ORIGINAL CONTRIBUTION

Remote postconditioning by humoral factors in effluent from ischemic preconditioned rat hearts is mediated via PI3K/Akt-dependent cell-survival signaling at reperfusion

**L. Breivik · E. Helgeland · E. K. Aarnes ·
J. Mrdalj · A. K. Jonassen**

Abstract Short non-lethal ischemic episodes administered to hearts prior to (ischemic preconditioning, IPC) or directly after (ischemic postconditioning, IPost) ischemic events facilitate myocardial protection. Transferring coronary effluent collected during IPC treatment to un-preconditioned recipient hearts protects from lethal ischemic insults. We propose that coronary IPC effluent contains hydrophobic cytoprotective mediators acting via PI3K/Akt-dependent pro-survival signaling at ischemic reperfusion. Ex vivo rat hearts were subjected to 30 min of regional ischemia and 120 min of reperfusion. IPC effluent administered for 10 min prior to index ischemia attenuated infarct size by $\geq 55\%$ versus control hearts ($P < 0.05$). Effluent administration for 10 min at immediate reperfusion (reperfusion therapy) or as a mimetic of pharmacological postconditioning (remote postconditioning, RIPost) significantly reduced infarct size compared to control ($P < 0.05$). The IPC effluent significantly increased Akt phosphorylation in un-preconditioned hearts when administered before ischemia or at reperfusion, while pharmacological inhibition of PI3K/Akt-signaling at reperfusion completely abrogated the cardioprotection offered by effluent administration. Fractionation of coronary IPC effluent revealed that cytoprotective humoral mediator(s) released during the conditioning phase were of hydrophobic nature as all hydrophobic fractions with molecules under 30 kDa significantly reduced infarct size versus the control and hydrophilic fraction-treated hearts ($P < 0.05$). The total hydrophobic effluent fraction significantly reduced infarct size independently of temporal administration (before ischemia, at reperfusion or as remote postconditioning). In conclusion, the IPC effluent retains strong cardioprotective properties, containing hydrophobic mediator(s) < 30 kDa offering cytoprotection via PI3K/Akt-dependent signaling at ischemic reperfusion.

Keywords Postconditioning · Preconditioning · Cardioprotection · Ischemia · Reperfusion · Akt

Introduction

Impaired coronary circulation caused by a myocardial ischemic episode will lead to compromised hemodynamic function and ultimately cell death. Rapid restoration of coronary blood flow to the infarct-related coronary artery by thrombolysis or primary coronary angioplasty is effective interventions for limiting myocardial infarct size and improving clinical outcomes [20, 39]. Although early reperfusion is essential to limit infarct size, it may paradoxically induce cell death (lethal reperfusion injury) [2]. Two ways of protecting the heart against reperfusion-induced injury are ischemic preconditioning (IPC) [22] and ischemic postconditioning (IPost) [40], applying transient non-lethal episodes of myocardial ischemia and reperfusion either before or after the index ischemic event, respectively. Another strategy for achieving cardioprotection is remote ischemic preconditioning (RIPC), whereby brief ischemia in one distant region or organ protects the heart from a sustained episode of ischemia [26]. Remote IPost (RIPost) is a more clinically relevant cardioprotective strategy, and has been shown to be effective in experimental studies when the remote ischemic

L. Breivik (✉) · E. Helgeland · E. K. Aarnes · J. Mrdalj ·
A. K. Jonassen
Department of Biomedicine, Faculty of Medicine and Dentistry,
University of Bergen, Bergen, Norway
e-mail: lars.breivik@biomed.uib.no

conditioning was applied after the onset of myocardial ischemia [15].

There is strong evidence that one or more factors released in response to brief episodes of ischemia during IPC can interact with receptors, locally or at a distance, triggering a cytoprotective response. Dickson et al. [6] found that IPC-induced cardioprotection could be transferred between individuals by transfusion of whole blood from preconditioned to non-preconditioned rabbits and that effluent from preconditioned isolated ex vivo rabbit hearts offered protection to non-preconditioned hearts [5]. Furthermore, Serejo et al. [28] recently showed that cardioprotective factors released into coronary IPC effluent from ex vivo rat hearts are thermolabile hydrophobic substances with molecular weights higher than 3.5 kDa offering cytoprotection via PKC activation in the recipient heart. In addition, Lang et al. [17] suggested that mediator(s) released during IPC in rat hearts are smaller than 8 kDa.

IPC and IPost reduce infarct size when studied in a wide variety of experimental models including rat, rabbit, dog, pig and primates by sharing similar mechanisms and exerting most of their cytoprotection at reperfusion [9, 12, 26, 37]. IPC activates PI3-kinase which facilitates phosphorylation and activation of the protein kinase Akt through its mediator PDK-1 [1]. Akt is part of the reperfusion injury salvage kinases (RISK) that confer cardioprotection when activated at reperfusion [9]. IPost activates similar signaling pathways to IPC [9] and is thereby also dependent on RISK activation to exert its protection [35], although the involvement of RISK has lately been proven to be species dependent [27, 30]. The underlying mechanism of RIPC and RIPost remains unclear, but many of the signaling pathways underlying myocardial preconditioning and postconditioning have been implicated in RIPC [11].

Numerous studies have focused on characterizing the signaling mechanisms involved in IPC. However, the precise adaptive response triggered by agents released during the short reperfusion episodes of IPC remains unresolved. Identification of such mediators would be of great importance for the development of pharmacological therapies to enhance myocardial tolerance to ischemia–reperfusion-induced stress in patients with acute myocardial infarction (AMI) [38]. The aims of this study were therefore to (1) investigate the protective capabilities of the IPC effluent when delivered at ischemic reperfusion and its potential to induce remote postconditioning (RIPost); (2) determine if coronary IPC effluent mediates its protection through a PI3K/Akt-dependent cytoprotective signaling pathway; (3) characterize the protective mediator(s) by fractionation of effluent based on different properties such as hydrophobicity and size.

Methods

Langendorff perfusion procedure

All experiments were approved by the Norwegian State Commission for Laboratory Animals, and carried out in accordance with the European Communities Council Directive of 1986 (86/609/EEC).

Male Wistar rats (250–350 g, $n = 183$) fed a standard diet were heparinized (200 IU) and anesthetized with sodium pentobarbital (50 mg/kg) i.p. The hearts were excised, placed in ice-cold Krebs–Heinseleit buffer (KHB) and rapidly mounted onto a Langendorff perfusion system. KHB (pH 7.4, oxygenated with 95% O_2/5% CO_2) contained in mM: 118 NaCl, 4.7 KCl, 1.8 $CaCl_2$, 1.2 $KH_2 \cdot PO_4$, 1.2 $MgSO_4$, 25.2 $NaHCO_3$, 11.0 glucose. Perfusion pressure was maintained at 100 cmH_2O, and the myocardial temperature kept at 37.5°C. A water-filled latex balloon, connected to a hydrostatic pressure transducer (SP844, Memscap, Norway) and coupled to a high performance data acquisition system (PowerLab 8/30, Chart Pro software-MLS250, Australia), was inserted into the left ventricle and inflated to set an end-diastolic pressure of 5–10 mmHg. Coronary flow (CF) was measured by timed collection of effluent over 1 min. A 3-0 silk suture was passed around the main branch of the left coronary artery, and threaded through a small vinyl tube to form a snare. Regional ischemia (RI) was achieved by pulling the snare, and confirmed by a substantial fall in left ventricular developed pressure (LVDP) and CF.

