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Remote ischemic preconditioning confers late protection against myocardial ischemia-reperfusion injury in mice by upregulating interleukin-10

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Abstract

Remote ischemic preconditioning (RIPC) induces a prolonged late phase of multi-organ protection against ischemia-reperfusion (IR) injury. In the present study, we tested the hypothesis that RIPC confers late protection against myocardial IR injury by upregulating expression of interleukin (IL)-10. Mice were exposed to lower limb RIPC or sham ischemia. After 24 h, mice with RIPC demonstrated decreased myocardial infarct size and improved cardiac contractility following 30-min ischemia and 120-min reperfusion (I-30/R-120). These effects of RIPC were completely blocked by anti-IL-10 receptor antibodies. In IL-10 knockout mice, RIPC cardioprotection was lost, but it was mimicked by exogenous IL-10. Administration of IL-10 to isolated perfused hearts increased phosphorylation of the protein kinase Akt and limited infarct size after I-30/R-120. In wild-type mice, RIPC increased plasma and cardiac IL-10 protein levels and caused activation of Akt and endothelial nitric oxide synthase in the heart at 24 h, which was also blocked by anti-IL-10 receptor antibodies. In the gastrocnemius muscle, RIPC resulted in immediate inactivation of the phosphatase PTEN and activation of Stat3, with increased IL-10 expression 24 h later. Myocyte-specific PTEN inactivation led to increased Stat3 phosphorylation and IL-10 protein expression in the gastrocnemius muscle. Taken together, these results suggest that RIPC induces late protection against myocardial IR injury by increasing expression of IL-10 in the remote muscle, followed by release of IL-10 into the circulation, and activation of protective signaling pathways in the heart. This study provides a scientific basis for the use of RIPC to confer systemic protection against IR injury.

Keywords

Remote ischemic preconditioning; Interleukin-10; Reperfusion injury; Phosphatase and tensin homologue deleted on chromosome ten; Stat3

Introduction

Reperfusion is the most effective strategy to save the ischemic tissue, but it can cause additional damage, leading to cell dysfunction and death [23]. Ischemia-reperfusion (IR)

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Conflict of interest None.

injury has been observed in the heart, brain, liver, and kidney [23, 42]. Remote ischemic preconditioning (RIPC), induced by several episodes of brief ischemia and reperfusion at a distance, confers systemic multi-organ protection against IR injury [3, 9, 10, 13, 15, 17, 32, 34, 43, 44, 50]. RIPC was developed from the concept of local IPC, in which cardioprotection was generated by briefly occluding and reopening a coronary artery [2, 20, 28, 33]. Similar to local IPC, RIPC has two temporal phases of protection. The first one occurs immediately after PC and lasts for 4–6 h; the second one appears 24 h after PC and is sustained for 48–72 h, termed late RIPC [21, 27, 45]. Recent clinical trials have suggested that late RIPC reduces cardiac and hepatic injury after IR [42, 46]. However, the underlying mechanism is still poorly understood.

Late RIPC has been shown to inhibit inflammatory responses [27, 42]. It suppresses expression of proinflammatory genes in the myocardium and decreases adhesion and exocytosis of neutrophils in reperfused tissues [16, 36]. Interleukin (IL)-10 is a potent anti-inflammatory cytokine [26]. The IL-10 receptor (R) is composed of at least two subunits R1 and R2. The ligand-binding subunit IL-10R1 has high affinity with IL-10 ($K_d \sim 35$ –200 pM). IL-10R1 is expressed in all IL-10-responsive cells; monoclonal antibodies against IL-10R1 block IL-10 activities [26, 31]. IL-10R2 is ubiquitously expressed in cells. IL-10 inhibits inflammation by decreasing production of chemokines and cytokines [26]. IL-10 can also directly activate pro-survival signaling pathways [22, 35]. It has been reported that IL-10 mediates protection against myocardial IR injury [12, 14, 30, 49].

