

Research Paper

Involvement of the δ -opioid receptor in exercise-induced cardioprotection

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New Findings

- **What is the central question of this study?**
Does the δ -opioid receptor trigger exercise-induced cardioprotection against ischaemia–reperfusion injury?
- **What is the main finding and its importance?**
In exercised hearts, the δ -opioid receptor appears to trigger cardioprotection against ischaemia–reperfusion-induced tissue necrosis but not apoptosis.

Abstract

Endogenous opioids mediate exercise-induced cardioprotection against ischaemia–reperfusion (IR) injury, although the opioid receptor subtype mediating this effect is unknown. We investigated whether the δ -opioid receptor mediates exercise-induced cardioprotection against IR injury. Endogenous opioids are produced in various tissues, including the heart and skeletal muscle; therefore, we also sought to identify the effect of exercise on circulating endogenous opioid as well as transcript, protein and receptor expression in heart and skeletal muscle. Male Sprague–Dawley rats ($n = 73$) were assigned randomly to treadmill exercise or sedentary treatments. Cardiac tissue and serum were harvested 0, 20 and 120 min following exercise and from sedentary animals ($n = 32$) to quantify effects on proenkephalin and δ -opioid receptor mRNA and protein levels, as well as serum enkephalin. Skeletal muscle (soleus) was harvested at identical time points for determination of proenkephalin protein and mRNA. A separate group of rats ($n = 41$) were randomly assigned to sham operation (Sham; surgical control), sedentary (Sed), exercise (Ex) or exercise + δ -opioid receptor antagonist (ExD; naltrindole, 5 mg kg⁻¹ I.P.) and received IR by left anterior descending coronary artery ligation *in vivo*. After IR, tissues were harvested to quantify treatment effects on necrosis and apoptosis. Cardiac proenkephalin mRNA expression increased following exercise (0 min, $P = 0.03$; 120 min, $P = 0.021$), while soleus expression was unaffected. Exercise-induced changes in serum enkephalin were undetectable. After IR, tissue necrosis was elevated in Sed and ExD hearts ($P < 0.001$ and $P = 0.003$, respectively) compared with the Sham group, while the Ex group was partly protected. After IR, apoptosis was evident in Sed hearts ($P = 0.016$), while Ex and ExD hearts were protected. Data suggest that cardioprotective opioids are produced by the heart, but not by the soleus. After IR, the δ -opioid receptor may mediate, in part, cardioprotection against necrosis but not apoptosis.

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Introduction

Ischaemic heart disease, including myocardial infarction, is the leading cause of death in industrialized nations. In contrast, an active lifestyle is linked with both decreased morbidity and increased survival rate associated with cardiovascular disease and myocardial infarction (Roger *et al.* 2011). Scientific inquiry reveals that brief exposure to exercise (1–3 days of moderate-intensity exercise) preconditions the heart against tissue injury and death resulting from ischaemia–reperfusion (IR; Locke *et al.* 1995; Taylor *et al.* 1999; Demirel *et al.* 2001; Brown *et al.* 2003, 2005; Quindry *et al.* 2005, 2010; Chicco *et al.* 2007; Dickson *et al.* 2008; French *et al.* 2008). The beneficial cellular adaptations due to exercise preconditioning are multifaceted and include upregulation of several cardiac-specific factors including, but not limited to, manganese superoxide dismutase (MnSOD), glutathione reductase, mitochondrial ATP-sensitive K⁺ (K_{ATP}) channels and sarcolemmal K_{ATP} channels (Werns *et al.* 1992; Yamashita *et al.* 1999; Hamilton *et al.* 2004, 2005; Quindry *et al.* 2010). Additionally, recent investigations suggest that endogenous opioids play a role in exercise-induced preconditioning against tissue death (Dickson *et al.* 2008; Michelsen *et al.* 2012).

Independent of exercise, the role of opioid compounds in pharmacological and ischaemic preconditioning models of cardioprotection against necrotic and apoptotic cell death is well documented (Sun *et al.* 1996; Liu *et al.* 2001; Schultz & Gross, 2001; van den Brink *et al.* 2003; Huang *et al.* 2009; Maslov *et al.* 2009). Based upon ischaemic and pharmacological models of preconditioning, the δ -opioid receptor is central to protection against IR injury (Liu *et al.* 2001; Gross & Peart, 2003; Maslov *et al.* 2009). Exercise preconditioning represents a unique and sustainable mechanism of cardioprotection, and it is believed that common mediators between models of preconditioning may exert an effect via different signalling mechanisms. Whether δ -opioid receptor activation is involved in an exercise-induced model of cardioprotection remains unknown. Given the sustainable nature of the exercise stimulus for evoking a cardioprotected phenotype, identification of δ -opioid receptor-based protection may be beneficial from both scientific and clinical perspectives.

