Across-Species Transfer of Protection by Remote Ischemic Preconditioning With Species-Specific Myocardial Signal Transduction by Reperfusion Injury Salvage Kinase and Survival Activating Factor Enhancement Pathways

Andreas Skyschally, Sabine Gent, Georgios Amanakis, Christiane Schulte, Petra Kleinbongard, Gerd Heusch

Rationale: Reduction of myocardial infarct size by remote ischemic preconditioning (RIPC), that is, cycles of ischemia/reperfusion in an organ remote from the heart before sustained myocardial ischemia/reperfusion, was confirmed in all species so far, including humans.

Objective: To identify myocardial signal transduction of cardioprotection by RIPC.

Methods and Results: Anesthetized pigs were subjected to RIPC (4×5/5 minutes hindlimb ischemia/reperfusion) or placebo (PLA) before 60/180 minutes coronary occlusion/reperfusion. Phosphorylation of protein kinase B, extracellular signal–regulated kinase 1/2 (reperfusion injury salvage kinase [RISK] pathway), and signal transducer and activator of transcription 3 (survival activating factor enhancement [SAFE] pathway) in the area at risk was determined by Western blot. Wortmannin/U0126 or AG490 was used for pharmacological RISK or SAFE blockade, respectively. Plasma sampled after RIPC or PLA, respectively, was transferred to isolated bioassay rat hearts subjected to 30/120 minutes global ischemia/reperfusion. RIPC reduced infarct size in pigs to 16±11% versus 43±11% in PLA (% area at risk; mean±SD; P<0.05). RIPC increased the phosphorylation of signal transducer and activator of transcription 3 at early reperfusion, and AG490 abolished the protection, whereas RISK blockade did not. Signal transducer and activator of transcription 5 phosphorylation was decreased at early reperfusion in both RIPC and PLA. In isolated rat hearts, pig plasma taken after RIPC reduced infarct size (25±5% of ventricular mass versus 38±5% in PLA; P<0.05) and activated both RISK and SAFE. RISK or SAFE blockade abrogated this protection.

Conclusions: Cardioprotection by RIPC in pigs causally involves activation of signal transducer and activator of transcription 3 but not of RISK. Protection can be transferred with plasma from pigs to isolated rat hearts where activation of both RISK and SAFE is causally involved. The myocardial signal transduction of RIPC is the same as that of ischemic postconditioning. (Circ Res. 2015;117:279-288. DOI: 10.1161/CIRCRESAHA.117.306878.)

Key Words: myocardial infarction ■ myocardial ischemia ■ reperfusion injury ■ signal transduction

Remote ischemic preconditioning (RIPC), that is, brief episodes of ischemia/reperfusion in an organ remote from the heart before sustained myocardial ischemia and subsequent reperfusion, reduces myocardial infarct size (IS). This cardioprotective maneuver is operative in all species tested so far, including humans.1–3 Repetitive inflation/deflation of a blood pressure cuff around a limb is easily feasible, safe, and effective in reducing IS in patients undergoing elective percutaneous coronary intervention5 or coronary artery bypass graft surgery6 and in patients having an acute myocardial infarction.7,8 We have recently shown in patients undergoing coronary artery bypass graft surgery that RIPC by 3 cycles of 5 minutes arm ischemia/5 minutes reperfusion not only attenuated periprocedural myocardial injury, as reflected by the reduced area under the curve of troponin I release, but also reduced all-cause mortality and the rate of major adverse cardiac and cerebrovascular events during follow-up for up to 4 years9; similar data on improved clinical outcome have been reported for patients with acute myocardial infarction when undergoing RIPC before reperfusion.10

At present, it is not understood how the remote stimulus is transferred from the ischemic/reperfused organ or limb to the heart. On the other hand, the identification of such humoral transfer factor would be an attractive pharmacological

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target to be reinforced in situations where cardioprotection is needed, for example, in acute myocardial infarction, complex cardiovascular surgery, or interventions. There is experimental evidence for both, a neuronal or a humoral transfer, as well for their interaction. Clearly, humoral transfer of a cardioprotective signal from one individual to another is possible, even if the recipient is an isolated heart preparation from a different species. The transfer factor(s) appear to be <15 kDa in size because it is still operative when dialyzed through a membrane with such cut-off. Some studies have identified specific factors, such as stromal cell-derived factor 1, nitrite, micro-RNA-144, in specific models and species, but no unequivocal candidate transfer factor has yet emerged. More systematic studies using proteomic approaches to identify the protective humoral transfer factor in plasma have also failed. Apart from the transfer signal, the recruited cardioprotective signaling pathways within the target myocardium are unclear in detail. To what extent RIPC shares established cardioprotective signaling pathways, such as the reperfusion injury salvage kinase (RISK) pathway or the survival activating factor enhancement (SAFE) pathway, with other conditioning strategies remains still to be clarified.

Against this background of a so far largely elusive search for the enigmatic humoral transfer factor of RIPC’s protection, we have now taken a novel, retrograde approach to characterize the properties of the upstream humoral transfer factor by the downstream myocardial signal transduction which it may activate. To this effect, we have used our experimental protocol (SAFE) pathway with stattic for SAFE blockade. In pigs, the antagonists were given before the RIPC maneuver and covered both the potential release of the protective factor and its potential action on the ischemic/reperfused myocardium. Stattic could not be used in pigs in situ because of its high toxicity. In the isolated rat hearts, the antagonists were given before infusion of plasma from pigs that had undergone RIPC or a PLA procedure and thus only antagonized the action of the cardioprotective signal on the ischemic/reperfused recipient heart.

Methods

The experimental protocols were approved by the Bioethical Committee of the district of Düsseldorf (pigs: GI240/11; rats: B1322/12).

