Exercise enhances myocardial ischemic tolerance via an opioid receptor-dependent mechanism

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Exercise enhances myocardial ischemic tolerance via an opioid receptor-dependent mechanism. Am J Physiol Heart Circ Physiol 294: H402–H408, 2008. First published October 19, 2007; doi:10.1152/ajpheart.00280.2007.—Exercise enhances myocardial ischemic tolerance via an opioid receptor-dependent mechanism. Am J Physiol Heart Circ Physiol 294: H402–H408, 2008. First published October 19, 2007; doi:10.1152/ajpheart.00280.2007.—Exercise increases serum opioid levels and improves cardiovascular health. Here we tested the hypothesis that opioids contribute to the acute cardioprotective effects of exercise using a rat model of exercise-induced cardioprotection. For the standard protocol, rats were randomized to 4 days of treadmill training and 1 day of vigorous exercise (day 5), or to a sham exercise control group. On day 6, animals were killed, and global myocardial ischemic tolerance was assessed on a modified Langendorff apparatus. Twenty minutes of ischemia followed by 3 h of reperfusion resulted in a mean infarct size of 42 ± 4% in hearts from sham exercise controls and 21 ± 3% (P < 0.001) in the exercised group. The cardioprotective effects of exercise were gone by 5 days after the final exercise period. To determine the role of opioid receptors in exercise-induced cardioprotection, rats were exercised according to the standard protocol; however, just before exercise on days 4 and 5, rats were injected subcutaneously with 10 mg/kg of the opioid receptor antagonist naltrexone. Similar injections were performed in the sham exercise control group. Naltrexone had no significant effect on baseline myocardial ischemic tolerance in controls (infarct size 43 ± 4%). In contrast, naltrexone treatment completely blocked the cardioprotective effect of exercise (infarct size 40 ± 5%). Exercise was also associated with an early increase in myocardial mRNA levels for several opioid system genes and with sustained changes in a number of genes that regulate inflammation and apoptosis. These findings demonstrate that the acute cardioprotective effects of exercise are mediated, at least in part, through opioid receptor-dependent mechanisms that may include changes in gene expression.

Like exercise, brief tissue ischemia has an acute cardioprotective effect, known as ischemic preconditioning (IPC). Several mediators of IPC have been identified, among them the endogenous opioids (3, 13, 15, 29, 40, 45). In contrast, little is known about the mediators of exercise-induced preconditioning. Our group and others have shown that natural or synthetic opiates can evoke preconditioning in cultured cardiac myocytes (45), isolated hearts (3, 41), isolated jejunal (47), and intact animals (34). Furthermore, several groups have reported that endogenous opioids (known to reduce pain, regulate the immune system, and modulate cardiac function) are released during exercise (23, 35, 43). Together, these data suggest that opioids released during exercise might contribute to its cardioprotective effects. To test this hypothesis, we used a rat model of exercise-induced cardioprotection and a broadly effective pharmacological opioid receptor antagonist. We also examined the effect of exercise on expression of a number of opioid system components and on an array of genes related to inflammation and apoptosis.

METHODS

Animals. Animals were maintained and killed in accordance with the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animals Resources (National Institutes of Health Publication, Vol. 25, 1996) and the guidelines of the Animal Care and Use Committee (ACUC) at the University of Iowa. All animal protocols were reviewed and approved by the University of Iowa Animal Care and Use Committee.

Standard rat exercise protocol. Male Sprague-Dawley rats (250–350 g) were randomized to a sham exercise control group (placed on treadmill without belt movement) or to an exercise group. For the latter, animals were exercised every morning for 15 min on a treadmill (Columbus Instruments, Columbus, OH) at a speed of 15 m/min for four consecutive mornings. The first 4 days of moderate exercise as described above were used to train and to prepare the animals for a vigorous period of exercise occurring the morning of day 5 that consisted of an exercise period lasting 25 min at 25 m/min. After the last exercise or sham exercise period (24 h), hearts were harvested, and myocardial ischemic tolerance was assessed.