Fundamental to the rationale that endogenous opioids mediate exercise-induced preconditioning against IR injury, exercise elicits an acute increase in circulating opioid compounds (Kraemer *et al.* 1985). Endogenous opioids include three families of peptides, the β -endorphins, dynorphins and enkephalins, the last of which has the greatest affinity for the δ -opioid receptor (van den Brink *et al.* 2003; Gianoulakis, 2009). Elevated transcript expression of opioid precursor molecules and receptors, including proenkephalin and the

δ -opioid receptor, has been observed following exercise in cardiac tissue (Dickson *et al.* 2008). Whether an acute exercise stimulus also results in increased protein expression of these molecules and receptors is currently unknown. Nonetheless, administration of naltrexone, a non-selective opioid receptor antagonist, abolishes the cardioprotective effect of exercise (Dickson *et al.* 2008; Michelsen *et al.* 2012). The specific opioid receptor subtype and corresponding opioid molecule involved has yet to be identified. Moreover, non-cardiac sources of endogenous opioids, including skeletal muscles, have not been examined in the context of cardioprotective exercise.

In light of this scientific rationale, we employed an established model of cardioprotective treadmill exercise in male Sprague–Dawley rats with a twofold purpose. Our first aim was to determine whether the δ -opioid receptor is involved in exercise-mediated cardioprotection against IR injury by administration of naltrindole, a selective pharmacological inhibitor of the δ -opioid receptor, in combination with an established model of treadmill running and IR infarction *in vivo*. Our second aim was to examine postexercise δ -opioid receptor and proenkephalin upregulation within the left ventricle. Given that the soleus muscle is important for successful treadmill running in rats, additional experiments were performed on soleus muscle to quantify potential increases of proenkephalin transcript in non-cardiac tissue.

Methods

Ethical approval

The experimental protocol was approved by the Auburn University Institutional Animal Care and Use Committee. Rats were housed in the Biological Research Facility of Auburn University. All animals were housed in a reverse light–dark cycle (12 h–12 h; lights off at 10 am and lights on at 10 pm), received unrestricted access to water and were fed a standard chow diet *ad libitum*. In total, 73 male Sprague–Dawley rats were randomly assigned into two study arms; either a ‘time-course trial’ ($n = 32$) or ‘ischaemia–reperfusion’ ($n = 41$), and further randomized into treadmill-exercised or sedentary treatment groups.

Treadmill exercise and sedentary treatments

Cardioprotective exercise was elicited by three consecutive days of forced treadmill running for 30 min at 30 m min⁻¹ and 0% gradient. Exercise habituation was performed over three consecutive days to orient rats to treadmill exercise at the prescribed exercise intensity. Treadmill habituation involved 10 min of running, increasing by 10 min day⁻¹ to a final duration of 30 min on day 3. Rats rested for 1 day between the habituation and exercise protocol. Rats

Table 1. Primers for real-time PCR analysis

Proenkephalin	Forward	TCAGGAAAGATTGTCCTGCTGGT
	Reverse	TTGGAAAGAAGAATGCGCCTGTGG
δ -Opioid receptor	Forward	TGAAGACGGCCACCAACATCTACA
	Reverse	TTCCATCAGGTAAGTGGCGCTCT

assigned to the sedentary treatment group were placed on a motionless treadmill for a time equivalent to the habituation and exercise treatment.

Exercise time course study arm

Exercised (Ex) rats assigned to the exercise time course study arm were anaesthetized with 1–4% isoflurane by inhalation and killed via cardiac excision immediately (0 min, $n = 8$) or at 20 (20 min, $n = 8$) or 120 min (120 min, $n = 8$) following the third and final bout of cardioprotective exercise; sedentary control animals (Sed, $n = 8$) were killed in the same manner. Body weight was measured immediately before the rats were killed. Blood serum, heart and soleus tissues were collected, immediately frozen in liquid nitrogen and stored at -80°C for subsequent mRNA and Western blot analysis.

Extraction and analysis of mRNA. Primers for proenkephalin (PENK), δ -opioid receptor and β -actin mRNA were obtained from Integrated DNA Technologies (Coralville, IA, USA) and are provided in Table 1. RNA was isolated from snap-frozen left ventricular and soleus tissue from the ‘exercise time course study arm’ using the Qiagen RNeasy Mini kit (Valencia, CA, USA), according to the Animal Tissue Protocol. The RNA yield and purity were determined by Nano Drop (Thermo Scientific, Waltham, MA, USA). Complementary DNA was synthesized from RNA using Verso cDNA kit (Thermo Scientific), following the manufacturer’s instructions. Quantitative PCR was performed with a reaction solution prepared with SYBR green quantitative PCR kit (SA Biosciences, Valencia, CA, USA) combined with $10\ \mu\text{M}$ of each primer and 50 ng complementary DNA to a final volume of $25\ \mu\text{l}$. Instrument cycles were as follows: one cycle at 95°C for 2 min, 35 cycles of 95°C for 30 s followed by an elongation step of 62°C for 30 s, and a third cycle of 72°C for 5 min. Fluorescence signals were monitored sequentially for each cycle at the end of each elongation step. The specificity of RT-PCR products was confirmed by analysis of melting curves and by omission of the complementary DNA template. All samples were normalized to β -actin mRNA using the $\Delta\Delta C_T$ method.

