**RISK and SAFE signaling pathway interactions in remote limb ischemic
perconditioning in combination with local ischemic postconditioning**

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Abstract

Local ischemic postconditioning (IPost) and remote ischemic preconditioning (RIPer) are promising methods to decrease ischemia-reperfusion (I/R) injury. We tested whether the use of the two procedures in combination led to an improvement in cardioprotection through a higher activation of survival signaling pathways.

Methods: Rats exposed to myocardial I/R were allocated to one of the following four groups: Control, no intervention at myocardial reperfusion; IPost, three cycles of 10-sec coronary artery occlusion followed by 10-sec reperfusion applied at the onset of myocardial reperfusion; RIPer, 10-min limb ischemia followed by 10-min reperfusion initiated 20 min after coronary artery occlusion; IPost+RIPer, IPost and RIPer in combination.

Results: Infarct size was significantly reduced in both IPost and RIPer ($34.25 \pm 3.36\%$ and $24.69 \pm 6.02\%$, respectively) groups compared to Control ($54.93 \pm 6.46\%$, both $p < 0.05$). IPost+RIPer (infarct size = $18.04 \pm 4.86\%$) was significantly more cardioprotective than IPost alone ($p < 0.05$). RISK pathway (Akt, ERK1/2, and GSK-3 β) activation was enhanced in IPost, RIPer, and IPost+RIPer groups compared to Control. IPost+RIPer did not enhance RISK pathway activation as compared to IPost alone, but instead increased phospho-STAT-3 levels, highlighting the crucial role of the SAFE pathway. In IPost+RIPer, a SAFE inhibitor (AG490) abolished cardioprotection and blocked both Akt and GSK-3 β phosphorylations, whereas RISK inhibitors (wortmannin or U0126) abolished cardioprotection and blocked STAT-3 phosphorylation.

Conclusion: In our experimental model, the combination of IPost and RIPer improved cardioprotection through the recruitment of the SAFE pathway. Our findings also indicate that cross talk exists between the RISK and SAFE pathways.

Keywords: postconditioning, preconditioning, cardioprotection, SAFE, RISK, reperfusion injury

Introduction

Acute myocardial infarction (AMI) is a leading cause of morbidity and mortality worldwide [41]. Although highly beneficial, prompt myocardial reperfusion is paradoxically associated with cellular injury including cardiomyocyte death [46]. Therefore, novel treatment strategies are required to protect the heart during myocardial reperfusion [15]. Local ischemic postconditioning (IPost), during which repeated brief episodes of ischemia/reperfusion (I/R) are applied at the onset of reperfusion, has emerged as a promising cardioprotective therapy against lethal reperfusion injury [61]. IPost has been shown to exert its cardioprotective effect by activating intrinsic pro-survival signaling cascades such as reperfusion injury salvage kinase (RISK) pathway and the recently-described survivor activating factor enhancement (SAFE) pathway [3-5, 16, 23, 35, 56]. However, in the setting of an AMI, the clinical application of IPost is limited to patients undergoing coronary angioplasty. In this regard, remote ischemic pre-, per-, and postconditioning are attractive new strategies to prevent myocardial injury. In each case, cardioprotection can be achieved by transient episodes of I/R applied to an organ or to tissue remote from the heart before, during, or immediately after myocardial ischemia [19, 25, 31, 48]. These new strategies have been shown to be effective in reducing myocardial reperfusion injuries in both experimental and clinical settings [2, 7, 13, 30, 38, 40], although their mechanisms are largely unknown. The aims of the present study were threefold: 1) to examine whether remote ischemic perconditioning (RIPer) was as effective as IPost in the same experimental model of AMI; 2) to determine whether the combination of IPost and RIPer allowed for additional cardioprotection compared to the administering of either treatment alone; 3) to clarify the intracellular signaling pathways involved in these cardioprotective strategies.

Methods

All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No 85 (23), revised 1996). Our regional Animal Care and Use Committee approved the protocol.

Surgical preparation

For all experiments, male Wistar rats, 8-10 weeks of age, weighing 250-300g, were used. All animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and endotracheally intubated with a 16-gauge tube. Animals were ventilated using a small animal ventilator (SAR-830 A/P, CWE). Body core temperature was continuously monitored throughout the surgical procedure and maintained at 36-38°C using a homeothermic blanket set, connected to a temperature control unit (HB101/2 RS, Bioseb, France). The chest was opened via a median sternotomy. The pericardium was removed, the heart was exposed, and a 7-0 monofilament suture (Premio 7.0, Peters Surgical) was placed around the proximal portion of the left anterior descending coronary artery (LAD) and passed through a short piece of tubing (PE50) in order to create a reversible snare. Following stabilization of the heart, coronary occlusion was initiated by clamping the snare onto the epicardial surface directly above the coronary artery. Ischemia was confirmed by epicardial cyanosis below the suture and dyskinesia of the ischemic region. After 40 min of occlusion, reperfusion was achieved by loosening the snare and confirmed by a marked hyperemic response at reperfusion.

Study groups and experimental protocol

For myocardial infarct size and apoptosis analysis, rats underwent 40 min of LAD occlusion followed by 2 hours of reperfusion. Rats were randomly assigned to one of four groups (Fig. 1): Control (no further intervention, n=6); local ischemic postconditioning (IPost, n=7), *i.e.*,

three cycles of 10-sec ischemia followed by 10-sec reperfusion initiated at the onset of reperfusion as previously described [32, 33, 54, 60]; remote ischemic preconditioning (RIPer, n=6), *i.e.*, 10-min limb ischemia followed by 10-min reperfusion initiated 20 min after coronary artery occlusion [10, 43]; combination of local ischemic postconditioning and remote ischemic preconditioning (IPost+RIPer, n=6). RIPer was achieved using a thin elastic rubber band placed around the upper third of one hindlimb in order to occlude arterial blood flow for 10 min followed by 10-min reperfusion. Limb ischemia was confirmed by a change in the color of the skin and a decrease in under-skin limb temperature. Following limb reperfusion, the skin color returned to pink and the under-skin temperature to baseline.

