0.4 ±0.2* |
| Reperfusion 10 min | 7.4 ±0.9*          | 22.8 ±3.0*                             | 9.7 ±1.1*                              | 34.3 ±6.1* |
| Reperfusion 30 min | 6.9 ±0.8*          | 19.1 ±3.8*                             | –                                      | –         |
| Sham (n = 6/8) | Analogue to        |                                        |                                        |
| Baseline   | 12.8 ±1.4          | 84.9 ±9.1                              | 12.2 ±0.7                              | 95.1 ±3.2 |
| After intervention | 12.7 ±1.3          | 87.7 ±9.0                              | 12.0 ±0.7                              | 95.0 ±3.6 |
| Ischemia 5 min | 12.4 ±1.3          | 85.6 ±9.1                              | 11.8 ±0.7                              | 94.0 ±3.7 |
| Ischemia 25 min | 11.9 ±1.3          | 84.5 ±9.4                              | 11.3 ±0.9                              | 88.0 ±4.9 |
| Reperfusion 10 min | 11.7 ±1.3          | 83.3 ±8.5                              | 10.9 ±0.8                              | 83.7 ±4.4 |
| Reperfusion 30 min | 11.5 ±1.1          | 83.3 ±8.3                              | –                                      | –         |

Mean coronary perfusate flow (CF<sub>mean</sub>) and maximal developed left ventricular pressure (LVDP<sub>max</sub>) of isolated perfused rat hearts with ischemia/reperfusion (I/R) and I/R with plasma taken from pigs after placebo (pPlacebo), with plasma taken from pigs after remote ischemic preconditioning (pRIPC), with local ischemic preconditioning (IPC), with tumor necrosis factor α (TNF-α) infusion and without I/R (sham). CF<sub>mean</sub> and LVDP<sub>max</sub> were analyzed at different time points: at baseline, after plasma infusion/IPC/TNF-α infusion (intervention), at 5/25 min ischemia, and at 10/30 min reperfusion. Mean ± SEM; *p < 0.05 vs. baseline; †p < 0.05 vs. I/R.
Mitochondrial function

ADP-stimulated complex I respiration was reduced after I/R compared to sham. With placebo pig plasma ADP-stimulated complex I respiration was not different from that with I/R. RIPC pig plasma improved ADP-stimulated complex I respiration over that with placebo pig plasma and I/R, as did IPC and TNF-α (Figure 2 A). Mitochondrial complex IV respiration and maximal oxygen uptake of uncoupled mitochondria were not different between groups, reflecting an equal loading of viable mitochondria (Figures 2 B).

The mitochondrial ATP production was decreased after I/R compared to sham. Placebo pig plasma did not cause any difference to I/R. The RIPC pig plasma increased ATP production over that with placebo pig plasma and I/R. ATP production was increased with IPC and TNF-α over that with I/R (Figure 3).

The CRC of mitochondria was reduced after I/R compared to sham. CRC with placebo pig plasma was comparable with that after I/R. RIPC pig plasma improved CRC over that with placebo pig plasma and I/R. Local IPC and TNF-α improved CRC over that with I/R (Figure 4 A). With cyclosporine A, CRC was not different between groups (Figure 4 B).

Mitochondrial ROS production after I/R was reduced compared to sham. ROS production with placebo pig plasma was comparable with that in I/R. RIPC pig plasma reduced the ROS production. Local IPC and TNF-α also reduced the ROS production (Figure 5).

Discussion

In the present study, improved mitochondrial function at early reperfusion was associated with cardioprotection by humoral transfer of RIPC pig plasma to isolated perfused rat hearts, supporting the notion that mitochondria are a myocardial target organelle of RIPC’s protection.

The extent of infarct size reduction and the improvement of mitochondrial function by RIPC pig plasma were comparable to those induced by local IPC or TNF-α infusion, whereas the recovery of rat heart LVDP during reperfusion was better with local IPC than in response to RIPC pig plasma or TNF-α. These findings suggest that improved mitochondrial function plays no important role in the functional recovery of the rat heart, except for the reduction of infarct size. The IPC is a most effective local ischemic conditioning maneuver and is associated with improved mitochondrial function [40, 41]. The cytokine TNF-α activates intracellular cardioprotective signaling [42, 43], which converges at the mitochondria [21]. The TNF-α has an ambivalent role in myocardial ischemia/reperfusion injury and during myocardial protection [32]. Both IPC [44–46] and remote ischemic postconditioning [47] depress the I/R-induced in-
increase of endogenous TNF-α in rodents. On the other hand, exogenous TNF-α mimics IPC cardioprotection when administered prior to ischemia [48], and protection by IPC is lost in TNF-α-KO mice [49]. Cardioprotection by exogenous TNF-α is dose-dependent; whereas lower doses are protective, higher TNF-α doses even increase infarct size [34]. Thus, TNF-α has bidirectional effects: TNF-α at low concentrations acts as a signaling molecule, but TNF-α at higher concentrations induces irreversible cell damage. The protection by TNF-α is also associated with restoration of mitochondrial respiration [48] and inhibition of mPTP opening [50].

Prior studies suggested that RIPC is also associated with improved mitochondrial respiration [25–28] but did not look for an association with infarct size reduction. Here, we confirmed improved respiration of mitochondria isolated from rat hearts after RIPC pig plasma transfer, and cardioprotection by RIPC was evidenced in the RIPC plasma donor pigs as well as in the plasma acceptor rat hearts by infarct size reduction.