Western blotting. Ventricular tissue sections and soleus tissue were homogenized 1:20 in 10 mM PBS with

0.1 mM EDTA and 5% protease inhibitor cocktail (Sigma #P2714) with glass on glass apparatus and centrifuged at $10,000\ \text{g}$ for 10 min at 4°C . Homogenized samples were diluted to $2.6\ \mu\text{g}\ \mu\text{l}^{-1}$ protein concentration in SDS sample buffer and β -mercaptoethanol, and separated by electrophoresis on precast gels (Lonza, Houston, TX, USA). Proteins were transferred to polyvinylidene fluoride membranes, which were blocked for 2 h at room temperature before primary antibody application. The following antibodies were purchased from Abcam (Cambridge, MA, USA); δ -opioid receptor (#ab66318); and Leu-enkephalin (#ab8902). Primary antibody (1° ; Cell Signaling, Danvers, MA, USA) was prepared 1:1000 in PBS-T 1% Super Block (Thermo Scientific), added to each membrane to cover and incubated overnight at 4°C . Secondary antibody (2° ; Cell Signaling #7074 and Abcam #6027) was diluted 1:2000 and 1:3000, respectively, in PBS-T 1% Super Block and applied for 2 h at room temperature. Membranes were imaged in enhanced chemiluminescent substrate (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions and digitally imaged and analysed (Kodak Gel Logic 2200, Kingsport, TN, USA).

Serum measures. Serum total enkephalin was measured using a commercially available enzyme-linked immunosorbent assay kit (Antibodies-Online, Atlanta, GA, USA), following the manufacturer’s instructions.

Ischaemia-reperfusion study arm

A separate group of rats were randomly designated to exercise or sedentary groups for the IR study arm. Further subsets of exercised and sedentary rats were then selected to receive naltrindole (Sigma-Aldrich), a selective δ -opioid receptor antagonist or vehicle (sterile saline). Naltrindole was administered for three consecutive days at a dose of $5\ \text{mg}\ \text{kg}^{-1}$ in sterile saline by i.p. injection 15 min before either exercise or sedentary treatments (Table 2). Anaesthetized rats from sedentary sham (Sham, $n = 8$), sedentary (Sed, $n = 9$), sedentary with δ -opioid receptor antagonist (SedD, $n = 4$) exercised (Ex, $n = 10$) and exercised with δ -opioid receptor antagonist (ExD, $n = 10$) treatment groups received either sham or IR surgery 24 h after the final exercise bout.

Ischaemia-reperfusion and sham surgery. Rats were weighed and anaesthetized with $80\ \text{mg}\ \text{kg}^{-1}$ sodium pentobarbital (i.p.; Vortech Pharmaceuticals, Dearborn, MI, USA) and ventilated with room air through a tracheotomy tube. Blood pressure and ECG were continuously monitored via computer software connected with a pressure transducer attached to a saline-filled catheter inserted into the right carotid artery (Biopac

Table 2. Exercise and injection protocol

Day	1	2	3	4	5	6	7
Exercise duration (min)	10	20	30	0	30	30	30
Injections					15 min before treadmill placement		
Phase	Habituation			Rest		Exercise stimulus	

Student Lab Pro 2005, Camino Goleta, CA, USA). A catheter was placed in the right jugular vein, and a surgical plane of anaesthesia was maintained with supplemental doses of sodium pentobarbital throughout the experimental procedures. Body temperature was maintained at 37°C with a heated water blanket. The heart was exposed by left thoracotomy, and ischaemia was induced by placing a reversible ligature around the left anterior descending coronary artery. Rats were exposed to an IR challenge *in vivo* consisting of 50 min ischaemia followed by 120 min reperfusion. Sham-operated animals underwent identical surgery, except that the ligature passed under the coronary vessel was not tightened during the experimental period (170 min non-ischaemic surgical control, which is equal to the ischaemic and reperfusion durations for all groups receiving IR). Reperfusion was initiated by loosening the ligature in sedentary and exercised animals. Following 120 min of reperfusion, ligatures were retightened for all animals, and Evans Blue dye was infused through the arterial catheter for visualization of the ischaemic area at risk, defined as non-perfused tissue. In the case of hearts from Sham animals, the ligature was tightened at the end of the 170 sham experiment and Evan Blue dye infused as described for IR groups. Using this technique in Sham control animals, the amount of ventricular tissue that would have been at risk during IR could be determined. Hearts from Sham and IR-treated groups were then immediately excised, weighed and processed for infarct area analysis and apoptotic markers, as detailed in the following subsections. The same surgeon performed surgical procedures, and all subsequent analyses were performed in blinded conditions.