Experimental protocols

The experimental protocols are shown in Fig. 1. Baseline values for functional parameters were obtained after 10 min of stabilization. All hearts were subjected to the ischemia/reperfusion (I/R) protocol consisting of 30 min of regional ischemia (RI = index ischemia) and 120 min of reperfusion. The IPC protocol consisted of 3× 5 min of global ischemia (GI) with 5 min intermittent reperfusions prior to RI. Coronary IPC effluent (∼150 ml) was collected during the intermittent reperfusion periods. Fresh effluent was used for each experiment. Since studies show that coronary effluent from non-preconditioned hearts does not influence the infarct size when administered to recipient hearts [28], we did not repeat these experiments in this study.

Initially, effects of coronary IPC effluent were studied during two temporal distinct administration periods, before and after index ischemia (Fig. 1a). IPC effluent was administered 10 min prior to index ischemia (Eff_{Pre}) and for 10 min at reperfusion (Eff_{Rep}). Subsequently, IPC effluent was administered to simulate ischemic postconditioning
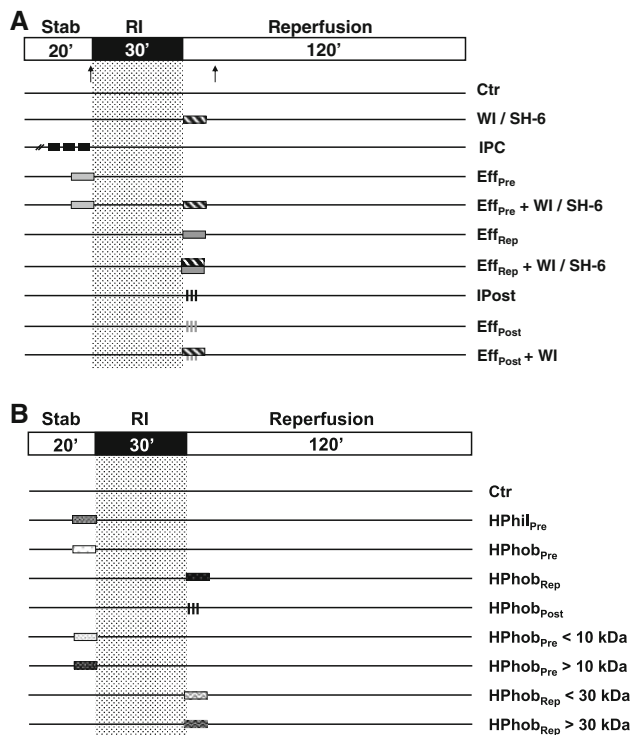
Fig. 1 Experimental protocols. All groups were subjected to 30 min of regional ischemia (RI) followed by 120 min of reperfusion. Hearts receiving no other treatments constitute the control group (Ctr). **a** The IPC group was exposed to $3\times$ 5 min global ischemia (GI), with 5 min intermittent reperfusion periods prior to RI. To test the temporal cytoprotective efficacy of IPC effluent in recipient hearts, freshly collected effluent was administered for 10 min prior to RI (Eff$_{Pre}$) or for 10 min at onset of ischemic reperfusion (Eff$_{Rep}$). Furthermore, to mimic ischemic postconditioning, the IPC effluent was administered for $3\times$ 30 s with 30 s intermittent KHB perfusion at immediate reperfusion (Eff$_{Post}$). Ischemic postconditioning (IPost) was achieved by $3\times$ 30 s of global ischemia (GI) at early ischemic reperfusion. The PI3K-inhibitor WI (1 μM) and the Akt-inhibitor SH-6 (10 μM) were administered for 10 min at immediate onset of ischemic reperfusion in conjunction with 10 min of pre- or post-ischemic treatment with IPC effluent (Eff$_{Pre}$ + WI/SH-6, Eff$_{Rep}$ + WI/SH-6 or Eff$_{Post}$ + WI). **b** The IPC effluent was fractionated in respect to charge and size. Effluent collected during IPC was first separated into a hydrophilic (HPhil) and a hydrophobic (HPhob) fraction. All total effluent fractions were administered for either 10 min prior to ischemia (HPhil$_{Pre}$ and HPhob$_{Pre}$) or for 10 min at reperfusion (HPhob$_{Rep}$ and HPhob$_{Post}$). The hydrophobic fraction was further fractionated by a 10 or 30 kDa size filter resulting in four hydrophobic fractions with molecules below 10/30 kDa (HPhob$_{Pre}$ < 10 and HPhob$_{Rep}$ < 30) or above 10/30 kDa (HPhob$_{Pre}$ > 10 and HPhob$_{Rep}$ > 30). *Arrows* indicate time points for tissue sampling. *Solid lines* KH buffer perfusion

(RIPost) by perfusing the coronary effluent for $3\times$ 30 s at early reperfusion (Eff$_{Post}$). The ischemic postconditioning (IPost) protocol consisted of $3\times$ 30 s GI intermitted by 30 s reperfusion periods. Furthermore, to explore if the cardioprotective properties mediated by the coronary IPC effluent were dependent on PI3K/Akt-dependent signaling at reperfusion, the PI3K-inhibitor Wortmannin (WI; 1 μM)

(Tocris Bioscience, UK) and the Akt-inhibitor SH-6 (10 μM) (Enzo Life Sciences, New York, USA) were administered for 10 min at ischemic reperfusion (Eff$_{Pre}$ + WI/SH-6 and Eff$_{Rep}$ + WI/SH-6 and Eff$_{Post}$ + WI; Fig. 1a). Since WI attenuated the cytoprotective effect of the effluent administered as a RIPost stimulus, and further that SH-6 abolished the IPC effluent-induced cardioprotection at reperfusion, we did not include experiments with effluent RIPost and the SH-6 inhibitor.

To further delineate the properties of putative cytoprotective mediator(s) released into collected IPC effluent, another set of experiments was performed (Fig. 1b). Firstly, effluent was separated into hydrophilic and hydrophobic fractions and administered either prior to ischemia (HPhil$_{Pre}$ and HPhob$_{Pre}$) or at reperfusion (HPhob$_{Rep}$ and HPhob$_{Post}$). The hydrophobic effluent was further fractionated using spin columns containing cut-off filters of either 10 or 30 kDa, resulting in four different fractions that were administered to recipient hearts either prior to ischemia (HPhob$_{Pre}$ < 10 and HPhob$_{Pre}$ > 10) or at reperfusion (HPhob$_{Rep}$ < 30 and HPhob$_{Rep}$ > 30).

Fractionation of IPC effluent

Coronary effluent from isolated ex vivo Langendorff perfused rat hearts exposed to IPC was collected during the intermittent reperfusion periods. The effluent was taken through a series of preparation steps in order to purify putative mediators of IPC-mediated cardioprotection. C18 solid phase extraction cartridges (Waters Corp., USA) were conditioned with 6 ml 100% acetonitrile (ACN) followed by equilibration with 6 ml of ddH$_2$O. Thereafter, IPC effluent was applied to the cartridge at a flow rate of 1 ml/min. Following this, the cartridge was washed with 10 ml water to elute unbound hydrophilic compounds (hydrophilic fraction). Hydrophobic proteins were eluted with 6 ml of 60% ACN. To remove the organic phase, eluted sample was evaporated under a stream of nitrogen. The hydrophobic eluted fraction was then either re-suspended in KHB for perfusion or re-suspended in ddH$_2$O and subjected to spin columns with either 10 or 30 kDa cut-off filter (Sartorius Stedim Biotech, France) and thereafter diluted in KHB (total volume of 150 ml KHB) before perfusion. All fractions were perfused to untreated recipient hearts for 10 min either prior to index ischemia (HPhil$_{Pre}$ or HPhob$_{Pre}$) or at reperfusion (HPhob$_{Rep}$ or HPhob$_{Post}$) (Fig. 1b).

