Peripheral Nociception Associated With Surgical Incision Elicits Remote Nonischemic Cardioprotection Via Neurogenic Activation of Protein Kinase C Signaling

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Background—Although remote ischemic stimuli have been shown to elicit cardioprotection against ischemia/reperfusion injury, there is little known about the effects of nonischemic stimuli. We previously described a remote cardioprotective effect of nonischemic surgical trauma (abdominal incision) called remote preconditioning of trauma (RPCT). In the present study, we elucidate mechanisms underlying this phenomenon.

Methods and Results—We used a murine model of myocardial infarction to evaluate ischemia/reperfusion injury, and either abdominal surgical incision, or application of topical capsaicin, to elicit cardioprotection. We show that the cardioprotective effect of RPCT is initiated by skin nociception, and requires neurogenic signaling involving spinal nerves and activation of cardiac sensory and sympathetic nerves. Our results demonstrate bradykinin-dependent activation and repression, respectively, of PKCe and PKCd in myocardium after RPCT, and we show involvement of the KATP channels in cardioprotection. Finally, we show that topical application of capsaicin, which selectively activates C sensory fibers in the skin, mimics the cardioprotective effect of RPCT against myocardial infarction.

Conclusions—Nontraumatic nociceptive preconditioning represents a novel therapeutic strategy for cardioprotection with great potential clinical utility.

Key Words: apoptosis ■ capsaicin ■ infarction ■ nervous system ■ remote preconditioning ■ signal transduction ■ sympathetic

Cardiac ischemia/reperfusion (I/R) injury contributes significantly to morbidity and mortality throughout the world.1 Over the past 2 decades, various strategies for protecting the heart against myocardial infarction (MI) and I/R dysfunction have been developed.2–4 Ischemic preconditioning (IPC), especially, has been extensively studied in multiple species and in the clinical setting.5–9 Importantly, remote IPC that results from brief episodes of ischemia occurring at a distant organ site has been shown to be cardioprotective.4,10,11 Previously, we observed that infarct size after in vivo I/R was altered by nonischemic surgical trauma. Vascular surgery performed to catheterize the carotid artery increased infarct size, whereas transverse abdominal incision resulted in a significantly decreased infarct size.12 To describe this nonischemic preconditioning phenomenon, we coined the term “remote preconditioning of trauma” (RPCT).

It has been demonstrated that bradykinin (BK), adenosine, opioids, and norepinephrine (NE) all have roles in remote IPC.10,13–16 BK is one of several oligopeptides called kinins that are produced by sympathetic nerve endings (ie, synaptosomes), myocytes, and endothelial cells in the heart.17–19 The actions of BK are mediated by two major receptor subtypes, BK receptors 1 and 2 (BK1R and BK2R).20,21 BK1R are inducible by inflammatory stimulation or tissue injury, and BK1R seem to play an injurious role in myocardial I/R.22 BK2R is constitutively expressed and mediates most of the physiological actions of kinins. Several studies demonstrate that activation of BK2R is involved in both IPC and in remote IPC.10,23 Previous studies demonstrate that endogenous BK activates sympathetic cardiac afferents during I/R and that this reduces cardiac dysfunction and MI.10,24–26 Calcitonin gene-related peptide (CGRP) and substance P are released along with BK from sensory nerves and

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can act on cardiac sympathetic nerves to provoke NE release. There is evidence that BK-induced protection requires protein kinase C (PKC) activation. In particular, PKC-δ has been shown to be a critical mediator of postischemic cardiomyocyte necrosis and contractile dysfunction after I/R, and PKCε is a mediator of cardioprotection. There is currently nothing known about the molecular initiators that instigate cardioprotection after RPCT. The fact that cardioprotection against MI mediated by RPCT does not require TNF-α suggests that the mechanism is not the same as IPC. To delineate the mechanism of RPCT, we performed pharmacological, genetic, biochemical, and physiological analyses in this study. Our results show that an abdominal incision elicits cardioprotection against MI via stimulation of peripheral nociception. Nociception triggers neurogenic signaling via spinal nerves, which activates the sympathetic nervous system in the heart and elicits activation of PKCe and inhibition of PKCδ in a BK2R-dependent manner. Activation of the mitochondrial K$_{ATP}$ (mitoK$_{ATP}$) channels is required for cardioprotection. Direct activation of C sensory fibers in skin using topical capsaicin mimics the cardioprotective effect of RPCT, supporting that peripheral nociceptor stimulation has great clinical potential.

**Materials and Methods**

**Experimental Protocols**

Mice were maintained in accordance with institutional guidelines, the Guide for the Care and Use of Laboratory Animals (NIH, revised 1985), and the Position of the American Heart Association on Research Animal Use (1984). Wild-type (B6129SF2/J F1) and BK2 receptor knockout mice (B6129SF2/J F1, strain 101045) were obtained from the Jackson Laboratories (Bar Harbor, Me). All groups of mice consisted of males and females distributed equally among groups; post hoc analyses confirmed previous results that there were no gender-related differences in these studies.