Recently, we reported that IL-10 protein expression is negatively regulated by phosphatase and tensin deleted on chromosome ten (PTEN) in the heart [30]. PTEN inactivation can increase phosphorylation of Stat3, a transcription factor for IL-10 expression [38, 52]. In this study, we investigated the hypothesis that RIPC confers late protection against IR injury by upregulating expression of IL-10 in ischemic skeletal muscle. We have found that RIPC limits myocardial infarct size and improves cardiac contractility through the IL-10 signaling pathway 24 h later; and that the cardioprotection is associated with elevated plasma and cardiac IL-10 levels as well as increased expression of IL-10 in the preconditioned skeletal muscle.

Materials and methods

Animals

All experiments were performed with age-matched male mice. At the time of the experiments, mice were 9–12 weeks old. Wild-type (WT, C57BL/6) mice and IL-10 KO mice (B6.129P2-*Il10*^{tm1Cgn/J}) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Myocyte-specific PTEN knockout (PKO) mice (*Pten*^{loxP/loxP}; *ckm-Cre*^{+/-}) were generated by loxp-cre technology [53]. *Pten*^{loxP/loxP}; *ckm-Cre*^{-/-} mice were used as the control (CON) for PKO. Their phenotypes were described previously [53]. All procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committee and conformed to 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).

Materials

Anti-mouse IL-10R1 monoclonal antibody (RA) was obtained from BioLegend (San Diego, CA, USA) to block IL-10 binding. RA (5 µg) was diluted in 200 µl of saline and administered to mice by intra-peritoneal injection twice, immediately after RIPC and 30 min before prolonged ischemia. Mouse IL-10 recombinant protein was purchased from eBioScience (San Diego, CA, USA). To induce cardioprotection, IL-10 was added into the

perfu-sion buffer of isolated hearts (final concentration 50 ng/ml) or injected into mice [10 ng in 200 μ l phosphate buffered saline (PBS), i.p.]. LY294002 (LY), a phosphatidylinositol-3 (PI3) kinase inhibitor, was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used to inhibit the effect of IL-10 in isolated perfused hearts. LY was added into the perfusion buffer at a final concentration of 10 μ mol/l over 15 min before 30-min ischemia. Stat3 Inhibitor VII (STI) was obtained from EMD, Inc. (San Diego, CA, USA). To inhibit Stat3 activity, STI was injected into mice at a dose of 0.1 mg/kg (i.p.) 30 min before RIPC in WT mice or collecting hearts in PKO mice. Antibodies against PTEN, Akt, p-Akt (Ser 473), p-Stat3 (Tyr705), and Stat3 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against IL-10, caveolin-3, endothelial nitric oxide synthase (eNOS), and p-eNOS (Ser 1177) were obtained from BD Biosciences (San Jose, CA, USA).

In situ ischemia and reperfusion mouse model

In vivo IR injury was measured in a mouse model. Mice were anesthetized with pentobarbital (70 mg/kg, i.p.) as described previously [6]. After mice had no response to tail pinch, trachea intubation was performed and mechanical ventilation was established. Anesthesia was maintained by repeated injection of pentobarbital (one-third of initial dose, every 45 min). A left thoracotomy was carried out. The left coronary artery was identified visually, and a 7-0 suture was placed around the artery about 2 mm below the left auricle. The electrocardiogram (ECG) was recorded continuously. The mice were warmed to 37 °C with a heating plate and kept continuously at this temperature, monitored with a rectal probe. A loop was made in the suture around the coronary artery and tightened over a small piece of polyethylene tubing. Occlusion of the left coronary artery resulted in changes in heart color (pallor) and ECG (ST-segment elevation and widening of the QRS complex). Reperfusion was accomplished by loosening the loop, confirmed by return of red color to the region and improved cardiac contraction. Mice were subjected to 30 min of ischemia and 120 min of reperfusion (I-30/R-120) with or without preceding RIPC. At the end of the experiment, animals were euthanized by transecting the aorta and removing the heart for determination of infarct size.