Determination of infarct size

At the end of each experiment, the silk suture was securely tightened and a 0.2% (w/v) Evans Blue solution (Sigma Chemicals Co., USA) infused to visualize the risk zone.

Hearts were thereafter frozen at $-20°C$ before being cut into 2-mm thick slices parallel to the atrioventricular groove and stained with 1% triphenyl-tetrazolium-chloride (TTC, Sigma Chemicals Co, USA) in phosphate buffer (pH 7.4) for 20 min at 37°C. The slices were then immersed in 4% (v/v) formalin to enhance contrast of the stain, before being placed between two glass plates and digitalized. Area at risk (AAR, i.e. area not stained by Evans Blue), live cells (TTC positive, i.e. red) and infarcted area (TTC negative) were determined in a blinded fashion using a computerized planimetry program (Planimetry⁺ v2.0; Erik Traasdahl, ENK, Norway). Infarct size is presented as the ratio between the infarcted area and AAR in percent. There were no significant differences between the different treatment groups in the relative size of the AAR (volume of area at risk/volume of left ventricle) (Table 1).

Table 1 Ratio of volume of area at risk (AAR) and left ventricle (LV) by Evans Blue staining

Group	AAR/LV
Ctr	0.43 ± 0.03
Ctr + WI	0.53 ± 0.06
Ctr + SH-6	0.41 ± 0.04
IPC	0.46 ± 0.02
Eff$_{Pre}$	0.50 ± 0.03
Eff$_{Pre}$ + SH-6	0.48 ± 0.04
Eff$_{Pre}$ + WI	0.34 ± 0.03
Eff$_{Rep}$	0.39 ± 0.03
Eff$_{Rep}$ + SH-6	0.36 ± 0.03
Eff$_{Rep}$ + WI	0.47 ± 0.02
IPost	0.52 ± 0.03
Eff$_{Post}$	0.46 ± 0.03
Eff$_{Post}$ + WI	0.43 ± 0.05
HPhil$_{Pre}$	0.42 ± 0.03
HPhob$_{Pre}$	0.43 ± 0.09
HPhob$_{Rep}$	0.39 ± 0.05
HPhob$_{Post}$	0.36 ± 0.04
HPhob$_{Pre}$ < 10	0.43 ± 0.03
HPhob$_{Pre}$ > 10	0.45 ± 0.02
HPhob$_{Rep}$ < 30	0.45 ± 0.03
HPhob$_{Rep}$ > 30	0.46 ± 0.01

Values represent mean $\pm$ SEM

Ctr ischemia–reperfusion controls; *IPC* ischemic preconditioning, 3× 5 min; *Eff$_{Pre}$/HPhob$_{Pre}$* 10 min effluent administration prior to ischemia; *Eff$_{Rep}$/HPhob$_{Rep}$* 10 min effluent administration at reperfusion; *IPost* ischemic postconditioning, 3× 30 s; *Eff$_{Post}$/HPhob$_{Post}$* postconditioning mimetic, 3× 30 s; *WI* 10 min Wortmannin (1 μM); *SH-6* 10 min SH-6 (10 μM) at reperfusion; *HPhil* effluent not bound to C18 column; *HPhob* total effluent eluted from C18 column; *< or >10 or 30 kDa* total hydrophobic fraction further separated by size exclusion column

Immunoblot analysis

Levels of Akt (total-Akt, Santa Cruz Biotechnology, California, USA) and its phosphorylation at Ser⁴⁷³ (p-Akt, Cell Signal, Massachusetts, USA) were determined by western blotting. Heart tissue was harvested at stabilization (control baseline, C_B), at the end of IPC protocol or at 15 min of reperfusion (time points indicated by arrow in Fig. 1a). Furthermore, in order to explore the potential of pre-ischemic effluent administrations ability to modulate Akt phosphorylation at reperfusion, a separate group for WB only were administered effluent for 10 min prior to index ischemia and the tissue harvested after these hearts had been reperfused for 15 min (Eff$_{Pre+Rep}$). At the end of the experiment, AAR of the left ventricle was excised and snap frozen in liquid nitrogen. Homogenization of cardiac tissue, protein quantification, sample preparation (40 μg per lane) and electrophoresis were performed as previously described [14]. Densitometric analysis was performed using Quantity One software (Bio-Rad, CA, USA) and phosphorylation of Akt was expressed as the ratio between p-Akt and total-Akt. Results normalized against baseline hearts, C_B. GADPH was used as loading control (Santa Cruz Biotechnology, California, USA).

Statistical analysis

Values are presented as mean $\pm$ standard error of the mean (SEM). Infarct sizes and AAR were tested for group differences by one-way analysis of variance (ANOVA) combined with Fisher's post hoc test. Comparisons of LVDP, HR and CF were performed with ANOVA combined with Bonferroni post hoc test. To test for immunoblot differences, Student's *t* test for independent samples was used. For all tests, $P < 0.05$ was considered statistically significant.

Results

IPC effluent induces remote postconditioning by reducing infarct size when administered at reperfusion

Administration of IPC effluent from the onset of reperfusion significantly reduced infarct size in un-preconditioned recipient hearts compared to controls (Eff$_{Rep}$: $25 \pm 4\%$ vs. Ctr: $54 \pm 5\%$, $P < 0.05$; Fig. 2). Furthermore, the infarct size reduction was comparable to that of hearts subjected to ischemic preconditioning (IPC) or treated with IPC effluent prior to index ischemia (Eff$_{Pre}$) (IPC: $21 \pm 2\%$, Eff$_{Pre}$: $24 \pm 6\%$ vs. Eff$_{Rep}$: $25 \pm 4\%$, ns; Fig. 2).
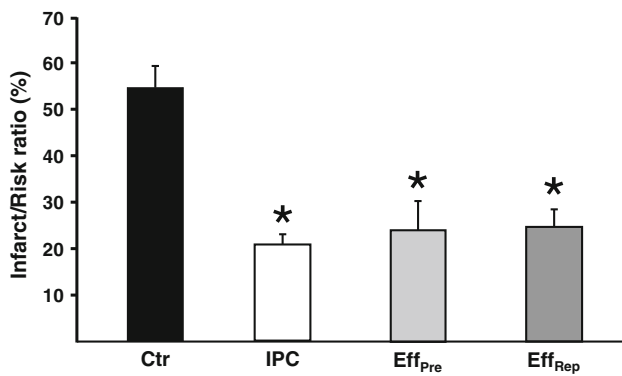
Fig. 2 The temporal effect of treatment with IPC effluent on myocardial infarct size. Infarct size is expressed as percentage of the region at risk of infarction (area at risk). Effluent from preconditioned donor hearts (IPC) administered either 10 min prior to index ischemia (Eff$_{Pre}$) or for 10 min at ischemic-reperfusion (Eff$_{Rep}$) significantly reduced infarct size in non-preconditioned recipient hearts as compared to controls (Ctr). *Bars* represent mean $\pm$ SEM. $N \geq 7$ in each group. $*P < 0.05$ versus Ctr group
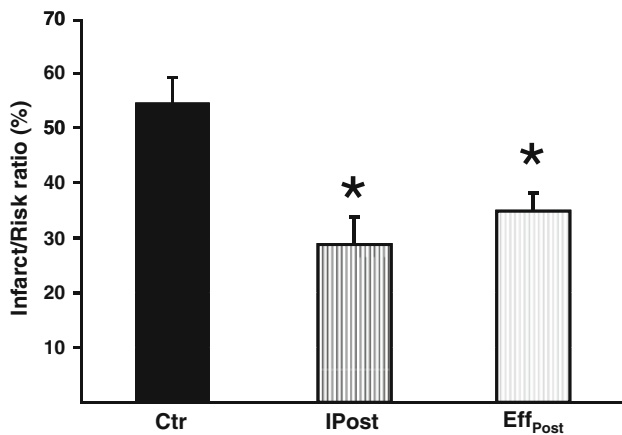
Fig. 3 The effect of administering IPC effluent as a mimetic of ischemic postconditioning (RIPost) on myocardial infarct size. Treatment of recipient hearts with IPC effluent for $3 \times$ 30 s at ischemic reperfusion (Eff$_{Post}$) reduced infarct size to the same extent as ischemic postconditioning (IPost), and both groups had significantly reduced infarct size as compared to the control group (Ctr). *Bars* represent mean $\pm$ SEM. $N \geq 7$ in each group. $*P < 0.05$ versus Ctr