To determine the involvement of RISK and SAFE pathways in IPost-, RIPer-, and IPost+RIPer-induced cardioprotection, we administered several pharmacological inhibitors: wortmannin (Wort), an inhibitor of PI3K/Akt pathway; U0126, an inhibitor of MEK1/2-ERK1/2 pathway; AG490, an inhibitor of JAK/STAT pathway. Thus, eight further groups were added to the study (n=6 rats/group): IPost+Wort (15 µg/kg); IPost+U0126 (200µg/kg); RIPer+Wort; RIPer+U0126; IPost+RIPer+Wort; IPost+RIPer+U0126; IPost+RIPer+AG490 (3mg/kg) (Fig. 1). Intravenous bolus injection of pharmacological inhibitors was performed 25 min prior to myocardial reperfusion [44].

Area at risk and infarct size determination

At the end of the 2-hour reperfusion period, infarct size was measured in all groups. The heart was removed, and the LAD was reoccluded using the monofilament suture kept in place. The heart was then retrogradely perfused with Evans blue (1%) in order to delineate the area at risk. Hearts were then cut into five to six slices from apex to base followed by incubation at 37°C in a 1% solution of phosphate-buffered 2,3,5-triphenyltetrazolium chloride

(TTC) so as to delineate infarcted myocardium. Slices were then fixed in formalin 10%, and infarct size was quantified by computerized planimetry using image J software. Area of necrosis (AN) was expressed as a percentage of the area at risk (AAR) and the area at risk as a percentage of the total ventricular area (LV).

Evaluation of apoptosis

The detection of apoptotic cells in four to seven hearts from each group was carried out using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. Following infarct size assessment, LV tissues from the ischemic zone and from the remote myocardium were fixed in formalin for 24 hours and embedded in paraffin, and 5- μ m sections were obtained. The sections were then deparaffinized and rehydrated with xylene and graded alcohol series. The sections were stained using the *in situ* DeadEnd™ Colorimetric Apoptosis Detection System (Promega, Madison, WI) according to the manufacturer's instructions. In short, tissue sections were washed in PBS and then fixed in 4% paraformaldehyde solution prior to incubation in 20 μ g/ml proteinase K for 10 min. Sections were washed in PBS and incubated with terminal deoxynucleotidyl transferase enzyme in a humidified chamber at 37°C for 60 min in order to incorporate biotinylated nucleotides at the 3'-OH DNA ends. The reaction was terminated by transferring the slices to 2X saline sodium citrate. Endogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide. Finally, streptavidin horseradish peroxidase was bound to the biotinylated nucleotides, and peroxidase activity in each section was demonstrated by the application of a stable chromogen diaminobenzidine. When using this procedure, apoptotic nuclei were stained dark brown. The sections were counterstained with hematoxylin for total nuclei counting. Staining was viewed using an Olympus BX40 microscope and analyzed. Two sections from each myocardial sample were randomly selected, and 10 microscopic

fields per section were evaluated by a blind observer. In each field, the nuclei were counted, and the percentage of TUNEL-positive nuclei was calculated.

Immunoblotting of survival kinases

In an additional set of experiments, rat hearts underwent 40-min coronary occlusion and were collected at 15-min reperfusion for analysis of protein phosphorylation [35, 59]. Rats were randomly assigned to one of four groups: Control (n=6); local ischemic postconditioning (IPost, n=5); remote ischemic preconditioning (RIPer, n=7); combination of local ischemic postconditioning and remote ischemic preconditioning (IPost+RIPer, n=6). Western blots were performed on myocardium from the area at risk. The ventricular tissue at risk was excised and freeze-clamped in liquid nitrogen (between stainless steel tongues precooled with liquid nitrogen) before being stored at -80°C. Frozen myocardial tissue samples were powdered in a mortar and pestle precooled to the temperature of liquid nitrogen.

Approximately 100mg of powdered ventricular tissue were used for protein extraction. Frozen myocardial tissue samples were homogenized on ice in 0.5ml ice-cold lysis buffer containing 30mM HEPES, 20mM KCl, 2.5mM EGTA, 2.5mM EDTA, 40mM sodium fluoride, 4mM sodium pyrophosphate, 1mM sodium orthovanadate, 10% glycerol, 1% Nonidet P-40, a phosphatase inhibitor cocktail (Sigma), and a protease inhibitor cocktail (Complete mini, Roche Applied Science, Mannheim, Germany). The homogenate was centrifuged at 13,000rpm at 4°C for 1 hour, and the resulting supernatant was collected. Protein concentration was determined using Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Aliquots of the supernatant containing equal amounts of proteins (40µg) were heated to 95°C for 5 min in sample loading buffer. Proteins were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Amersham Bioscience). After the nonspecific binding sites with 5% nonfat milk were

blocked for 1 hour in Tris-buffered saline Tween (TBST) containing 20mM Tris-HCl, 137mM NaCl (pH 7.6), and 0.1% Tween-20, the membranes were incubated overnight at 4°C with rabbit antibodies against ⁴⁷³Ser-phospho-Akt, total Akt, phospho-ERK1/2, total ERK1/2, ⁹Ser-phospho-GSK-3β, total GSK-3β, ⁷⁰⁵Tyr-phospho-STAT-3, and total STAT-3 (all from Cell Signaling Technology). Beta-actin (Sigma Aldrich) was used as a loading control. After being washed in TBST, the membranes were incubated for 1 hour at room temperature with horseradish peroxidase conjugated anti-rabbit IgG secondary antibody (1/2000, Santa Cruz Biotechnologies), and the bound antibody was detected using an enhanced chemiluminescence Western blotting kit (Santa Cruz Biotechnologies). The densities of the bands with appropriated molecular mass (60kDa for Akt, 42/44kDa for ERK1/2, 46kDa for GSK-3β, and 80kDa for STAT-3) were determined semi-quantitatively using a lumino-image analyzer, LAS-3000 mini (Fujifilm, Tokyo, Japan).

Chemicals

Pentobarbital was obtained from Céva Santé Animal. Wortmannin and U0126 were purchased from Sigma-Aldrich and AG490 from Tocris. Wortmannin, U0126, and AG490 were dissolved in DMSO and diluted into saline in order for the vehicle to constitute less than 1% of the total volume injected.

