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Bioassays of Humoral Cardioprotective Factors Released by Remote Ischemic Conditioning in Patients Undergoing Coronary Artery Bypass Surgery

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Abstract

Remote ischemic conditioning (RIC) induces the release of circulating cardioprotective factors and attenuates myocardial ischemia/reperfusion injury. Evidence for such humoral cardioprotective factor(s) is derived from transfer with plasma (derivatives) from one individual undergoing RIC to another individual's heart, even across species. With transfer into an isolated perfused heart, only a single plasma (derivative) sample can be studied with infarct size as endpoint, and therefore the comparison of samples before and after RIC or between RIC and placebo is hampered by the inter-individual variation of infarct sizes in isolated perfused hearts. We therefore developed a preparation of cardiomyocytes from a single mouse heart, where aliquots of the same heart can undergo hypoxia/reoxygenation (H/R) with exposure to buffer, RIC, or placebo samples without or with pharmacological blockade. To validate this approach, we used plasma dialysates taken before and after RIC from patients undergoing coronary bypass grafting who had experienced protection by RIC (troponin release ↓ by 28% vs placebo). The cardiomyocyte bioassay had little variation after H/R with buffer (mean ± standard deviation; 7% ± 2% viable cells) and demonstrated preserved viability after RIC (15% ± 5% vs 6% ± 3% before). For comparison, infarct size in isolated mouse hearts after global ischemia and reperfusion was 22% ± 14% of left ventricular mass after versus 42% ± 14% before RIC. Stattic, an inhibitor of signal transducer and activator of transcription (STAT)3 protein, abrogated protection in the cardiomyocytes. We have thus established a cardiomyocyte bioassay to analyze RIC's protection which minimizes inter-individual variation and the use of animals.

Keywords

cardioprotection, ischemia/reperfusion, myocardial ischemia, remote ischemic conditioning, reperfusion injury

Introduction

Ischemic conditioning by brief cycles of coronary occlusion/reperfusion reduces not only infarct size in the dependent myocardium, but also at a distance in neighboring myocardium.[1] In fact, brief cycles of ischemia/reperfusion in a tissue or organ even further remote from the heart protect from myocardial ischemia/reperfusion injury.[2,3] Remote ischemic conditioning (RIC) is a systemic phenomenon, which is elicited not only by ischemia/reperfusion but also by trauma and chemical or electrical sensory nerve stimulation. RIC is elicited from a variety of tissues and organs, and it also protects not only the heart but also a variety of other parenchymal organs.[4,5] The signal transfer between the tissue of origin and the tissue of protection is through humoral and neuronal pathways which interact.[5,6] RIC has been demonstrated in all species tested so far, including humans. A number of studies has evidenced cardioprotection in patients undergoing elective or primary interventional or surgical coronary revascularization,[3,7] but not all studies were positive.[3,8] In an individual patient undergoing a RIC protocol,

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cardioprotection is difficult to assess. When measuring biomarkers such as troponin one does not know what the troponin release would have been without RIC. The same is true for imaging techniques which measure infarct size and have the advantage over biomarkers to provide also information on the area at risk.[9]

Evidence for humoral transfer of cardioprotective factors in RIC has been provided by transfer of plasma or plasma derivatives from one individual undergoing RIC to another individual's heart preparation.[10-14] However, when using an isolated perfused heart undergoing ischemia/reperfusion as a bioassay of cardioprotective transfer, only a single plasma/ plasma derivative sample can be tested with infarct size as endpoint. Thus, the comparison of samples before and after RIC or of RIC versus placebo, respectively, entails inevitably the inter-individual variation inherent to infarct sizes in different isolated hearts which is considerable. We therefore developed a bioassay using isolated cardiomyocytes from a single mouse heart. Aliquots of cardiomyocytes can then be subjected in parallel to viability and buffer controls, exposure to RIC and placebo plasma/plasma derivatives without or with pharmacological blockade, thus avoiding inter-individual differences between different mouse hearts. More specifically, we compared the variation of bioassays using isolated perfused hearts undergoing ischemia/reperfusion with infarct size as endpoint and of isolated cardiomyocyte preparations from a single heart undergoing hypoxia/reoxygenation (H/R) with viability as endpoint. We evaluated the ability of the respective bioassay to detect the transfer of cardioprotective factors from a cohort of patients who underwent coronary bypass grafting and had protection by RIC as evidenced by reduced troponin release.[15,16]

Methods

Patient Cohort

The study conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local Institutional Review Board of the University of Essen Medical School (#13-5507). Patients gave their written informed consent. Patients were recruited as part of an extended randomized placebo-controlled single center trial and underwent CABG surgery under isoflurane anesthesia (NCT01956708, date of registration: October 8, 2013). Inclusion and exclusion criteria have been reported previously.[15,16] For the present analysis, samples from 40 consecutive patients who were enrolled between October 2014 and October 2015 were used. These patients underwent CABG surgery in a non-campus hospital and had less complex surgery than those in the entire cohort. RIC was induced by 3 cycles of 5 min right upper arm ischemia (by inflation of a blood pressure cuff to 200 mm Hg)/5 min reperfusion (by cuff deflation) after induction of general anesthesia and before skin incision. For placebo, the cuff was left deflated for 30 min. Venous blood samples were collected at baseline prior to RIC or placebo, and 30 min after completion of RIC or placebo, respectively. Additional venous blood samples were withdrawn at 1, 6, 12, 24, 48, and 72 h after surgery for the measurement of serum cardiac troponin T (cTnT). The cTnT area under the curve (AUC) calculated.[15,16]