A logical consequence of increased ADP-stimulated respiration is increased ATP production [51]. Exactly such increased ATP production was evident in parallel to the increased respiration by RIPC pig plasma, local IPC and TNF-α infusion.

Improved mitochondrial respiration after local ischemic preconditioning in anesthetized pig hearts was also associated with increased CRC of mitochondria isolated at 10 min reperfusion [52]. As in our previous study on ischemic preconditioning, the improved mitochondrial respiration was associated with an increased CRC in the present study, indicating inhibition of mPTP opening. Opening of the mPTP induces cell death; therefore the inhibition of mPTP opening is decisive for cardiomyocyte survival at early reperfusion [29–31, 53]. Inhibition of mPTP opening by cyclosporine A attenuates reperfusion injury in animals [54, 55], as it also did in a small cohort of patients with acute myocardial infarction [56]. However, this proof-of-concept study was not confirmed by two recent large-scaled randomized trials, CIRCUS and CYCLE, where cyclosporine A neither reduced biomarker release nor improved clinical outcome of patients with acute myocardial infarction [57, 58]. The reasons for the discrepancy between the proof-of-concept and the two phase III trials are not clear in detail, but may include different patient inclusion criteria, notably an increased time from symptom onset to reperfusion, greater pre-existing protection by more widespread use of novel P2Y12 antagonists, a different vehicle used to dissolve cyclosporine A in CIRCUS but not in CYCLE, inadequate delivery of the drug to the myocardium at early reperfusion, and a potential false-positive type I error [59].

In contrast to our study, humoral transfer of RIPC rabbit plasma dialysate to isolated neonatal rabbit hearts did not affect the mPTP opening of mitochondria isolated after 30 min reperfusion [28]. The opening of mPTP could have been
missed due to a late observation time point during reperfusion [60] or by absence of cardioprotection, which was not assured [28].

In an in vivo murine model with RIPC cardioprotection, S-nitrosation of mitochondrial complex I reduced its activity and finally reduced myocardial ROS production [19]. Here, the transfer of RIPC pig plasma attenuated I/R induced mitochondrial ROS production after improved ADP-stimulated complex I respiration, indicating that mitochondria are involved in the reduction of ROS formation.

The RIPC-induced factor(s) which may contribute to modulation and preservation of mitochondrial function have not been identified yet. In an isolated neonatal rabbit heart model, remote cardioprotection by IPC effluent (from isolated rabbit hearts) preserved mitochondrial integrity and function in an adenosine receptor dependent mechanism. Although infarct size reduction could be mimicked by adenosine, IPC effluent contained additional factor(s) contributing to modulation of the mitochondrial response to I/R injury [61]. In our model, RIPC activated the survival activating factor enhancement (SAFE) pathway, notably signal transducer and activator of transcription 3 (STAT3) in the pig myocardium and both the reperfusion injury salvage kinase (RISK) and the SAFE pathway in the rat myocardium [18]. The activation of mitochondrial STAT3 was causally involved in infarct size reduction by ischemic postconditioning in pigs and associated with preservation of mitochondrial complex I respiration and CRC [52]. Whether RIPC activates mitochondrial STAT3 in association with improved mitochondrial function requires further investigation.

In this study we demonstrated improved mitochondrial function associated with infarct size reduction in isolated perfused rat hearts after humoral transfer of RIPC. However, a causal relationship between preserved mitochondrial function by RIPC and infarct size reduction remains to be established.

The extent of cardioprotection and improvement of mitochondrial function by RIPC pig plasma was comparable to that induced by local IPC. However, other algorithms for IPC and for RIPC but also changes in the concentration of humoral factor transfer to the isolated rat heart may influence the extent of protection. Placebo pig plasma tended to reduce infarct size and increase mitochondrial ATP production compared to the values observed in hearts without plasma infusion. This slight protection could be caused by the plasma protein content itself, e.g. in isolated perfused rat hearts, trace amounts (0.0004%) of bovine or rat serum albumin protected against I/R injury [62]. Also, plasma derived hormones [63] or cytokines [43] can induce cardioprotection. Species-specific differences in hormone or cytokine levels between pig and rat plasma may further contribute to protection; e.g. in pig plasma/serum, the concentration of the cytokine TNF-α is higher (50–150 pg/ml) [64–66] than in rat plasma (~10 pg/ml) [67, 68].

Mitochondrial function was measured at one time point of early reperfusion, and these results are not transferable to the whole reperfusion period [69]. However, the first minutes of reperfusion after the sustained ischemia have been emphasized as critical for signal transduction of cardioprotection and mitochondrial function [22, 69]. Mitochondrial function was assessed using a mixture of mitochondrial fractions, the subsarcolemmal and the interfibrillar mitochondria. Prior studies used subsarcolemmal mitochondria [19, 28] or saponin-permeabilized cardiac fibers [26, 27]. Subsarcolemmal mitochondria are more vulnerable to ischemic injury and to mitochondrial calcium overload than interfibrillar mitochondria [70, 71].

All measurements of mitochondrial function were made at physiological temperature of 37°C. At this temperature, respiration rates are increased and may not be comparable to mitochondrial respiration measured at 25°C [72].

In conclusion, the present study provides evidence that myocardial protection by across-species transfer of RIPC pig plasma to isolated perfused rat hearts is associated with improved mitochondrial function at early reperfusion, supporting the notion that mitochondria are an intracellular target of protection by RIPC.

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Conflict of interest
The authors declare no conflict of interest.

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