Sustained global ischemia of isolated buffer-perfused hearts. Animals were anesthetized with intramuscular ketamine (70 mg/kg) and xylazine (10 mg/kg). The hearts were removed using midline thoracotomy and immediately immersed in chilled physiological saline solution [PSS (in mM): 118 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4·7H2O, 11 glucose, and 2.5 CaCl2 anhydrous]. Within 2 min of excision, the hearts were installed on a modified Langendorff apparatus. PSS was equilibrated with 95% O2–5% CO2, warmed to 37°C, and delivered by retrograde perfusion at a constant flow rate of 10.220.33.5 on October 23, 2017 http://ajpheart.physiology.org/ Downloaded from http://ajpheart.physiology.org/ by 10.220.33.5 on October 23, 2017

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pressure of 80 mmHg. A latex balloon was inserted in the left ventricle (LV) through the left atrium and adjusted to an end-diastolic pressure of 5–10 mmHg at baseline. The LV balloon volume was then maintained at the same volume (isovolumic) throughout the experiment.

Heart rate was maintained at 240 beats/min by right ventricular pacing (Grass Stimulator, Quincy, MA). Throughout the experiments, hearts were immersed in PSS warmed to 37°C. LV pressure (monitored via the LV balloon) was recorded at baseline and at the end of the reperfusion period, whereas coronary flow rate was assessed at similar time points by the timed collection of coronary effluent. Hearts were always hung in pairs (one control and one exercised) to minimize variability. After 20 min of baseline perfusion, all hearts underwent a period of 20 min of sustained global ischemia followed by 3 h of reperfusion.

**Determination of infarct size.** At the end of each experiment, right ventricular tissue was trimmed from each heart, and the remaining tissue was weighed. The tissue was then cut into four transverse slices, and the slices were incubated for 20 min in a 1% solution of triphenyltetrazolium chloride (Sigma, St. Louis, MO) to distinguish necrotic from intact myocardium (14). Tissues were stored for 48 h in 10% buffered formalin, and the hearts were photographed. Photographic images of the heart slices (Fig. 1) were projected and traced at approximately 5–10× magnification. The total area of necrosis in each slice was quantified by a blinded observer using planimetry (SigmaScan; Systat, Richmond, CA). Values were corrected for the weight of the slice, and the total corrected area was calculated for each heart. The area of necrosis was then expressed as a percentage of the total LV weight.

**Antagonist treatment.** Two groups of animals, n = 8 each, were exercised or served as sham exercise controls as described above, except that, on days 4 and 5, all animals received a subcutaneous injection of naltrexone (10 mg/kg) 15 min before placement on the treadmill. On day 6, myocardial ischemic tolerance and infarct size were determined as described above.

**Duration of exercise-induced cardioprotection.** To determine the duration of the cardioprotective effect evoked by exercise, we assessed ischemic tolerance on animals that had undergone the standard exercise protocol (n = 10) or sham exercise (n = 10) at 5 days after the last exercise period (day 10). Infarct sizes in these hearts were compared with sham exercise controls as described above.

**Real-time PCR.** Hearts were harvested from animals undergoing the standard exercise protocol or sham exercise protocol as described above. Whole hearts were minced in ice-cold Dulbecco’s phosphate-buffered saline without calcium and magnesium. Total RNA was then isolated from minced tissues by Dounce homogenization in 5 ml of TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. mRNA levels were measured using a Stratagene MX3005 QPCR Instrument (Stratagene, La Jolla CA). Reaction solutions (final volume 20 μl) were prepared using reagents from the one-step SYBR Green Quantitative RT-PCR kit (Sigma-Aldrich, St. Louis, MO) combined with 0.25 μM of each primer (Table 1) and 100 ng of total RNA. The instrument program was as follows: 42°C for 30 min, 94°C for 2 min, and 40 cycles of 95°C for 15 s followed by 60°C for 1 min. Fluorescent signals were monitored sequentially for each sample tube once per cycle at the end of the elongation step. The specificity of the RT-PCR products was confirmed by analysis of melting curves and by omission of the reverse transcriptase. All samples were run in duplicate or triplicates.
cated and were normalized to 18S rRNA (6, 7) using the ΔCt method: normalized sample value = 2^{ΔCt_{18S rRNA} - ΔCt_{target mRNA}}.