Determination of ventricular infarct area. Hearts from rats receiving Sham and IR surgeries were cut into four 1.5 mm cross-sections and incubated at 37°C for 12 min in 1% triphenyltetrazolium chloride (Sigma). Each heart cross-section was digitally photographed, and images were computer analysed (Kodak Gel Logic 2200) in blinded conditions to determine the ischaemic area at risk, defined as the non-perfused area and infarct area (necrotic tissue area). Tissue cross-sections were then further designated for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay or Western blotting. The infarct area was normalized to the area at risk to minimize

percentage infarct variations that may be magnified by between-animal differences in the area at risk.

Apoptotic cell death/TUNEL. Tissue cross-sections designated for the TUNEL assay were coated in Richard Allen Scientific-50 embedding medium (Waltham, MA, USA) and frozen gradually in 2-methylbutane chilled with liquid nitrogen. Frozen heart samples were sliced into 8 μm cross-sections and fixed in 10% formalin. Histological sections were incubated with rabbit anti-laminin (Sigma-Aldrich) followed by a secondary antibody conjugated to a Texas Red fluorescent tag (Vector Laboratories, Burlingame, CA, USA). Histological sections were incubated with TUNEL label reaction mixture (cell death detection kit; Roche, Indianapolis, IN, USA). Slide covers were mounted with 4',6-diamidino-2-phenylindole mounting medium (Vector Laboratories). Apoptotic nuclei were imaged via fluorescence microscopy within the whole tissue (Nikon Eclipse Ti-U, Melville, NY, USA) and expressed relative to tissue area.

Western blotting. Tissue cross-sections designated for Western blotting were quickly dissected into non-ischaemic (perfused, blue tissues) and ischaemic (non-perfused tissue), rapidly frozen in liquid nitrogen and stored at -80°C . Western blotting was performed as described for the exercise time course study arm. The following antibodies for apoptotic markers were purchased from Cell Signaling: Bax (#2772); Bcl-2 (#2876); αII spectrin (#2122); and cleaved αII spectrin (#2121); and expressed relative to $\beta\text{-actin}$ (#4970).

Statistical analysis

One-way ANOVA was used to analyse between-group differences for area at risk, infarct area, apoptotic tissue death (TUNEL), tissue protein and serum measures. Messenger RNA expression levels were analysed using REST software (Qiagen). Two-way repeated-measures ANOVA was used to compare group differences between variables in perfused and non-perfused tissue samples. Significance was set *a priori* to $P \leq 0.05$; significant group differences were determined via Tukey's *post hoc* test.

Table 3. Animal characteristics

IR	Sham	Sed	Ex	ExD	SedD
Body weight (g)	348.6 ± 5.8	351.0 ± 10.7	373.8 ± 5.3	375.2 ± 6.9	341.0 ± 5.9
Heart weight (g)	0.97 ± 0.03	1.05 ± 0.03	1.07 ± 0.02	1.04 ± 0.03	1.00 ± 0.05

Abbreviations: Sham, sedentary sham; Sed, sedentary; SedD, sedentary + 8 opioid receptor antagonist; Ex, exercised; ExD, exercised + 8 opioid receptor antagonist.

Results

Animal characteristics

Animal characteristic data are presented in Table 3. In the exercise time course study arm, no significant differences existed between treatments for body weight. In the IR study arm, between-group differences did not exist for either body or heart weight.

Real-time RT-PCR and protein expression of opioid-related compounds and receptors

Relative mRNA expression levels from left ventricular tissue are presented in Fig. 1A and B for proenkephalin and δ -opioid receptor, respectively. No differences existed between groups for δ -opioid receptor mRNA levels examined in ventricular tissues. Cardiac proenkephalin mRNA levels were elevated compared with sedentary control animals at 0 and 120 min ($P=0.031$ and $P=0.025$, respectively). In contrast to the findings in ventricular tissue, no differences were noted between treatments for proenkephalin mRNA expression from soleus ($P=0.105$; Fig. 1C). Protein levels of Leu-enkephalin and δ -opioid receptor, as well as serum enkephalin were not significantly different between groups (data not shown).