**Surgical Procedures**

Mice were subjected to surgical protocols as delineated in Figure 1. A minimally traumatic mouse model was used for in vivo studies of I/R injury and RPCT as described previously. All mice were continuously monitored by electrocardiography, and mice without evidence of ischemia and timely reperfusion were excluded from the studies (1%); survival in this study was 96%. Coronary occlusion was for 45 minutes. In experiments in which infarct size was the end point, infarct size was determined at 4 or 24 hours after reperfusion, as previously described, and is presented as area of the infarct normalized to the area of the region at risk. For all studies, the region-at-risk was not significantly different between groups (Table 1, supplement). The 4-hour time point was used only in the spinal transection and related control studies to prevent the mice from regaining consciousness in that study, for ethical reasons. Abdominal incision was used as the nonischemic stimulus for RPCT as previously described. The incision was through the skin, subcutaneous, fat, muscle, and peritoneum, and was 2 cm in length through the abdominal midline; we refer to this as the RPCT stimulus. Afterward, the incision was sutured immediately using 7-0 polypropylene sutures. For skin incision, the incision was made anatomically in the same location but care was taken to cut the skin only. Sham control groups were used for the abdominal and skin incisions. In these groups, mice were treated exactly the same, except with a control gel base without capsaicin.

Nociception-induced preconditioning was elicited by application of topical capsaicin ($\approx$150 μL of 0.1%; Chattem, Inc) along the same line as surgical incision, using a mask that exposes a strip of skin $2\times1$ cm through the umbilicus. Sham control mice for this study were treated exactly the same, except with a control gel base without capsaicin.

To determine the role of the central nervous system versus spinal ganglia, we transected the spinal cord at different levels 15 minutes before the abdominal incision. Mice were anesthetized and bone markers were identified at the thoracic vertebra C7 and T7 levels. The spinal cords of C7 and T7 were transected by a sharp knife with limited bleeding under a microscope, followed by immediate abdominal incision. I/R 15 minutes later, and infarct size determination 4 hours later. Sham controls were subjected to the same surgical
manipulations and procedures (ie, anesthesia, shaving, opening the skin and muscle, exposing the spinal column), but without actually cutting the cord.

**Neuronal Tracing**

To track the connection between nerve cells from the sensory fibers of the skin to the spine, a fluorescent dye, 1,1’-dioactetyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI), was injected subcutaneously at the abdominal incision level (thoracic vertebra T9-T10 of spine). One week after the injection, mice were dissected. The sectioned spinal cord (40 μm) and whole-mount dorsal root ganglion were placed under a confocal microscope for direct visualization and image capture.

**Pharmacological Agents**

Details of treatment with pharmacological agents, dosage, route, suppliers, and vehicle controls are provided in the supplement, because of space limitation. Briefly, the agents used were the BK2R-antagonist Hoe140 (50 μg/kg, intravenous), the ganglionic blocker hexamethonium (20 mg/kg, intravenous), K<sub>ATP</sub> channel inhibitors 5HD (100 μmol/L/kg, intravenous), glibenclamide (0.3 mg/kg, intravenous), and CGRP antagonist CGRP5-37 (3 nmol/kg). Capsaicin (0.1%; Chattem, Inc) was applied topically (150 μL over a 1×2 cm area; supplement). In all studies, vehicle control experiments were performed with the specific vehicle required for each agent referenced.

**Morphological and Histological Assessments**

Please see the supplement and literature for details.

**Cell Fractionation and Immunoblotting**

Please see the supplement and literature for details.

**Statistical Analysis**

Group size was determined by Power analysis, as described. For parameters that require quantification and evaluation for statistical significance, results were expressed as mean±SEM. Statistical significance (P values) was determined using the Student t test (2-tailed distribution and 2-sample unequal variance) with the Bonferroni correction. For multiple group comparisons, 1-way analysis of variance followed by Fisher post hoc test was used. P≤0.05 was considered statistically significant.

**Results**

**Remote Preconditioning of Trauma Attenuates Infarct Size and Reduces Apoptosis After I/R**

We observed a 5- to 6-fold reduction in infarct size after RPCT (Figure 2A; 55.3±3.4% sham, versus 10.2±6.3% RPCT; P=0.05; n=11). We assessed the extent of apoptosis in both sham and RPCT hearts after I/R, using in situ end labeling of DNA fragmentation (TUNEL staining) and an ELISA-based nucleosome assay. TUNEL results revealed, relative to shams, a significantly decreased proportion of TUNEL-positive nuclei in the myocardium of mice subjected to RPCT (Figure 2B, 2C; 33.6±2.5% versus 4.2±0.01%; P≤0.05; n=4). Results of the quantitative nucleosome assay showed that levels of mononucleosomes and oligonucleosomes were significantly decreased, relative to sham, after RPCT (Figure 2D).