Statistics

All values were expressed as mean±SEM. Statistical analyses were performed using StatView 5.0 software. Differences between groups were evaluated using one-way ANOVA, followed by *post hoc* Fisher's least significant difference (LSD)-corrected multiple comparison test. *P* values <0.05 were considered statistically significant.

Results

RIPer combined with IPost shown to be more protective than IPost alone

The area at risk/LV ratio (AAR/LV) did not differ among the groups (Fig. 2). As compared with Control, both IPost and RIPer treatments significantly decreased infarct size (AN/AAR=54.93±6.46% in Control, 34.25±3.36% in IPost, and 24.69±6.02% in RIPer, both $p<0.05$ vs. Control; Fig. 2). Similarly, both IPost and RIPer treatments significantly reduced the number of TUNEL-positive cells observed after 2 hours of reperfusion in the area at risk (20.59±5.9% and 17.8±2.91%, respectively) as compared with Control (37.23±5.89%, both $p<0.05$; Fig. 3). On the contrary, neither treatment affected the level of apoptotic nuclei in the remote myocardium (Fig. 3). Interestingly, the combination of treatments, IPost+RIPer, was significantly more effective in reducing infarct size as compared with IPost alone (18.04±4.86% vs. 34.25±3.36%, $p<0.05$).

RIPer combined with IPost involved RISK and SAFE pathways

There was no significant difference in total Akt, ERK1/2, GSK-3 β and STAT-3 protein levels in all study groups. Therefore, phospho-Akt, phospho-ERK1/2, phospho-GSK-3 β and phospho-STAT-3 levels were expressed as densitometric levels normalized by their total protein levels [11, 54].

As illustrated in Fig. 4a, phospho-Akt levels at 15 min of reperfusion were significantly enhanced in IPost compared with Control ($p<0.05$). Furthermore, there was a trend towards increased levels of phospho-ERK1/2 compared with Control ($p=0.11$; Fig. 4b). Logically, GSK-3 β phosphorylation as a downstream target of Akt and ERK1/2 was significantly increased in IPost as compared with Control ($p<0.05$; Fig. 4c).

Akt, ERK1/2, and GSK-3 β phosphorylations were significantly increased in RIPer as compared with Control (all $p<0.05$; Fig. 4a, b, and c).

The IPost+RIPer treatment significantly increased Akt, ERK1/2, and GSK-3 β as compared with Control (all $p<0.05$; Fig. 4a, b, and c). While the IPost+RIPer treatment was more effective in decreasing infarct size than IPost alone, none of the tested proteins from the RISK pathway (Akt, ERK1/2, and GSK-3 β) were significantly more phosphorylated in IPost+RIPer than in IPost alone (Fig. 4), suggesting that another signaling pathway might play a role in the enhanced cardioprotection afforded by this combination. When testing the SAFE pathway, phospho-STAT-3 levels were not significantly increased following IPost or RIPer administered alone. Interestingly, the IPost+RIPer combination highly increased phospho-STAT-3 levels as compared with the Control, IPost, and RIPer groups (all $p<0.05$) (Fig. 5).

SAFE and RISK pathway interactions

In order to further explore the respective role of RISK and SAFE pathways in the cardioprotection induced by IPost, RIPer, and IPost+RIPer, pharmacological inhibitors were administered. As shown in Fig. 4d, the protective effect of IPost was completely abolished by the treatment with wortmannin, an inhibitor of PI3K/Akt signaling pathway, and with U0126, an inhibitor of MEK1/2. Similarly, wortmannin and U0126 fully prevented RIPer-mediated cardioprotection (Fig. 4d). The protective effect of IPost+RIPer was completely abolished by the treatment with wortmannin and U0126 (Fig. 4d). Surprisingly, cardioprotection induced by the IPost+RIPer combination was also completely abolished by AG490, a pharmacological inhibitor of the JAK/STAT pathway (Fig. 6).

In order to clarify whether RISK and SAFE pathways interact, we examined phosphorylation of survival kinases from RISK (Akt, ERK1/2, and GSK-3 β) and SAFE (STAT-3) pathways following administration of pharmacological inhibitors (wortmannin and U0126 as inhibitors of RISK, and AG490 as an inhibitor of SAFE). In the presence of

AG490, IPost+RIPer-induced phosphorylation of ERK1/2 was significantly reduced ($p<0.05$; Fig. 7b). Akt phosphorylation was also reduced, although the difference did not reach statistical significance ($p=0.10$; Fig 7a). Phosphorylation of the downstream GSK-3 β induced by the IPost+RIPer combination was abolished in the presence of AG490 ($p<0.05$; Fig 7c). Conversely, IPost+RIPer-induced STAT-3 phosphorylation was completely abolished in the presence of wortmannin or U0126 (both $p<0.05$ vs. IPost+RIPer; Fig. 7d).

Discussion

The present study shows that RIPer was as effective as IPost in decreasing myocardial reperfusion injury in a rat model of I/R. Additionally, the IPost+RIPer combination was superior to IPost alone through recruitment of the SAFE pathway. Our present findings also indicate that cross talk exists between the RISK and SAFE signaling pathways in this model.

The cardioprotective effect of RIPer was first reported by Kerendi *et al.* in 2005 [30]. In a rat model of myocardial I/R, a single 5-min episode of renal artery occlusion followed by renal reperfusion 1 min prior to myocardial reperfusion reduced myocardial infarct size by nearly 50%. Later protocols using limb ischemia as a stimulus of RIPer reported cardioprotective effect in various animal models including rats [59], rabbits [38], and pigs [2]. Furthermore, remote pre- and preconditioning have been shown to be applicable to the clinical setting [1, 7, 17, 26, 40, 47, 49, 55]. Remote conditioning stimuli, consisting of transient limb ischemia applied before or during a prolonged ischemic insult, reduced vascular I/R injury in humans [40]. More recently, Botker *et al.* tested the effect of RIPer in patients with AMI [7]. Interestingly, intermittent arm ischemia with four cycles of 5-min inflation and 5-min deflation of a blood-pressure cuff taking place in the ambulance during transfer to primary PCI was shown to significantly increase myocardial salvage [7]. While the

mechanisms of local IPost have been extensively studied, the protective mechanisms underlying remote ischemic conditioning are still poorly understood. It is assumed that soluble mediators transported through the circulation play a critical role [9], although they remain to be identified [19, 39].