In addition, mimicking pharmacological postconditioning by administering IPC effluent to recipient hearts for $3 \times$ 30 s at onset of reperfusion (RIPost) significantly reduced infarct size as compared to control hearts (Eff$_{Post}$: $35 \pm 3\%$ vs. Ctr: $54 \pm 5\%$, $P < 0.05$; Fig. 3). There was no statistical difference in infarct size comparing the IPost and Eff$_{Post}$ groups (IPost: $30 \pm 5\%$ vs. Eff$_{Post}$: $35 \pm 3\%$, ns; Fig. 3).

Lastly, comparing the infarct sizes in all IPC effluent-treated groups, it is apparent that the effluent confers similar degree of cytoprotection independent of whether treatment was administered before or after the index ischemic insult (Eff$_{Pre}$: $24 \pm 6\%$, Eff$_{Rep}$: $25 \pm 4\%$ and

Eff$_{Post}$: $35 \pm 3\%$, ns; Figs. 2, 3). Furthermore, coronary IPC effluent conveys cardioprotection comparable to IPC hearts, revealing that the putative cytoprotective mediator(s) could lead to a very promising therapeutic strategy for patients being revascularized after AMI.

The cytoprotection offered by IPC effluent at reperfusion is mediated by PI3K/Akt-dependent cell-survival signaling in recipient heart

IPC is known to activate the pro-survival kinase Akt directly after the IPC protocol [34]. Interestingly, administration of IPC effluent prior to index ischemia caused a significant increase in Akt phosphorylation compared to hearts exposed to IPC treatment only (Eff$_{Pre}$: 5.2 ± 0.2 AU vs. IPC: 2.0 ± 0.2 AU, $P < 0.05$; Fig. 4a, b), indicating an increased potential of the IPC effluent to activate the pro-survival Akt-signaling as compared to the IPC stimulus itself. Furthermore, administration of IPC effluent at reperfusion elevated the Akt-phosphorylation compared to I/R control hearts (Eff$_{Rep}$: 7.2 ± 0.2 AU vs. Ctr$_{Rep}$: 2.0 ± 0.5 AU, $P < 0.05$; Fig. 4c, d). Treatment with pre-ischemic IPC effluent and analysis of Akt phosphorylation at reperfusion (Eff$_{Pre+Rep}$) also increased phosphorylation status of Akt as compared to the I/R control group (Eff$_{Pre+Rep}$: 4.2 ± 0.6 AU vs. Ctr$_{Rep}$: 2.0 ± 0.5 AU, $P < 0.05$; Fig. 4c, d).

Total Akt or GADPH did not alter during the experiments (Fig. 4a, c), implying that increased relative phosphorylation is a result of changes in Akt phosphorylation and not alterations in the total kinase level or unequal loading. Exploring the phosphorylation status of STAT-3, a member of the Survivor Activating Factor Enhancement (SAFE) pathway [18], we found no regulation (results not shown).

Hausenloy et al. [9] have previously shown that Akt phosphorylation at reperfusion is essential for IPC-induced protection, since inhibition of this kinase during early reperfusion abrogated the IPC-mediated reduction in infarct size. Therefore, to elucidate whether IPC effluent exerts its cardioprotective effect through an PI3K/Akt-dependent signaling pathway activated at reperfusion, hearts were administered fresh IPC effluent for 10 min either prior to index ischemia (Eff$_{Pre}$) or at reperfusion (Eff$_{Rep}$ or Eff$_{Post}$), and co-administered the PI3K-inhibitor WI or the Akt-inhibitor SH-6 for 10 min at reperfusion (see experimental protocol Fig. 1). The protective effect of IPC effluent was completely abolished by co-administering WI or SH-6 at reperfusion compared to the Eff$_{Pre}$ group (Eff$_{Pre}$ + WI: $51 \pm 7\%$, Eff$_{Pre}$ + SH-6: $60 \pm 4\%$ vs. Eff$_{Pre}$: $24 \pm 6\%$, $P < 0.05$; Fig. 5) and the Eff$_{Rep}$ group (Eff$_{Rep}$ + WI: $56 \pm 12\%$, Eff$_{Rep}$ + SH-6: $54 \pm 8\%$ vs. Eff$_{Rep}$: $25 \pm 4\%$, $P < 0.05$; Fig. 5) and Eff$_{Post}$ group (Eff$_{Post}$ + WI: $50 \pm 11\%$ vs. Eff$_{Post}$: $35 \pm 3\%$, $P < 0.05$)
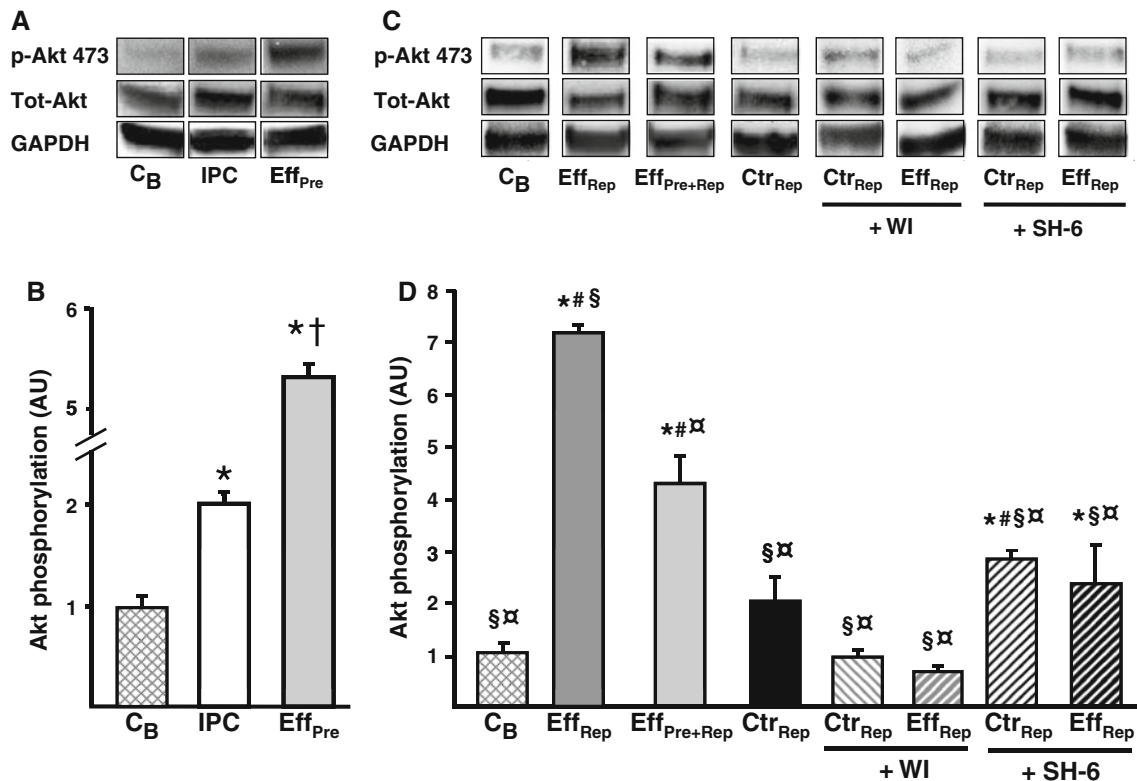
Fig. 4 Phosphorylation status of myocardial Akt in recipient hearts exposed to treatment with IPC effluent. **a** Representative immunoblots of Akt phosphorylation (Ser^{473}) showing the effects of treatment with IPC effluent (Eff_{Pre}) in recipient hearts (tissue harvested at end of treatment), as compared to hearts exposed to the standard IPC protocol (IPC) and baseline hearts (C_B). **c** Representative immunoblots of Akt phosphorylation (Ser^{473}) in pre-ischemic IPC effluent-treated hearts that was also reperfused for 15 min ($Eff_{Pre+Rep}$) and reperfusion treatment with IPC effluent (Eff_{Rep}) in recipient hearts as compared to ischemic reperfused control hearts (Ctr_{Rep}) (tissues harvested at 15 min of reperfusion). Administering the PI3K-inhibitor WI (1 μM) and the Akt-inhibitor SH-6 (10 μM) for 10 min at reperfusion abrogated Akt phosphorylation in IPC effluent-treated hearts. Total Akt and GADPH indicates equal loading. **b, d** Densitometric analysis of total and phosphorylated Akt immunoblots expressed in arbitrary units (AU). p-Akt expressed as a ratio of total Akt with $C_B = 1$. *Bars* represent mean $\pm$ SEM. $N \geq 3$ in each group. $*P < 0.05$ versus C_B, $^{†}P < 0.05$ versus IPC, $^{§}P < 0.05$ versus $Eff_{Pre+Rep}$, $^{¤}P < 0.05$ versus Eff_{Rep}, $^{#}P < 0.05$ versus Ctr_{Rep}