**Neurogenic Transmission Is Required for Cardioprotection After RPCT**

To determine the possible role of a neurogenic pathway, we first addressed whether sympathetic ganglionic transmission is required for cardioprotection against MI by RPCT. Results show that administration of the sympathetic ganglionic blocker hexamethonium (20 mg/kg) before the RPCT stimulus abrogated the protective effect of RPCT against MI (Figure 3A; 9.5±1.2 RPCT versus 55.9±1.9 RPCT hexamethonium; P≤0.05; n=7).

Next, we transected the spinal cord at 2 different levels, vertebral levels C7 and T7, before RPCT (Figure 3B). Transection of the spinal cord at C7 had no effect on RPCT (Figure 3B white bars; 12.1±3.1%, sham versus 10.3±2.2%;
C7 transection; P<0.05; n=4). However, transection at T7 abolished the cardioprotective effects of RPCT against MI (Figure 3B black bars; 13.4±2.5%, sham, versus 53.4±2.8%; T7 transection; P<0.05; n=6).

We then tested whether the abdominal incision, if limited to the skin layer (location of sensory nerves), was still capable of triggering cardioprotection. The results (Figure 3C) show that incision of skin alone is sufficient to elicit cardioprotection against MI (7.3±3.2% wall incision versus 10.4±2.5%; T7 transection; P<0.05; n=6).

Next, we pretreated the abdominal incision site with lidocaine (1%) 15 minutes before abdominal incision. This treatment completely abrogated the cardioprotective effect of RPCT against MI (Figure 3D; 54.0±3.9% lidocaine RPCT versus 7.2±2.5% RPCT vehicle; P<0.05; n=7) supporting a critical role for pain sensation (nociception) in initiating RPCT.

It is known that sensory fibers from the abdominal skin project to the spinal cord at the vertebral T9-T10 levels, whereas the sensory nerves innervating the heart project to the vertebral T1-5 level43,44 (Figure 3A). Therefore, we investigated the possibility that sensory nerves originating in the skin or muscle underneath the skin at the incision site may connect to a higher level in the spinal cord. We found that dye injection (Dil) in skin at the abdominal midline (T9-10 vertebral level) labeled spinal neurons of the dorsal horns at both the vertebral T1-T5 level (Figure 3E), as well as at the T9-10 level.

Cardioprotection of RPCT Is Dependent on BK and β-Adrenergic Receptors

To elucidate whether RPCT requires BK/BK2R signaling, we treated mice with the BK2R-selective antagonist Hoe140 (50 μg/kg) 15 minutes before abdominal incision. Inhibition of BK2R, relative to vehicle-treated RPCT controls, abolished the protective effect of RPCT on infarct size (Figure 4A; 10.4±2.5% RPCT vehicle versus 52.4±2.4% RPCT Hoe140; P<0.05; n=7). Furthermore, RPCT did not have a cardioprotective effect against myocardial infarction in BK2R knockout mice (Figure 4B; 55.3±3.4% BK knockout sham versus 54.8±2.4% BK knockout RPCT; P>0.05; n=7).

To assess the role of β-adrenergic signaling after RPCT, we treated groups of mice with propanolol (2 mg/kg) or...
vehicle and subjected them to RPCT, followed by 45 minutes of coronary occlusion 15 minutes later (Figure 4C). Analysis of infarct size assessed 24 hours later demonstrated complete blockade of the cardioprotection afforded by RPCT against MI (54.4 ± 4.7 propanolol versus 10.4 ± 5.7 vehicle; *P = 0.05 vs. saline RPCT; n = 7). To determine the involvement of sensory nerve transmission, we assessed the requirement for CGRP using the antagonist CGRP5-37 (3 nmol/kg; Figure 4D). Blockade of CGRP prevented cardioprotection against MI after RPCT (53.2 ± 3.8 CGRP5-37 versus 7.2 ± 5.2 vehicle; *P = 0.05 vs. vehicle RPCT; n = 7).

Cardioprotection of RPCT Is Dependent on PKC Activity and Associated With Activation of PKCε and Repression of PKCδ

Groups of mice were treated with chelerythrine (5 mg/kg, intravenous) or vehicle 15 minutes before RPCT (Figure 4E) and infarct size assessed (45-minute ischemia, 24-hour reperfusion). The results demonstrate that blockade of PKC abrogates the cardioprotection afforded by RPCT (58.3 ± 4.5 chelerythrine versus 9.5 ± 1.2 vehicle; *P = 0.05; n = 7).

We next determined the alterations of PKC activity in myocardium after RPCT, measured by the ratio of the membrane-associated to cytoplasm-associated fraction of PKC. Quantitative immunoblotting demonstrated that PKCε is activated, whereas PKCδ activity is repressed 15 minutes after the RPCT stimulus (Figure 5A–D). There was no effect on PKCζ activity. Importantly, the results of similar experiments using BK2R knockout hearts demonstrated that ablation of BK2R prevented the RPCT-induced effects on PKCε and PKCδ (Figure 5E–H).