Multiple signaling pathways have been demonstrated to participate in infarct reduction by IPost [28]. During early reperfusion, IPost can activate prosurvival kinases, including Akt and ERK1/2, which are key components of the RISK pathway. This activation results in the inhibition of the mitochondrial permeability transition pore (mPTP) mediated through the phosphorylation and subsequent inhibition of GSK-3 β [20, 24, 27]. More recently, IPost has been shown to activate the signal transducer and activator of transcription-3 (STAT-3) as part of the SAFE pathway [3, 5, 6, 16, 29, 35-37]. Emerging evidence indicates that remote conditioning may share common mechanistic signaling pathways with IPost [19, 45, 57, 58]. Heidbreder *et al.* [21] suggested that within the remote organ, the activation of the mitogen-activated protein kinases (MAPKs), namely p38, ERK1/2, and JNK, may contribute to remote preconditioning-induced cardioprotection [21]. They demonstrated that remote preconditioning induced by occlusion of the mesenteric artery prior to coronary artery occlusion activated MAPKs within the intestinal tissue but not within the myocardium [21]. Moreover, pharmacologic inhibition of these kinases abolished cardioprotection [21]. Using an *ex vivo* rat heart model, the recent work by Breivik *et al.* [8] demonstrated that effluent collected during ischemic preconditioning, when administered as a stimulus of remote postconditioning, could protect untreated recipient hearts from reperfusion-induced injury via a PI3K/Akt-dependent pathway [8].

Hence, activation of the RISK pathway is a commonly-admitted mechanism involved in local ischemic pre- and postconditioning [18, 20]. However, in a porcine model of AMI, Schwartz *et al.* reported that Akt and ERK activation through IPost did not have any protective effects against reperfusion injuries [50]. Conversely, Skyschally *et al.* revealed IPost-induced cardioprotection in pigs in the absence of any significant increase in ERK1/2 and Akt phosphorylation [51]. In the latter study, the use of RISK pathway inhibitors did not abolish IPost-induced cardioprotection [51]. Moreover, isolated perfused mouse hearts with non-inhibitable forms of GSK-3 could still be cardioprotected by IPost [42]. All together, these controversial results have suggested the existence of an alternative signaling pathway and given this context, IPost was proposed to protect the ischemic heart from myocardial reperfusion injury through activation of either the RISK or SAFE pathway [12, 16, 22].

In the present study, the IPost+RIPer combination decreased infarct size to a larger degree than IPost alone. Similarly, Xin *et al.* [59] recently showed that the combination of IPost (six cycles of 10-sec coronary reocclusion followed by 10-sec reperfusion) and RIPer (four cycles of 5-min limb ischemia followed by 5-min reperfusion) resulted in lower infarct size as compared with either procedure alone. The authors showed that Akt and ERK1/2 phosphorylations were increased when both strategies were used in combination [59]. The SAFE pathway was not explored in this study. In our work, the enhanced cardioprotective effect observed with the IPost+RIPer combination was not associated with enhanced activation of the RISK pathway but instead with increased phospho-STAT-3 levels, inferring a key role of the SAFE pathway. It must be underlined that the IPost and RIPer protocols used in the Xin *et al.* study notably differed from those used in our study. Interestingly, Boengler *et al.* demonstrated that mitochondrial STAT-3 could potentially contribute to cardioprotection by inhibiting mPTP [6]. Combined strategies of myocardial ischemic conditioning may

increase tolerance of the myocardium to reperfusion injury by improving the balance between death and survival signals.

Recent data suggests that the RISK and SAFE pathways likely interact in mediating IPost-induced cardioprotection [12]. In the study by Goodman *et al.*, administration of a STAT-3 inhibitor prevented the cardioprotective effects of IPost in a mouse model of I/R and abolished IPost-induced phosphorylation of both Akt and STAT-3 [12]. These findings highlight that Akt may be a downstream target of STAT-3. Using cardiomyocytes and isolated perfused hearts, Suleman *et al.* [53] previously demonstrated that both prosurvival signaling pathways, PI3K/Akt and JAK/STAT-3, closely interacted during ischemic preconditioning to promote maximal protection. Suleman's study showed that inhibition of STAT-3 activation using AG490 inhibited Akt activation, whereas inhibition of Akt using wortmannin decreased STAT-3 phosphorylation [53]. Both wortmannin and AG490 abolished the protection afforded by preconditioning in isolated perfused hearts [53]. In addition, Gross *et al.* reported a possible interaction between PI3K/Akt and SAFE pathways [14]. Using an *in vivo* rat heart model of I/R in conjunction with a H9C2 cell culture model, the authors showed that opioid-induced cardioprotection occurred via activation of both JAK/STAT-3 and PI3K/Akt pathways [14]. Opioid-induced Akt phosphorylation could be blocked by AG490, whereas opioid-induced STAT-3 phosphorylation could be inhibited by wortmannin, suggesting an interaction between both pathways [14].

In our work, both RISK and SAFE pathways were activated using the IPost+RIPer combination. A cross talk between RISK and SAFE pathways was suggested, since the inhibition of either of them totally abolished the cardioprotective effect obtained with the combination. We were able to confirm this interaction between RISK and SAFE when an

inhibitor of SAFE abolished Akt and GSK-3 β phosphorylation and conversely, when inhibitors of RISK abolished STAT-3 phosphorylation. These findings indicate that cross talk exists in this model between the RISK and SAFE signaling pathways as summarized in Fig. 8.

