Myocardial preconditioning factors evoke mesenteric ischemic tolerance via opioid receptors and $K_{\text{ATP}}$ channels

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Dickson, Eric W., Robert J. Tubbs, William A. Porcaro, Won Jae Lee, David J. Blehar, Robert E. Carraway, Chad E. Darling, and Karin Przyklenk. Myocardial preconditioning factors evoke mesenteric ischemic tolerance via opioid receptors and $K_{\text{ATP}}$ channels. Am J Physiol Heart Circ Physiol 283: H22–H28, 2002. First published February 14, 2002; 10.1152/ajpheart.01055.2001.—We have shown that a reverse-phase concentrate generated from the effluent of preconditioned (PC) rabbit hearts evokes a cardioprotective effect in virgin acceptor hearts. With the use of a model of sustained (1 h) simulated ischemia in isolated, spontaneously contracting rabbit jejunum, our current aims were to 1) determine whether protective factor(s) released from PC hearts can improve ischemic tolerance in noncardiac tissue; and 2) obtain preliminary insight into the mediator(s) involved in triggering and eliciting this remote protection. Recovery of contractile force following reoxygenation (our index of ischemic tolerance) was enhanced in jejunal segments pretreated with concentrate generated from PC hearts (33 ± 3% of baseline, $P < 0.01$) versus segments that received no concentrate (21 ± 2%) and segments treated with concentrate from normoxic hearts (16 ± 3%; $P < 0.01$). Protection achieved with PC concentrate was attenuated by coadministration of naloxone or glibenclamide, thereby implicating the involvement of opioids and ATP-sensitive potassium channels. Moreover, evaluation of purified subfractions of the crude PC concentrate identified a specific bioactive fraction that may participate in triggering the improved jejunal ischemic tolerance.

ischemia-reperfusion; ischemia; myocardium

MYOCARDIUM EXPOSED TO BRIEF ISCHEMIA acquires an increased resistance to future prolonged ischemic insults by a process referred to as ischemic preconditioning (PC) (18). The phenomenon of PC is not limited to myocardium, but rather has been documented in many other tissues, including the small intestine (15), brain, (1, 2), kidney (28), and even in intact animals (23). The precise mechanisms responsible for PC-induced protection have not yet been fully elucidated but appear to involve the release of several paracrine/autocrine factors, including adenosine, norepinephrine, bradykinin, and unidentified opioids (22). Acting independently and/or synergistically, these substance bind cell surface receptors, purportedly leading to the activation and translocation of protein kinase C (reviewed in Refs. 22 and 26) and, ultimately, to the opening of mitochondrial and/or sarcolemmal ATP-sensitive potassium (K$_{\text{ATP}}$) channels (14).

The first evidence that the protective effects of preconditioning extend beyond the site of the initial PC stimulus was presented by Przyklenk et al. (21), who found that brief episodes of ischemia in one coronary bed rendered remote, virgin myocardium resistance to infarction. Remote PC has similarly been described in other organs, including the skeletal muscle and brain, with intermittent ischemia of one pectoris muscle (17) or brain hemisphere (2) reportedly preconditioning the contralateral side. Other investigators have further extended this concept of “PC at a distance” and have shown that intermittent ischemia of noncardiac tissue, including mesenteric (13), kidney (13, 20), and skeletal muscle (3), can also induce improved ischemic tolerance in the heart. Although the mediator(s) of this protective stimulus, presumably “communicated” from one organ or vascular bed to a remote site, remains to be elucidated, both neuronal (13, 24) and humoral (8, 19) factors have been implicated.

Previous work from our laboratory has focused on a paradigm of PC at a distance evoked via collection and transfer of whole blood (8), coronary effluent (6, 7), or hydrophobic constituents of coronary effluent (5) from preconditioned rabbit hearts to virgin acceptor hearts. Indeed, we found that the acceptor cohorts exhibited an increased resistance to infarction (5, 6, 8), elicited at least in part by opioid receptor activation (5). Our current aims were to 1) establish whether the concentrate generated from PC rabbit hearts, previously shown to be cardioprotective, can similarly improve
ischemic tolerance in a noncardiac (i.e., mesenteric) tissue known to be well supplied with opioid receptors; and 2) obtain preliminary insight into the mediator(s) involved in triggering and eliciting this remote protection. Using a model of simulated ischemia in isolated, spontaneously contracting rabbit jejunum, we report significant protection, assessed by postischemic recovery of contractile performance, in mesenteric tissue pretreated with concentrate generated from PC myocardium. Evidence obtained by coadministration of the pharmacological antagonists naltrexone or glibenclamide implicates the involvement of two classic preconditioning pathways (opioid receptor stimulation and KATP channel activation) in jejunal protection. Finally, preliminary evaluations of purified subfractions of the crude PC concentrate suggest that a specific bioactive fraction released during brief myocardial ischemia-reperfusion may participate in triggering the improved jejunal ischemic tolerance.