Role of KATP Channels in Cardioprotection After RPCT

Administration of 5HD (100 μg/kg, intravenous) completely eliminated the protective effect of RPCT against MI (Figures 6, 8; 1 ± 1.5 RPCT saline versus 51.3 ± 1.6 RPCT 5HD; *P = 0.05; n = 8), whereas cardioprotection was partially abrogated by glibenclamide (300 μg/kg, intravenous; 8.1 ± 1.5 RPCT saline versus 38.9 ± 2.8 RPCT glibenclamide; *P = 0.05; n = 7).
Topical Capsaicin Mimics Cardioprotection Against MI After RPCT

To determine whether direct chemical stimulation of sensory C-fibers in the skin elicits cardioprotection similar to that of RPCT, we applied capsaicin topically (0.1%) to the abdominal midline, along the same line used for the abdominal incision. The mice were subjected to a 45-minute coronary occlusion/reperfusion, and infarct size was measured 24 hours later (Figure 7). Infarct size was significantly reduced by topical capsaicin (51.2 ± 1.46 sham versus 7.48 ± 1.99 capsaicin; *P<0.05; n=6).

Discussion

There is recent evidence supporting the cardioprotective effects of remote ischemic stimuli and the mechanisms by which these produce cardioprotection.4,10,11,13 However, there is nothing known concerning the effect of remote nonischemic stimuli, including surgical injury, on cardioprotection or myocardial I/R injury. We published the first report12 to our knowledge describing the effects of remote nonischemic surgical stimuli on cardiac I/R injury and showed that, depending on the site, surgical incisions can be either cardioprotective or injurious. We showed that an abdominal surgi-

Figure 5. RPCT leads to a significant increase in PKCε translocation (B; membrane/cytoplasmic ratio; *P<0.05) and decrease in PKCa translocation (D; *P<0.05; n=6) in wild-type mice, whereas PKCa activation was not significantly affected (C). However, in BK2R knockout mice, RPCT did not affect PKC translocation (E–H; n=6).
Herein, we delineate major aspects of the mechanism underlying the phenomenon related to nociceptive stimulation (supplement). We also have evidence that there is a postconditioning phenomenon related to ventricular dysfunction after I/R (see supplement).

Non RPCT. We determined that RPCT reduces infarction, in part by reducing apoptosis (Figure 2), and that protection extends to ventricular dysfunction after I/R (see supplement). We also have evidence that there is a postconditioning phenomenon related to nociceptive stimulation (supplement). Herein, we delineate major aspects of the mechanism underlying RPCT.

Neurological Basis of Cardioprotection by RPCT

Although some evidence supports that a neurogenic pathway is involved in the cardioprotection of remote IPC, other evidence implicates diffusible humoral factors.7,8 We reported that administration of hexamethonium, a ganglionic blocker that inhibits impulse transmission from the preganglionic neurons to the postganglionic neurons of both the sympathetic and parasympathetic systems, abrogates the protection of RPCT against MI (Figure 3A), supporting a neurogenic mechanism. Our results with spinal transection (Figure 3B) also support a neurogenic mechanism and rule out an essential diffusible humoral factor as the cause of cardioprotection after RPCT. We also demonstrated that a shallow skin incision is sufficient to initiate RPCT (Figure 3C). Our result (Figure 3D) that lidocaine completely blocks RPCT supports that peripheral nociception via skin sensory fibers is required for RPCT. These peripheral nerves are essentially the axons of the dorsal root ganglion neurons. We propose that after nociceptive stimulation, peripheral nerve depolarization leads to a dorsal root reflex at the T9–10 vertebral level of the spinal cord, the level of the abdominal incision. Our results with spinal transection (Figure 3B) demonstrate that RPCT after stimulation of sensory nerves at the T9–10 vertebral level requires an intact spine to the T7 level. That spinal integrity above C7 is not required demonstrates that the central nervous system is not involved in RPCT. The dorsal root reflex at T9–10 likely activates spinal nerves at higher levels, leading to activation of the cardiac nerves, which mediate the cardioprotection. This is consistent with evidence that a dorsal root reflex can activate the dorsal horn neurons at higher levels of the spinal cord.45 Furthermore, it is known that action potentials can travel antidromically along the dorsal root and activate the sensory fibers innervating the heart.46,47

that sensory fibers in the heart activate the cardiac sympathetic nervous system,48 and that cardioprotection is elicited by activation of adrenergic neurons after spinal cord stimulation.49 To support the dorsal root reflex hypothesis, we performed retrograde nerve tracing studies with Dil to determine if the sensory nerves along the abdominal midline connect to the dorsal root at T9–10 and T1–5. We did see fluorescence at both levels (Figure 3E). These results are consistent with 2 interpretation: (1) that a dorsal root reflex activates sensory neurons innervating the heart and stimulates the cardiac sympathetic system as suggested and as supported by other studies; or (2) that a dorsal root reflex may directly activate preganglionic neurons in the lateral horn of the spine that activate the sympathetic nervous system. Future studies will test functionally the involvement of the dorsal root reflex in RPCT.