Limitations

In the present study, we tested only one IPost protocol using three cycles of 10-sec ischemia/10-sec reperfusion applied at the onset of reperfusion, and only one RPer protocol with one cycle of 10-min ischemia/10-min reperfusion initiated 20 min before the end of myocardial ischemia. Even if these protocols were previously validated in rat models of AMI [10, 32, 33, 43, 54, 60], differing results might have been obtained when using other protocols. As controversial results were reported with rat models testing IPost [52], our results must be further confirmed in others species. Another limitation of the study lies in the timing of the protein phosphorylation assessment. We chose 15-min reperfusion for assessing reperfusion salvage kinases, since both RISK and SAFE signaling pathways are activated at early reperfusion, and as changes in phosphorylation levels had already been described at 15-min reperfusion [35, 59]. Also, our study did not explore the role of TNF- α , a critical component of the SAFE pathway [34]. Finally, data obtained with pharmacological inhibitors must be interpreted with caution, as these inhibitors likely act in a concentration-dependant manner. Lastly, it should be noted that AG490 is known to block JAK2, which is upstream of STAT-3.

Conclusion

In our experimental model, RPer was as effective as IPost in decreasing reperfusion injuries, while the IPost+RPer combination improved cardioprotection through recruitment of the

SAFE pathway. In addition, our data reinforces the existing body of evidence suggesting a possible cross talk between the RISK and SAFE pathways. Further experimental and clinical studies are required to confirm that the combination of local and remote conditioning strategies is efficacious in further protecting the myocardium from reperfusion injuries.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Ali ZA, Callaghan CJ, Lim E, Ali AA, Nouraei SA, Akthar AM, Boyle JR, Varty K, Kharbanda RK, Dutka DP, Gaunt ME (2007) Remote ischemic preconditioning reduces myocardial and renal injury after elective abdominal aortic aneurysm repair: a randomized controlled trial. *Circulation* 116:198-105 doi: 10.1161/circulationaha.106.679167
2. Andreka G, Vertesaljai M, Szantho G, Font G, Piroth Z, Fontos G, Juhasz ED, Szekely L, Szelid Z, Turner MS, Ashrafian H, Frenneaux MP, Andreka P (2007) Remote ischaemic postconditioning protects the heart during acute myocardial infarction in pigs. *Heart* 93:749-752 doi: 10.1136/hrt.2006.114504
3. Boengler K, Buechert A, Heinen Y, Roeskes C, Hilfiker-Kleiner D, Heusch G, Schulz R (2008) Cardioprotection by ischemic postconditioning is lost in aged and STAT3-deficient mice. *Circ Res* 102:131-135 doi: 10.1161/CIRCRESAHA.107.164699
4. Boengler K, Heusch G, Schulz R Mitochondria in Postconditioning (2011). *Antioxid Redox Signal* 14:863-881 doi: 10.1089/ars.2010.3309
5. Boengler K, Hilfiker-Kleiner D, Drexler H, Heusch G, Schulz R (2008) The myocardial JAK/STAT pathway: from protection to failure. *Pharmacol Ther* 120:172-185 doi: 10.1016/j.pharmthera.2008.08.002
6. Boengler K, Hilfiker-Kleiner D, Heusch G, Schulz R (2010) Inhibition of permeability transition pore opening by mitochondrial STAT3 and its role in myocardial ischemia/reperfusion. *Basic Res Cardiol* 105:771-785 doi: 10.1007/s00395-010-0124-1
7. Botker HE, Kharbanda R, Schmidt MR, Bottcher M, Kaltoft AK, Terkelsen CJ, Munk K, Andersen NH, Hansen TM, Trautner S, Lassen JF, Christiansen EH, Krusell LR, Kristensen SD, Thuesen L, Nielsen SS, Rehling M, Sorensen HT, Redington AN,

- Nielsen TT (2010) Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: a randomised trial. *Lancet* 375:727-734 doi: 10.1016/S0140-6736(09)62001-8
8. Breivik L, Helgeland E, Aarnes EK, Mrdalj J, Jonassen AK (2011) Remote postconditioning by humoral factors in effluent from ischemic preconditioned rat hearts is mediated via PI3K/Akt-dependent cell-survival signaling at reperfusion. *Basic Res Cardiol* 106:135-45 doi: 10.1007/s00395-010-0133-0
 9. Dickson EW, Reinhardt CP, Renzi FP, Becker RC, Porcaro WA, Heard SO (1999) Ischemic preconditioning may be transferable via whole blood transfusion: preliminary evidence. *J Thromb Thrombolysis* 8:123-129
 10. Dong JH, Liu YX, Ji ES, He RR (2004) [Limb ischemic preconditioning reduces infarct size following myocardial ischemia-reperfusion in rats]. *Sheng Li Xue Bao* 56:41-46
 11. Ghaboura N, Tamareille S, Ducluzeau PH, Grimaud L, Loufrani L, Croue A, Tourmen Y, Henrion D, Furber A, Prunier F (2011) Diabetes mellitus abrogates erythropoietin-induced cardioprotection against ischemic-reperfusion injury by alteration of the RISK/GSK-3beta signaling. *Basic Res Cardiol* 106:147-162 doi: 10.1007/s00395-010-0130-3
 12. Goodman MD, Koch SE, Fuller-Bicer GA, Butler KL (2008) Regulating RISK: a role for JAK-STAT signaling in postconditioning? *Am J Physiol Heart Circ Physiol* 295:H1649-1656 doi: 10.1152/ajpheart.00692.2008
 13. Gritsopoulos G, Iliodromitis EK, Zoga A, Farmakis D, Demerouti E, Papalois A, Paraskevaidis IA, Kremastinos DT (2009) Remote postconditioning is more potent