Measurement of left ventricular pressure

To monitor left ventricular function, a 26 gauge needle was used to make a stab wound near the left ventricular apex after exposure of the heart. A Mikro-tip catheter (Millar Instruments, Houston, Texas, USA) was inserted through the stab wound into the left ventricle. Left ventricular pressure was directly measured with the Powerlab data acquisition system and displayed on a computer. Left ventricular developed pressure [LVDP = systolic pressure (LVSP) - end-diastolic pressure (LVEDP)], heart rate (HR), positive maximal LVP derivative ($+dp/dt_m$), and negative maximal LVP derivative ($-dp/dt_m$) were automatically calculated using Chart 5 software.

Assessment of myocardial infarct size

Myocardial infarct size was assessed to measure the extent of IR injury. The loop around the coronary artery was retightened as described previously [6]. 0.5 % Evans blue solution was injected into the aorta. The dye passed into the coronary arteries and distributed throughout the non-risk ventricular wall proximal to the coronary artery ligature. The heart was frozen, and then cut transversely into five sections, each of which was weighed and incubated in 1.5 % triphenyltetrazolium chloride (TTC) for 15 min at 37 °C. Two sides (A and B) of each section were photographed. Infarct area, area at risk (AAR), and LV area were measured by computerized planimetry (Image J, NIH, Bethesda, MD, USA). Infarct size (IS) was presented as a percentage of LV (IS/LV) or AAR (IS/AAR).

RIPC

RIPC was performed 24 h before prolonged ischemia. To determine whether a brief period of ischemia and reperfusion in one limb induced cardioprotection 24 h later, the left femoral artery was exposed at the inguinal ligament and separated from the femoral vein and nerve. A microvessel clip was used to occlude the artery under a stereo microscope. Ischemia was confirmed by pallor of the paw. Reperfusion was followed by clip removal and confirmed by return of blood supply and restoration of normal color. Three cycles of 5-min ischemia and 5-min reperfusion (I-5/R-5) were applied. Sham control (CON) had the same treatment as RIPC, except that no ischemia was applied. Mice were allowed to wake up, and no drug or analgesics were given in the time period between RIPC and protein analysis or prolonged ischemia except for administration of RA in some experiments.

Measurement of plasma IL-10

Plasma IL-10 levels were measured by mouse IL-10 enzyme-linked immunosorbent assay (ELISA) kit (Bio-Legend, San Diego, CA, USA). Blood was drawn into a heparinized syringe and immediately separated in a centrifuge. The plasma was aspirated and assayed in accordance with the manufacturer's instructions.

Mouse Langendorff preparation

Experiments in isolated perfused mouse hearts were performed to measure the direct effect of IL-10 on IR injury as described previously [54]. After the chest was opened, the heart was excised and the ascending aorta was cannulated with a blunt needle. The heart was perfused at a constant pressure of 100 cm H₂O with Krebs-Henseleit buffer (in mmol/l, glucose 17, NaCl 120, NaHCO₃ 25, CaCl₂ 2.5, KCl 5.9, MgSO₄ 1.2, and EDTA 0.5), which was maintained at 37 °C and bubbled continuously with a mixture of 95 % O₂ and 5 % CO₂. Global ischemia was induced by cessation of perfusion, followed by reperfusion. Before hearts were subjected to I-30/R-120, IL-10 was added into the perfusion line by a syringe pump according to the coronary flow rate over 15-min in the presence or absence of LY or RA.

Assessment of myocardial infarct size in isolated perfused hearts

At the end of experiments, mouse hearts were perfused with 1 % TTC (dissolved in saline) for 1 min and then incubated with 1 % TTC at 37 °C for 15 min [54]. After freezing at -80 °C, hearts were sectioned transversely into 5 slices. Slices were incubated with 10 % formalin for 30 min. Both sides (A and B) of each slice were photographed. The areas of red and white color were measured by computerized planimetry (Image J, NIH, Bethesda, MD, USA). IS was calculated as a percentage of LV (IS/LV): total weight of white tissue/weight of LV × 100 %.