Experiments in Pigs

Experimental Preparation

Göttinger minipigs (weight, 29.9±2.2 kg; age, 15±2 months) were sedated with flunitrazepam (0.4 mg/kg, Rohypnotol; Roche, Grenzach-Wyhlen). Anesthesia was induced by etomidate (0.5 mg/kg, Hypnomidat; Janssen-Cilag, Neuss) and sufentanil (1 µg/kg IV, Sufenta; Janssen-Cilag, Neuss). Anesthesia was maintained by artificial ventilation with isoflurane (2%) in oxygen-enriched air. This anesthesia is identical to that used in our institution for patients undergoing surgical coronary revascularization. The pigs were placed on a heated table and covered with drapes to prevent hypothermia. Esophageal temperature was thus kept at 36.9±0.9°C. One jugular vein was cannulated with a teflon catheter for volume replacement and intravenous drug administration. ECG-lead II was continuously monitored. The left hindlimb was shaved, and a tourniquet was placed around it for later induction of RIPC. After completion of the RIPC or PLA maneuver (see below), respectively, the common carotid arteries were cannulated to measure arterial pressure and to withdraw blood from the descending thoracic aorta as reference for the regional blood flow measurement. After a left lateral thoracotomy, the heart was exposed and instrumented with a micromanometer (P7; Konigsberg Invest., Pasadena, CA) in the left ventricle. A teflon catheter was placed in the left atrium for the injection of colored microspheres. A silk suture was placed around the left anterior descending (LAD) coronary artery distal to its second diagonal branch for later coronary occlusion. Ventricular fibrillation during the protocol was immediately terminated by electric countershock.

Experimental Protocols

RIPC (n=15)

RIPC was induced by tightening of the tourniquet around the left hindlimb; pale skin was taken to indicate leg ischemia. The tourniquet was released after 5 minutes, and the hindlimb was reperfused for 5 minutes. Skin blush indicated reperfusion. The ischemia/reperfusion cycle in the leg was repeated 4 times in total, just after induction of anesthesia and establishment of venous access. Then, the surgical preparation was completed. One hour after the RIPC maneuver, 100 to 120 mL arterial blood was withdrawn and sampled in vials containing Lithium–heparin and immediately centrifuged at 4°C with 800 g for 10 minutes. Separated plasma was centrifuged at 4°C with 4500 g for additional 10 minutes. The separated plasma was stored at −80°C for later use and again centrifuged for 10 minutes at 4500 g and filtered (0.2 µm pore size) before use. The sampled blood volume was replaced with saline. In preliminary experiments, we had ascertained that storage of plasma for up to 83 days at −80°C did not attenuate its IS reducing potential. After measurement of systemic hemodynamics and regional myocardial blood flow, myocardial drill biopsies (2–4 g) were taken from the area at risk. Samples were immediately snap-frozen in liquid nitrogen and stored at −80°C for later Western blot analysis.

Then, the suture around the LAD was carefully tightened against a soft silicone plate for 60 minutes. At 5 minutes ischemia, systemic hemodynamics and regional myocardial blood flow were measured again.
Reperfusion was induced by release and quick removal of the suture and confirmed by the disappearance of the light blue color and the reappearance of red color on the surface of the reperfused myocardium. Myocardial biopsies were again sampled at 10 minutes reperfusion, and systemic hemodynamics were measured at 30, 60, and 120 minutes reperfusion. Reperfusion was continued for 3 hours before the experiment was terminated.

**PLA** *(n=13)*

The experimental protocol was identical to that of that for RIPC, except that the RIPC maneuver was omitted.

**RIPC With Pharmacological Blockade of the RISK Pathway** *(RIPC+RISK-BL; n=3)*

The experimental protocol was identical to that for RIPC, except that the RIPC maneuver and the subsequent protocol were performed in the presence of blockade of the RISK pathway. RISK blockade was induced 10 minutes before the RIPC maneuver by intravenous injection of the phosphatidylinositol (4,5)-bisphosphate-3-kinase inhibitor wortmannin27 (300 µg/kg bolus) and the mitogen-ERK-activator-kinase 1/2 inhibitor U012628 (3 mg/kg bolus+15 µg/kg per min continuous infusion up to 10 minutes reperfusion).

**RIPC With Pharmacological Blockade of the SAFE Pathway** *(RIPC+SAFE-BL; n=3)*

The experimental protocol was identical to that for RIPC, except that the RIPC maneuver and the subsequent protocol were performed in the presence of blockade of the SAFE pathway. SAFE blockade was induced by repetitive intravenous infusion of 10 mg/kg each of the janus kinase 2 inhibitor AG490 at 10 minutes before the RIPC maneuver, at 10 minutes before LAD occlusion, and at 10 minutes before reperfusion. AG490 was dissolved in 8 mL dimethylsulfoxide+6 mL saline and slowly administered for 10 minutes using a syringe pump.

We omitted additional protocols with only ischemia/reperfusion to avoid the presence of RISK or SAFE pathway blockade because we have already shown before that blockade of these pathways per se does not alter IS.31,33

**IS in In Situ Pig Hearts**

At the end of each experiment, the LAD was reoccluded, and 5 mL blue dye (Patentblau V, Guerbet GmbH, Sulzbach, Germany) was quickly injected into the left atrium to delineate the area at risk as remaining unstained. Infarcted tissue was demarcated by triphenyl tetrazolium chloride staining. The area at risk was calculated as fraction of the left ventricle, and the IS was calculated as fraction of the area at risk.

**Experiments in Isolated Rat Hearts**

**Experimental Preparation**

Male Lewis rats (250–400 g) were anesthetized by an intraperitoneal injection of sodium pentobarbital (Narcome, Merial, Hallbergmoos, Germany). The injection was supplemented with 1000 IU heparin to attenuate coagulation. The heart was rapidly excised and placed in modified Krebs–Henseleit buffer (in mmol/L: NaCl, 118.0; KCl, 4.7; MgSO4, 1.6; KH2PO4, 1.2; glucose, 5.6; NaHCO3, 24.9; sodium pyruvate, 2.0; CaCl2, 2.0; gassed with 95% O2 and 5% CO2 in a 37°C prewarmed reservoir) connected to the aortic cannula. During the entire experiment, the hearts were continuously immersed in 37°C warm buffer to avoid hypothermia. Hearts were allowed to stabilize for 20 minutes before a protocol was started.