- than classic postconditioning in reducing the infarct size in anesthetized rabbits. *Cardiovasc Drugs Ther* 23:193-198 doi: 10.1007/s10557-009-6168-5
14. Gross ER, Hsu AK, Gross GJ (2006) The JAK/STAT pathway is essential for opioid-induced cardioprotection: JAK2 as a mediator of STAT3, Akt, and GSK-3 beta. *Am J Physiol Heart Circ Physiol* 291:H827-834 doi: 10.1152/ajpheart.00003.2006
 15. Hausenloy DJ, Baxter G, Bell R, Botker HE, Davidson SM, Downey J, Heusch G, Kitakaze M, Lecour S, Mentzer R, Mocanu MM, Ovize M, Schulz R, Shannon R, Walker M, Walkinshaw G, Yellon DM (2010) Translating novel strategies for cardioprotection: the Hatter Workshop Recommendations. *Basic Res Cardiol* 105:677-686 doi: 10.1007/s00395-010-0121-4
 16. Hausenloy DJ, Lecour S, Yellon DM (2011) Reperfusion Injury Salvage Kinase and Survivor Activating Factor Enhancement Prosurvival Signaling Pathways in Ischemic Postconditioning: Two Sides of the Same Coin. *Antioxid Redox Signal* 14:893-907 doi: 10.1089/ars.2010.3360
 17. Hausenloy DJ, Mwamure PK, Venugopal V, Harris J, Barnard M, Grundy E, Ashley E, Vichare S, Di Salvo C, Kolvekar S, Hayward M, Keogh B, MacAllister RJ, Yellon DM (2007) Effect of remote ischaemic preconditioning on myocardial injury in patients undergoing coronary artery bypass graft surgery: a randomised controlled trial. *Lancet* 370:575-579 doi: 10.1016/S0140-6736(07)61296-3
 18. Hausenloy DJ, Tsang A, Yellon DM (2005) The reperfusion injury salvage kinase pathway: a common target for both ischemic preconditioning and postconditioning. *Trends Cardiovasc Med* 15:69-75 doi: 10.1016/j.tcm.2005.03.001
 19. Hausenloy DJ, Yellon DM (2008) Remote ischaemic preconditioning: underlying mechanisms and clinical application. *Cardiovasc Res* 79:377-386 doi: 10.1093/cvr/cvn114

20. Hausenloy DJ, Yellon DM (2006) Survival kinases in ischemic preconditioning and postconditioning. *Cardiovasc Res* 70:240-253 doi: 10.1016/j.cardiores.2006.01.017
21. Heidbreder M, Naumann A, Tempel K, Dominiak P, Dendorfer A (2008) Remote vs. ischaemic preconditioning: the differential role of mitogen-activated protein kinase pathways. *Cardiovasc Res* 78:108-115 doi: 10.1093/cvr/cvm114
22. Heusch G (2009) No risk, no ... cardioprotection? A critical perspective. *Cardiovasc Res* 84:173-175 doi: 10.1093/cvr/cvp298
23. Heusch G, Boengler K, Schulz R (2008) Cardioprotection: nitric oxide, protein kinases, and mitochondria. *Circulation* 118:1915-1919 doi: 10.1161/CIRCULATIONAHA.108.805242
24. Heusch G, Boengler K, Schulz R (2010) Inhibition of mitochondrial permeability transition pore opening: the Holy Grail of cardioprotection. *Basic Res Cardiol* 105:151-154 doi: 10.1007/s00395-009-0080-9
25. Heusch G, Schulz R (2002) Remote preconditioning. *J Mol Cell Cardiol* 34:1279-1281 doi: 10.1006/jmcc.2002.2093
26. Hoole SP, Heck PM, Sharples L, Khan SN, Duehmke R, Densem CG, Clarke SC, Shapiro LM, Schofield PM, O'Sullivan M, Dutka DP (2009) Cardiac Remote Ischemic Preconditioning in Coronary Stenting (CRISP Stent) Study: a prospective, randomized control trial. *Circulation* 119:820-827 doi: 10.1161/CIRCULATIONAHA.108.809723
27. Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN, Sollott SJ (2004) Glycogen synthase kinase-3 β mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest* 113:1535-1549 doi: 10.1172/JCI19906
28. Kaur S, Jaggi AS, Singh N (2009) Molecular aspects of ischaemic postconditioning. *Fundam Clin Pharmacol* 23:521-536 doi: 10.1111/j.1472-8206.2009.00733.x

29. Kelly RF, Lamont KT, Somers S, Hacking D, Lacerda L, Thomas P, Opie LH, Lecour S (2010) Ethanolamine is a novel STAT-3 dependent cardioprotective agent. *Basic Res Cardiol* 105:763-770 doi: 10.1007/s00395-010-0125-0
30. Kerendi F, Kin H, Halkos ME, Jiang R, Zatta AJ, Zhao ZQ, Guyton RA, Vinten-Johansen J (2005) Remote postconditioning. Brief renal ischemia and reperfusion applied before coronary artery reperfusion reduces myocardial infarct size via endogenous activation of adenosine receptors. *Basic Res Cardiol* 100:404-412 doi: 10.1007/s00395-005-0539-2
31. Kharbanda RK (2010) Cardiac conditioning: a review of evolving strategies to reduce ischaemia-reperfusion injury. *Heart* 96:1179-1186 doi: 10.1136/hrt.2009.179101
32. Kin H, Wang NP, Mykytenko J, Reeves J, Deneve J, Jiang R, Zatta AJ, Guyton RA, Vinten-Johansen J, Zhao ZQ (2008) Inhibition of myocardial apoptosis by postconditioning is associated with attenuation of oxidative stress-mediated nuclear factor-kappa B translocation and TNF alpha release. *Shock* 29:761-768 doi: 10.1097/SHK.0b013e31815cfd5a
33. Kin H, Zhao ZQ, Sun HY, Wang NP, Corvera JS, Halkos ME, Kerendi F, Guyton RA, Vinten-Johansen J (2004) Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. *Cardiovasc Res* 62:74-85 doi: 10.1016/j.cardiores.2004.01.006
34. Lacerda L, McCarthy J, Mungly SF, Lynn EG, Sack MN, Opie LH, Lecour S (2010) TNFalpha protects cardiac mitochondria independently of its cell surface receptors. *Basic Res Cardiol* 105:751-762 doi: 10.1007/s00395-010-0113-4