(data not shown). SH-6 is a phosphatidylinositol (PI) analog that inhibits Akt without affecting the activity of its upstream kinase PDK-1 [16], and has also been shown to prevent phosphorylation of Akt at Ser^{473} [4, 32]. The inhibitors themselves had no effect on infarct size (WI: 54 ± 8%, SH-6: 52 ± 3% vs. Ctr: 54 ± 5%, ns). Furthermore, both WI and SH-6 significantly reduced Akt phosphorylation in IPC effluent-treated hearts (Eff_{Rep} + WI: 0.7 ± 0.1 AU, Eff_{Rep} + SH-6: 2.8 ± 0.2 AU vs. Eff_{Rep}: 7.2 ± 0.2 AU, $P < 0.05$; Fig. 4c, d).

Functional parameters are shown in Tables 2 and 3. There were no major group differences concerning baseline values, values at the start of RI or at reperfusion (Tables 2, 3).

Cardioprotective potency of fractionated IPC effluent

To further characterize potential humoral factor(s), the coronary IPC effluent was separated into hydrophilic and hydrophobic fractions, and the hydrophobic fraction was further divided based on particulate size (> or <10 or 30 kDa). The total hydrophobic effluent fraction administered for 10 min before hearts were subjected to 30 min of regional ischemia ($HPhob_{Pre}$) resulted in a significantly reduced infarct size as compared to hydrophilic fraction-treated hearts and controls ($HPhil_{Pre}$: 59 ± 4%, Ctr: 54 ± 5% vs. $HPhob_{Pre}$: 28 ± 5%, $P < 0.05$; Fig. 6). Furthermore, administration of the total hydrophobic effluent fraction at ischemic reperfusion also resulted in significantly reduced infarct size as compared to controls ($HPhob_{Rep}$: 29 ± 3%, $HPhob_{Post}$: 22 ± 4% vs. Ctr: 54 ± 5%, $P < 0.05$; Fig. 6). We further fractionated the hydrophobic IPC effluent into four fractions with particulate size > or <10 or 30 kDa. All fractions below 30 kDa afforded cardioprotection as compared to control hearts ($HPhob_{Pre}$ < 10: 27 ± 4%, $HPhob_{Pre}$ > 10: 37 ± 5%, $HPhob_{Rep}$ < 30: 36 ± 6% vs. Ctr: 54 ± 5%, $P < 0.05$; Fig. 6). However, the cytoprotective mediator in the IPC effluent appears to be of a size less than 30 kDa as the
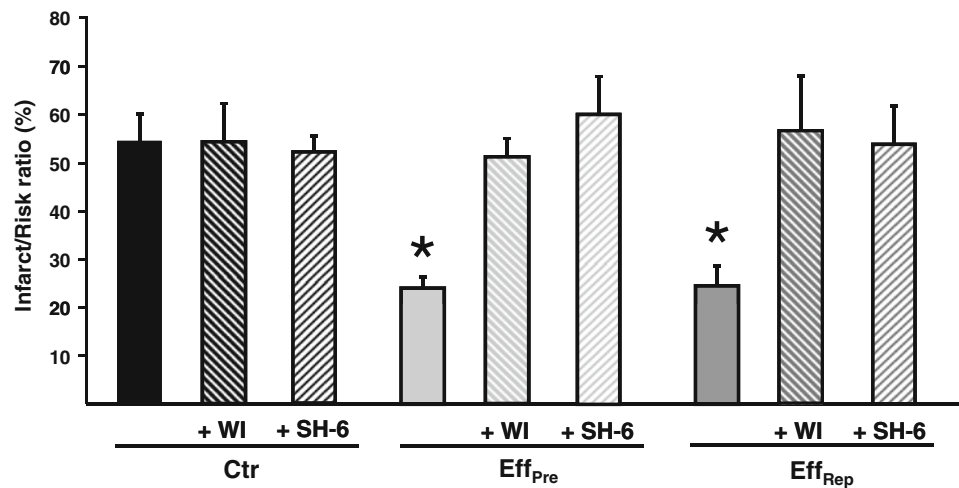
Fig. 5 The effect of inhibiting PI3K and Akt upon reperfusion in IPC effluent-treated recipient hearts. When administering the PI3K-inhibitor WI and the Akt-inhibitor SH-6 at the onset of reperfusion in IPC effluent pre-ischemic (Eff$_{Pre}$) or post-ischemic (Eff$_{Rep}$ and Eff$_{Post}$) treated hearts, the cardioprotective effect of the effluent was completely abolished. *Ctr* control; *Eff$_{Pre}$* 10 min effluent administration prior to RI; *Eff$_{Rep}$* 10 min effluent administration after RI; *Eff$_{Post}$* effluent administration 3× 30 s at start of reperfusion; *WI* 10 min Wortmannin (1 μM) at reperfusion; *SH-6* 10 min SH-6 (10 μM) at reperfusion. *Bars* represent mean ± SEM. $N \geq 6$ in each group. *$P < 0.05$ versus Ctr

group with particulate fraction above this size failed to offer any cardioprotection (HPhob$_{Rep}$ > 30: 52 ± 4% vs. Ctr: 54 ± 5%, ns).