Isolation of adult cardiomyocytes

Adult cardiomyocytes were isolated to measure expression of IL-10R1 as described previously [47]. The heart was excised and then mounted on the perfusion apparatus. After 5 min of perfusion with low Ca²⁺, 150 Units/ml of type-II collagenase was added and perfused for 15 min. LV tissue was minced and allowed to digest in perfusate for 15 min. The digested heart was filtered through a 200-μm nylon mesh, placed in a conical tube, and spun at 100 rpm to allow viable myocytes to settle. Serial washes were used to remove nonviable myocytes and digestive enzymes until the concentration of Ca²⁺ was gradually increased to 1.8 mM in the perfusion buffer.

Immunoprecipitation and Western blot analysis

Hearts or skeletal muscle were homogenized in lysate buffer (in mmol/l: pH 7.5 Tris 20, NaCl 150, EDTA 1, EGTA 1, PMSF 1, Na₃VO₄ 1, 1% Triton). To determine the association of IL-10R1 with caveolin-3, IL-10R1 was immuno-precipitated from 300 µg of heart lysates with 20 µl of anti-IL-10R1 antibody overnight, followed by incubation with protein G-agarose beads for 2 h at 4 °C. The precipitates were washed three times with lysate buffer and then dissolved in sample buffer. Proteins were separated on a precast NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and transferred to a nitrocellulose membrane. Proteins were detected using primary antibodies against caveolin-3, followed by horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence.

Statistical analysis

Data are presented as mean ± standard error of the mean. The difference among groups was analyzed by using Student's *t* test or two-way ANOVA with Tukey's post hoc test. Differences were considered significant if $p < 0.05$.

Results

Late RIPC confers protection against myocardial IR injury via the IL-10 signaling pathway

To determine whether RIPC induces late protection via the IL-10 signaling pathway, WT mice were exposed to lower limb RIPC or CON. At 24 h post-RIPC, mice were subjected to myocardial I-30/R-120 (Fig. 1a). Late RIPC decreased infarct size compared to CON (Fig. 1b, c). This infarct-limiting effect was completely blocked by RA, but RA alone had no effect on infarct size (Fig. 1b, c). Consistent with its effect on infarct size, late RIPC caused an increase in LVDP, $+dp/dt_m$, and heart rate, which was reversed by RA (Fig. 1d, e; Table S1). To further determine whether IL-10 is involved in late RIPC, IL-10 KO mice were exposed to CON or RIPC. After 24 h, mice were subjected to myocardial I-30/R-120 (Fig. 2a). There was no significant difference in infarct size between CON and RIPC (Fig. 2a). To examine whether IL-10 is sufficient to induce cardio-protection in IL-10 KO mice, IL-10 KO mice were treated with mouse recombinant IL-10 for 30 min, followed by myocardial I-30/R-120. IL-10 significantly decreased infarct size in IL-10 KO mice (Fig. 2b). IL-10 protein was constitutively expressed in WT mouse hearts, but it was undetectable in IL-10 KO mouse hearts (Fig. 2c). These results suggest that RIPC induces late protection against IR injury, and that this effect is mediated through the IL-10 signaling pathway.

IL-10 induces protection through the PI3K/Akt signaling pathway in isolated perfused hearts

Since RA effectively blocked the effect of RIPC, to determine whether IL-10 receptors are expressed in cardiomyocytes, IL-10R1 protein levels were analyzed in lysates from murine cardiomyocytes and whole hearts by immunoblotting. Lysates from macrophages were used as a positive control. IL-10R1 was detected in cardiomyocytes and hearts (Fig. 3a). To confirm its constitutive expression, IL-10R1 was pulled down from heart lysates and immunoblotted with antibodies against caveolin-3, a myocyte-specific protein. IL-10R1 was associated with caveolin-3 in heart lysates, but caveolin-3 was undetectable in macrophage lysates, supporting that IL-10R1 is expressed in cardiomyocytes (Fig. 3b). To determine whether IL-10 decreases myocardial infarct size through the PI3K/Akt signaling pathway, isolated hearts from WT mice were treated with IL-10 in the absence or presence of LY or RA (Fig. 4a). IL-10 significantly increased Akt phosphorylation, and this effect was inhibited by LY and RA (Fig. 4b). Consistent with this result, IL-10 treatment decreased myocardial infarct size and LVEDP after I-30/R-120, whereas LVDP was not affected (Fig. 4c, d; Table S2). LY and RA blocked the effects of IL-10 (Fig. 4c, d), supporting the notion

that IL-10 is cardioprotective against IR injury, and that this effect is mediated through the PI3K/Akt signaling pathway.