**Experimental Protocols**

Common to all protocols in the isolated bioassay rat hearts is the infusion of plasma which had been taken during the experiments in pigs. The plasma was added via a syringe pump to the perfusate (1:10 volume ratio) before passing the heat exchanger. The plasma dilution and the volume and timing of its infusion into rat hearts had been elaborated and optimized in preliminary experiments. When experiments were performed with specific blockade of either the RISK or the SAFE pathway, the perfusate containing the respective blocker substances was freshly prepared in a second preheated reservoir, and perfuse reservoirs were then switched by a mechanical valve. The efficacy of the respective signaling pathway blockade was estimated by Western blot analysis of myocardial samples taken from the rat heart at the end of each protocol. Sampled tissue was snap-frozen in liquid nitrogen and stored at −80°C.

**RIPC Plasma** *(pRIPC; n=15)*

Plasma taken from a pig subjected to a RIPC maneuver was infused for 8 minutes. After brief washout of plasma, global ischemia was induced for 30 minutes by full stop of retrograde perfusion. The plasma infusion was continued during the initial 5 minutes of 120 minutes reperfusion at constant pressure (65–68 mm Hg).

**RIPC Plasma+In Vitro Blockade of the RISK Pathway** *(pRIPC+RISK-BL; n=5)*

Wortmannin and U0126 were dissolved in dimethylsulfoxide and added to the perfusate at a final concentration of 1 µmol/L wortmannin and 1 µmol/L U0126 for 20 minutes before plasma infusion. Thereafter, the protocol was identical to that for pRIPC.

**RIPC Plasma+In Vitro Blockade of the SAFE Pathway** *(pRIPC+SAFE-BL; n=4)*

Statcic was dissolved in dimethylsulfoxide and added to the perfusate at a final concentration of 10 µmol/L starting at 20 minutes before plasma infusion and continued up to the end of the experiment. The protocol was otherwise identical to that for pRIPC.

**PLA Plasma** *(pPLA; n=13)*

The protocol was identical to that for pRIPC, except that plasma taken from a pig subjected to a PLA maneuver was used.

**PLA Plasma+In Vitro Blockade of the RISK Pathway** *(pPLA+RISK-BL; n=5)*

The protocol was identical to that for pRIPC with RISK blockade, except that plasma taken from a pig subjected to a PLA maneuver was used.

**PLA Plasma+In Vitro Blockade of the SAFE Pathway** *(pPLA+SAFE-BL; n=4)*

The protocol was identical to that for pRIPC with SAFE blockade, except that paired comparisons for the same plasma without and with RISK or SAFE blockade, respectively, were possible.

At the end of each protocol, the apex of the heart (≈50 mg) was cutoff and quickly frozen in liquid nitrogen for later analysis by Western blot. The rest of the heart was frozen in Cryomatrix (Thermo Fisher Scientific, Germany) at −20°C and cut into transverse 1-mm thick slices. Infarcted tissue was demarcated by staining with 2% triphenyl tetrazolium chloride solution containing 5% dextran at 37°C for 20 minutes. Stained slices were weighed and photographed from both sides. The total slice area and the infarcted area were measured by computer-assisted planimetry. After normalization for weight, IS was calculated as fraction of ventricular mass.
Western Blot Analysis of Myocardial Samples (Pig and Isolated Rat Heart Experiments)

Protein aliquots of 20 µg for pig myocardium or 30 µg for rat myocardium were electrophoretically separated on precasted sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels (BioRad, Munich, Germany) and transferred to polyvinylidene fluoride membranes. After blocking, membranes were incubated with antibodies (Online Table I) directed against the phosphorylated forms of protein kinase B (AKT), extracellular-signal-regulated kinase 1/2 (ERK1/2), STAT3, and STAT5. After incubation with the respective secondary antibodies, immunoreactive signals were detected by chemiluminescence and quantified with ChemCam/LabImage1D software (INTAS, Göttingen, Germany). Membranes were reprobed for detection of the respective total form of each protein. Immunoreactivities of phosphorylated protein were normalized to those of the respective total form of the protein.

Investigators assessing IS and performing Western blot analyses were blinded to the RIPC versus PLA protocol or the origin of the plasma, respectively.

Statistics

All data are mean±SD. Area at risk and IS were compared by one-way ANOVA. Hemodynamics, transmural blood flow, and the time courses of protein phosphorylation were analyzed by two-way ANOVA for repeated measures (SigmaStat 3.5, Erkrath, Germany). When a significant difference was detected, individual mean values were compared by Fisher least significant difference post hoc tests. Differences were considered significant at the level of P<0.05.

Experiments in Pigs

Three pigs were retrospectively excluded from analysis. In one pig each of the RIPC and the PLA group, ischemia was not severe enough (transmural blood flow, >0.06 mL/min per g). In one pig from the PLA group, the suture for LAD occlusion was not fixed tightly enough such that there was no infarct at all. Nevertheless, the plasma samples taken from these pigs before ischemia were used in the isolated rat heart experiments.

Systemic Hemodynamics in Pigs

Heart rate was not different between groups and remained unchanged throughout the protocol. Left ventricular pressure decreased with the onset of ischemia and remained below preischemic values up to the end of the protocol (Table 1).

Regional Myocardial Blood Flow, Area at Risk, and IS in Pigs

Transmural blood flow in the area at risk was not different between groups before ischemia and during ischemia (Table 2). The area at risk was not different between groups (Table 2). RIPC reduced IS when compared with PLA (Figure 1). RIPC protected against infarction also in the presence of in vivo RISK blockade (RIPC+RISK-BL), whereas RIPC’s protection was completely abolished in the presence of in vivo SAFE blockade (RIPC+SAFE-BL; Figure 1).

### Table 1. Systemic Hemodynamics in Pigs

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<th>dP/dt&lt;sub&gt;max&lt;/sub&gt;, mmHg/s</th>
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Mean=SD. +RISK-BL/+SAFE-BL indicates RIPC in the presence of reperfusion injury salvage kinase (RISK) pathway or survival activating factor enhancement (SAFE) pathway blockade, respectively; dP/dt<sub>max</sub>, maximal rate of rise of left ventricular pressure; HR, heart rate; isch55, 5/55 min ischemia; LVP<sub>max</sub>, maximal left ventricular pressure; PLA, placebo; preischemia, 5 min before ischemia; rep 30/60/120, 30/60/120 min reperfusion; and RIPC, remote ischemic preconditioning.