Discussion

To summarize, our data demonstrate that collected coronary IPC effluent contains humoral factor(s) of a hydrophobic character < 30 kDa in size that offers strong cytoprotective properties, significantly reducing infarct size when administered at early ischemic reperfusion in recipient rat hearts, either given as a reperfusion therapy or as pharmacological mimetic of ischemic postconditioning (remote IPost). Moreover, these data suggest that this cardioprotection is partly mediated via PI3K/Akt-dependent cell-survival signaling at ischemic reperfusion.

Our results are in agreement with experiments performed by Serejo et al. [28] and Dickson et al. [5] in the ex vivo rat and rabbit heart, respectively. These studies demonstrate that coronary effluent from donor hearts subjected to IPC confers cardioprotection in isolated buffer-perfused ex vivo recipient hearts administered prior to index ischemia. However, coronary reperfusion is the only means of limiting infarct size, provided that it occurs early after coronary occlusion, but may paradoxically directly result in tissue injury (lethal reperfusion injury). In order to explore if collected IPC coronary effluent could reduce lethal reperfusion-induced injury, we administered IPC effluent for 10 min at immediate reperfusion. Our results confirm that IPC effluent limits reperfusion-induced injury

by proving able to offer cytoprotection similar to pre-ischemic treatment even when administered as reperfusion therapy (Fig. 2). In order to further evaluate the temporal requirements of the IPC effluent, it was administered as a postconditioning stimulant at early reperfusion (experimental protocol, Fig. 1), drastically reducing the total treatment time and volume. Nevertheless, exposing the recipient hearts to ~85% less effluent over a shorter time period still offered substantial cardioprotection in un-pre-conditioned hearts, also validating the phenomena of remote postconditioning (RIPost).

The study by Serejo et al. [28] indicated that the protective effect afforded by IPC effluent disappeared when heated or left in RT for 24 h, while addition of protease inhibitors prevented this time-dependent break down and effluent purification by reverse phase chromatography suggested that the cardioprotective factor(s) could be of a hydrophobic "protein nature". Our results confirm these findings as administration of total isolated hydrophobic fraction prior to index ischemia or at reperfusion, as reperfusion therapy or RIPost treatment, reduced infarct size by 35–40% compared to control and hydrophilic fraction-treated group. Further fractionation of the total hydrophobic effluent into low and high molecular weight fractions, with either a 10 or 30 kDa cut-off, revealed that both 10 kDa fractions, but only the low weight fractions of 30 kDa cut-off, retained the ability to offer cardioprotection comparable to the cytoprotection afforded by the total hydrophobic faction (Fig. 6). Thus, we propose that the factor(s) responsible for the protective capabilities have a size either under or close to 30 kDa (depending on the

Table 2 Functional parameters recorded during the experimental protocol investigating the temporal effects of administering the IPC effluent to un-preconditioned recipient hearts

Group	10 min stab	5 min RI	30 min rep	120 min rep
LVDP (mmHg)				
Ctr	155 ± 22	$87 \pm 8^{\#}$	109 ± 12	$78 \pm 8^{\#}$
Ctr + WI	160 ± 12	$73 \pm 11^{\#}$	71 ± 9	$67 \pm 6^{\#}$
Ctr + SH-6	149 ± 17	$78 \pm 13^{\#}$	$112 \pm 9^{\#}$	$84 \pm 10^{\#}$
IPC	160 ± 11	$73 \pm 7^{\#}$	$98 \pm 7^{\#}$	$70 \pm 5^{\#}$
Eff_{Pre}	171 ± 21	$73 \pm 14^{\#}$	$87 \pm 2^{\#}$	$67 \pm 5^{\#}$
Eff_{Pre} + SH-6	177 ± 17	$113 \pm 11^{\#}$	$68 \pm 8^{\#}$	$51 \pm 3^{\#}$
Eff_{Pre} + WI	148 ± 12	$73 \pm 9^{\#}$	$81 \pm 7^{\#}$	$66 \pm 6^{\#}$
Eff_{Rep}	177 ± 9	$84 \pm 14^{\#}$	$104 \pm 10^{\#}$	$77 \pm 9^{\#}$
Eff_{Rep} + SH-6	156 ± 8	$103 \pm 9^{\#}$	$93 \pm 7^{\#}$	$74 \pm 6^{\#}$
Eff_{Rep} + WI	162 ± 17	$111 \pm 18^{\#}$	$97 \pm 18^{\#}$	$69 \pm 13^{\#}$
IPost	150 ± 10	$68 \pm 11^{\#}$	$78 \pm 9^{\#}$	$59 \pm 7^{\#}$
Eff_{Post}	157 ± 19	$80 \pm 14^{\#}$	106 ± 9	$61 \pm 8^{\#}$
Eff_{Post} + WI	156 ± 11	$99 \pm 15^{\#}$	$94 \pm 15^{\#}$	$85 \pm 10^{\#}$
CF (ml/min)				
Ctr	13.4 ± 1.7	$9.1 \pm 1.3^{\#}$	$9.1 \pm 0.8^{\#}$	$6.2 \pm 0.7^{\#}$
Ctr + WI	12.9 ± 0.6	$7.3 \pm 0.6^{\#}$	10.5 ± 0.8	$7.3 \pm 0.6^{\#}$
Ctr + SH-6	12.8 ± 0.4	$7.9 \pm 0.4^{\#}$	$6.2 \pm 1.6^{\#}$	$6.1 \pm 1.1^{\#}$
IPC	14.2 ± 0.9	$8.0 \pm 0.7^{\#}$	12.1 ± 1.1	$7.3 \pm 0.9^{\#}$
Eff_{Pre}	13.3 ± 0.5	$6.9 \pm 0.8^{\#}$	$8.0 \pm 0.5^{\#}$	$6.5 \pm 0.6^{\#}$
Eff_{Pre} + SH-6	17.2 ± 3.2	$8.7 \pm 1.2^{\#}$	$6.8 \pm 0.8^{\#}$	$6.2 \pm 1.1^{\#}$
Eff_{Pre} + WI	15.8 ± 1.5	$7.8 \pm 0.8^{\#}$	$10.8 \pm 1.0^{\#}$	$7.1 \pm 1.0^{\#}$
Eff_{Pre}	13.6 ± 1.0	$8.1 \pm 1.0^{\#}$	$10.2 \pm 0.4^{\#}$	$7.5 \pm 0.4^{\#}$
Eff_{Pre} + SH-6	14.9 ± 0.9	$8.9 \pm 0.9^{\#}$	$7.8 \pm 0.6^{\#}$	$6.5 \pm 0.6^{\#}$
Eff_{Rep} + WI	15.6 ± 0.6	$7.6 \pm 1.1^{\#}$	$9.3 \pm 1.5^{\#}$	$7.1 \pm 1.4^{\#}$
IPost	12.3 ± 0.8	$7.2 \pm 0.5^{\#}$	$8.2 \pm 0.9^{\#}$	$5.9 \pm 0.3^{\#}$
Eff_{Post}	13.8 ± 0.7	$8.4 \pm 0.6^{\#}$	$10.4 \pm 0.7^{\#}$	$7.6 \pm 0.6^{\#}$
Eff_{Post} + WI	14.1 ± 0.6	$8.9 \pm 0.8^{\#}$	11.4 ± 0.9	$8.7 \pm 0.4^{\#}$
HR (beats/min)				
Ctr	328 ± 21	294 ± 34	268 ± 31	254 ± 17
Ctr + WI	300 ± 12	288 ± 12	258 ± 43	293 ± 23
Ctr + SH-6	318 ± 17	276 ± 16	299 ± 18	246 ± 15
IPC	295 ± 22	283 ± 17	270 ± 13	242 ± 9
Eff_{Pre}	321 ± 12	278 ± 19	285 ± 15	270 ± 14
Eff_{Pre} + SH-6	294 ± 18	250 ± 11	236 ± 11	226 ± 29
Eff_{Pre} + WI	297 ± 13	298 ± 25	339 ± 23	289 ± 23
Eff_{Rep}	275 ± 23	273 ± 15	276 ± 19	268 ± 14
Eff_{Rep} + SH-6	256 ± 17	238 ± 17	224 ± 17	236 ± 12
Eff_{Rep} + WI	277 ± 32	248 ± 30	261 ± 30	241 ± 28
IPost	298 ± 15	266 ± 13	261 ± 9	233 ± 6
Eff_{Post}	280 ± 7	230 ± 16	293 ± 15	239 ± 14
Eff_{Post} + WI	316 ± 16	304 ± 24	301 ± 23	278 ± 33