Late RIPC increases plasma and cardiac IL-10 levels and activates the Akt/eNOS signaling pathway

Since IL-10 mediated cardioprotection, to determine whether RIPC regulates IL-10 protein levels in the blood and heart and its downstream signaling pathway at the late phase, mice were exposed to CON or RIPC. Plasma IL-10 levels were measured 24 h after RIPC (Fig. 5a). RIPC significantly increased plasma IL-10 levels (Fig. 5b), while in the heart, RIPC also increased IL-10 protein levels, and this effect was blocked by RA (Fig. 5c). The same heart lysates were analyzed for phosphorylation of Akt and eNOS. RIPC increased their phosphorylation which was reversed by RA (Fig. 5d, e). Thus, these results suggest that late protection of RIPC may be attributed to activation of the Akt/eNOS signaling pathway in the heart via upregulation of plasma and cardiac IL-10 levels.

Late RIPC induces IL-10 expression in the gastrocnemius muscle via the Stat3 signaling pathway

To determine the potential source of plasma IL-10 and the molecular mechanism that regulates IL-10 production in RIPC, mice were exposed to CON or RIPC. RIPC rapidly decreased PTEN protein levels in the ischemic gastrocnemius muscle (Fig. 6a). In separate experiments, 30 min before exposure to CON or RIPC, mice were treated with STI or vehicle. RIPC increased phosphorylation of Stat3 in the gastrocnemius muscle, which was inhibited by STI (Fig. 6b). After 24 h, RIPC increased IL-10 expression in the skeletal muscle, and STI inhibited this effect (Fig. 6c). These results suggest that RIPC inactivates PTEN and upregulates IL-10 expression in the gastrocnemius muscle through Stat3.

PTEN inactivation increases IL-10 expression in the gastrocnemius muscle through the Stat3 signaling pathway

We previously reported that PTEN protein levels are decreased in the skeletal muscle and heart of PKO mice [53]. To determine whether PTEN inactivation is sufficient to induce IL-10 expression in the skeletal muscle, PKO and CON mice were treated with STI or vehicle. After 30 min, gastrocnemius muscles from PKO and CON mice were isolated for measurement of p-Stat3 and Stat3. PTEN inactivation significantly increased Stat3 phosphorylation (Fig. 7a). After 24 h, IL-10 protein levels were analyzed. PTEN inactivation increased expression of IL-10 protein in the gastrocnemius muscle (Fig. 7b). These effects were blocked by Stat3 inhibition, suggesting that PTEN inactivation induces IL-10 expression in the muscle through the Stat3 signaling pathway. IL-10 protein levels are increased in the heart of PKO mice [31]. To determine whether PKO mice are resistant to myocardial IR injury in an in vivo animal model, we exposed PKO and CON mice to I-30/R-120 without RIPC. Myocardial infarct size was decreased in PKO mice compared with CON mice (Fig. 7c). Therefore, PTEN inactivation increases induction of IL-10 protein through the Stat3 signaling pathway, contributing to myocardial protection.