METHODS

These experiments were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and were in accordance with National Institutes of Health Guide for the Use of Laboratory Animals (NIH Publication, Vol. 25, 1996).

Protocol 1: Transfer of Precipitate from Heart to Mesenteric Tissue

New Zealand White rabbits (1–1.5 kg, 6–8 wk of age; n = 40) were anesthetized with a single intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg) and ventilated by mask with 100% oxygen. As described in detail below, the hearts were excised and buffer perfused for the collection of coronary effluent, while jejunal segments were harvested and subjected to simulated ischemia-reperfusion.

Isolated buffer-perfused heart preparation. Immediately upon excision, the hearts were submersed in chilled physiological saline solution (PSS) consisting of the following (in mM): 118 NaCl, 4.7 KCl, 24 NaHCO3, 1.2 KH2PO4, 1.2 MgSO4·7H2O, 11 glucose, and 2.5 CaCl2 anhydrous and placed on a modified Langendorff apparatus. PSS equilibrated with 95% O2-5% CO2 was warmed to 38°C and delivered by retrograde perfusion at a constant pressure of 80 mmHg. The hearts were paced at 210 beats/min via electrodes applied to the right ventricle (Grass Stimulator; Quincy, MA), and heart temperature was maintained at 37–38°C.

Reverse-phase concentration of coronary effluent. After stabilization, each heart received either 50 min of normoxic perfusion (95% O2-5% CO2) or five 5-min episodes of PC ischemia interspersed with 10 min of reperfusion. Coronary effluent released from the hearts was collected on ice and maintained at 10°C throughout the preparation. The methods used to generate concentrate from the coronary effluent have been reported previously (5). Briefly, the effluent was applied to a hydrophobic matrix (Sep-Pak C-18; Waters Milford, MA) by roller pump. The cartridge was desalted with distilled water and adsorbed material eluted from the cartridge with 80% acetonitrile. The acetonitrile solution was flash frozen with liquid nitrogen and lyophilized, and the resultant material (PC or normoxic concentrate) was stored at −40°C until use.

Simulated ischemia-reperfusion in the isolated buffer-bathed jejunal preparation. The jejunum (proximal end 2 times the stomach length after the gastroduodenal junction and ending 3 stomach lengths thereafter) was removed and immediately submersed and irrigated (intraluminal) with chilled PSS. As described previously (30), jejunal segments 3 cm in length (−4 per animal) were cut and tied at both ends to prevent leakage of mucosal secretions into the bathing fluid. Each segment was then placed in a separate 25-ml tissue bath (Harvard Apparatus; Holliston, MA) containing PSS maintained at 38°C and equilibrated with 95% O2-5% CO2. Each segment was connected to a force-displacement transducer and recorder (Grass Instruments; Quincy, MA). A basal tension of 2.5 g was applied and generated force recorded continuously. Segments without spontaneous contractile activity were removed before randomization. All segments were stabilized for 20 min before experimentation.

After measurement of baseline parameters and a 20-min intervention period (both described in Treatment groups), all jejunal segments were subjected to 1 h of simulated ischemia followed by 30 min of reoxygenation (Fig. 1). Simulated ischemia was achieved by replacing the PSS with glucose-and oxygen-free medium (in mM: 129 NaCl, 4.7 KCl, 24 NaHCO3, 1.2 KH2PO4, 1.2 MgSO4·7H2O, 11 glucose, and 2.5 CaCl2 anhydrous, continuously equilibrated with 95% N2-5% CO2). To further deplete energy stores, acetylcholine (final concentration: 1 μM) was added to the buffer at the beginning of ischemia and at 30 min into the ischemic insult. Reoxygenation was accomplished by replacing the glucose- and oxygen-free buffer with standard PSS.