To summarize the neurogenic aspect of our findings, peripheral sensory nerve stimulation leads, via spinal nerves, to activation of the cardiac sensory nerves, which, in part through CGRP release, stimulate sympathetic nerves. Release of NE and BK from the sympathetic nerves underlies cardioprotective response in the myocardium after RPCT.

Signaling Pathway Underlying the Cardioprotection of RPCT

BK is both a hormone and a neurotransmitter, and it is secreted from sympathetic nerves in the heart.45,48 BK/BK2R has been shown to be an important mediator of remote IPC10 through inhibition of AP10,23 and is known to trigger NE release from cardiac sympathetic nerves.10,48 Release of substance P and CGRP from afferent nerves can act on sympathetic nerves to stimulate release of NE and BK, and both can act on cardiomyocytes, which possess both βAR and BK2R.17 Both of these G-coupled receptor systems have been shown to activate PKC in cardiomyocytes; particularly, PKCε is known to be an essential mediator of IPC.29 Our results demonstrate that PKCe and PKCδ are activated and preserved, respectively, and that PKCe is unaffected by a RPCT stimulus. PKCε is thought to work, at least in part, through repression of proapoptotic pathways including via activation of KATP channels.27,29 Conversely, PKCδ has been shown to be pro-cell death.30,31,49 Our results demonstrate that both the activation of PKCe and inhibition of PKCδ after RPCT are BK2R-dependent (Figure 5), and that the cardioprotection against MI of RPCT is PKC-dependent, BK2R-dependent (Figure 4), and that the action of the mitoKATP and perhaps the sarcaN channels (Figure 6) are required for RPCT. These results are consistent with a PKC-mediated cardioprotection elicited by neurogenic stimulation of cardiomyocytes after RPCT.

Our results, interpreted in the light of recent discoveries by others, cited herein, support a proposed mechanism of RPCT (Figure 8). We propose that nociceptive stimulation of sensory nerves in the skin of the abdomen triggers a neurogenic signal (initiator of RPCT) that is transmitted via nerve fibers and causes a dorsal root reflex that activates spinal nerves higher in the spinal cord, ultimately leading to activation of the cardiac sympathetic nervous system. Most likely (and supported by our results with CGRP blockade), the stimulation of cardiac sensory
nerves triggers the activation of the cardiac sympathetic system, which involves NE and BK release and activation of βAR and BK2R in myocardiun. Finally, the signal results in activation of PKCe and mitoKATP and inhibition of PKCδ, which together mediate cardioprotection against MI (mediators of RCT; Figure 8).

**Novel Cardioprotective Phenomenon**

The cardioprotective effect of RCT against MI is the most powerful noted to date in the mouse (80% decrease in infarct size). Although PKCe and PCKδ have been previously implicated in cardioprotection, the clear-cut activation of a protective isoform (PKCe) and repression of a presumably injurious isoform (PKCδ) are unique to RCT thus far. The ability of a nonischemic surgical stimulus to elicit this form of cardioprotection is completely novel. Although the mechanism may in some aspects be similar to cardioprotection afforded by remote IPC and spinal stimulation, the protection that we observe is much more powerful (80% reduction in infarct size compared to 45% reduction), is TNF-α-independent, and therefore is mechanistically different from IPC. As we demonstrate (Figure 7), remote nociceptive stimulation can be accomplished by chemical stimulation (capsaicin) of skin sensory nerves. This and the fact that spinal cord stimulation for angina was found to be effective and provided functional benefit support that this study is of high clinical relevance. Capsaicin is FDA-approved, inexpensive, widely available, and used topically to treat pain. Most importantly, topical capsaicin has no known serious adverse effects and could be easily applied in an ambulance or emergency room setting, well in advance of coronary reperfusion. If proven efficacious in humans, this simple therapy has the potential to reduce myocardial injury in the setting of I/R, thereby reducing the extent and consequences of acute MI.

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**Disclosures**

Drs Jones, Ren and Weintraub are coinvestors of provisional patent entitled “Methods of Preventing Ischemic Injury Using Peripheral Nociceptive Stimulation.”

**References**


**Supplemental Methods**

**Surgical Procedures**

Surgical procedures are described in detail in the manuscript. For all studies, the region-at-risk was not significantly different between groups (Table S1).