*P<0.05 vs preischemia.
**Protein Phosphorylation in Pig Myocardium**

The levels of phosphorylated AKT and ERK1/2, normalized to their respective total protein, were higher at 10 minutes reperfusion than before ischemia, but not different between RIPC and PLA. With RIPC+RISK-BL, levels of phosphorylated AKT and ERK1/2 were attenuated, both before ischemia and at 10 minutes reperfusion (Figure 2). The level of phosphorylated STAT3, normalized to total STAT3 protein, was increased at 10 minutes reperfusion versus before ischemia only with RIPC, but not with PLA or RIPC in the presence of SAFE blockade by AG490 (Figure 2). AG490 did not attenuate the increased phosphorylation of AKT and ERK1/2 (Online Figures I and II). The level of phosphorylated STAT5, normalized to total STAT5 protein, decreased with reperfusion in both groups (Online Figure III).

Membranes and chemiluminescence signals for AKT, ERK1/2, and STAT3 are displayed in Online Figures IV to VI.

**Experiments in Isolated Rat Hearts**

**Coronary Flow and Left Ventricular Developed Pressure in Isolated Rat Hearts**

Coronary flow and left ventricular developed pressure were not different between groups at baseline. Infusion of pig plasma slightly decreased developed pressure in all groups. Developed pressure ceased ≈5 minutes after the onset of global ischemia and recovered only partially during reperfusion. Coronary flow at reperfusion was lower than baseline but not different between pRIPC and pPLA. Except for pRIPC+RISK-BL, coronary flow at reperfusion was lower in the presence of RISK and SAFE blockade than in the respective untreated groups (Online Table II).

**IS in Isolated Rat Hearts**

IS was decreased with infusion of plasma taken from pigs subjected to RIPC (pRIPC) when compared with infusion of plasma from pigs subjected to PLA (pPLA; Figure 3). The average IS with pPLA was similar to that observed after 30 minutes ischemia and 2 hours reperfusion without plasma infusion (Online Figure VII). The protective effect of pRIPC was abolished when either RISK blockade, that is, pretreatment with wortmannin and U0126, or SAFE blockade, that is, pretreatment with static, were induced in the isolated bioassay rat heart before plasma infusion (pRIPC+RISK-BL and pRIPC+SAFE-BL). The RISK or SAFE blockade, respectively, did not affect IS when the isolated rat hearts were treated with plasma taken from pigs subjected to PLA (pPLA+RISK-BL and pPLA+SAFE-BL; Figure 3).

**Protein Phosphorylation in Rat Myocardium**

In myocardial samples from rat hearts after 120 minutes reperfusion, the levels of phosphorylated AKT and ERK1/2, normalized to their respective total protein, were higher with pRIPC than with pPLA. The increases in AKT and ERK1/2 phosphorylation were abolished when RISK blockade was induced before pRIPC infusion (Figure 4). RISK blockade did not affect STAT3 (Online Figure VIII). The level of phosphorylated STAT3, normalized to total STAT3 protein, was higher with pRIPC than with pPLA, and this increase was abolished in the presence of SAFE blockade by static (Figure 4). SAFE blockade did not affect AKT or ERK1/2 (Online Figure IX). The level of phosphorylated STAT5, normalized to total STAT protein, was not different between pRIPC and pPLA (Online Figure X).

Membranes and chemiluminescence signals for AKT, ERK1/2, and STAT3 are displayed in Online Figures XI to XIII.

**Discussion**

**Major Findings**

We have taken a novel, retrograde approach to identify the still enigmatic humoral transfer factor of RIPC’s protection by...
characterizing its properties through the downstream myocardial signal transduction which it might activate. Using this approach, we could first demonstrate that the myocardial signal transduction pathways recruited by the humoral transfer factor of remote preconditioning are identical to those recruited by local postconditioning, in both isolated perfused rat hearts and in pigs in situ. Although admittedly we could not identify the ultimate factor, our findings will further narrow down the exhaustive and painful search for such needle in the haystack and facilitate its identification.

Methodological Considerations

In the present study, we confirmed RIPC protection against myocardial infarction in our clinically relevant pig model,26 in line with the recent CAESAR recommendations for experimental studies on cardioprotection.34 In our current study, we have used the same anesthetic regime as was used in our studies on RIPC in patients undergoing surgical coronary revascularization.10 More specifically, we used isoflurane and not propofol because propofol interferes with the cardioprotection by RIPC.35,36 Of note in this context, the two studies on RIPC in patients undergoing cardiac surgery under cardiopulmonary bypass (ERICCA and RipHeart), which have recently presented preliminary neutral results at the hot line sessions of the American College of Cardiology and the German Cardiac Society, respectively, have used propofol in the majority of patients. To further elucidate the underlying myocardial signal transduction of RIPC, we have collected pig plasma after completion of the RIPC maneuver or PLA, respectively, and transferred one or more potentially protective blood-borne factor(s) from the in vivo pig model to isolated perfused rat hearts, which were used as an in vitro bioassay. A technical advantage of such bioassay is the abundance of plasma for use in testing different signaling pathways. Plasma taken from pigs after the RIPC maneuver and diluted 1:10 still reduced IS in the isolated bioassay rat heart, whereas diluted plasma taken from pigs after PLA did not. The rationale for taking the plasma with 1 hour delay after the RIPC procedure was again

Figure 2. Phosphorylation of protein kinase B (AKT) at ser473 and extracellular signal–regulated kinase 1/2 (ERK1/2) at thr202/tyr204 (reperfusion injury salvage kinase [RISK] pathway) and STAT3 at tyr705 (survival activating factor enhancement [SAFE] pathway) in pig myocardium from the area at risk taken preischemia (black bars) and at 10 minutes reperfusion (gray bars). Placebo (PLA): n=4; remote ischemic preconditioning (RIPC): n=4; RIPC+RISK-BL: n=3; RIPC+SAFE-BL: n=3; mean±SD; *P<0.05 vs respective preischemia value; +P<0.05 vs PLA; #P<0.05 vs RIPC. BL indicates blockade.