Values represent mean $\pm$ SEM

Ctr ischemia–reperfusion controls; *IPC* ischemic preconditioning, 3× 5 min; *Eff_{Pre}* 10 min effluent administration prior to ischemia; *Eff_{Rep}* 10 min effluent administration at reperfusion; *IPost* ischemic postconditioning, 3× 30 s; *Eff_{Post}* effluent postconditioning, 3× 30 s; *WI* 10 min Wortmannin (1 μM); *SH-6* 10 min SH-6 (10 μM) at reperfusion

$^{\#}$ $P < 0.05$ versus Stab

Table 3 Functional parameters recorded during the experimental protocol investigating the effects of administering fractionated IPC effluent (charge and size) to un-preconditioned recipient hearts

| Group	10 min stab	5 min RI	30 min rep	120 min rep
LVDP (mmHg)				
Ctr	155 ± 22	$87 \pm 8^{\#}$	109 ± 12	$78 \pm 8^{\#}$
$HPhil_{Pre}$	153 ± 9	$87 \pm 8^{\#}$	$96 \pm 9^{\#}$	$69 \pm 9^{\#}$
$HPhob_{Pre}$	151 ± 6	$89 \pm 18^{\#}$	$100 \pm 5^{\#}$	$87 \pm 5^{\#}$
$HPhob_{Rep}$	168 ± 20	$89 \pm 8^{\#}$	90 ± 16	$71 \pm 11^{\#}$
$HPhob_{Post}$	143 ± 8	$78 \pm 8^{\#}$	$90 \pm 5^{\#}$	$73 \pm 5^{\#}$
$HPhob_{Pre} < 10$	143 ± 15	$62 \pm 9^{\#}$	$101 \pm 8^{\#}$	$74 \pm 7^{\#}$
$HPhob_{Pre} > 10$	149 ± 13	$81 \pm 13^{\#}$	111 ± 9	$83 \pm 7^{\#}$
$HPhob_{Rep} < 30$	173 ± 9	$83 \pm 8^{\#}$	$107 \pm 5^{\#}$	$82 \pm 5^{\#}$
$HPhob_{Rep} > 30$	160 ± 15	$100 \pm 13^{\#}$	$83 \pm 6^{\#}$	$64 \pm 7^{\#}$
CF (ml/min)				
Ctr	13.2 ± 1.4	$8.8 \pm 1.2^{\#}$	$8.6 \pm 0.8^{\#}$	$5.8 \pm 0.7^{\#}$
$HPhil_{Pre}$	13.6 ± 0.5	$8.4 \pm 0.8^{\#}$	10.2 ± 0.9	$7.6 \pm 0.8^{\#}$
$HPhob_{Pre}$	13.7 ± 0.5	$8.2 \pm 2.4^{\#}$	$9.9 \pm 2.7^{\#}$	$7.9 \pm 1.9^{\#}$
$HPhob_{Rep}$	16.4 ± 0.7	$10.6 \pm 0.5^{\#}$	12.2 ± 1.3	$10.4 \pm 1.5^{*,\#}$
$HPhob_{Post}$	13.2 ± 1.1	$7.6 \pm 1.0^{\#}$	9.7 ± 1.3	$7.9 \pm 1.1^{\#}$
$HPhob_{Pre} < 10$	14.6 ± 1.6	$7.4 \pm 1.2^{\#}$	$11.9 \pm 1.5^{\#}$	$9.4 \pm 1.3^{\#}$
$HPhob_{Pre} > 10$	14.3 ± 0.9	$7.3 \pm 1.0^{\#}$	11.5 ± 1.1	$8.6 \pm 0.8^{\#}$
$HPhob_{Rep} < 30$	17.1 ± 1.0	$10.0 \pm 2.1^{\#}$	$11.8 \pm 2.4^{\#}$	$9.7 \pm 2.2^{\#}$
$HPhob_{Rep} > 30$	15.4 ± 1.1	$9.1 \pm 0.7^{\#}$	$10.3 \pm 0.9^{\#}$	$8.0 \pm 0.7^{\#}$
HR (beats/min)				
Ctr	320 ± 15	291 ± 24	249 ± 23	230 ± 24
$HPhil_{Pre}$	299 ± 13	265 ± 27	257 ± 16	266 ± 16
$HPhob_{Pre}$	306 ± 11	283 ± 15	299 ± 12	265 ± 7
$HPhob_{Rep}$	330 ± 13	312 ± 5	315 ± 11	304 ± 9
$HPhob_{Post}$	329 ± 26	339 ± 18	334 ± 18	$328 \pm 17^{*}$
$HPhob_{Pre} < 10$	294 ± 10	264 ± 13	287 ± 15	269 ± 10
$HPhob_{Pre} > 10$	298 ± 11	274 ± 6	274 ± 8	287 ± 10
$HPhob_{Rep} < 30$	303 ± 10	272 ± 6	255 ± 28	253 ± 28
$HPhob_{Rep} > 30$	290 ± 15	273 ± 14	241 ± 19	234 ± 18

Values represent mean $\pm$ SEM

Ctr ischemia–reperfusion controls; *IPC* ischemic preconditioning, 3× 5 min; *Eff_{Pre}/$HPhob_{Pre}$* 10 min effluent administration prior to ischemia; *Eff_{Rep}/$HPhob_{Rep}$* 10 min effluent administration at reperfusion; *IPost* ischemic postconditioning, 3× 30 s; *Eff_{Post}/$HPhob_{Post}$* postconditioning mimetic, 3× 30 s; *WI* 10 min Wortmannin (1 μM); *SH-6* 10 min SH-6 (10 μM) at reperfusion. *HPhil* effluent not bound to C18 column; *HPhob* total effluent eluted from C18 column; *< or >10 or 30 kDa* total hydrophobic fraction further separated by size exclusion column

* $P < 0.05$ versus Ctr, $^{\#}$ $P < 0.05$ vs. Stab

efficiency of the columns used). Since both 10 kDa fractions were protective, this suggests that the effluent either contains more than one factor with protective capabilities, with at least one of them being of a smaller and one of a larger size than the size of the filter used (10 kDa), or that the factor(s) is of a size close to the columns 10 kDa cutoff limit, causing it to be present in both fractions.