Real-time PCR microarray analysis. The following four groups were used in this study (n = 8 each): baseline control, baseline exercise, ischemia-reperfusion (I/R) control, and I/R exercise. For all groups, hearts were harvested 24 h after the vigorous exercise period (day 6). For baseline groups, LV RNA was isolated from hearts without further intervention according to the manufacturer’s recommendations using TRIzol and the Absolutely RNA kit from Stratagene. For I/R groups, hearts from control and exercised animals were hung in pairs on a modified Langendorff apparatus and underwent 20 min of global ischemia followed by 30 min of reperfusion. Shorter reperfusion times were used to see acute changes in gene expression before significant tissue damage occurred. RNA was then isolated from LV tissues using the Stratagene kit. Samples for each group were pooled and analyzed according to the manufacturer’s recommendations using the “Rat Inflammatory Cytokines and Receptors” array and the “Rat Apoptosis” array in conjunction with the RT2 Profiler PCR Array System from SuperArray Bioscience (Frederick, MD).

Endpoints and statistics. In all studies, cardiac function was evaluated by comparing coronary flow and LV-developed pressure (LVDP; maximal systolic-diastolic) among groups at baseline and after ischemia by two-way ANOVA. If significant F-values were obtained, subsequent pairwise comparisons were made using the Newman-Keul’s post hoc test. Infarct sizes for each protocol were analyzed according to the manufacturer’s recommendations using TRIzol and the Absolutely RNA kit from Stratagene. Data for each group are presented as means ± SE, and P values of ≤0.05 were considered statistically significant.

RESULTS

Exercise is cardioprotective in rats. Based on previous reports (31), we designed initial studies to confirm that exercise induced a cardioprotective effect in rats. In our first set of experiments, nine animals underwent the standard exercise protocol, and nine animals served as sham exercise controls. The results from these experiments revealed that exercise was associated with a significant reduction in infarct size. To verify these initial results, we duplicated the experiments using nine additional animals for each group and again found that exercise induced a significant cardioprotective effect. Data were subsequently combined for this report (n = 18 for control and exercise groups).

Using the standard exercise protocol, we observed no significant differences in body weight between control and exercised animals (data not shown). Similarly, no differences were observed in baseline coronary flow or LVDP between control and exercised hearts (Table 2). In control hearts, 20 min of ischemia followed by 3 h of reperfusion resulted in a mean infarct size of 42 ± 4% (Fig. 2). Exercise induced a significant cardioprotective effect (infarct size 21 ± 3%; P < 0.001; Fig. 2). Although recovery of left ventricular function was almost 50% higher in the exercised group (Table 2), this did not prove to be significantly different from sham exercise controls. A similar lack of correlation between infarct size and cardiac function was seen in a rabbit model of enkephalin-mediated cardioprotection (15).

We next designed experiments to determine the sustainability of the cardioprotective effect. For these studies, rats were exercised according to the standard protocol. Hearts from control and exercised animals were then harvested 5 days after the last exercise period (day 10) and underwent global ischemia and reperfusion, and infarct sizes were determined. Under these conditions, the infarct sizes of hearts from exercised animals (31 ± 10%, n = 10) were not significantly different from those of controls (35 ± 15%).

Naltrexone blocks the cardioprotective effect of exercise in rats. To test the hypothesis that opioids contribute to the cardioprotective effects of exercise, animals were treated with naltrexone, a broadly effective opioid receptor antagonist. For