35. Lacerda L, Somers S, Opie LH, Lecour S (2009) Ischaemic postconditioning protects against reperfusion injury via the SAFE pathway. *Cardiovasc Res* 84:201-208 doi: 10.1093/cvr/cvp274
36. Lecour S (2009) Activation of the protective Survivor Activating Factor Enhancement (SAFE) pathway against reperfusion injury: Does it go beyond the RISK pathway? *J Mol Cell Cardiol* 47:32-40 doi: 10.1016/j.yjmcc.2009.03.019
37. Lecour S (2009) Multiple protective pathways against reperfusion injury: a SAFE path without Aktion? *J Mol Cell Cardiol* doi: 46:607-609 10.1016/j.yjmcc.2009.01.003
38. Li CM, Zhang XH, Ma XJ, Luo M (2006) Limb ischemic postconditioning protects myocardium from ischemia-reperfusion injury. *Scand Cardiovasc J* 40:312-317 doi: 10.1080/14017430600925292
39. Lim SY, Yellon DM, Hausenloy DJ (2010) The neural and humoral pathways in remote limb ischemic preconditioning. *Basic Res Cardiol* 105:651-655 doi: 10.1007/s00395-010-0099-y
40. Loukogeorgakis SP, Williams R, Panagiotidou AT, Kolvekar SK, Donald A, Cole TJ, Yellon DM, Deanfield JE, MacAllister RJ (2007) Transient limb ischemia induces remote preconditioning and remote postconditioning in humans by a K(ATP)-channel dependent mechanism. *Circulation* 116:1386-1395 doi: 10.1161/CIRCULATIONAHA.106.653782
41. Murray CJ, Lopez AD (1997) Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *Lancet* 349:1498-1504 doi: 10.1016/S0140-6736(96)07492-2
42. Nishino Y, Webb IG, Davidson SM, Ahmed AI, Clark JE, Jacquet S, Shah AM, Miura T, Yellon DM, Avkiran M, Marber MS (2008) Glycogen synthase kinase-3

- inactivation is not required for ischemic preconditioning or postconditioning in the mouse. *Circ Res* 103:307-314 doi: 10.1161/CIRCRESAHA.107.169953
43. Oxman T, Arad M, Klein R, Avazov N, Rabinowitz B (1997) Limb ischemia preconditions the heart against reperfusion tachyarrhythmia. *Am J Physiol* 273:H1707-1712
 44. Peart JN, Gross ER, Reichelt ME, Hsu A, Headrick JP, Gross GJ (2008) Activation of kappa-opioid receptors at reperfusion affords cardioprotection in both rat and mouse hearts. *Basic Res Cardiol* 103:454-463 doi: 10.1007/s00395-008-0726-z
 45. Pell TJ, Baxter GF, Yellon DM, Drew GM (1998) Renal ischemia preconditions myocardium: role of adenosine receptors and ATP-sensitive potassium channels. *Am J Physiol* 275:H1542-1547
 46. Piper HM, Garcia-Dorado D, Ovize M (1998) A fresh look at reperfusion injury. *Cardiovasc Res* 38:291-300
 47. Rentoukas I, Giannopoulos G, Kaoukis A, Kossyvakis C, Raisakis K, Driva M, Panagopoulou V, Tsarouchas K, Vavetsi S, Pyrgakis V, Deftereos S (2010) Cardioprotective role of remote ischemic periconditioning in primary percutaneous coronary intervention: enhancement by opioid action. *JACC Cardiovasc Interv* 3:49-55 doi: 10.1016/j.jcin.2009.10.015
 48. Saxena P, Newman MA, Shehatha JS, Redington AN, Konstantinov IE (2010) Remote ischemic conditioning: evolution of the concept, mechanisms, and clinical application. *J Card Surg* 25:127-134 doi: 10.1111/j.1540-8191.2009.00820.x
 49. Schmidt MR, Smerup M, Konstantinov IE, Shimizu M, Li J, Cheung M, White PA, Kristiansen SB, Sorensen K, Dzavik V, Redington AN, Kharbanda RK (2007) Intermittent peripheral tissue ischemia during coronary ischemia reduces myocardial infarction through a KATP-dependent mechanism: first demonstration of remote

- ischemic preconditioning. *Am J Physiol Heart Circ Physiol* 292:H1883-1890 doi: 10.1152/ajpheart.00617.2006
50. Schwartz LM, Lagranha CJ (2006) Ischemic postconditioning during reperfusion activates Akt and ERK without protecting against lethal myocardial ischemia-reperfusion injury in pigs. *Am J Physiol Heart Circ Physiol* 290:H1011-1018 doi: 10.1152/ajpheart.00864.2005
 51. Skyschally A, van Caster P, Boengler K, Gres P, Musiolik J, Schilawa D, Schulz R, Heusch G (2009) Ischemic postconditioning in pigs: no causal role for RISK activation. *Circ Res* 104:15-18 doi: 10.1161/CIRCRESAHA.108.186429
 52. Skyschally A, van Caster P, Iliodromitis EK, Schulz R, Kremastinos DT, Heusch G (2009) Ischemic postconditioning: experimental models and protocol algorithms. *Basic Res Cardiol* 104:469-483 doi: 10.1007/s00395-009-0040-4
 53. Suleman N, Somers S, Smith R, Opie LH, Lecour SC (2008) Dual activation of STAT-3 and Akt is required during the trigger phase of ischaemic preconditioning. *Cardiovasc Res* 79:127-133 doi: 10.1093/cvr/cvn067
 54. Tamarelle S, Ghaboura N, Treguer F, Khachman D, Croue A, Henrion D, Furber A, Prunier F (2009) Myocardial reperfusion injury management: erythropoietin compared with postconditioning. *Am J Physiol Heart Circ Physiol* 297:H2035-2043 doi: 10.1152/ajpheart.00472.2009
 55. Thielmann M, Kottenberg E, Boengler K, Raffelsieper C, Neuhaeuser M, Peters J, Jakob H, Heusch G (2010) Remote ischemic preconditioning reduces myocardial injury after coronary artery bypass surgery with crystalloid cardioplegic arrest. *Basic Res Cardiol* 105:657-664 doi: 10.1007/s00395-010-0104-5
 56. Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM (2004) Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol

3-kinase-Akt pathway. Circ Res 95:230-232 doi:
10.1161/01.RES.0000138303.76488.fe