Adenosine, opioids and bradykinin are endogenously released triggers of IPC that work in parallel, binding to
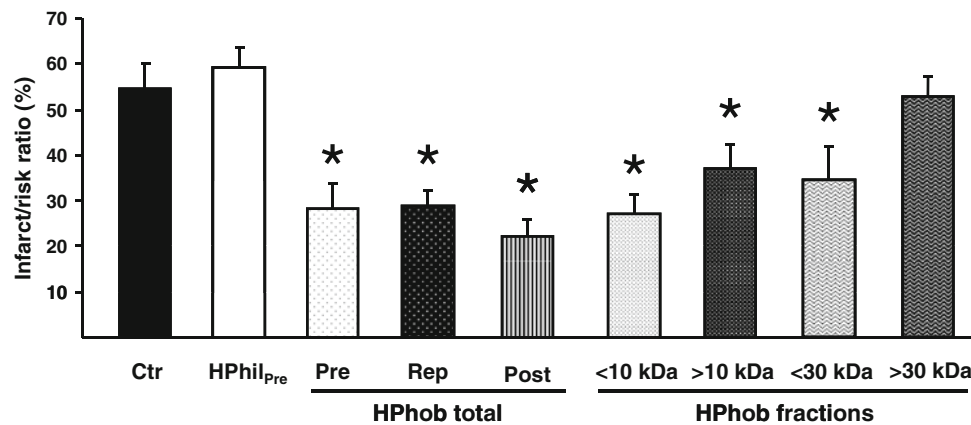
Fig. 6 The effect of fractionating the IPC effluent based on charge and size with regard to myocardial infarct size. Pre- or post-ischemic administration of the total hydrophobic fraction (HPhob$_{Pre}$, HPhob$_{Rep}$ and HPhob$_{Post}$) and the fractions containing proteins < or >10 kDa (HPhob$_{Pre}$ < 10 and HPhob$_{Pre}$ > 10) and <30 kDa (HPhob$_{Rep}$ < 30) significantly reduced infarct size in non-preconditioned recipient hearts as compared to hydrophilic effluent (HPhil) and control (Ctr)

hearts. The hydrophobic fraction containing proteins >30 kDa (HPhob$_{Rep}$ < 30) did not offer any cytoprotection. *HPhil* effluent not bound to C18 column; *HPhob* effluent eluted from C18 column; *total* entire hydrophobic fraction; < or >10/30 kDa total hydrophobic fraction further separated by a size exclusion column. *Bars* represent mean ± SEM. $N \geq 5$ in each group. *$P < 0.05$ versus Ctr

cell surface receptors initiating the IPC response (reviewed in [7]). Being released into the circulation, the triggers have also the potential to protect cells not directly subjected to IPC [26], which could at least in part explain the phenomenon of remote preconditioning (RIPC), in which preconditioning of non-cardiac tissue such as skeletal muscle [24] and kidney [33] will protect the heart from subsequent ischemic events. The mechanisms behind RIPC are believed to be shared with IPC and IPost [10], and include the release of cardioprotective autocoids such as adenosine [3], nitric oxide [29], activation of innate immunity [36], activation of RISK signaling [9], and the inhibition of the mitochondrial permeability transition pore (mPTP) [13]. Recently, RIPC was also shown to work via unidentified neurological or humoral pathways [19]. However, emerging evidences suggest that adenosine and other small molecules involved in IPC protection might not be responsible for the protective capabilities of the IPC effluent. Dickson et al. [5] compared adenosine levels in coronary effluent from preconditioned and control hearts, and found no difference in mean adenosine concentrations. They also failed to find a relationship between adenosine concentration and infarct size [5]. Using a 3.5 kDa dialysis membrane, Serejo et al. [28] excluded low molecular weight molecules (adenosine 267.24 Da, opioids 500–800 Da, and bradykinin 1,060.22 Da) without losing the protective effect. Our study confirms that the IPC effluent must contain cytoprotective substances larger than 3.5 kDa as the hydrophobic fraction > 10 kDa resulted in protection similar to the total hydrophobic and low molecular fractions. Furthermore, our results also suggest that smaller size molecules might not be necessary in

evoking the cytoprotective response, since the hydrophobic fraction > 10 kDa offered a reduction in infarct size comparable to the total hydrophobic fraction.

Activation of RISK signaling during reperfusion unites IPC, IPost and other agents that facilitate cardioprotection when offered either prior to ischemia or at reperfusion [10], and prolonging RISK activation by phosphatase inhibition during reperfusion also offers cytoprotection [8]. Of note, studies have questioned the importance of RISK in cardioprotection by IPost [27, 30] and in gentle reperfusion [23]. However, Akt is an important mediator of RISK signaling, and inhibition of the up-stream PI3-kinase with either Wortmannin or LY 294002 reduced Akt phosphorylation and abolished IPC-mediated protection [21, 32]. We have previously demonstrated that pharmacologically induced phosphorylation of PI3K/Akt in the first few min of reperfusion after a sustained ischemic insult is cardioprotective [14]. In the present study, we demonstrate that activation of Akt-dependent pro-survival signaling at reperfusion is required for the pre-ischemic IPC effluent-induced protection. Inhibiting PI3K/Akt-signaling at the onset of reperfusion in hearts pretreated with IPC effluent completely abolished the protection (Fig. 5). This PI3K/Akt-dependent cytoprotection offered by pre-ischemic effluent treatment was paralleled by a significantly increase in Akt phosphorylation both pre- and post-ischemia (Fig. 4). Administration of the effluent as a reperfusion therapy also resulted in increased Akt-phosphorylation, and the protection was abolished by PI3K/Akt-signaling inhibition. Taken together, our data indicate that factor(s) retaining cytoprotective properties in IPC effluent are conveying cardioprotection via activation of Akt-dependent signaling that can be

modulated at reperfusion after a lethal ischemic insult. We could not prove involvement of the SAFE pathway [18], which is thought to interact with RISK signaling at reperfusion, as the IPC effluent failed to modulate the status of STAT3 phosphorylation (results not shown). In addition to activation of Akt, the IPC effluent has previously been shown to lose its protective effect when co-administered with the PKC inhibitor chelerythrine [28]. PKC has been implicated as an early mediator of pre-conditioning [31], and may contribute to Akt phosphorylation at the time of reperfusion [25], and thereby inducing cardioprotection which may include inhibition of mito-chondrial permeability transition pore (mPTP) opening [9].

In conclusion, effluent collected during ischemic pre-conditioning of ex vivo rat hearts protects untreated reci-pient hearts from reperfusion-induced injury via a PI3K/Akt-dependent pathway that can be modulated at ischemic reperfusion. The IPC effluent has the ability to offer cytoprotection when administered as a stimulus of remote postconditioning (RIPost) or as a reperfusion therapy. The molecules responsible for the effluents protective abilities are below or close to 30 kDa and at least one of them are larger than 10 kDa. Furthermore, our results demonstrate that the effluent or its mediator(s) has a strong cardiopro-tective action implying a great potential as adjunct reper-fusion therapy for patients with AMI.

Conflict of interest The authors declare that they have no conflict of interest.

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