Table 2. Cardiac function before and after ischemia

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<th>Baseline</th>
<th>Percent of Baseline After 2-h Reperfusion*</th>
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<td></td>
<td>Coronary Flow, ml/min</td>
<td>LVDP, mmHg</td>
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<tr>
<td>Sham exercise (n = 18)</td>
<td>20.1 ± 3.9 (18.1, 22.1)</td>
<td>102.2 ± 2.4 (89, 115.3)</td>
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<tr>
<td>Exercise (n = 18)</td>
<td>20.3 ± 4.4 (18.2, 22.5)</td>
<td>107.8 ± 21.0 (97.4, 18.3)</td>
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<td>Sham exercise + naltrexone (n = 8)</td>
<td>16.6 ± 2.6 (14.4, 18.8)</td>
<td>84.4 ± 24.2 (64.2, 104.7)</td>
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<tr>
<td>Exercise + naltrexone (n = 8)</td>
<td>21.8 ± 4.6 (17.9, 25.6)</td>
<td>86.0 ± 28.8 (61.9, 110.1)</td>
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Values are means ± SE with 95% confidence intervals in parentheses; n, no. of subjects. LVDP, left ventricular developed pressure. *P < 0.05 relative to preischemia control.

Fig. 2. Determination of infarct size in a rat exercise model. Male Sprague-Dawley rats were randomized to sham exercise or exercise groups. The latter were exercised for 5 days as described in METHODS. For naltrexone-treated groups, animals were injected subcutaneously on days 4 and 5 with 10 mg/kg of naltrexone, just before placing the animals on the treadmill. After the last exercise period (24 h), hearts were harvested and underwent global ischemia (20 min) and reperfusion (3 h). Infarct sizes were then determined using TTC staining. Values are expressed as %infarct size and represent mean ± SE; n = 18 animals for untreated and 8 for naltrexone-treated groups, respectively. *P < 0.001.
coronary flow and LVDP, there were no significant baseline differences between hearts from naltrexone-treated exercised animals and naltrexone-treated controls (Table 2). For naltrexone-treated controls, infarct size was similar (43 ± 4%; Fig. 2) to untreated controls, suggesting that naltrexone had no effect on baseline myocardial ischemic tolerance. In contrast, naltrexone treatment appeared to completely block the cardioprotective effect of exercise (infarct size 40 ± 5%; Fig. 2). As in untreated animals, there was no significant difference in the recovery of left ventricular function between naltrexone-treated exercise and control groups (Table 2).

**Effect of exercise on opioid precursor and receptor expression.** To determine whether exercise acutely alters expression of opioid precursors [preproenkephalin (pPENK), prodynorphin (pPD), proopiomelanocortin (POMC)] and/or opioid receptors [δ-opioid receptor (DOR), κ-opioid receptor (KOR), μ-opioid receptor (MOR)], rats were exercised according to the standard protocol. On day 5, hearts were harvested at the end of the exercise period (0 h) and at 8 and 24 h later. Hearts from sham exercise control animals were harvested in parallel. Total RNA was prepared, and mRNAs were measured using real-time PCR. When normalized to 18S rRNA, pPENK, pPD, and POMC mRNA control levels were 100 ± 23 × 10⁻⁵, 4.2 ± 1.4 × 10⁻⁵, and 0.34 ± 0.14 × 10⁻⁵, respectively (n = 15). In the case of the receptors, DOR (18 ± 11 × 10⁻⁵) and KOR (2.6 ± 1.0 × 10⁻⁵) mRNAs were readily detectable, whereas MOR mRNA levels were considerably lower (0.025 ± 0.012 × 10⁻⁵). After exercise, we observed an immediate but transient increase in mRNA levels for all opioid genes (Fig. 3).

**Effect of exercise on expression of genes related to cardioprotection.** We next examined the effect of exercise on expression of several genes previously reported to correlate with improved ischemic tolerance. For these studies, hearts were harvested on day 6 of the standard exercise protocol, and total RNA was assayed using real-time PCR. Under these conditions, we found no apparent differences in expression of the genes that were tested (Table 3).

Based on these results, we then chose to use quantitative real-time PCR microarrays, both as a hypothesis-generating tool and to identify candidate genes for future study. For microarray analysis, LV mRNA from eight replicate animals was pooled. We reasoned that this would provide an averaging of values and would more readily identify differences. The following four groups were studied: baseline control, baseline exercise, I/R control, and I/R exercise. Only changes that were more than twofold control levels are reported (Table 4). Additional discussion of these results can be found in the discussion.