Figure 3. Infarct size in isolated rat hearts with infusion of pig plasma. pPLA: plasma taken from pigs after placebo (PLA); pRIPC: plasma taken from pigs after remote ischemic preconditioning (RIPC). Subsets of pPLA and pRIPC samples (squares and diamonds) were retested in rat hearts subjected to reperfusion injury salvage kinase (RISK; +RISK-BL; squares) or survival activating factor enhancement (SAFE; +SAFE-BL; diamonds) blockade, respectively, before plasma infusion. Open symbols: individual data points; closed symbols: means±SD; *P<0.05 vs pRIPC. BL indicates blockade.
to mimic the time course of our RIPC protocol in patients undergoing cardiac surgery. In preliminary studies, we found no evidence that plasma taken with less delay caused greater protection.

The samples for Western blot analysis were taken from pig and rat hearts, in which IS was also determined by triphenyl tetrazolium chloride staining such that there was evidence for protection or lack of it in each instance. In the pig hearts, the first samples taken before myocardial ischemia reflect not a truly naive baseline but the situation ≈1 hour after the RIPC protocol or PLA, respectively, such that activation of AKT, ERK1/2, or STAT3 at earlier time points after the RIPC stimulus might have been missed. Nevertheless, just before myocardial ischemia, there was no difference in the myocardial expression and phosphorylation status of AKT, ERK1/2, and STAT3 between pigs which had undergone RIPC or PLA.

The increased phosphorylation of AKT, ERK1/2, and STAT3 at 10 minutes reperfusion in biopsy samples from pig hearts probably reflects a situation where activated proteins might play a causal role in attenuating reperfusion injury, and we could ascertain such causal role for STAT3 through Western blot analysis combined with a pharmacological antagonist approach. In contrast in the isolated rat hearts, tissue for Western blot analysis was taken only after 2 hours reperfusion when the increased phosphorylation levels probably are no longer representative of earlier time points when protection has occurred. Nevertheless, the Western blot analysis after 2 hours reperfusion still provided evidence that the pharmacological antagonists indeed hit their targets.

Myocardial Signal Transduction of RIPC

The focus of our study was on those myocardial signal transduction cascades that have already been identified in cardioprotection by ischemic preconditioning and postconditioning, that is, AKT and ERK1/2 as central elements of RISK and STAT3 as a central element of SAFE. As in our previous studies on ischemic postconditioning, we assessed the time courses of phosphorylation of AKT, ERK1/2, STAT3, and STAT5 by the RIPC maneuver in sequential biopsies taken from myocardium at risk in pigs. The observed IS reduction by RIPC was not associated with a greater increase in phosphorylation of AKT and ERK1/2 at reperfusion than with PLA. RIPC in the presence of pharmacological RISK blockade was still protective, although the increases in AKT and ERK1/2 phosphorylation at reperfusion were largely attenuated, consistent with our previous study in which RISK activation was not mandatory to confer protection by ischemic postconditioning. Consequently, the activation of the RISK pathway was apparently not causal for cardioprotection by RIPC. In contrast, a recent study in pigs suggested an involvement of AKT in RIPC’s cardioprotection; however, the findings of this particular study are somewhat ambiguous because protection by RIPC was still observed when the phosphorylation of AKT was abrogated by the adenosine antagonist 8-sulfophenyltheophylline.

An obvious increase in the phosphorylation of STAT3 at reperfusion was observed only with RIPC and not with PLA in pigs. The pretreatment of pigs with AG490, a blocker of janus kinase 2, attenuated such increase in STAT3.

Figure 4. Phosphorylation of protein kinase B (AKT) at ser473 and extracellular signal–regulated kinase 1/2 (ERK1/2) at thr202/tyr204 (reperfusion injury salvage kinase [RISK] pathway) and STAT3 at tyr705 (survival activating factor enhancement [SAFE] pathway) in rat myocardium subjected to infusion of plasma taken from pigs subjected to placebo (pPLA) or remote ischemic preconditioning (pRIPC), respectively, and in the presence of RISK blockade (pRIPC+RISK-BL), n=5 each and in the presence of SAFE blockade (pRIPC+SAFE-BL), n=3 each. Mean±SD; *P<0.05 vs pPLA; #P<0.05 vs pRIPC.
phosphorylation at reperfusion with RIPC and resulted in a complete loss of RIPC protection, supporting a causal role of STAT3 in RIPC protection. This observation is again consistent with our previous study which has shown a causal role of STAT3 in cardioprotection by ischemic postconditioning in pigs.31

Plasma taken from pigs after the RIPC maneuver and diluted 1:10 still reduced IS in the isolated bioassay rat heart, whereas diluted plasma taken from pigs after PLA did not. With RIPC plasma, the phosphorylation of AKT and ERK1/2 in the rat myocardium at reperfusion was greater than with PLA plasma. RISK blockade in the isolated bioassay rat heart before infusion of pRIPC abolished the activation of both RISK and IS reduction. Such causal involvement of RISK in cardioprotection in rodent hearts is consistent with many previous studies.22,39 The lack of protection with plasma taken from pigs in which the RIPC maneuver was performed in the presence of a RISK blockade is then expected (Online Figure VII). Plasma taken from pigs subjected to a RIPC maneuver when compared with PLA plasma also increased the phosphorylation of STAT3 in the isolated bioassay rat heart. This observation supports the notion that the SAFE pathway is also involved in cardioprotection in rodent hearts.40,41 Consequently, blockade of STAT3 phosphorylation by static abrogated the protective effect of pRIPC. Plasma taken from pigs in which the RIPC maneuver was performed in the presence of SAFE blockade also did not protect isolated bioassay rat hearts from infarction. However, it cannot be distinguished whether the SAFE pathway blockade in the pig before the RIPC maneuver prevented the release of a protective factor and thus its transfer to the isolated rat heart or whether the residual amounts of AG490 in the pig plasma prevented protection in the rat heart per se.