57. Weinbrenner C, Schulze F, Sarvary L, Strasser RH (2004) Remote preconditioning by infrarenal aortic occlusion is operative via delta1-opioid receptors and free radicals in vivo in the rat heart. Cardiovasc Res 61:591-599 doi: 10.1016/j.cardiores.2003.10.008
58. Wolfrum S, Schneider K, Heidbreder M, Nienstedt J, Dominiak P, Dendorfer A (2002) Remote preconditioning protects the heart by activating myocardial PKCepsilon-isoform. Cardiovasc Res 55:583-589
59. Xin P, Zhu W, Li J, Ma S, Wang L, Liu M, Wei M, Redington AN (2010) Combined local ischemic postconditioning and remote preconditioning recapitulate cardioprotective effects of local ischemic preconditioning. Am J Physiol Heart Circ Physiol 298:H1819-1831 doi: 10.1152/ajpheart.01102.2009
60. Zatta AJ, Kin H, Yoshishige D, Jiang R, Wang N, Reeves JG, Mykytenko J, Guyton RA, Zhao ZQ, Caffrey JL, Vinten-Johansen J (2008) Evidence that cardioprotection by postconditioning involves preservation of myocardial opioid content and selective opioid receptor activation. Am J Physiol Heart Circ Physiol 294:H1444-1451 doi: 10.1152/ajpheart.01279.2006
61. Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, Vinten-Johansen J (2003) Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 285:H579-588 doi: 10.1152/ajpheart.01064.2002

Figure captions

Fig. 1 Experimental protocols. All groups were subjected to 40-min coronary artery occlusion followed by either 120-min reperfusion for infarct size measurement and apoptosis or 15-min reperfusion for protein phosphorylation analysis. Ischemic postconditioning (IPost) was induced by three cycles of 10-sec myocardial ischemia/10-sec myocardial reperfusion. Remote ischemic preconditioning (RIPer) was achieved by 10-min limb ischemia followed by 10-min reperfusion. Pharmacological inhibitors (wortmannin, U0126, and AG490) were administered as a bolus 25 min prior to myocardial reperfusion

Fig. 2 a Representative sections of triphenyltetrazolium chloride (TTC)-stained heart following 40-min ischemia and 120-min reperfusion. **b** Tabulated data from TTC staining. Bar graph showing area of necrosis (AN) expressed as a percentage of area at risk (AAR) and AAR as a percentage of total LV area (LV). All data is expressed as mean $\pm$ SEM. * p <0.05 vs. Control group, # p <0.05 vs. IPost group. n=6-7 in each group

Fig. 3 a Representative pictures from TUNEL-stained heart tissue sections from ischemic area. Apoptotic cardiomyocyte nuclei appear brown-stained, whereas TUNEL-negative nuclei appear blue with hematoxylin. Heavy staining of numerous TUNEL-positive nuclei was observed in control hearts. **b** Bar graph showing TUNEL-positive nuclei expressed as a percent of total nuclei in tissue sections from each group. * p <0.05 vs. Control hearts. n=4-7 hearts in each group

Fig. 4 a to c Western blot analysis of Akt (**a**), ERK1/2 (**b**), and GSK-3 β (**c**) phosphorylation in rat hearts subjected to ischemia-reperfusion. *Top*: Representative immunoblots of phosphorylated Akt (p-Akt) and total Akt (**a**); phosphorylated ERK1/2 (p-ERK1/2) and total

ERK1/2 (**b**); phosphorylated GSK-3 β (p-GSK-3 β) and total GSK-3 β (**c**) in LV homogenates from hearts subjected to ischemia-reperfusion. *Bottom*: Bar graphs showing mean $\pm$ SEM of the densitometry of p-Akt-to-Akt ratio (**a**), p-ERK1/2-to-ERK1/2 ratio (**b**), and p-GSK-3 β -to-GSK-3 β ratio (**c**). All data is expressed as mean $\pm$ SEM. * p <0.05 vs. Control; n=5-7 in each group. **d** Bar graph showing area of necrosis (AN) expressed as a percentage of area at risk (AAR) for each group in the absence or presence of wortmannin (wort) and U0126. All data is expressed as mean $\pm$ SEM. * p <0.05 vs. Control; # p <0.05 vs. IPost; † p <0.05 vs. RIPer and £ p <0.05 vs. IPost+RIPer; n=6-7 in each group

Fig. 5 Western blot analysis of STAT-3 phosphorylation. Top: Example of immunoblots of phosphorylated STAT-3 (p-STAT-3), total STAT-3 and actin in LV homogenates from hearts subjected to ischemia-reperfusion. *Bottom*: Bar graphs showing means $\pm$ SEM of the densitometry of p-STAT-3-to-STAT-3 ratio.* p <0.05 vs. Control group; # p <0.05 vs. IPost; † p <0.05 vs. RIPer; n=5-7 in each group. Note that example of immunoblots does not exactly reflect the mean value of all samples.

Fig. 6 Effect of AG490 on infarct size in IPost+RIPer combination. **a** Representative sections of rat hearts stained with triphenyltetrazolium chloride (TTC) after 40-min coronary artery occlusion and 120-min reperfusion. **b** Tabulated data from triphenyltetrazolium chloride staining. Area of necrosis (AN) expressed as a percentage of area at risk (AAR) * p <0.05 vs. Control; £ p <0.05 vs. IPost+RIPer; n= 6 in each group

Fig. 7 Western blot analysis of Akt (**a**), ERK1/2 (**b**), GSK-3 β (**c**), and STAT-3 (**d**) phosphorylations in the absence or presence of pharmacological inhibitors wortmannin (wort),

U0126 and AG490. * $p < 0.05$ vs. Control; £ $p < 0.05$ vs. IPost+RIPer; n= 6 in each group. Note that examples of immunoblots do not exactly reflect the mean value of all samples.

Fig. 8 Schematic representation illustrating the possible interaction between reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE) pathways at the early myocardial reperfusion phase in rats using IPost+RIPer combination

GSK-3 β : glycogen synthase kinase 3 beta; ERK1/2: extracellular regulated kinase; STAT-3: signal transducer and activator of transcription-3; mPTP: mitochondrial permability transition pore; Wort: wortmannin