For baseline expression, more differences were observed (exercise vs. control) with the Rat Inflammatory Cytokines and Receptors array than with the Rat Apoptosis array. For the former (baseline cytokines and receptors), these included exercise-dependent increases in expression of anti-inflammatory cytokines [macrophage inflammatory protein (MIP)-1β, MIP-2, interleukin (IL)-10, stromal cell-derived factor (Sdf1), IL-4], growth factors [transforming growth factor (TGF)-β], and cytokine receptors [C-C chemokine receptor (CCR)1, CCR9]. For the latter (baseline apoptosis), only three genes showed changes with exercise [death-associated protein kinase 1 (Dapk1), baculovirus inhibitory repeat sequence clb (Birc1b), LIM homeobox protein 4 (Lhx4)].

For expression following ischemia and reperfusion, many more differences in expression were observed using the same samples in the apoptosis array (I/R apoptosis). Overall, there was downregulation (indicated by minus signs) of proapoptotic genes such as the caspases and of intermediates and mediators of proapoptotic pathways [caspase and RIP adaptor with death domain (Crad), FAS-associated protein with death domain...
The first evidence that opioids were involved in cardioprotection came from studies of IPC, where it was shown that the cardioprotective effect of intermittent brief myocardial ischemia was completely blocked by the nonspecific opioid antagonist naloxone (40). Several studies have since provided additional pharmacological evidence for the involvement of opioid receptors and receptors. Note, however, that Scyd1 at baseline is 200-fold higher in tissue from exercised animals.

These data identify potential changes in gene expression that may contribute to exercise-induced cardioprotection. Additional studies are underway to expand upon these findings.

DISCUSSION

The first evidence that opioids were involved in cardioprotection came from studies of IPC, where it was shown that the cardioprotective effect of intermittent brief myocardial ischemia was completely blocked by the nonspecific opioid antagonist naloxone (40). Several studies have since provided additional pharmacological evidence for the involvement of opioid receptors in IPC in animals (13, 16, 41) and in humans (3). Moreover, studies with receptor subtype-specific antagonists have placed increasing importance on the DOR both in animals (1, 39) and in humans (46).

Our pharmacological data provide evidence that endogenous opioids also contribute to the cardioprotective effects of exercise, thus providing the first link between the known cardio-protective effects of exercise, opioid-induced cardioprotection, and exercise-related elevations in serum opioid levels. These data provide a strong rationale for future studies to determine which of the three major classes of opioids (enkephalins, dynorphins, endorphins) and opioid receptors (δ, κ, or μ) are involved. The cardioprotective effect of exercise in our model was lost within 5 days of the last exercise period. Consistent with a more transient effect in our model is the observation that initiation of naltrindole treatment abrogated the protective effect in hearts measured 72 h after the start of treatment. In contrast, with the use of more rigorous exercise models, the cardioprotective effects are sustained for >9 days (30). We speculate that this difference in sustainability may relate to greater training effects in the rigorous exercise model relative to our model, which involves only a single vigorous exercise period.

Previous studies showed that ischemia and ischemic-like stresses upregulate proenkephalin (PENK) expression in rat heart and cultured ventricular cardiomyocytes (37, 44) and increase cardiac opioid release and serum opioid levels in animal and human models (5, 23, 27, 28, 35, 42, 43). In our model, we observed an acute but transient increase in expression of the opioid precursor and receptor genes. This suggests that exercise has a broad effect on the opioid system in rat hearts. The extent to which these acute changes in transcription contribute to exercise-dependent cardioprotection remains to be determined.