Animals and Materials

All procedures involving animals were conducted in accordance with the German laws for animal welfare and the regulations of the local governmental Animal Care and Use Committee of the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany, and are reported in accordance with the ARRIVE guidelines.[17] The experimental protocols in isolated buffer-perfused mouse hearts and cardiomyocytes as well as the methods for the measurement of coronary flow (CF), left ventricular (LV) pressure and the quantification of infarct size and cardiomyocyte viability were standard[9] and have been described in detail previously.[13,18] Male C57Bl6/J mice, 20-27 g, 2.5 $\pm$ 0.5 months from Charles River (Sulzfeld, Germany, n = 177 in total) were used. Mice were housed in temperature- (23 °C $\pm$ 1 °C) and light-controlled (inverse 12:12 hour light-dark cycle) conditions. Food and water were provided ad libitum. Unless otherwise specified, materials were obtained from Sigma-Aldrich (Deisenhofen, Germany).

Plasma-Dialysate Preparation

Blood samples, collected before and after RIC or placebo, respectively, were centrifuged at 800 xg for 10 min to obtain plasma. The obtained plasma samples were again centrifuged at 2400 xg for 10 min before being placed into a dialysis tube with a pore size of 12-14 kDa (SpectraPor, Spectrum Europe, Breda, The Netherlands) and dialyzed against a 10-fold volume of saline buffer for 24 h using an orbital shaker. At least 6 mL of plasma was required to prepare a sufficient volume of plasma-dialysate for perfusion in the Langendorff apparatus. For the isolated perfused heart, a modified Krebs-Henseleit buffer[18] was used (in mmol/L: NaCl 118.0, KCl 4.7, $MgSO_4$ 16.0, KH_2PO_4 1.2, glucose 5.6, sodium pyruvate 2.0), dialysate was titrated to $CaCl_2$ 2.0 and 24.0 NaHCO3, filtered with a 5 µm pore-sized syringe filter and gassed during prewarming to 37 °C with 95% O_2 and 5% CO_2, pH 7.40. For the isolated cardiomyocytes,[13] 1 mL plasma was dialyzed against a 10-fold volume of modified Tyrode buffer (containing in mmol/L: 125.0 NaCl, 5.4 KCl, 1.2 NaH_2PO_4, 20.0 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, 5.0 taurine, 15.0 glucose, 2.5 creatine, 0.5 $MgCl_2$) for 24 h using an orbital shaker. The dialysate was titrated to 1.0 $CaCl_2$ and gassed with 100% oxygen, pH 7.4 before use. The plasma-dialysate from one patient in the placebo group was excluded from further experiments in isolated perfused mouse hearts, because precipitation of calcium and pH >7.9 occurred during the dialysis process, therefore two mouse hearts were not included in the analysis. Due to the limited volume of plasma samples, plasma-dialysates from only 13 patients with RIC and 11 patients with placebo were analyzed in the cardiomyocyte bioassay.

Isolated Perfused Mouse Hearts

Mice were sacrificed by cervical dislocation, their hearts isolated and perfused at constant pressure (80 mm Hg) with modified Krebs-Henseleit buffer.[18] Heart rate was set to 500 beats/min by right atrial pacing. CF was measured with an inline ultrasonic flowprobe (TS410, Transsonic Systems Inc., Ithaca, NY, USA) above the aortic cannula. A fluid-filled cling-film balloon was inserted into the LV cavity and connected to a pressure transducer (Codan-PVB, Lensahn, Germany) to measure LV pressure. End-diastolic LV pressure was set to 1-7 mm Hg at baseline by graded balloon inflation during the initial 5 min perfusion. Left ventricular developed pressure (LVDP) was calculated as the difference between peak and end-diastolic LV pressure. CF, end-diastolic and peak LV pressure were continuously recorded (LabChart 8, AD Instruments Pty LTD, New South Wales, Australia). Hearts were allowed to stabilize for 10-20 min. CF and LVDP were calculated as mean values during the last min each of the stabilization period (baseline), the last min of plasma-dialysate infusion, at 5 and 25 min ischemia and at 10, 30 and 60 min reperfusion, respectively. Preparations with CF <1.0 mL/min or >5.0 min or LVDP <60 mm Hg at baseline were excluded. Eighteen isolated heart preparations did not meet baseline criteria and were thus excluded from further analysis. After CF and LVDP were recorded at baseline, plasma-dialysate or buffer was infused over 15 min before hearts were subjected to 30 min global zero-flow ischemia and 120 min reperfusion. For time control (TC), hearts were perfused for a duration equal to that of an experimental protocol, i.e., 150 min. The temperature of the perfusion buffer was monitored with probes in the aortic cannula throughout the experiment and kept between 37.5 °C and 37.8 °C by heat exchangers. Hearts were continuously warmed in a 37.5 °C to 37.7 °C humidified chamber. After termination of the protocol, hearts were frozen at −20 °C and cut into transverse 1 mm thick slices. Infarcted tissue was demarcated by staining with 0.09 mol/L sodium phosphate buffer containing 1.0% triphenyl tetrazolium chloride (TTC) at 37 °C for 5 min. Stained slices were photographed from both sides. The total slice area and the infarcted areas were quantified by computer-assisted planimetry (ImageJ 1.48v, National Institutes of Health, Bethesda, Maryland, USA), and infarct size was calculated as percent of the sum of left and right ventricular mass (% of ventricular mass).[18] Inter-individual coefficients of variation were calculated for infarct sizes and expressed as percent of the mean values.