Discussion

In this study, we report three important findings. First, we demonstrate that late protection of RIPC is mediated by IL-10, as RIPC protection was blocked by IL-10 receptor antibodies and lost in IL-10 KO mice, and IL-10 alone was sufficient to induce cardioprotection. Second, we show that RIPC increased plasma and cardiac IL-10 levels 24 h later, and that late RIPC activated Akt signaling in the heart through IL-10 receptors. Thus, IL-10, as a humoral factor, activates pro-survival signaling in the heart after RIPC. Third, we demonstrate that RIPC inactivated PTEN and increased Stat3 phosphorylation and IL-10

expression in the ischemic skeletal muscle. PTEN inactivation by gene manipulation had similar effects on Stat3 phosphorylation and IL-10 expression. Moreover, the induction of IL-10 was blocked by Stat3 inhibition in RIPC mice and PTEN KO mice. Thus, RIPC induces IL-10 expression in the preconditioned muscle through the Stat3 signaling pathway, probably via inactivation of PTEN. Taken together, our study has demonstrated for the first time that RIPC confers late protection of the heart against IR injury by upregulating expression of IL-10 in the transiently ischemic skeletal muscle through the Stat3 signaling pathway.

It has been reported that RIPC suppresses inflammatory responses, especially neutrophil function [16, 36, 42]. Although inducible NOS has been implicated in late protection of local IPC, RIPC had no effect on its expression in the heart [8, 45]. In the present study, we demonstrate that IL-10 mediates late RIPC. IL-10 has been shown to inhibit expression of proinflammatory cytokines and decrease leukocyte adhesion to vascular endothelium and infiltration through the vessel wall [12, 18, 30, 31]. Moreover, IL-10 induces protection against IR injury in the heart and brain by promoting cell survival [18, 22, 35, 49]. Because there are two protective phases of RIPC, each of which may be related to IL-10, two doses of RA were administered. Based on the experimental result (Fig. 5c), the first dose probably played the major role in blocking RIPC. However, the second dose might also have a modest impact since antibodies can rapidly enter the blood through lymphatic drainage [1]. In IL-10 KO mice, RIPC was unable to confer protection. Loss of RIPC effect in IL-10 KO mice could have resulted from increased expression of proinflammatory cytokines [24]. However, these cytokines are known to be modulated by IL-10 [26]. This supports the notion that IL-10 is required for suppression of proinflammatory cytokines and myocardial recovery after IR. Humoral factors have previously been implicated in the early phase of RIPC [7, 20]. Our study is the first to show that IL-10 acts as a humoral factor to protect the heart from IR injury in the late phase of RIPC. Previous studies have suggested that humoral factors smaller than 30 kDa mediate the early protection of RIPC [4], which is consistent with the 18 kDa molecular weight of IL-10 [26]. Whether IL-10 is involved in early RIPC needs further investigation.

We demonstrate that late RIPC activates the Akt signaling pathway. It has been reported that the PI3K/Akt signaling pathway is involved in early RIPC and IL-10-mediated protection against IR injury [4, 19, 35]. In the present study, we showed that RA blocks the phosphorylation of eNOS and Akt in the heart induced by RIPC. eNOS is required for late protection of local IPC [48]. It is one of the downstream targets of Akt [11, 25]. Therefore, our study suggests that the activation of Akt/eNOS may also contribute to the late protection of RIPC. Targeting this signaling pathway may have therapeutic benefits.

We report that increased expression of IL-10 in the preconditioned skeletal muscle is associated with elevated plasma and cardiac IL-10 levels. It has been reported that IL-10 expression is dependent on PTEN activity in the heart [31]. In the present study, IL-10 expression is increased in the skeletal muscle of PKO mice. This confirms the notion that myocyte-specific PTEN inactivation induces expression of IL-10 in the skeletal muscle, although it still needs to be determined whether IL-10 is directly released from myocytes in PKO mice. We further demonstrate that RIPC downregulates PTEN protein levels in the preconditioned skeletal muscle. This result is consistent with previous reports, in which PTEN activity was shown to be decreased in the heart and brain by local IPC [5, 51]. PTEN is ubiquitously expressed in tissues [25]. Thus, our studies suggest that IPC-induced PTEN inactivation contributes not only to local protection by activating the Akt signaling pathway, but also to remote protection by increasing plasma IL-10 levels. Since IL-10 receptors have high affinity for IL-10, increased plasma IL-10 may raise its tissue levels. Furthermore, IL-10 has been shown to increase its own expression in tissues [31, 38]. Therefore, RIPC-

induced protective signaling may be amplified through IL-10. Further studies are needed to determine whether increased plasma and cardiac IL-10 is released from the preconditioned skeletal muscle myocytes.