Infarct area

Area at risk and tissue infarct area are presented in Fig. 2A and B, respectively. Significant between-group differences existed for tissue necrosis ($P < 0.001$), as quantified by triphenyltetrazolium chloride staining. Compared with the Sham group, Sed and ExD animals had greater tissue necrosis ($P < 0.001$ and $P = 0.003$, respectively). Tissue necrosis was not different between Sham and Ex groups. Ischaemia–reperfusion-induced necrosis was attenuated in Ex compared with Sed ($P = 0.003$). Ventricular necrosis values were not different between Sed and SedD treatments ($P = 0.935$, data not shown).

Apoptosis

Tissue levels of apoptosis measured via the TUNEL assay are presented in Fig. 3. Between-group differences existed for tissue apoptosis ($P = 0.013$), with Sed exhibiting more TUNEL-positive nuclei than Sham ($P = 0.016$).

In contrast, TUNEL values from Ex and ExD treatments were not different from Sham. After IR, TUNEL-positive nuclei were significantly fewer in Ex rats compared with Sed ($P = 0.035$), but ExD hearts did not differ significantly from either Ex or Sed. No difference existed for TUNEL-positive nuclei between Sed and SedD (data not shown). Western blot analysis for apoptotic markers revealed that the cleaved/intact α II spectrin ratio was elevated in Sed ischaemic tissue compared with Sham tissue ($P < 0.001$; Fig. 4A), but not in either exercise treatment. Ischaemic tissues from both Ex and ExD groups were significantly different from Sed ischaemic tissue ($P = 0.028$ and $P = 0.010$, respectively). No differences existed in the cleaved/intact α II spectrin ratio between groups in non-ischaemic tissues. Western blot analysis of the Bax/Bcl-2 ratio did not reveal treatment differences between groups or between ischaemic and non-ischaemic tissues (Fig. 4B).

Discussion

Exercise-induced cardioprotection against IR injury is a well-studied phenomenon and includes multiple observations of tissue-sparing effects against both necrosis and apoptosis (Demirel *et al.* 2001; Hamilton *et al.* 2004, 2005; Brown *et al.* 2005; Dickson *et al.* 2008; Quindry *et al.* 2010, 2012). The known mechanisms of protection are endogenous to the ventricular myocardium and include upregulation of MnSOD and glutathione reductase (Hamilton *et al.* 2004, 2005), mitochondrial and sarcolemmal K_{ATP} channels (Brown *et al.* 2005; Chicco *et al.* 2007; Quindry *et al.* 2010, 2012) and calcium-handling proteins (Hamilton *et al.* 2003; Collins *et al.* 2005). An investigation by Dickson *et al.* (2008) was the first to suggest that exercise preconditioning may also be mediated by an opioid receptor-dependent mechanism. In their study, administration of a non-selective pharmacological inhibitor of opioid receptors mitigated exercise protection against IR-induced tissue necrosis. Left unknown was whether activation of the δ -opioid receptor, a mechanism central to non-exercise cardiac preconditioning (Liu *et al.* 2001; Gross & Peart, 2003; Maslov *et al.* 2009), is involved in exercise-mediated cardioprotection.

In the present study, a selective pharmacological agent was administered to exercised animals (ExD)

before cardioprotective exercise. The findings suggest that the δ -opioid receptor is responsible, in part, for exercise-induced cardioprotection against IR-induced tissue necrosis, although statistical similarities between ExD and Ex groups temper this conclusion. The findings from the present study highlight several novel insights into cardioprotection against IR injury, as will be discussed in the following sections.