Isolated Mouse Cardiomyocytes

Cardiomyocyte preparation. Mice were sacrificed as described above, their hearts isolated and perfused with modified Tyrode buffer (in mmol/L: 113.0 NaCl, 4.7 KCl, 0.6 KH_2PO_4, 0.6, Na_2HPO4, 1.2 $MgSO_4$, 12.0 $NaHCO_3$, 10.0 $KHCO_3$, 10.0 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, 30.0 taurine, 5.5 glucose, and 10.0 2,3-butanedione monoxime, pH 7.42 at 36.5 °C) at constant flow of 3 mL/min for 3 min. Liberase (Liberase TM Research Grade, Roche, Basel, Switzerland, 175 µg/ml), trypsin (75 µg/ml) and 12.5 µmol/L $CaCl_2$ were subsequently added to the perfusion buffer, and hearts were digested for 4 min. Atrial and connective tissue was removed and discarded, ventricles were sectioned, and cells were re-suspended in Tyrode buffer containing 10% bovine calf serum (gibco, Thermo Fisher Scientific, Waltham, USA) and 12.5 µmol/L $CaCl_2$. Cardiomyocytes were isolated, separated from tissue residues by filtering through a nylon mesh filter (200 µm pore size, Millipore, Billerica, USA), and $CaCl_2$ was slowly titrated at 20 °C to a final concentration of 1 mmol/L (5 steps of 10 min duration each). Cardiomyocytes were kept in modified Tyrode buffer (containing in mmol/L: 125.0 NaCl, 5.4 KCl, 1.2 NaH_2PO_4, 20.0 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, 5.0 taurine, 15.0 glucose, 2.5 creatine, 0.5 $MgCl_2$, and 1.0 $CaCl_2$, gassed with 100% oxygen, pH 7.4) at 20 °C under normoxic conditions for 5 min before viability was determined at baseline. Cardiomyocytes were stained with 0.5% trypan blue, and 300-700 cells per sample were analyzed in non-overlapping visual fields using light microscopy at 40 x magnification (Leica DMLB microscope, Leica, Bensheim, Germany). Viability was expressed as the percentage of rod-shaped, unstained cardiomyocytes over the total number of cells. Cardiomyocyte isolations with a viability of <60% at baseline were discarded (n = 7 preparations with viabilities ranging between 45% and 59%).

Intra-individual variation of cardiomyocyte viability after H/R. Whereas the above experiments using patient plasma in isolated perfused heart and cardiomyocyte preparations were performed along with the patient recruitment, a separate series on the variation of viability in isolated cardiomyocyte preparations was performed in June and July 2021. Isolated cardiomyocytes from one single mouse heart were prepared as described above (n = 15) and divided into 5 aliquots each, respectively. Cardiomyocyte aliquots from one single heart were either incubated with buffer (n = 5 isolations, 750 µL buffer/aliquot) or plasma-dialysate taken before or after RIC from patients (n = 5 isolations with 750 µL plasma-dialysate/aliquot, each) for 30 min, respectively, and subjected to H/R. Hypoxia was induced for 50 min by exposing cardiomyocytes to glucose-free, non-gassed buffer (in mmol/L: 119.0 NaCl, 120.0 KCl, 5.0 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 20.0 sodium lactate, pH 6.5) and sealing with mineral oil. Cells were kept in solution at 20 °C where they sedimented. Reoxygenation was induced by removal of oil and glucose-free buffer and by adding reoxygenation buffer with an osmolality of 250 mosm/L (in mmol/L: 88.0 NaCl, 5.4 KCl, 1.2 NaH_2PO4, 12.0 $NaHCO_3$, 20.0 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, 5.0 taurine, 15.0 glucose, 2.5 creatine, 0.5 $MgCl_2$, and 1.0 $CaCl_2$, gassed with 100% oxygen, pH 7.4) for 5 min. In separate experiments (n = 4), the oxygen partial pressure during hypoxia was continuously measured using the oxygraph-2 k (O2 k, OROBOROS Instruments, Austria). The oxygen partial pressure at 1 min hypoxia was 66 ± 2 mm Hg and decreased within 10 min

hypoxia to 10 ± 0 mm Hg. Incubation with reoxygenation buffer increased the oxygen partial pressure back to 153 ± 3 mm Hg. The cardiomyocyte viability of each aliquot was determined after H/R. Viabilities of aliquots obtained from one single heart were used to calculate the mean viabilities ± standard deviations per aliquot and per group (incubation with buffer, plasma-dialysate before RIC, plasma-dialysate after RIC). **Intra**-individual coefficients of variation of viabilities were calculated per aliquot, and **inter**-individual coefficients of variation were calculated per group, respectively, and expressed as percent of the respective mean values.