PTEN negatively regulates Stat3 activity and expression of IL-10 in the infarcted heart [31]. In the present study, we demonstrate that PTEN inactivation induces IL-10 expression through the Stat3 signaling pathway by the use of a specific Stat3 inhibitor. However, the part of the PTEN/Stat3 pathway upstream from Stat3 which is stimulated by RIPC or PTEN deletion would not be affected by Stat3 inhibition. As expected, the levels of Stat3 phosphorylation and IL-10 expression varied in the different experimental groups due to different activities of protein upstream from Stat3. In PKO mice, PTEN inactivation not only increases IL-10 expression but also stimulates growth and proliferation in myocytes, which likely include arterial smooth muscle cells [29, 31, 53]. This could cause vascular remodeling and increase arterial resistance. It was noticed that PKO mice appeared to have a larger infarct size than RIPC mice despite the similarity in IL-10 expression in these two groups. Although the underlying mechanism is unknown, tissue remodeling due to PTEN inactivation may impair IL-10-induced protection against IR injury. It has been reported that femoral nerve transection or the nitric oxide donor *S*-nitroso-*N*-acetylpenicillamine abolishes the cardioprotective effect of RIPC [39]. PTEN is sensitive to reactive oxygen species [5]. Therefore, further investigation is needed to determine whether blocking the neural pathway affects production of reactive oxygen species and then oxidation and degradation of PTEN in the preconditioned muscle. Activation of Stat3 has been reported in the heart after local IPC, and is required for the protection of IPC [37, 40]. Stat3 signaling may crosstalk with Akt signaling in the heart [40, 41]. More studies are needed to determine whether IL-10 connects the signaling between Stat3 and Akt in local IPC.

In conclusion, RIPC confers late myocardial protection against IR injury in a murine model by upregulating expression of IL-10 in the transiently ischemic skeletal muscle, with circulation of released IL-10 to the heart. Since RIPC is a simple and almost no-cost procedure with a prolonged period of late protection, it is potentially very attractive for preventing IR injury in patients with a high risk of myocardial infarction or stroke since it can be applied non-invasively through a blood pressure cuff on the arm or leg. RIPC might also be useful in generating systemic protection before planned surgical procedures that require temporary occlusion of blood supply. Although more studies are needed to determine whether other factors are involved in late RIPC, endogenous IL-10 appears to be a potent mediator of its protection. Since IL-10 has also been shown to inhibit inflammation and adverse remodeling [18, 30, 31], the benefit of RIPC may extend beyond the prevention of IR injury.

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Abbreviations

eNOS	Endothelial nitric oxide synthase
IL-10R1	Interleukin 10 receptor subunit 1
IS	Infarct size
IR	Ischemia–reperfusion
LVDP	Left ventricular developed pressure

LY	LY294002
PI3K	Phosphatidylinositol-3-kinase
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
PKO	Myocyte-specific PTEN knockout
RA	Anti-IL-10 receptor antibody
RIPC	Remote ischemic preconditioning
Stat3	Signal transducer and activator of transcription 3
STI	Stat3 inhibitor

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Fig. 1. RIPC induces late protection through the IL-10 signaling pathway. **a** Experimental protocol. Mice were exposed to lower limb RIPC, three cycles of I-5/R-5 or sham (CON), followed by treatment with or without anti-IL-10R1 antibody (RA). After 24 h, mice were subjected to myocardial I-30/R-120. **b** Representative images of cardiac sections from one infarcted heart per group. **c** Myocardial infarct size (IS) as a percentage of left ventricle (LV) or area at risk (AAR). **d** LV developed pressure (LVDP). **e** Maximal positive LV dp/dt ($+dp/dt_m$). * $p < 0.01$ versus CON or CON/RA or RIPC/RA, $N = 8$.

Fig. 2.