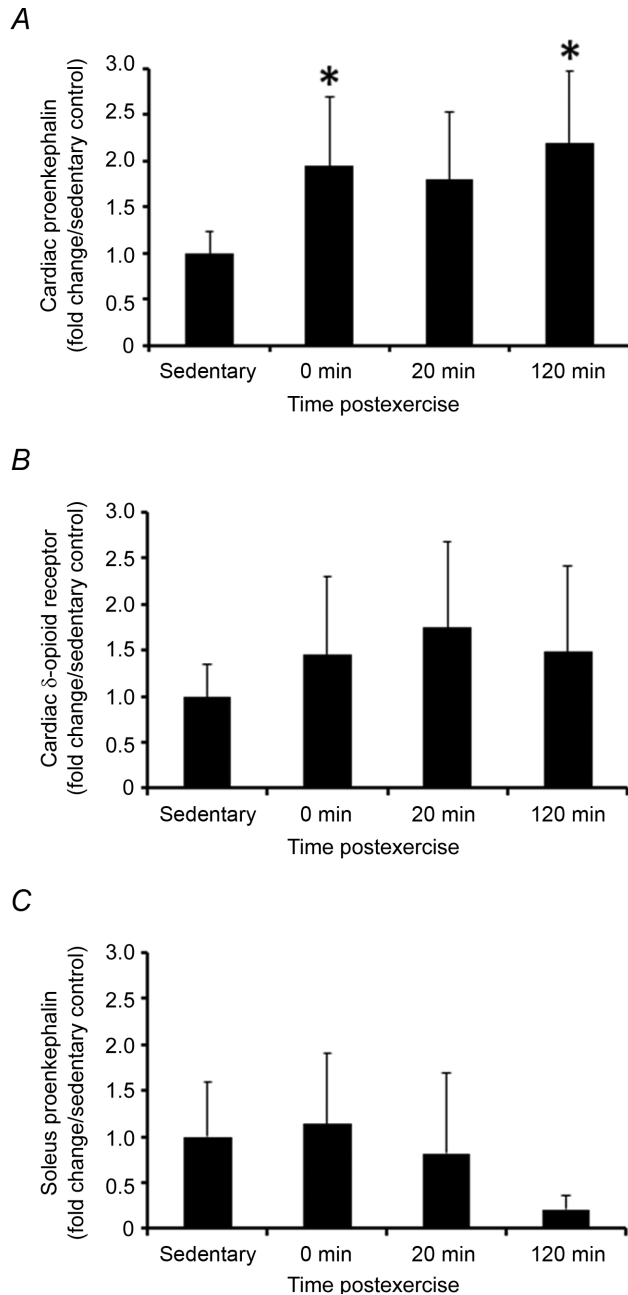


Figure 1. Cardiac (A and B) and soleus muscle mRNA expression (C), expressed as multiples of sedentary control values

Data represent group means \pm SEM ($n = 8$). * $P \leq 0.05$; $P = 0.031$ Sed at 0 min; $P = 0.025$ Sed at 120 min.

Exercise 'dose'

An important consideration for exercise-induced cardioprotection research is the 'dose' and intensity required to achieve the protected phenotype. Early exercise preconditioning studies demonstrated improved maintenance of ventricular pressure during IR following a 10 week protocol of treadmill exercise at $\sim 70\%$ of maximal oxygen consumption ($\dot{V}_{O_2 \max}$; Powers *et al.* 1998). Subsequent research demonstrated equivalent protection with a short duration (3–5 days) of similar exercise intensity (Demirel *et al.* 2001). In fact, a single bout of exercise for 30 min protects against IR-induced necrotic tissue death (Yamashita *et al.* 1999). Given these short time frames, exercise preconditioning is likely to reflect acute biochemical alterations within the myocardium and occurs independent of structural alterations to the coronary vasculature or myocardial architecture (Lennon *et al.* 2004a,b,c; Quindry *et al.* 2005). Moreover, the intensity threshold required to elicit exercise-induced cardioprotection is relatively low in comparison to functional capacity. While findings from a few studies indicate that long-term exercise at an intensity of 55–60% $\dot{V}_{O_2 \max}$ is not effective in eliciting an exercise-induced cardioprotective response (Starnes *et al.* 2005), other findings reveal that exercise intensities of 55 and 75% of $\dot{V}_{O_2 \max}$ are equally protective against IR-induced losses in ventricular contractility (Lennon *et al.* 2004a). While variations in the exercise protocol intensity may account for disparate findings among studies, they suggest collectively that the protected phenotype is stimulated in a threshold-dependent fashion (Demirel *et al.* 2001; Lennon *et al.* 2004a; Starnes *et al.* 2005).

Despite the wealth of evidence to support the positive effect of exercise on cardiovascular function, long-term exercise at an extreme intensity is associated with an elevation in markers of oxidative stress, tissue damage and markers of apoptotic tissue death (Benito *et al.* 2011). Adverse cardiac remodelling that occurs as a result of extreme endurance exercise is termed 'athletes' heart' and is reviewed extensively elsewhere (O'Keefe *et al.* 2012). In contrast to findings from extreme exercise, we employed a short-term moderate-intensity exercise protocol to elicit a protected phenotype, while avoiding both potential negative effects associated with extreme exercise.

Exercise and δ -opioid-mediated preconditioning

Ischaemia–reperfusion was marked by tissue necrosis in Sed hearts compared with sham-operated animals. Exercise protected against tissue necrosis in that Ex animals were not different from the Sham group. Administration of naltrindole, a specific δ -opioid receptor antagonist, in the ExD group partly abolished the protection against tissue necrosis, evidenced by tissue

necrosis levels significantly elevated in comparison to Sham. Interpretation of the present finding that tissue-sparing protection was partial is based on the finding that ExD was not statistically different from either Ex or Sed groups, whereas a significant difference was observed between Ex and Sed. Despite a finding of partial protection, there is rationale to suspect that involvement of the δ -opioid receptor due to exercise preconditioning is clinically significant. First, infarct size is directly related to cardiac function and mortality; therefore, even a modest reduction in infarct is likely to provide beneficial health outcomes (Pfeffer *et al.* 1979, 1985; Miller *et al.* 1995). Second, exercise preconditioning against IR-induced injury is multifaceted; therefore, an incomplete ablation of protection does not necessarily preclude the importance of a specific mechanism. Further

investigation is warranted to determine the means by which δ -opioid receptor activation mediates exercise preconditioning against IR-induced necrotic tissue death.