The signal transduction of cardioprotection is highly species dependent. In small rodents, RISK activation is mandatory to confer protection,22 but SAFE activation seems to play also an important role.23,37 Apparently, there is a close interaction of RISK and SAFE pathways in the rat heart such that either blockade abrogates protection, consistent with previous studies.42,43 In contrast, RISK activation is not necessary for cardioprotection in pigs33 or humans,44 but an activation of STAT is certainly involved.35,44 However, species differences are also evident even within the SAFE signal transduction scheme. Cardioprotection by ischemic postconditioning in pigs requires the activation of STAT3,31 whereas cardioprotection by RIPC in humans is characterized by an activation of STAT5 and not STAT3.44 Such species differences in myocardial signal transduction of cardioprotection were also confirmed in our current experiments. Only STAT3 activation was causally involved in cardioprotection by RIPC in the pig, whereas phosphorylation of STAT5 decreased with reperfusion and was not different between RIPC and PLA. In the isolated bioassay rat heart, both the RISK and the SAFE pathways were activated by plasma taken from pigs after a RIPC maneuver. Only STAT3, but not STAT5, was activated in the isolated bioassay rat heart. Obviously, the blood-borne factor(s) present in the plasma of pigs after RIPC was/were able to activate both the RISK and the SAFE pathways. Whether this transfer factor is a protein, a micro-RNA, or an exosome, which again might carry proteins or micro-RNAs, is not clear at this point. The transfer with plasma (our study) seems to exclude cellular elements, and the transfer with dialysate when using a 15-kDa cutoff membrane13 seems to exclude larger particles and molecules.

Our study now is the first to provide evidence that in both isolated rat hearts and pigs in situ, the myocardial signal transduction of RIPC is identical to that of local ischemic postconditioning. Obviously, there is a point of convergence for signal transduction of various conditioning forms, also upstream of mitochondria.23 Whether such point of convergence is genuine to the cardiomyocyte or to some other cellular compartment, for example, some sort of resident cell which is activated to release a paracrine factor which then acts on the cardiomyocyte, is unclear at present. Also, an additional involvement of neuronal pathways either during the initiation of the RIPC stimulus, its transfer, or the installation of protection in the pig myocardium cannot be excluded.45,46

Acknowledgments

This study will in part be used in the thesis of Christiane Schulte.

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Disclosures

None.

References

Enhancement (SAFE) pathway against reperfusion injury: does it go
proceed?


nervous system in cardioprotection by remote preconditioning in isoflu-

The neural and humoral pathways in remote limb ischemic preconditioning. Basic Res Cardiol. 2010;105:651–

11. Dickow EW, Lorbar M, Porcaro WA, Fenton RA, Reinhardt CP, Gysenbergh AP, Przyklenk K. Rabbit heart can be “preconditioned” via

10. Heusch G. Cardioprotection: chances and challenges of its translation to the

9. Ibáñez B, Heusch G, Ovize M, Van de Werf F. Evolving therapies for myo-
1471. doi: 10.1016/j.jacc.2015.02.032.

8. Heusch G, Skyschally A, Schulz R. The in-situ pig heart with re-
gional ischemia/reperfusion - ready for translation. J Mol Cell Cardiol.

phosphoinosit-
de 3-kinase by covalent modification of Lys-802, a residue involved in

of remote ischemic preconditioning during coronary artery bypass graft surgery.


2. Jones SP, Tang XL, Guo Y, et al. The NHLBI-sponsored Consortium for pro-
clinicaAl assEssment of cARdioprotective therapies (CAESAR): a new paradigm for rigorous, accurate, and reproducible evaluation of pu-
tative infarct-sparing interventions in mice, rabbits, and pigs. Circ Res.

artery bypass graft surgery with isoflurane but not propofol - a clinical
6571.2010.02585.x.

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What Is Known?

• Brief cycles of ischemia/reperfusion in a limb or an organ remote from the heart reduce myocardial infarct size resulting from subsequent coronary occlusion/reperfusion, that is, there is cardioprotection by remote ischemic preconditioning (RIPC).
• Protection by RIPC is also recruitable in humans undergoing interventional or surgical coronary revascularization.
• The protection by RIPC can be transferred with plasma from one individual to another.

What New Information Does This Article Contribute?

• In pigs, RIPC protection is mediated by activation of the signal transducer and activator of transcription 3 in the myocardium, similar to that by ischemic preconditioning.
• In isolated rat hearts, plasma from pigs which have undergone a RIPC protocol reduces infarct size.
• Infarct size reduction by RIPC plasma in isolated rat hearts is mediated by activation of protein kinase B, extracellular signal–regulated kinase, and signal transducer and activator of transcription 3.

RIPC, that is, repeated brief cycles of ischemia/reperfusion in a limb or organ remote from the heart, reduces infarct size after coronary artery occlusion/reperfusion. Such remote cardioprotection is also recruitable in humans with acute myocardial infarction or during interventional or surgical coronary revascularization. The protection by RIPC can be transferred with plasma or a plasma dialysate from one individual to another, but the nature of the humoral cardioprotective mediator(s) has not yet been identified. We used a retrograde approach to characterize the cardioprotective humoral RIPC mediator by the signal transduction which it activates in the myocardium. In pigs undergoing RIPC by repeated hindlimb ischemia/reperfusion, infarct size reduction was mediated by activation of signal transducer and activator of transcription 3 in the myocardium. Plasma from pigs undergoing RIPC reduced infarct size in isolated rat hearts by activation of protein kinase B, extracellular signal–regulated kinase, and signal transducer and activator of transcription 3. Apparently, the myocardial signal transduction which is activated by RIPC is species-specific and identical to that of ischemic postconditioning in pigs and rats.