Incubation with plasma-dialysates from individual patients. Cardiomyocyte aliquots from each isolation were either incubated for 30 min with RIC plasma-dialysate from blood taken before or after RIC, respectively, with plasma-dialysate from blood taken before or after placebo, respectively, or with modified Tyrode buffer (750 µL/aliquot) and subjected to H/R. Plasma samples from one individual patient were tested in cardiomyocytes isolated from one individual mouse heart. We have demonstrated in our previous study, that signal transducer and activator of transcription (STAT)3 activation in murine myocardium is causally involved in the cardioprotection transferred with human humoral factors released in response to RIC.[18] Thus, all plasma-dialysates were incubated in the absence and presence of 1 µmol/L of the STAT3 blocker stattic (Tocris, Bio-Tech GmbH, Wiesbaden, Germany). The stattic concentration had been optimized in preliminary experiments to not impact on cardiomyocyte viability per se. In viability control (VC) experiments, cardiomyocytes were incubated for a duration equal to that of a H/R protocol i.e., 85 min. Modified Tyrode buffer was replaced with fresh oxygenated Tyrode buffer in VC experiments at the same time points when buffer was changed in H/R experiments, i.e., after 30 min and after 80 min of incubation. In separate experiments (n = 4) the oxygen partial pressure (oxygraph-2 k) was 152 ± 1 mm Hg after 30 min and decreased over the subsequent 50 min to 64 ± 1 mm Hg. Reoxygenation with modified Tyrode buffer restored oxygen partial pressure back to 163 ± 1 mm Hg. Cardiomyocyte samples were taken again at 85 min after H/R or VC, respectively, and viability was quantified. Plasma-dialysates before and after RIC, respectively, and before and after placebo, respectively, were used without or with stattic.

Statistical Analysis

Data are presented as means ± standard deviations. Experiments in isolated perfused mouse hearts and cardiomyocytes were performed using contemporary block randomization. Investigators performing experiments in isolated perfused mouse hearts and cardiomyocytes and analyzing infarct size, cardiomyocyte viability, and time courses of CF and LVDP in isolated hearts were blinded with respect to the nature of the plasma-dialysate (RIC/placebo and before/after, respectively). The Kolmogorov-Smirnov-test was used to test for normal distribution of all data. Patient demographics and intraoperative characteristics were compared between RIC and placebo using unpaired Student's t test (continuous data) or 2-tailed Fisher's exact test (categorical data). The cTnT AUC was compared between RIC and placebo by unpaired Student's t test. Two-way (time, group) ANOVA for repeated measures was used to analyze CF and LVDP in isolated perfused mouse hearts. Two-way (time, blocker) ANOVA for repeated measurements was used to analyze cardiomyocyte viability after incubation with plasma-dialysate and H/R. Fisher's least-significant-difference post-hoc tests were used when the two-way ANOVA indicated a significant difference. One-way Kruskal-Wallis ANOVA on ranks was used to analyze infarct size in isolated perfused hearts. Individual mean values of data sets were compared by multiple comparisons procedures using Dunn's method when ANOVA on ranks indicated a significant difference. Differences were considered significant at the level of $P < .05$ (SigmaStat 3.5, Erkrath, Germany).

Results

Patient Demographics and Perioperative cTnT AUC

Demographics and intra- and postoperative characteristics were not different between patients undergoing RIC and placebo, respectively (Table 1). The preoperative serum cTnT concentration did not differ between RIC and placebo. The cTnT AUC was reduced by 28% with RIC than with placebo (20.4 ± 9.0 versus 28.4 ± 14.0 ng/mL × 72 h, $P = .0233$), reflecting cardioprotection by RIC in this patient cohort.

CF, LVDP, and Infarct Size in Isolated Hearts

Baseline CF and LVDP of the isolated perfused mouse hearts were not different between TC, buffer, RIC and placebo and before and after RIC or placebo, respectively (Table 2). With infusion of plasma-dialysate after RIC, the recovery of LVDP at 10, 30 and 60 min reperfusion was better than with plasma-dialysate before RIC (Table 2). The recovery of CF during reperfusion was not different between isolated perfused mouse hearts with RIC and placebo and before and after RIC or placebo, respectively.

With VC, only negligible infarction (4% ± 2%) was detected (Figure 1). With buffer infusion and global ischemia/reperfusion infarct size was 42% ± 9% (Figure 1). Infarct size was 42% ± 14% of ventricular mass with plasma-dialysate before RIC and significantly less (22% ± 14%) after infusion of plasma-dialysate after RIC (Figure 1). Infusion of plasma-dialysate taken before or after placebo, respectively, had no impact on infarct size (before placebo: 39% ± 14% versus after placebo: 40% ± 13%, Figure 1).

Cardiomyocyte Viability After H/R

The yield of viable cardiomyocytes over all cells ranged between 60% and 74% at baseline and was not different between isolations (Figures 2 and 3). The viability of cardiomyocyte aliquots from one single heart incubated with buffer

Table 1. Patient Demographics and Intraoperative Characteristics.[a,b]