**Pharmacological Inhibitors**

Groups of mice were treated with the relatively selective BK2R-antagonist Hoe140 (50 µg/kg, i.v.)\(^{1-3}\) or with saline solution 15 min prior to abdominal incision (Fig. 1). Mice were subjected to I/R challenge (45 min coronary artery occlusion, 24h reperfusion) and infarct size was determined. The ganglionic blocker hexamethonium (Hex, 20mg/kg, iv.)\(^{1,4}\), \(K_{\text{ATP}}\) channel inhibitors: 5HD (100uM/kg, iv)\(^{5,6}\) and glibenclamide (Glib, 0.3mg/kg, iv)\(^{7}\) were administered 15 min prior to the RPCT stimulus (Fig. 1)\(^{5,7}\). Hex, Hoe140, 5HD and Glib were obtained from Sigma (St. Louis, MO). Glibenclamide was dissolved in DMSO and then diluted to 10% DMSO in saline. The calcitonin gene related peptide (CGRP) antagonist CGRP5-37 (3nmol/kg)\(^{8,9}\) was given by iv, 15 min prior to RPCT. Lidocaine (100 ml of 1%, in saline) was administrated by sub-cutaneous (subQ) injection at the abdominal level 5 min prior to skin incision, and saline injection was used as a control. Propanolol (2mg/kg)\(^{10,11}\) was administered by i.v. 15 min prior to RPCT. In all studies, vehicle controls were done with the specific vehicle required for each agent referenced above. For all of the abovementioned agents, the vehicle control was the same as the vehicle used for each drug. Glibenclamide, was 10% DMSO in saline, while for
all other agents, saline was used. Capsaicin (0.1 % cream, Chattem, Inc.; 150 µl for a 25g mouse) was administered topically to a 1.0 X 2.0 cm area, centered upon the umbilicus, using a mask. Capsaicin was applied 15 min before coronary occlusion, to mimic the time of the early RPCT stimulus (as in Fig. 1, protocol 2 for RPCT). Vehicle control was a topical gel without capsaicin.

**Morphological and Histological Assessments**

Hematoxylin and eosin staining were performed on wild type mice subjected to RPCT or to sham surgery (Fig. 1). The mice were euthanized 24 h after surgery and the hearts were perfused with PBS and then fixed by perfusion with 10% formalin in PBS. Hearts were post-fixed overnight, embedded in paraffin, sectioned, stained and examined microscopically. *In situ* DNA fragmentation was assessed using the DeadEnd™ Fluorometric TUNEL system (Promega, Madison, WI), followed by staining with an anti-sarcomeric actins antibody (Sigma, St. Louis, MO) and DAPI (Nitrogen, South San Francisco, CA) \(^{12,13}\). TUNEL-positive (green) nuclei were counted from 10 randomly chosen microscopic fields of the biventricular section \((n=5)\) and were expressed as a percentage of total nuclei (both blue and green staining nuclei; approximately 400 nuclei counted per field) in the same fields. For independent quantitative measure of apoptosis in the hearts after I/R (with and without RPCT 15 min prior to I/R), DNA fragmentation was determined by a cell-death-detection ELISA kit (Roche Applied Science, Indianapolis, IN), which measures the content of cryptozoic mono- and oligo-nucleosomes (180 base pair nucleotides or multiples) by employing the sandwich enzyme immunoassay technique. Results were normalized to the standard provided in the kit and expressed as a fold-increase over control \(^{14,15}\).
Cell Fractionation and Immunoblotting

Mice were euthanized 15 min after the RPCT stimulus, the heart excised and LV samples (~ 0.5g) flash-frozen in liquid nitrogen until used. Frozen tissues were powdered at liquid nitrogen temperature and homogenized in buffer A (5 mM TRIS, 4 mM EGTA, 2 mM EDTA, 5 mM dithothreitol, 1 mM phenylmethyl sulfonylefluoride and EDTA-free protease inhibitor [1 tablet/10 ml]). The homogenate was centrifuged at 100,000 X g for 30 minutes at 4°C and the supernatant was used as the cytosolic fraction. The pellet was re-homogenized with homogenization buffer A containing 1% Triton X, incubated on ice for 20 minutes and then centrifuged at 100,000X g for 30 minutes at 4°C. The new supernatant was used as the membrane-associated fraction. Protein concentration was determined via a Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Cytosolic and membrane-associated fractions (50mg) were electrophoresed on a 10% SDS-PAGE gel. After transfer to membranes, immunoblotting analysis was performed with PKC isoform-specific primary antibodies against PKCa, PKCe and PKCd (Cell Signaling, Boston, MA). The membrane was next incubated with HRP-conjugated secondary mouse (Bio-Rad, Hercules, CA) or rabbit (Santa Cruz, Santa Cruz, CA) antibody. Membranes were developed using ECL-PLUS Western Blotting Detection kit (Amersham Pharmacia Biotech, Piscataway, NJ), and densitometry of protein bands was quantitated using a Fluorchem 8800 gel imager. Transfer efficiency and loading equality were examined by staining the membrane with 0.1% Ponceau S in 5% acetic acid.
Stained membranes were digitally scanned and densitometric analysis of major bands used to normalize signal intensity for quantitative analysis as previously described\(^\text{16, 17}\).