Novelty and Significance

Across-Species Transfer of Protection by Remote Ischemic Preconditioning With Species-Specific Myocardial Signal Transduction by Reperfusion Injury Salvage Kinase and Survival Activating Factor Enhancement Pathways
Andreas Skyschally, Sabine Gent, Georgios Amanakis, Christiane Schulte, Petra Kleinbongard and Gerd Heusch

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Supplemental Material

Across-species transfer of protection by remote ischemic preconditioning with species-specific myocardial signal transduction by RISK and SAFE pathways

Andreas Skyschally, PhD
Sabine Gent, PhD
Georgios Amanakis, MD
Christiane Schulte, MSc
Petra Kleinbongard, PhD
Gerd Heusch, MD, PhD

Institute for Pathophysiology, West German Heart and Vascular Center, University of Essen Medical School, Essen, Germany
Non-standard abbreviations and acronyms used in the supplemental material

AKT       protein kinase B  
ERK1/2    extracellular-signal-regulated kinase 1/2 
IS        infarct size   
PLA       placebo     
STAT      signal transducer and activator of transcription 
RIPC      remote ischemic preconditioning  
RISK      reperfusion injury salvage kinases 
SAFE      survival activating factor enhancement 

Expanded Methods

Regional myocardial blood flow in pig myocardium

Regional myocardial blood flow was measured using colored microspheres¹. In brief, microspheres (2-5·10⁶; 15 µm diameter) were injected into the left atrium. During the injection, arterial blood was withdrawn at a constant rate (5 ml/min over 3 min) via a teflon-coated catheter placed in the descending thoracic aorta. The colored microspheres were recovered from myocardial tissue samples and the reference blood sample by overnight digestion in 1 mol/l KOH and subsequent filtration. The dye was dissolved from the recovered spheres, and the dye concentration was measured using a fluorescence photometer (Varian Eclipse, Agilent Technologies, Böblingen, Germany). Transmural myocardial blood flow in the central area at risk was calibrated against the reference withdrawal and normalized for the weight of the tissue sample.


Infarct size (IS) in in-situ pig hearts

At the end of each experiment, the left anterior descending coronary artery was re-occluded, and 5 ml blue dye (Patentblau V, Guerbet GmbH, Sulzbach, Germany) was quickly injected into the left atrium to delineate the area at risk as remaining unstained. The heart was then arrested by electrical induction of fibrillation, removed from the chest and sectioned from base to apex into 5 transverse slices in a plane parallel to the atrioventricular groove. Both sides of each myocardial slice were photographed, and the slice shape and the unstained area at risk were traced manually on transparent film. Slices were then immersed in 0.09 mol/l sodium phosphate buffer containing 1% triphenyl tetrazolium chloride (Sigma-Aldrich Chemie GmbH, München, Germany) and 8% dextran for 20 min at 37°C to demarcate viable from infarcted tissue. The infarcted triphenyl tetrazolium chloride-negative areas were traced on the same transparent film as the area at risk. The total slice area, the area at risk, and the infarcted area were measured by computer-assisted planimetry. After normalization for the weight of the tissue slices, the size of the area at risk was calculated as fraction of the left ventricle, and the IS was calculated as fraction of the area at risk.
Western blot analysis of tissue samples from pig and rat myocardium

Online Table I: Antibodies used for Western blot analyses

<table>
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<tr>
<th>Kinase / Phosphorylation site</th>
<th>Manufacturer</th>
<th>Order number</th>
<th>Source</th>
<th>Clonality</th>
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<td>#9271</td>
<td>rabbit</td>
<td>polyclonal</td>
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<tr>
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<td>Cell Signaling</td>
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<td>polyclonal</td>
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<tr>
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<td>rabbit</td>
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<tr>
<td>pSTAT3&lt;sub&gt;tyr705&lt;/sub&gt;</td>
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<td>mouse</td>
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<td>monoclonal</td>
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Online Table II: Hemodynamics in isolated bioassay rat hearts:

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<th>DP&lt;sub&gt;max&lt;/sub&gt; [mmHg]</th>
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<td>87.6 ± 21.5</td>
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<td>0.3 ± 0.1*</td>
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<tr>
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<td>9.0 ± 1.6*</td>
<td>28.2 ± 20.6*</td>
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<tr>
<td>rep30</td>
<td>8.0 ± 1.9*</td>
<td>32.1 ± 20.4*</td>
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<td>pPLA+RISK-BL (n=5)</td>
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<td>baseline</td>
<td>10.8 ± 1.2</td>
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<td>24.9 ± 20.6*</td>
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<td>pPLA+SAFE-BL (n=4)</td>
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<td>101.6 ± 12.7</td>
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<td>90.0 ± 11.2</td>
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<td>31.0 ± 23.2*</td>
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<td>25.8 ± 19.1*</td>
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<td>pRIPC +RISK-BL (n=5)</td>
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<td>21.1 ± 24.1*</td>
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<td>0.0 ± 0.0*</td>
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<tr>
<td>rep30</td>
<td>4.4 ± 0.2**†</td>
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</table>