Late protection of RIPC is lost in IL-10 KO mice and mimicked by IL-10 treatment. **a** Late RIPC in IL-10 KO mice. IL-10 KO mice were exposed to CON or RIPC. After 24 h, they were subjected to myocardial I-30/R-120. $N = 8$. **b** Effect of exogenous IL-10 on IR injury. IL-10 KO mice were treated with phosphate buffered saline (PBS) or IL-10 for 30 min, followed by I-30/R-120. * $p < 0.01$ versus PBS, $N = 8$. **c** IL-10 expression in the hearts of WT and IL-10 KO mice.

Fig. 3.

Cardiomyocytes express IL-10R1 under basal conditions. a IL-10R1 protein levels were analyzed by immunoblotting in heart lysates (lane 1) and cardiomyocyte lysates (lane 2). Macrophage lysates were used as a positive control (lane 3). b IL-10R1 association with caveolin-3 (Cav-3), a myocyte-specific protein, in the heart. IL-10R1 was immunoprecipitated with anti-IL-10R1 antibody, followed by immunoblotting with anti-Cav-3 antibody.

Fig. 4.

IL-10 induces protection in isolated mouse hearts through the PI3K/Akt signaling pathway.

a Isolated mouse hearts were treated with IL-10 in the presence or absence of the PI3K inhibitor LY or RA. **b** Phospho (p)-Akt and total Akt protein levels in heart lysates after 15 min of treatment. * $p < 0.01$ versus CON or IL-10/LY or IL-10/RA, $N = 4$. **c** and **d** Myocardial infarct size and LV end-diastolic pressure (LVEDP) after I-30/R-120. * $p < 0.05$ versus CON or IL-10/LY or IL-10/RA, $N = 6-8$.

Fig. 5.

Late RIPC increases plasma and cardiac IL-10 levels and activates the Akt/eNOS signaling pathway. **a** Mice were exposed to CON or RIPC. After 24 h, plasma and cardiac samples were collected. **b** Plasma IL-10 levels in CON and RIPC mice. $*p < 0.001$ versus CON, $N = 5$. **c** RIPC raised IL-10 protein levels in the heart, and RA blocked this effect. $*p < 0.01$ versus CON or RIPC/RA, $N = 4$. **d** and **e** RIPC increased phosphorylation of Akt and eNOS in the heart, which was blocked by RA. $*p < 0.01$ versus CON or RIPC/RA, $N = 4$.

Fig. 6.

RIPC decreases PTEN protein levels and induces expression of IL-10 through the Stat3 signaling pathway. **a** PTEN protein levels in the gastrocnemius muscle immediately after RIPC or CON. $*p < 0.01$ versus CON, $N = 4$. **b** Stat3 phosphorylation in the gastrocnemius muscle. Mice were treated with or without Stat3 Inhibitor (STI) for 30 min, followed by RIPC or CON. $*p < 0.05$ versus CON, $^{\dagger}p < 0.01$ versus CON or RIPC/STI, $N = 4$. **c** IL-10 protein expression in the gastrocnemius muscle. In parallel experiments, IL-10 protein levels were measured 24 h later. $*p < 0.01$ versus CON, $^{\dagger}p < 0.01$ versus CON or RIPC/STI, $N = 4$.

Fig. 7.

PTEN inactivation in myocytes upregulates IL-10 expression by increasing Stat3 activity. **a** Stat3 phosphorylation in the gastrocnemius muscle. Myocyte-specific PTEN knockout mice (*PKO*) and control (*CON*) mice were treated with or without STI for 30 min. * $p < 0.05$ versus CON, [†] $p < 0.01$ versus CON or PKO/STI, $N = 4$. **b** IL-10 expression in the gastrocnemius muscle in PKO and CON mice 24 h after treatment with or without STI. * $p < 0.05$ versus CON, [†] $p < 0.01$ versus CON or PKO/STI, $N = 4$. **c** Myocardial infarct size in PKO and CON mice after I-30/R-120. * $p < 0.01$ versus CON, $N = 8$.