**Cardiac Function Measurement in vivo**

Mice were anesthetized with sodium pentobarbital (90 mg/kg i.p.) as described\(^\text{15, 18}\). As previously described\(^\text{19, 20}\), tracheotomy was performed with a short length of PE-90 connected to a mouse miniventilator. For the measurement of myocardial function, the right carotid artery was dissected and cannulated with a 1.4-Fr Millar MIKRO-TIP transducer (SPR-407, Millar Instruments, Houston, TX) after the mouse had been intubated, and before the chest was opened. The tip of the transducer was advanced through the ascending aorta and into the left ventricle under constant monitoring of the blood pressure wave. The transducer was then anchored in place with 7-0 sutures. The mice were allowed to stabilize for 10 min before the abdominal incision. 15 min after abdominal incision, the coronary artery was occluded, as described above. In total, the mice were subjected to 45 min of ischemia and 45 min reperfusion. Pressure signals were continuously collected and analyzed on a Macintosh G4 microcomputer, using PowerLab software (AD Instruments, Colorado Springs, CO)\(^\text{19, 20}\). Heart rate, left ventricular pressure (LVP), maximal and minimal change in ventricular pressure over time (dP/dt\(_{\text{max}}\) and dP/dt\(_{\text{min}}\)) were continuously monitored to evaluate myocardial contractile function pre-ischemia (defined as baseline level) and during ischemic as well as during the post-ischemic recovery period. Data was analyzed for timepoints selected at 1 or 2 min intervals (1 min intervals bracketing the time of coronary occlusion and the time of reperfusion). For each time point, the mean maximal and minimal dP/dt were compared.
between groups. 8 wild type mice were employed for the functional studies, sham control group (n=4) and early RPCT group (n=4). The data was plotted as average dP/dt_{max} or dP/dt_{min}, normalized to pre-ischemic levels.

**Non-Ischemic Remote Postconditioning**

We examined whether a remote non-ischemic stimulation (abdominal incision) administered at the time of reperfusion is as protective as the early phase of RPCT. Instead of making the incision prior to coronary occlusion, in these experiments, mice were subjected to 45-min coronary occlusion and the abdominal incision was administered 15 min prior to reperfusion. Infarct size was determined after 24 hours reperfusion.

**Results**

**RPCT elicits a transient myocardial stunning-like effect that is protective against I/R-induced LV dysfunction**

In order to determine whether RPCT affects ventricular function, we performed continuous LV pressure measurement using an indwelling catheter beginning at 15 min prior to RPCT, through ischemia (45 min coronary occlusion), and for 45 min after reperfusion. We observed that RPCT caused an immediate 33% reduction of dP/dt_{max}, dP/dt_{min}, and a significant reduction in heart rate (data not shown). However, unlike stunning, both effects were extremely transient, reversing completely in the first few minutes of the 45 min ischemic period. Upon initiation of ischemia in sham treated mice,
there was a decrease in dP/dt\textsubscript{max} and an increase in dP/dt\textsubscript{min}. The recovery of function during the ischemic phase likely reflects compensation by the non-ischemic portion of the LV. There is an additional decrease in function at reperfusion, which was larger in the RPCT group. However, after RPCT, the function improved much more rapidly in the RPCT group relative to the sham. The values of dP/dt\textsubscript{max} in the RPCT-treated hearts recovered to the pre-ischemic baseline within 5 min, relative to 20 min for shams. The values of dP/dt\textsubscript{min} recovered to pre-ischemic baseline at 13 min post-reperfusion in the RPCT group while they never recovered to baseline in shams before the end of the 45 min reperfusion. Overall, RPCT resulted in significantly improved functional recovery (both systolic and diastolic) after I/R (Fig. 1S).

Remote non-ischemic postconditioning is as protective as early RPCT

We found that remote postconditioning of trauma is as protective as the early phase of RPCT. The infarct size was significantly different between the incision group (6.3±2.2%) and control group (52.6±2.8%, *P≤0.05, n=7) (Figure 2).

Discussion

The effect of RPCT on I/R-induced cardiac injury Interestingly, the RPCT stimulus, abdominal incision, elicits an immediate, though transient (minutes) reduction of cardiac contractility and relaxation. However, when I/R is initiated following RPCT, the prior (RPCT) stimulus reduces ischemia-dependent ventricular dysfunction (Fig. 1S).
Moreover, hearts from mice subjected to RPCT demonstrate better functional recovery after reperfusion compared to sham controls (Fig. 1S). Thus, although RPCT causes a transient stunning-like effect upon ventricular function, RPCT blunts the effects of I/R-induced injury on myocardial function relative to pre-ischemic levels.

Since RPCT elicits transient functional depression, it may have something in common with myocardial stunning\textsuperscript{21, 22}. Stunning is a condition of reduced myocardial function that is reversible. By the fact of its reversibility, stunning is associated with survival of myocardial cells during ischemia and/or I/R\textsuperscript{21}. This is similar to our results that RPCT elicits strong cardioprotective effects against apoptotic cell death and infarction post-I/R injury (Fig. 2). Some evidence suggests that chronic hypoperfusion associated with the onset of ischemia results in an imbalance between metabolic demand and supply of oxygen that creates depressed contractility and is associated with enhanced survival of cells, likely involving cellular adaptation and metabolic adjustment\textsuperscript{23}. Indeed we have observed shifts in the expression levels of metabolic proteins after RPCT (data not shown) and the transiently reduced heart rate could be an effect of BK signaling in the heart, resulting in transient hypoperfusion and reduced function. Future studies will investigate the mechanisms of these functional effects after RPCT but these are beyond the scope of the present study.