	RIC, n = 20	Placebo, n = 20	*P* value
Demographics			
Age, years	66 ± 11	68 ± 9	.57
Sex, (male)	14 (70%)	16 (80%)	.71
Body weight, kg	80 ± 12	82 ± 13	.64
Risk factors and comorbidities			
Chronic obstructive pulmonary disease	2 (10%)	0	.48
Diabetes mellitus	0 (0%)	0 (0%)	1.00
Hypertension	17 (85%)	15 (75%)	.69
Hypercholesterolemia	8 (40%)	5 (25%)	.50
Peripheral arterial disease	1 (5%)	1 (5%)	1.00
Renal disease, creatinine >200 mol/L	0	0	1.00
Cardiac status			
Angina CCS III to IV	10 (50%)	15 (75%)	.19
Previous myocardial infarction	2 (10%)	2 (10%)	1.00
Left ventricular ejection fraction, %	46 ± 6	48 ± 4	.48
Preoperative serum cTnT, ng/mL	0.01 ± 0.02	0.01 ± 0.01	.43
Medication			
ACE inhibitors or ARBs	16 (80%)	12 (60%)	.30
Aspirin	20 (100%)	19 (95%)	1.00
β-blockers	16 (80%)	15 (75%)	1.00
Clopidogrel	0	2 (10%)	.48
Statins	16 (80%)	19 (95%)	.34
Risk scores			
EuroSCORE II, %	3 ± 2[c]	2 ± 1	.04
Logistic EuroSCORE, %	4 ± 3[c]	2 ± 1	.013
Intraoperative characteristics			
Aortic cross-clamp duration, min	38 ± 14	41 ± 11	.42
Cardioplegia, mL	1311 ± 210	1342 ± 150	.60
Number of bypass grafts	3 ± 1	3 ± 1	.68
Reperfusion time, min	22 ± 9	27 ± 11	.17
Time from end of RIPC/placebo to cardioplegic arrest, min	67 ± 22	75 ± 44	.44
Time from end of RIPC/placebo to reperfusion, min	130 ± 32	150 ± 53	.17

Abbreviations: ACE, angiotensin-converting enzyme; ARBs, angiotensin-II-receptor blockers; CCS, Canadian Cardiovascular Society; EuroSCORE, European system for cardiac operative risk evaluation; cTnT, cardiac troponin T; reperfusion time, time from the release of aortic cross-clamp to end of cardiopulmonary bypass; RIC, remote ischemic conditioning.

[a]Data are means ± standard deviations or number (%).

[b]Demographics and intraoperative characteristics were compared using Student's *t* test (continuous data) or two-tailed Fisher's exact test (categorical data).

[c]$P < .05$ versus placebo; unpaired Student's *t* test.

or plasma-dialysate before RIC before being subjected to H/R, was comparable and ranged between 5% to 9% with buffer and 2% to 8% with plasma-dialysate before RIC. Incubation with plasma dialysate after RIC preserved viability better (11%-21%) than incubation with plasma-dialysate before RIC or buffer (Figure 2). In experiments, where plasma-dialysates before/after RIC from individual patients were tested in cardiomyocytes isolated from one single heart, incubation with plasma-dialysate after RIC preserved viability after H/R better (15% ± 5%) than with plasma-dialysate before RIC (6% ± 3%). In VC experiments, cardiomyocyte viability significantly decreased by 9% ± 5% from a baseline of 66% ± 6%. Stattic per se had no impact on cardiomyocyte viability after H/R (Figure 3). Stattic abrogated the protection by plasma-dialysate after RIC (8% ± 3%) (Figure 3A). With plasma-dialysate before or after placebo, no protection was observed (before placebo: 8% ± 2% vs after placebo: 7% ± 3%, Figure 3B).

Discussion

We confirm the release of humoral cardioprotective factors from patients undergoing CABG surgery under isoflurane anesthesia with prior RIC which we have reported before.[15,16] RIC's cardioprotection was evidenced via reduction of postoperative troponin release in the cohort undergoing RIC as compared to the placebo cohort. Prior larger phase III trials, which did not report reduced troponin release in cardiac surgery patients with RIC, were confounded by use of propofol anesthesia[19,20] which interferes with the protection by RIC.[21-23] The less pronounced reduction of postoperative troponin in the present study may be attributed to less injury from shorter ischemic cross-clamp duration in this particular cohort when compared to the larger cohorts of our previous studies, which involved a greater proportion of more complex surgery.[15,16] However, the focus of the present study was not on cardioprotection in patients per se, but on the release of circulating

Table 2. CF and LVDP of Isolated Buffer-Perfused Mouse Hearts.[a,b]

	Time	CF (mL/min)	LVDP (mm Hg)
TC (n = 5), time corresponding to data points in the other protocols	Baseline	2.6 ± 1.4	71 ± 15
	+7 min	2.7 ± 1.4	77 ± 11
	+25 min	2.6 ± 1.5	81 ± 36
	+40 min	2.6 ± 1.4	82 ± 34
	+50 min	2.5 ± 1.4	70 ± 13
	+60 min	2.6 ± 1.3	64 ± 13
Buffer (n = 24)	Baseline	3.0 ± 0.7	91 ± 16
	Buffer	2.9 ± 0.7	89 ± 15
	Isch5	0 ± 0[c]	0 ± 0[c]
	Isch25	0 ± 0[c]	0 ± 0[c]
	Rep10	2.9 ± 1.6	15 ± 17[c]
	Rep30	2.5 ± 1.3	27 ± 27[c]
	Rep60	2.4 ± 1.2	31 ± 23[c]
Plasma-dialysate before RIC (n = 20)	Baseline	2.6 ± 0.8	72 ± 19
	Dialysate	2.9 ± 0.8	77 ± 25
	Isch5	0 ± 0[c]	0 ± 1[c]
	Isch25	0 ± 0[c]	1 ± 1[c]
	Rep10	2.8 ± 1.1	14 ± 21[c]
	Rep30	2.5 ± 1.1	28 ± 21[c]
	Rep60	2.4 ± 1.2	30 ± 18[c]
Plasma-dialysate after RIC (n = 20)	Baseline	2.7 ± 0.6	82 ± 12
	Dialysate	3.3 ± 0.8	95 ± 22
	Isch5	0 ± 0[c]	1 ± 2[c]
	Isch25	0 ± 0[c]	1 ± 2[c]
	Rep10	3.0 ± 1.0	26 ± 23[c,d]
	Rep30	2.9 ± 0.7	49 ± 21[c,d]
	Rep60	2.8 ± 0.7	54 ± 16[c,d]
Plasma-dialysate before placebo (n = 19)	Baseline	2.3 ± 0.5	79 ± 15
	Dialysate	2.5 ± 0.5	88 ± 19
	Isch5	0 ± 0[c]	1 ± 1[c]
	Isch25	0 ± 0[c]	0 ± 0[c]
	Rep10	2.5 ± 0.6	28 ± 21[c]
	Rep30	2.4 ± 0.6	53 ± 17[c]
	Rep60	2.2 ± 0.6	51 ± 11[c]
Plasma-dialysate after placebo (n = 19)	Baseline	2.8 ± 1.1	81 ± 21
	Dialysate	3.0 ± 1.2	83 ± 20
	Isch5	0 ± 0[c]	1 ± 1[c]
	isch25	0 ± 0[c]	1 ± 1[c]
	Rep10	2.8 ± 1.1	21 ± 25[c]
	Rep30	2.7 ± 1.1	42 ± 26[c]
	Rep60	2.6 ± 1.1	46 ± 26[c]