In addition to prevention of tissue necrosis, exercise treatment resulted in a reduction in TUNEL-positive nuclei and cleaved/intact α II spectrin ratio within ischaemic tissue compared with sedentary animals. Ventricular tissues collected from ExD animals did not exhibit statistical differences in the mean levels of TUNEL-positive nuclei compared with Sham, Sed or Ex groups, while the cleaved/intact α II spectrin ratio in ischaemic tissue was different from only the Sed group. Our present findings for TUNEL and α II spectrin cleavage appear to confirm previous observations of exercise protection against apoptotic cell death, but do not support an essential role of the δ -opioid receptor in the observed protection. Involvement of the δ -opioid receptor in cardioprotection against tissue necrosis but not apoptosis is noteworthy. This point is important given the fact that experimental models of ischaemic and pharmacological preconditioning clearly implicate the δ -opioid receptor as an essential mediator against both necrotic and apoptotic tissue death (Liu *et al.* 2001; Schultz & Gross, 2001; Okubo *et al.* 2004; Dickson *et al.* 2006; Maslov *et al.* 2009). Finally, mean Bax/Bcl2 ratios were not different between ischaemic and non-ischaemic tissues of any group receiving IR. While only speculative, the relatively short reperfusion time frame may be too short to enable observation of consistent differences in this marker of apoptosis. An extended duration of reperfusion (e.g. 24–48 h) is recommended for future investigation in order to provide better resolution of ischaemic and non-ischaemic tissue responses for these biomarkers.

Exercise cardioprotection against one but not both tissue death outcomes is not necessarily surprising given evidence that mediators common to multiple forms of preconditioning may have different signalling mechanisms. For example, both the sarcolemmal K_{ATP} and mitochondrial K_{ATP} channels are implicated in protection against necrotic and apoptotic tissue death in the ischaemic preconditioning model (Patel *et al.* 2002; Gross & Peart, 2003). In an exercise model, however, the mitochondrial K_{ATP} channel is involved in protection against arrhythmia, while the sarcolemmal K_{ATP} channel is involved in protection against necrotic tissue death (Quindry *et al.* 2010, 2012). While not confirmatory in the present study, this broad observation between exercise and non-exercise modes of preconditioning adds to a growing scientific belief that the exercise stimulus is a unique form of protection (Frasier *et al.* 2011; Powers *et al.* 2014).

Important to the present study are several potential factors that may have contributed to a partial blunting of cardioprotection against tissue necrosis in the exercised rats receiving naltrindole. First, naltrindole was only administered before the 'exercise stimulus' sessions, but