Treatment groups. Before the onset of simulated ischemia, each segment was randomly assigned to receive 1 of 7 treatments: 1) PC concentrate (n = 25 segments); 2) normoxic concentrate (n = 8); 3) no concentrate (control: n = 44); 4) the nonspecific opioid receptor antagonist naltrexone (5 μM,
Sigma; St. Louis, MO) + PC concentrate (n = 10); 5) nalone (5 \mu M, n = 7); 6) the K\textsubscript{ATP} channel antagonist glibenclamide (5 \mu M Sigma) + PC concentrate (n = 7); or 7) glibenclamide (5 \mu M, n = 7). For groups treated with normoxic or PC concentrates, the lyophilized material was reconstituted in fresh buffer and added to the tissue bath at 15 min preischemia. All concentrate was administered within 2 days after being prepared, with concentrate generated from one normoxic-PC heart used to treat two jejunal segments. In groups randomized to receive nalone or glibenclamide, the agent was added to the tissue bath at 5 min before the administration of the normoxic PC concentrate or at the corresponding time point (20 min preischemia) in the drug-treated control groups. Doses of both agents were within the micromolar range used previously in isolated buffer-perfused heart preparations (5, 16). For glibenclamide, the agent was initially dissolved in dimethyl sulfoxide (DMSO, 12.5 mM), and 0.01 ml of the stock solution was added to the bath, yielding final concentrations of 5 \mu M of the antagonist and 0.04 vol% of DMSO. The vehicle for nalone was PSS. Control samples that did not receive concentrate were exposed to either PSS alone (n = 36) or PSS containing 0.04 vol% DMSO (n = 8) for a matched, 15-min time period. Of note, the PC-normoxic concentrate and pharmacological test agents were only present in the bath before, not during, simulated ischemia.

Data collection. Contractile performance following relief of sustained ischemia has been shown to be inversely related to the extent of jejunal necrosis in this model (30). Thus our primary end point was the repeated measurement of contractile force assessed by transient (1 min) exposure of the isolated jejunal segments to 1 \mu M acetylcholine (Fig. 1). Data were obtained at baseline, immediately preischemia (at the end of the 20-min intervention phase), and at 10, 20, and 30 min following reoxygenation, with all values normalized and expressed, for each segment as a percentage of the baseline response. In addition, because previous studies from our group have established that recovery of maximal contractile force remains approximately stable throughout the 30 min of reperfusion (30) (Fig. 1), the three measurements obtained after oxygenation were averaged and reported as a single, aggregate value.

Protocol 2: Evaluation of Purified Subfractions of Crude PC Concentrate

Having established the existence of a jejunal-protective factor(s) in the myocardial PC concentrate, our secondary aim was to obtain preliminary insight into the characteristics of the protective component(s) by generating and evaluating purified subfractions of the crude concentrate.

Preparation of subfractions. With the use of the same methods described for protocol 1, an additional eight rabbits were anesthetized. The hearts were rapidly excised, mounted on the Langendorff apparatus, and, after stabilization, subjected to PC ischemia. Coronary effluent was collected and crude concentrate was prepared. Purified fractions were generated by high-performance liquid chromatography (HPLC, Waters system; Milford, MA) with reverse-phase separations accomplished using a (7.8 × 100 mm) \mu-Bondapak C-18 column. The PC concentrate was applied to the column in 10 ml of trifluoroacetic acid (0.1%) followed by a linear gradient of acetonitrile from 0% to 75% over 40 min. Three specific fractions, with retention times of 15–20 min (fraction 1), 20–25 min (fraction 2), and 25–30 min (fraction 3) were assayed in the jejunal ischemia-reperfusion model based on initial screening, which revealed jejunal contractile effects (bioactivity).

Treatment groups. Isolated jejunal segments (~4 per animal) were prepared as in protocol 1 and were subjected to the standard 1 h of simulated ischemia followed by 30 min of reoxygenation. Segments were pretreated for 15 min with purified fraction 1, 2, or 3 (n = 8, 10, and 8 per group, respectively), with each jejunal segment receiving 50% of the HPLC fraction generated from a single heart.

Data collection. Maximum contractile force in response to acetylcholine challenge was measured and quantified as in protocol 1.

Statistical Analysis

For protocols 1 and 2, percent maximum contractile force was compared among groups by two-way ANOVA (for treatment and time) with repeated measures and, if significant F values were obtained, subsequent pairwise comparisons were made using the Newman-Keuls test. All data are expressed as means ± SE, and P values < 0.05 were considered statistically significant.

RESULTS

Protocol 1

Baseline contractile force for all jejunal segments enrolled in protocol 1 averaged 7.4 ± 0.3 g, with no significant differences among the seven groups. Treatment with either normoxic or PC concentrate was generally associated with a brief (~1 min) and modest (~25% of baseline) increase in spontaneous jejunal contraction (Fig. 2). This physiological effect was transient such that at the end of the intervention period (before the onset of sustained ischemia), maximal contractile force remained unchanged from baseline values and did not differ significantly among groups (Fig. 3A).