Abbreviations: Baseline, last min of stabilization period before ischemia/reperfusion; CF, coronary flow; isch5/25, 5/25 min of ischemia; LVDP, left ventricular developed pressure; n, number of mouse hearts; rep 10/30/60, 10/30/60 min reperfusion; RIC, remote ischemic conditioning.

[a] Data are means ± standard deviations.

[b] Baseline values for CF and LVDP and their time courses were analyzed by two-way ANOVA for repeated measures and Fisher's least significant difference post-hoc tests.

[c] $P < .05$ versus baseline, respectively.

[d] $P < .05$ versus before RIC.

cardioprotective factors and the methods of their detection. Our bioassay technique enables the analysis of plasma samples taken from an individual patient before and after the RIC procedure. When plasma dialysates are tested in isolated buffer-perfused hearts, the protection by RIC becomes evident in reduced infarct size but is always confounded by the inter-individual differences in infarct size between the bioassay hearts. However, when using aliquots from a single heart's isolated cardiomyocyte preparation, responses to dialysates from samples taken before versus after RIC can be compared without such confounding by inter-individual differences. In addition, viability controls and pharmacological blocker experiments can be performed in aliquots from the same heart to study the signal transduction and gain further insight into the nature of the human cardioprotective factor(s).

Several humoral cardioprotective factors have been identified.[24] The disturbing observation, that the inhibition or elimination of a single factor by blocking agents abrogated cardioprotection completely, although other identified factors were not considered,[25] may be, in part, explained by the use of isolated heart bioassays with a considerable inter-individual variance, where subtle differences in the degree of cardioprotection and its attenuation may have been obscured. The use of the cardiomyocyte bioassay permits to analyze the transfer of cardioprotection robustly in a smaller cohort of patients than would be needed with analyzing transfer to an isolated perfused heart bioassay. Since accompanying control experiments, but also additional blocker experiments can be performed in parallel, the cardiomyocyte bioassay has the potential to reduce the number of sacrificed animals.[26] The exclusion rate of preparations in both our bioassays that did not meet baseline criteria was similar in the present study: 127 animals were used for the isolated heart bioassay of which 20 preparations were excluded which corresponds to an exclusion rate of 16%. In the cardiomyocyte bioassay, 7 of the 50 mouse hearts, i.e., 14% were excluded. Nevertheless, the isolated cardiomyocyte model permits the use of at least 6 conditions in parallel using aliquots of a single heart, whereas the isolated perfused heart with infarct size as endpoint only permits the analysis of one condition. Although the exclusion rate of the isolated cardiomyocyte bioassay appears similar to that of the isolated heart bioassay, this bioassay was still superior in terms of the potential to save animals and reduce the number of used animals by a factor of 6 in our study when comparing 6 conditions. Also, the cardiomyocyte bioassay uses much less plasma-dialysate than the isolated heart such that 6 conditions in the cardiomyocyte rather than one condition in the isolated heart bioassay per patient sample can be compared. In the viability control experiments of the isolated cardiomyocyte preparation, oxygen partial pressure decreased over time whereas viability was almost fully preserved (Figure 3). The preserved viability might either reflect the fact that oxygen supply to the non-contracting cardiomyocytes at a temperature of 20 °C was still sufficient, or to a slowly developing state of hibernation.[27] In any event, the preserved viability in these experiments contrasted to the substantially reduced viability in the experiments with induced H/R, and this reduced viability was the target endpoint to study the cardioprotective properties of patient RIC plasma.

