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Running title: IPost combined to RIPer

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**Abstract**

Local ischemic postconditioning (IPost) and remote ischemic perconditioning (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±3.36% and 24.69±6.02%, respectively) groups compared to Control (54.93±6.46%, both $p<0.05$). IPost+RIPer (infarct size=18.04±4.86%) was significantly more cardioprotective than IPost alone ($p<0.05$). RISK pathway (Akt, ERK1/2, and GSK-3β) 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-3β 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, perconditioning, 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 endotrachealy 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 dyskinesis 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 perconditioning (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 perconditioning (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+RIPost+U0126; IPost+RIPost+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-µm sections were obtained. The sections were then deparaffinized and rehydrated with xylene and graded alcohol series. The sections were stained using the *in situ* DeadEndTM 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 20µg/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 hemotoxylin 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 perconditioning (RIPer, n=7); combination of local ischemic postconditioning and remote ischemic perconditioning (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 $^{473}$Ser-phospho-Akt, total Akt, phospho-ERK1/2, total ERK1/2, $^{9}$Ser-phospho-GSK-3β, total GSK-3β, $^{705}$Tyr-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-3β and STAT-3 protein levels in all study groups. Therefore, phospho-Akt, phospho-ERK1/2, phospho-GSK-3β 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-3β 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-3β 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-3β 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-3β) 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-3β) 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-3β 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 perconditioning 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-3β [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-3β 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 RIPer 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, RIPer was as effective as IPost in decreasing reperfusion injuries, while the IPost+RIPer 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.

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**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 perconditioning (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±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-3β (**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-3β (p-GSK-3β) and total GSK-3β (**c**) in LV homogenates from hearts subjected to ischemia-reperfusion. *Bottom*: Bar graphs showing mean±SEM of the densitometry of p-Akt-to-Akt ratio (**a**), p-ERK1/2-to-ERK1/2 ratio (**b**), and p-GSK-3β-to-GSK-3β ratio (**c**). All data is expressed as mean±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±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±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-3β (**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-3β: 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