In control segments, maximum contractile force following relief of ischemia recovered to a mean of only 21 ± 2% of baseline values (Fig. 3B), with no difference in response seen in the cohort that received PSS alone (20 ± 3%) versus the subset exposed to 0.04 vol% DMSO (23 ± 6%). Administration of normoxic concentrate had no effect on posts ischemic contractile performance, with the maximal contractile response to ace-

![Fig. 2. Original recording of contractile force from an isolated jejunal segment treated with crude concentrate from a preconditioned (PC) heart.](http://ajpheart.physiology.org/)
tylcholine averaging 16 ± 3% of baseline. In contrast, pretreatment with PC concentrate improved ischemic tolerance of the isolated jejunal segments; maximum contractile force following reoxygenation was 33 ± 3%, significantly greater ($P < 0.01$) than the results obtained in both control samples and those that received normoxic concentrate. Neither naloxone nor glibenclamide affected recovery of contractile performance in control segments. However, the beneficial effect of PC concentrate was attenuated by coadministration of either antagonist (Fig. 3B).

Protocol 2

Baseline maximal contractile force of all segments utilized in protocol 2 was comparable to that observed in protocol 1 (mean of 7.4 ± 0.5 g), with no significant differences among the three study cohorts.

In contrast to the modest physiological response evoked by administration of crude PC concentrate (Fig. 2), two of the three fractions purified by HPLC elicited robust and divergent alterations in spontaneous contraction. Specifically, treatment with fraction 1 (retention time of 15–20 min) resulted in at least a 50% decrease in developed force (Fig. 4A), whereas fraction 3 (retention time of 25–30 min) was associated with greater than a 50% increase in contractile force of jejunal segments (Fig. 4B). These physiological effects were, however, nonsustained (duration of ~2–3 min, Fig. 4) and, at the end of the 15-min treatment period, maximum contractile force in response to acetylcholine did not differ significantly from that observed at baseline (Fig. 5A). Treatment with fraction 2 (retention time of 20–25 min) had no discernible physiological effect (data not shown) and was incorporated as a “nonactive” control fraction.

Contractile performance in isolated jejunal segments pretreated with fractions 2 and 3 recovered to only 23% of baseline during the 30 min of reoxygenation (Fig. 5B), similar to the value of 21% observed for control segments.
segments in protocol 1. However, administration of fraction 1, the bioactive component of the crude PC concentrate that eluted at 15–20 min and elicited a transient depression in contractile force, evoked a significant improvement in ischemic tolerance, with maximum contractile force following relief of ischemia averaging 35 ± 5% of baseline (P < 0.05 vs. pooled data from fractions 2 and 3).

**DISCUSSION**

In this study, we make the novel observation that administration of a reverse-phase concentrate generated from the coronary effluent of preconditioned rabbit hearts significantly improves the ischemic tolerance of isolated, spontaneously contracting rabbit jejunal segments. We further report that two classic pathways implicated to play a role in myocardial ischemic PC, opioid receptor stimulation and KATP channel activation, also participate in evoking this transferred protection in mesenteric tissue.

**Increased Ischemic Tolerance in Isolated Jejunum**

It is well established that the intestine, like the heart, is susceptible to ischemia-induced injury (15, 30) and that this susceptibility is attenuated by ischemic PC (15). It has also been shown that communication of a remote PC response between mesenteric and myocardial tissue occurs (13), and thus this represents an appropriate target in which to evaluate the efficacy of transferred protection. Our specific choice of a 1-h sustained ischemic insult in the isolated jejunum, achieved by incubation in oxygen- and glucose-free medium, was based on previous studies by our group aimed at characterizing the temporal profile of ischemic injury in this preparation: shorter durations of ischemia (15 and 30 min) elicited minimal ischemic damage, whereas a more prolonged, 2-h ischemic episode resulted in near-total necrosis of the isolated jejunal segments. Moreover, concomitant oxygen and glucose deprivation, rather than either insult alone, was required to achieve significant injury in this model (30). Our previous experiments further demonstrated an expected, inverse correlation between maximal contractile force following relief of ischemia and “infarct size” (i.e., the extent of smooth muscle necrosis) in isolated jejunal segments (30), thereby providing the rationale for the use of recovery of contractile performance as our index of ischemic tolerance in this model. Furthermore, the spontaneously contracting jejunum preparation employed also provided physiological (contractile) information on purified fractions, potentially providing greater insight as to the identity of the protective factor(s).