The cardioprotective potential of plasma-dialysate from CABG surgery patients after RIC in the present study was comparable to that induced by plasma-dialysate from healthy

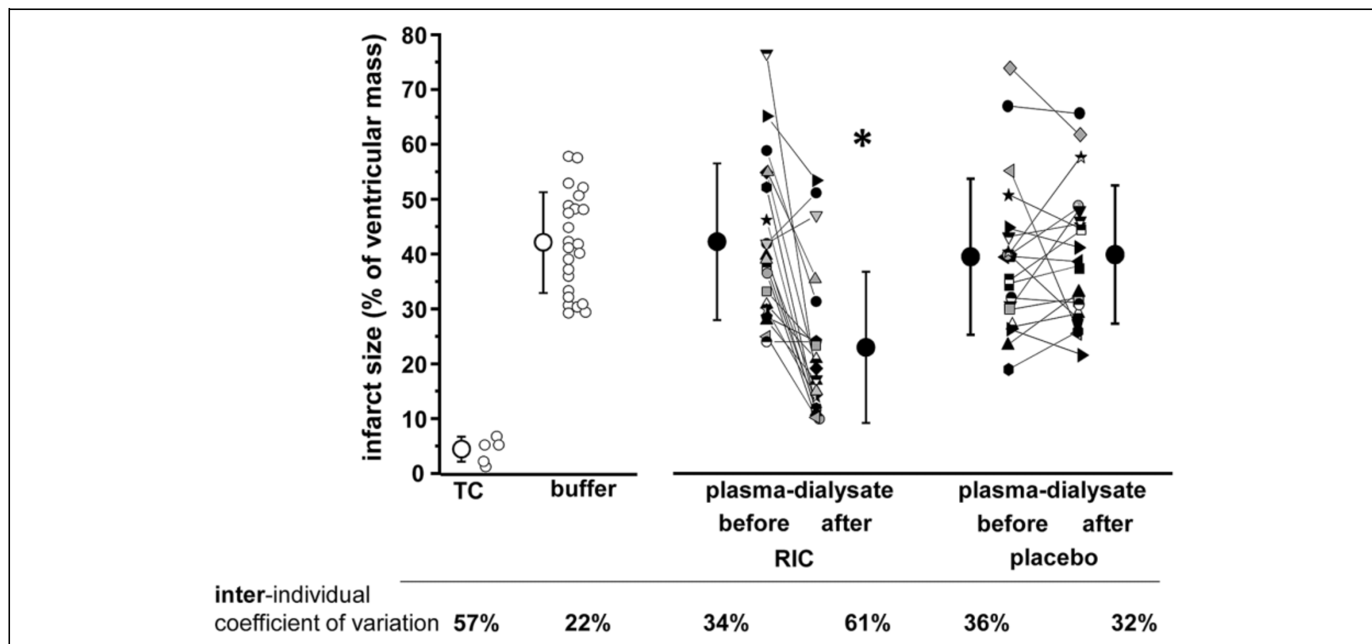

Figure 1. Infarct size in isolated perfused mouse hearts subjected to 30 min/120 min global zero-flow ischemia/reperfusion with infusion of plasma-dialysate before or after RIC and before or after placebo, respectively. Data are presented as means $\pm$ standard deviations. Samples from a single patient are reflected by use of identical symbols. Gray symbols indicate female, black symbols indicate male patients. RIC indicates remote ischemic conditioning. Left side: Infarct size in isolated perfused hearts with TC (time control) or GI/R (global ischemia/reperfusion). *P < .05 versus before RIC; one-way Kruskal-Wallis ANOVA on ranks.