pPLA: infusion of plasma from pigs subjected to a PLA protocol; pRIPC: infusion of plasma from pigs subjected to RIPC; +RISK-BL and +SAFE-BL: plasma infusion in the presence of RISK or SAFE blockade, respectively; plasma: after plasma infusion; isch5/25: 5/25 min ischemia; rep 10/30: 10/30 min reperfusion; CF<sub>mean</sub>: mean coronary perfusate flow; DP<sub>max</sub>: maximal developed left ventricular pressure; mean±SD; * p<0.05 vs. baseline; † p<0.05 vs. pPLA; ‡ p<0.05 vs. pRIPC
Online Figure I: Cross-over effects of SAFE blockade by AG490 on the phosphorylation of AKT in pig myocardium. Samples were taken at pre-ischemia (black bars) and 10 min reperfusion (grey bars). From top to bottom: Western blot membrane stained with Ponceau Red, immunoreactivity signals for AKT phosphorylated at ser473 and total form of AKT, and mean±SD of the pAKT/total AKT ratio in the two experimental groups. * p<0.05 vs. respective pre-ischemia value.
Online Figure II: Cross-over effects of SAFE blockade by AG490 on the phosphorylation of ERK1/2 in pig myocardium. Samples were taken at pre-ischemia (black bars) and 10 min reperfusion (grey bars). From top to bottom: Western blot membrane stained with Ponceau Red, immunoreactivity signals for ERK1/2 phosphorylated at thr202/tyr204 and total form of ERK1/2, and mean±SD of the pERK1/2/total ERK1/2 ratio in the two experimental groups. * p<0.05 vs. respective pre-ischemia value, # p<0.05 vs PLA & RIPC.
Online Figure III: Western blot analysis of STAT5 and its phosphorylation in myocardial samples from pigs subjected to PLA or RIPC. Samples were taken at pre-ischemia (black bars) and 10 min reperfusion (grey bars). From top to bottom: Western blot membrane stained with Ponceau Red, immunoreactivity signals for STAT5 phosphorylated at tyr694 and total form of STAT5, and mean±SD of the pSTAT5/total STAT5 ratio in the two experimental groups. * p<0.05 vs. respective pre-ischemia value.
Online Figure IV: Western blot analysis of AKT and its phosphorylation in myocardial samples from pigs subjected to PLA or RIPC and RIPC in the presence of RISK blockade (RIPC+RISK-BL). Samples were taken pre-ischemia and at 10 min reperfusion. From top to bottom: Western blot membrane stained with Ponceau Red, immunoreactivity signals for AKT phosphorylated at ser473, and total form of AKT.
Online Figure V: Western blot analysis of ERK1/2 and its phosphorylation in myocardial samples from pigs subjected to PLA or RIPC and RIPC in the presence of RISK blockade (RIPC+RISK-BL). Samples were taken pre-ischemia and at 10 min reperfusion. From top to bottom: Western blot membrane stained with Ponceau Red, immunoreactivity signals for ERK1/2 phosphorylated at thr202/tyr204, and total form of ERK1/2.
Online Figure VI: Western blot analysis of STAT3 and its phosphorylation in myocardial samples from pigs subjected to PLA or RIPC and RIPC in the presence of SAFE blockade using AG490 (RIPC+SAFE-BL). Samples were taken pre-ischemia and 10 min reperfusion. From top to bottom: Western blot membrane stained with Ponceau Red, immunoreactivity signals for STAT3 phosphorylated at tyr705, and total form of STAT3.
Online Figure VII: Infarct size (mean±SD) in isolated rat hearts; I/R: 30 min ischemia and subsequent 120 min reperfusion; pPLA: infusion of plasma taken from a pig subjected to PLA; pRIPC/pig RISK-BL and /pig SAFE BL: infusion of plasma taken from pigs in which RIPC was performed in presence of RISK or SAFE blockade, respectively.
Online Figure VIII: Cross-over effects of RISK blockade by wortmannin and U0126 on the phosphorylation of STAT3 in rat myocardium. Samples were taken at 2 h reperfusion. For the sake of completeness, the impact of RISK blockade on AKT and ERK1/2 is shown in the lower panel. Top: Western blot membrane stained with Ponceau Red, immunoreactivity signals for STAT3 phosphorylated at tyr705, and total form of STAT3, and mean±SD of the pSTAT3/total STAT3 ratio; bottom: immunoreactivity signals for AKT phosphorylated at ser 473, and total form of AKT, and ERK1/2 phosphorylated at thr202/tyr204, and total form of ERK1/2. Data are mean±SD; paired t-test.
Online Figure IX: Cross-over effects of SAFE blockade by stattic on the phosphorylation of AKT and ERK1/2 in rat myocardium. Samples were taken at 2 h reperfusion. For the sake of completeness, the impact of SAFE blockade on STAT3 is shown in the lower panel. Top: Western blot membrane stained with Ponceau Red, immunoreactivity signals for AKT phosphorylated at ser473, and total form of AKT, and ERK1/2 phosphorylated at thr202/tyr204, and total form of ERK1/2, and mean±SD of the pAKT/total AKT and pERK1/2/total ERK1/2 ratios; bottom: immunoreactivity signals for STAT3 phosphorylated at tyr705, and total form of STAT3. Data are mean±SD; paired t-test.
Online Figure X: Western blot analysis of STAT5 and its phosphorylation in myocardial samples from isolated rat hearts after treatment with plasma taken from pigs subjected to PLA (pPLA) or RIPC (pRIPC). Samples were taken at 2 h reperfusion. From top to bottom: Western blot membrane stained with Ponceau Red, immunoreactivity signals for STAT5 phosphorylated at tyr694, and total form of STAT5, and mean±SD of the pSTAT5/total STAT5 ratio in the two experimental groups.
Online Figure XI: Western blot analysis of AKT and its phosphorylation in myocardial samples from isolated rat hearts after treatment with plasma taken from pigs subjected to PLA (pPLA) or RIPC (pRIPC) and to RIPC-plasma with prior RISK blockade in the rat heart (pRIPC+RISK-BL). Samples were taken at 2 h reperfusion. From top to bottom: Western blot membrane stained with Ponceau Red, immunoreactivity signals for AKT phosphorylated at ser473, and total form of AKT.
Online Figure XII: Western blot analysis of ERK1/2 and its phosphorylation in myocardial samples from isolated rat hearts after treatment with plasma taken from pigs subjected to PLA (pPLA) or RIPC (pRIPC) and to RIPC-plasma with prior RISK blockade in the rat heart (pRIPC+RISK-BL). Samples were taken at 2 h reperfusion. From top to bottom: Western blot membrane stained with Ponceau Red, immunoreactivity signals for ERK1/2 phosphorylated at thr202/tyr204, and total form of ERK1/2.
Online Figure XIII: Western blot analysis of STAT3 and its phosphorylation in myocardial samples from isolated rat hearts after treatment with plasma taken from pigs subjected to PLA (pPLA) or RIPC (pRIPC) and to RIPC-plasma with prior SAFE blockade in the rat heart using statitic (pRIPC+SAFE-BL). Samples were taken at 2 h reperfusion. From top to bottom: Western blot membrane stained with Ponceau Red, immunoreactivity signals for STAT3 phosphorylated at tyr705, and total form of STAT3.