**Remote non-ischemic postconditioning**

To determine whether the nociception-inducible cardioprotection can be elicited by nociception as a postconditioning stimulus, we performed abdominal incision 30 min into
a 45-min coronary occlusion. Infarct size was measured 24 h after reperfusion and we observed a significant RPCT-like effect (Fig. S2). Importantly, the effect was as strong as that of early RPCT. This result supports that nociceptive stimulation can activate a powerful postconditioning effect, and along with evidence that cardioprotection can be elicited by chemically induced activation of peripheral nociception, supports the clinical potential of the results of this study.

**PKC and $K_{ATP}$ channel results**

PKC is known to elicit cardioprotection via activation of $K_{ATP}$ channels \(^4,24\). Therefore, we investigated the role of the $K_{ATP}$ channels in cardioprotection after RPCT using the inhibitors glibenclamide and 5HD. While glibenclamide is a non-selective $K_{ATP}$ channel antagonist, 5HD is relatively selective for the mito$K_{ATP}$ \(^6\). The cardioprotective effect of RPCT against MI is completely abrogated by 5HD, and there is significant reduction of protection after treatment with glibenclamide. These results suggest involvement of the mito$K_{ATP}$ channel, but do not rule out action of the sarcoplasmic $K_{ATP}$ (sar$K_{ATP}$). Our results are consistent with PKC$\varepsilon$ activation of mito$K_{ATP}$ as part of the mechanism of cardioprotection in the myocardium after RPCT.

Though Hassouna et al. \(^24\) found activation of PKC$\alpha$ downstream of mito$K_{ATP}$, we find no RPCT-associated change in PKC$\alpha$ activation and no association between PKC$\alpha$ activity and cardioprotection. Taken together with evidence that PKC$\varepsilon$ and PKC$\alpha$ activation may be flipped relative to mito$K_{ATP}$ in H$_2$S-induced cardioprotection, that is, PKC$\alpha$ upstream and PKC$\varepsilon$ downstream of mito$K_{ATP}$ \(^25\), this suggests that different
cardioprotective phenomena may act through PKCs though the detailed architecture of the signaling cascades vary with stimulus.\(^7\)

**Supplemental Tables, Figures and Figure Legends**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Procedure</th>
<th>Infarct/LV</th>
<th>Risk/LV</th>
<th>Infarct/Risk</th>
<th>(P) versus control (Risk/LV)</th>
<th>(P) versus control Infarct/LV</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A</td>
<td>RPCT</td>
<td>6.3 ± 3.7</td>
<td>57.2 ± 5.8</td>
<td>10.2 ± 6.3</td>
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<td></td>
<td>Sham</td>
<td>38.8 ± 3.2</td>
<td>55.5 ± 3.7</td>
<td>55.3 ± 3.4</td>
<td>Control for RPCT</td>
<td>Control for RPCT</td>
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<tr>
<td>7</td>
<td>Capsaicin</td>
<td>5.4 ± 3.1</td>
<td>58.7 ± 5.4</td>
<td>7.48 ± 2.0</td>
<td>0.616</td>
<td>(P=0.05)</td>
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<tr>
<td></td>
<td>Sham</td>
<td>33.9 ± 1.9</td>
<td>59.1 ± 2.5</td>
<td>51.7 ± 1.5</td>
<td>Control Capsaicin</td>
<td>Control Capsaicin</td>
</tr>
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Data presented as mean ± SE. The final two columns present results of t-tests comparing the size of the risk region and the normalized infarct size between each experimental and the appropriate control group. There were no significant differences in size of the risk region in experimental versus control groups. Abbreviations: Infarct/LV = mean area of the infarct divided by mean area of the left ventricle; Risk/LV = mean area of the risk region divided by mean area of the left ventricle; Infarct/Risk = mean area of the infarct divided by the mean area of the risk region; \(P\) versus control; the \(P\) value for the comparison between the indicated group and its control group (see Statistical Methods).

**Figure 1.** RPCT improves cardiac functional recovery during ischemia/reperfusion. Maximal rates of contractility (+dP/dt, A) and minimal rates of relaxation (-dP/dt, B) are expressed as a percentage of pre-ischemic values. \((n = 4, \*P \leq 0.05\) vs. no-RPCT controls, starting point is 1.0 = 100%).

**Figure 2.** Testing whether a remote non-ischemic postconditioning stimulus is as protective as the early phase of RPCT. We performed an experiment using an
abdominal incision administered during the last 15 min of the 45-min coronary occlusion. Thus, the nociceptive stimulus occurs just before reperfusion. The infarct size was reduced in mice subjected to postconditioning relative to sham control (*P ≤0.05 vs. control, n=7).

Supplemental References