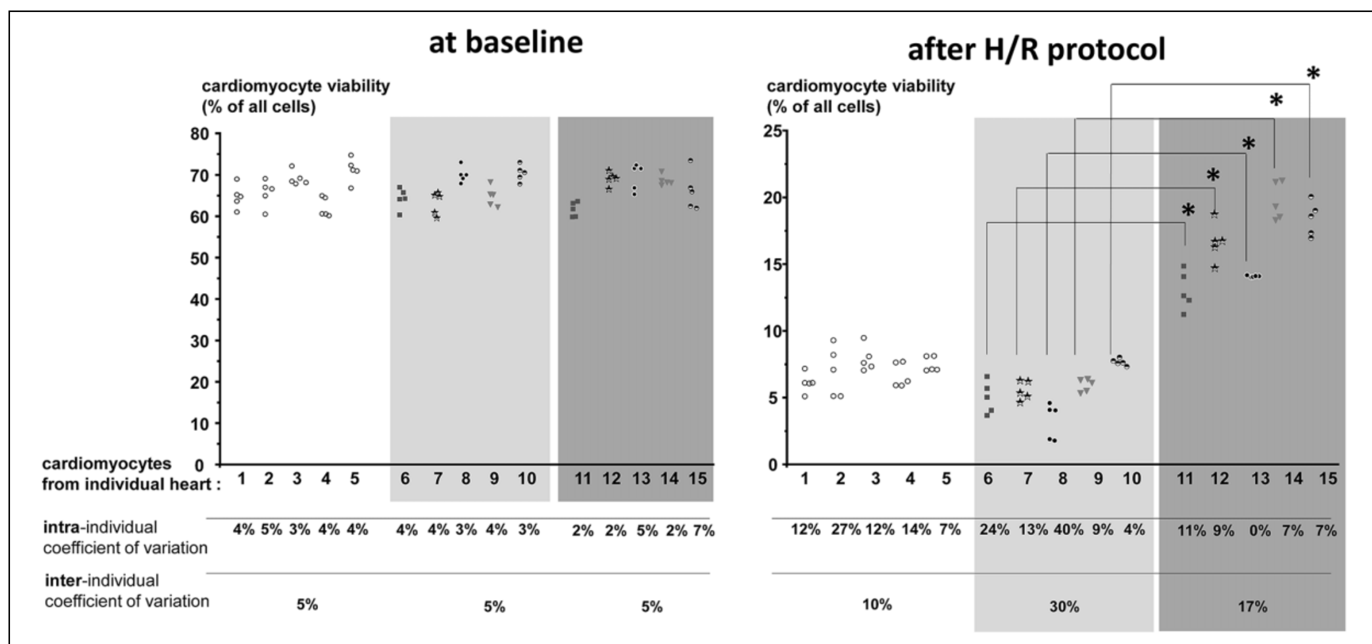

Figure 2. Viability of isolated adult ventricular mouse cardiomyocytes from one single heart, divided into 5 aliquots and subjected to a protocol of baseline followed by 50 min hypoxia/5 min reoxygenation with responses to incubation with buffer or plasma-dialysate before RIC or plasma-dialysate after RIC, respectively. Light gray highlighted indicates incubation with plasma-dialysate before RIC, gray highlighted indicates incubation with plasma-dialysate after RIC. H/R indicates hypoxia/reoxygenation; RIC, remote ischemic conditioning. *P < .01 versus plasma-dialysate before RIC; two-way ANOVA for repeated measures with Fisher's least-significant differences post-hoc tests.

volunteers in our previous study.[18] In human myocardium, cardioprotection by RIC is associated with STAT5 activation,[28] whereas the transfer of RIC's cardioprotection with plasma-dialysate of healthy volunteers causally involves STAT3 activation in the myocardium of the isolated perfused mouse hearts.[18] We now characterized RIC's humoral transfer of protection more

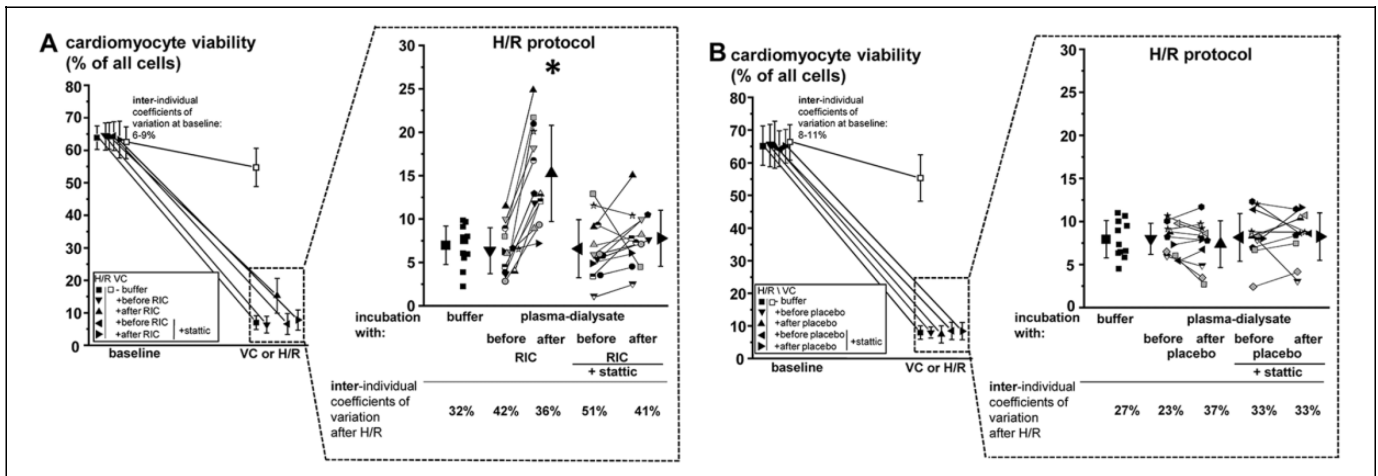

Figure 3. Viability of isolated adult ventricular mouse cardiomyocytes after 50 min hypoxia/5 min reoxygenation and incubation of cardiomyocytes from a single isolation with (A) plasma-dialysate prepared from a single individual patient's blood samples taken before and after RIC, or (B) incubation with plasma-dialysate from another patient's blood samples taken before and after placebo. Data are presented as means ± standard deviations. H/R indicates hypoxia/reoxygenation; RIC, remote ischemic conditioning; VC, viability control. **P* < .01 versus plasma-dialysate before RIC, respectively; two-way ANOVA for repeated measures with Fisher's least-significant differences post-hoc tests.

specifically as a cardiomyocyte phenomenon, associated with STAT3 activation in the isolated mouse cardiomyocytes. Whether or not our bioassay provides valuable information on cardioprotective factors of RIC not only in cardiosurgical patients, but also in patients with myocardial infarction undergoing remote ischemic perconditioning[29] remains to be seen in future studies. For cardiosurgical patients, the cardiomyocyte approach may open new strategies to optimize cardioprotection on an individual basis.

Authors' Note

The data generated during and/or analyzed in the current study are available from the corresponding author on reasonable request. Institution where the work reported was done: Institute for Pathophysiology, West German Heart and Vascular Center, University of Essen Medical School, Essen, Germany.

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Data of the present manuscript are scheduled to be part of P. Tüllers' MD thesis.

Author Contributions

HL, PK, and GH designed the experiments. HL, PT, and FB performed the experiments and analyzed the data. AZ performed anesthesia and MK performed surgery in patients. MT collected and analyzed the patient data. HL and PK drafted the manuscript and GH interpreted the data and critically revised the manuscript.

Declaration of Conflicting Interests

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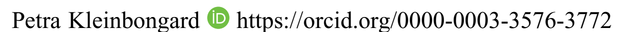
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