Changes in expression of a number of other genes have been reported to contribute to the protective effects of preconditioning. These include antioxidant enzymes, heat shock proteins, and regulators of apoptosis (10–12, 24, 26, 32, 48). Other studies suggest that one or more of these changes are not observed in all models of preconditioning and/or do not correlate with cardioprotection (21, 30, 38). Using real-time PCR, we examined expression of several of these genes in whole hearts harvested 24 h after vigorous exercise. Under the conditions of our study, we saw no apparent differences between control and exercised groups in expression of antioxidant enzymes [EsSOD, copper and zinc superoxide dismutase, manganese superoxide dismutase, catalase], heat shock proteins (Hsp 70), and regulators of apoptosis (p53, Bcl-2, Bax). Because preconditioning is a multifactorial process, it should be noted that our results do not preclude a role for these proteins in other models, including other models of exercise.
Because individual real-time PCR measurements did not identify potential candidates for future studies, we used real-time PCR microarrays. These arrays are preferable to membrane oligo arrays in that they are quantitative. In pooled samples of RNA from the LV of control and exercised animals, we observed a greater than twofold difference in expression of a number of genes (Table 4).

The most striking differences in the relative magnitude of expression were observed between baseline control and exercise groups using the “Rat Cytokines and Receptors” array (Table 4, baseline cytokines and receptors). Increases were observed in a number of cytokines/chemokines, including MIP-1β (97-fold), MIP-2 (14-fold), IL-10 (2-fold), Sdf1 (5,000-fold), Sgcd1/taftalkine (240-fold), IL-4 (2-fold), and IL-1β (30-fold). With the exception of IL-1β, these cytokines are generally anti-inflammatory and/or antiapoptotic. Moreover, IL-4 and IL-10 induce PENK expression in mononuclear cells (25). Interestingly, IL-1 has been shown to act as a stimulus of preconditioning (8, 20) and also upregulates PENK. Expression of several cytokine/chemokine receptors was also increased, including CCR1 (110-fold), CCR9 (2-fold), and IL-2Rβ (200-fold). Additionally, the growth factor TGF-β was increased twofold. Studies suggest that TGF-β may play a number of protective roles in response to ischemia and reperfusion (9, 22, 33) and increases PENK expression (25). In contrast to baseline samples, only two differences in gene expression were observed with this array following ischemia and reperfusion (Table 4, I/R cytokines and receptors). One of these is decreased expression of the proinflammatory, proapoptotic cytokine TNF (−3-fold; see Ref. 2); the apoptosis array also demonstrated a decrease in TNF (−2.5-fold).

For the Rat Apoptosis array, expression of only three genes was different when baseline samples were compared (Table 4, baseline apoptosis). Expression of Dapk1 was markedly lower (−48-fold), whereas expression of Birc1b (2.3-fold) and Lhx4 (3.6-fold) were higher in the exercise group. Reduced levels of Dapk1 are associated with resistance to apoptosis (18). Consistent with individual measurements (Table 3), baseline expression of the apoptosis-related genes p53, Bcl, and Bax were not different between baseline control and exercise groups (<2-fold).

The greatest number of differences in gene expression was observed between I/R exercise and I/R controls using the apoptosis array (Table 4, I/R apoptosis). Among genes with increased expression were the caspases (e.g., caspase 7, −15-fold), TNF (−2.5-fold), TNF receptor (−12), and a number of intermediates in proapoptotic signaling pathways (Cradd, Fadd, NFKB1, NoI3). Dffh (−3.6-fold) was also decreased in the I/R exercise sample relative to controls. Overall, downregulation of these genes would promote survival of tissues during ischemia and reperfusion (19). In contrast, expression of the proapoptotic protein Bax was higher (2.2-fold), and expression of the antiapoptotic protein Bcl was lower (−4.6) in the I/R exercise group relative to I/R controls. If these changes reflect a shift to a higher Bax-to-Bcl protein ratio, then that would favor apoptosis.

Taken together, microarray studies suggest that exercise induces changes in gene expression both at baseline and following ischemia and reperfusion. These data provide valuable insight that will direct our future studies.

Natural processes like hibernation, preconditioning, and exercise protect the heart and other organs from ischemic injury. Studies to determine the molecular mechanisms that underlie these processes will likely contribute to future development of therapeutic interventions to prevent and to treat ischemic diseases, including ischemic heart disease.

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REFERENCES

EXERCISE ENHANCES MYOCARDIAL ISCHEMIC TOLERANCE