We have previously shown that crude concentrate generated from the coronary effluent of PC rabbit hearts elicits significant cardioprotection when administered to virgin acceptor hearts, comparable in magnitude to that achieved with conventional PC ischemia (5). Our current results establish that this cardioprotective concentrate similarly evokes an improved ischemic tolerance in mesenteric tissue. The efficacy of protection achieved in isolated jejunal segments by transfer of crude PC concentrate is, arguably, modest, with postischemic contractile performance recovering to 33% versus 21% of baseline in PC concentrate-treated versus control samples, respectively. Although greater protection may, in theory, be achieved by modifying the cardiac PC protocol, the “dose” of PC concentrate delivered to each segment, the duration of the intervention period, and/or the length of the sustained ischemic challenge, we nonetheless demonstrate the successful transfer of a protective factor(s) from preconditioned heart to noncardiac tissue.

**Role of Opioid Receptors and KATP Channels**

One obvious question arising from these data is whether similar signaling pathways are involved in both conventional, PC-induced cardioprotection and the protection seen with transfer of PC concentrate from heart to the isolated jejunal segments. Among the host of mechanisms under investigation in the setting of myocardial PC, we chose, for two reasons, to focus on the potential role of opioid receptor stimulation. First, results obtained in our rabbit heart model of transferred protection imply that at least two opioids, Met and Leu-enkephalin, are present in the crude PC con-
centrate and, moreover, opioid receptor activation contributes to its cardioprotective effect (5). Second, the rabbit jejunum is rich in opioid receptors, including the δ-, κ-, and µ-subtypes (31) and, indeed, pharmacological evidence obtained using d-Ala2-d-Leu5 enkephalin further suggests that stimulation of opioid receptors, specifically, the δ-subtype, evokes a significant increase in ischemic tolerance in this preparation (30). Finally, from the overwhelming interest in the role of the mitochondrial and/or sarcolemmal KATP channel as either an end-effector and/or proximal signaling element in myocardial ischemic PC (26, 29), we further elected, as a second intervention, to explore the effects of glibenclamide on transferred protection. We found that coadministration of either naloxone or glibenclamide attenuated, but did not abrogate, the protective effects of crude PC concentrate on the isolated jejunal segments; i.e., recovery of contractile force of glibenclamide on transferred protection.

Although the current study does not yield a definitive answer, the results of protocol 2 may provide preliminary insight into the characteristics of the protective factor(s). Specifically, we found that a purified and highly bioactive subfraction of PC concentrate, isolated by HPLC with a retention time of 15–20 min and termed “fraction 1,” evoked an improvement in ischemic tolerance of isolated jejunal segments comparable to that obtained with the crude precipitate. The profound but transient depression in contractile force associated with administration of this fraction is consistent with the presence of one or more opioids, a concept supported by our finding that Met-enkephalin, a suggested mediator of myocardial preconditioning (27), has an elution time similar to fraction 1 (18–19 min) in the our described HPLC system. However, unlike Met-enkephalin, the depressive effects of fraction 1 are partially, but not fully, blocked by naloxone (unpublished data). Additional candidate compounds stemming from recent pilot studies include lipoxygenase metabolites, because we have noted that the depressive effect of fraction 1 is lost when the concentrate is generated from preconditioned rabbit hearts treated with the nonspecific lipoxygenase inhibitor nordihydroguaiaretic acid (2 μM; Cayman Laboratories). In this regard, it is interesting to note that metabolites of the lipoxygenase pathway (specifically, 12-hydroperoxyeicosatetraenoic acid) have been implicated to play a role in myocardial ischemic preconditioning (4). Definitive identification of the arachidonic acid metabolite(s) present in fraction 1 and, more importantly, their specific role in triggering transferred protection, either alone or in synergy with opioids and/or other factors, is the focus of intensive ongoing study in our laboratory.

Conclusions and Future Directions

Our results reveal that administration of a reverse-phase concentrate generated from the coronary effluent of preconditioned rabbit hearts evokes significant protection in noncardiac (mesenteric tissue) mediated at least in part by opioid receptor stimulation and KATP channel activation. Detailed characterization of the mediators and signaling pathways that participate in this transferred protection, including, among other issues, resolution of the precise opioid receptor subtypes and specific involvement of mitochondrial versus sarcolemmal KATP channels, await further investigation. Finally, although preliminary evidence suggests that a specific bioactive fraction of the crude PC concentrate, possibly containing opioids and or lipoxygenase metabolites, may serve to trigger the improved ischemic tolerance, the identity of the protective trigger(s) is, at present, unknown.

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REFERENCES

H28 PRECONDITIONING TRANSFERS FROM MYOCARDIUM TO MESENTERY