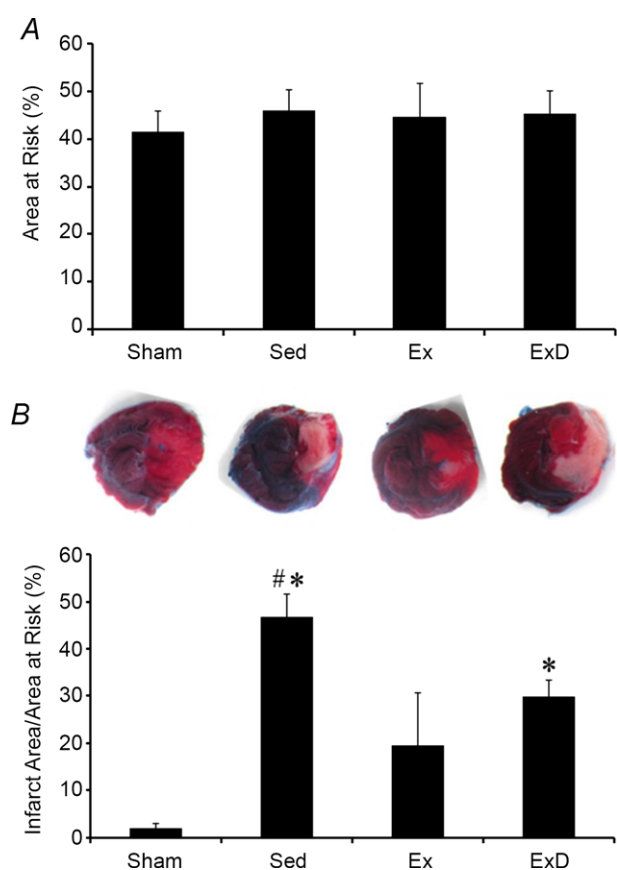


Figure 2. Mean area at risk and mean percentage infarct area at risk

A, area at risk, expressed as a percentage of the ischaemic area (pink, non-perfused) per total heart area. Data represent group means \pm SEM. **B**, representative images and infarct area (white) expressed as a percentage of area at risk (pink). Data represent group means \pm SEM. Sham, $n = 8$; sedentary (Sed), $n = 9$; exercise (Ex), $n = 10$; and exercise plus naltrindole (ExD), $n = 10$. * $P \leq 0.05$ different from Sham; # $P \leq 0.05$ different from Ex.

not during the treadmill habituation period. Given that exercise preconditioning may be elicited by as little as 1 day of exercise (Yamashita *et al.* 1999), it is possible that residual protection afforded during the habituation period impacted study outcomes. Second, this is the first study to employ a selective δ -opioid receptor antagonist during exercise preconditioning, and whether a larger dosage may have resulted in complete attenuation of cardioprotection in exercised hearts remains unknown. Nonetheless, the dosage used currently was based upon previous studies in a rat model of ischaemic and pharmacological preconditioning (Schultz *et al.* 1997). Preliminary studies indicate that this dosage completely blocks the δ -opioid receptor agonist, [D-Pen²⁵]-enkephalin, shown by multiple groups to provide robust protection against tissue necrosis and apoptosis (Sigg *et al.* 2002; Huang *et al.* 2009). The dosage used in this study is further supported by pharmacological studies examining the antagonistic effect of naltrindole on δ -specific agonists, including [D-Pen²⁵]-enkephalin, in responsiveness to pain (Crook *et al.* 1992). Follow-up studies are warranted to elucidate further the involvement of the δ -opioid receptor in exercise preconditioning against IR injury and to gain a better understanding of the mechanisms of exercise preconditioning.

Endogenous opioids and exercise

Circulating opioid compounds, including proenkephalin peptide F, are acutely elevated in response to exercise, peaking soon after exercise cessation (Kraemer *et al.* 1985). Likewise, transient increases in transcript expression of

opioid peptide precursors occur within the myocardium in response to exercise (Dickson *et al.* 2008). The myocardial expression of the proenkephalin gene results in a 31 kDa polypeptide precursor, which enters post-translational processing to yield the pentapeptides Met- and Leu-enkephalin and several other large peptides (van den Brink *et al.* 2003). Interestingly, the myocardium represents a major source of enkephalin production (Howells *et al.* 1986; van den Brink *et al.* 2003; Denning *et al.* 2008). However, to our knowledge it had not previously been determined whether exercise results in increased protein expression within the myocardium; therefore, we investigated whether elevated transcript expression of the δ -opioid receptor and proenkephalin as well as the protein levels of Leu-enkephalin and the δ -opioid receptor occurred in the minutes and hours immediately following exercise. Additionally, we aimed to gain preliminary insight into other potential source(s) of cardioprotective enkephalin peptides during exercise. To this end, we also looked for transcript and protein expression within the soleus as well as circulating enkephalin.

In agreement with the work of Dickson *et al.* (2008), we observed that proenkephalin transcript was elevated immediately and at 120 min after exercise, although the magnitude of the transcript increase was numerically lower in the present study. In contrast to prior findings of > 30-fold increase in cardiac δ -opioid receptor transcript expression immediately following exercise (Dickson *et al.* 2008), we did not find significant differences in cardiac δ -opioid receptor transcripts in rat hearts obtained between 0 and 120 min after exercise. Discrepancies in the outcome of δ -opioid transcript expression may be due to differences in the exercise protocols, although it should be noted that our protocol was of modestly greater intensity and duration. Future investigation should be directed at understanding whether δ -opioid receptor protein expression confirms transcript findings or whether constitutive receptor activity mediates protection.

While not currently confirmed, our findings of increased proenkephalin transcript levels in the myocardium but not soleus muscle of exercised rats suggest that endogenous opioid-dependent cardioprotection due to exercise may occur in a paracrine/autocrine fashion. However, we were unable to detect differences in myocardial Leu-enkephalin in our serum samples. A probable explanation for this occurrence includes the rapid degradation of peptides produced in the heart, lack of peptide storage and the fact that most forms of enkephalins remain in precursor or large protein form (peptide B or methionine-enkephalin-arginine-phenylalanine) and are not included in most standard measurements, including our own (van den Brink *et al.* 2003). Although the function of these larger peptides is unclear, it may be that

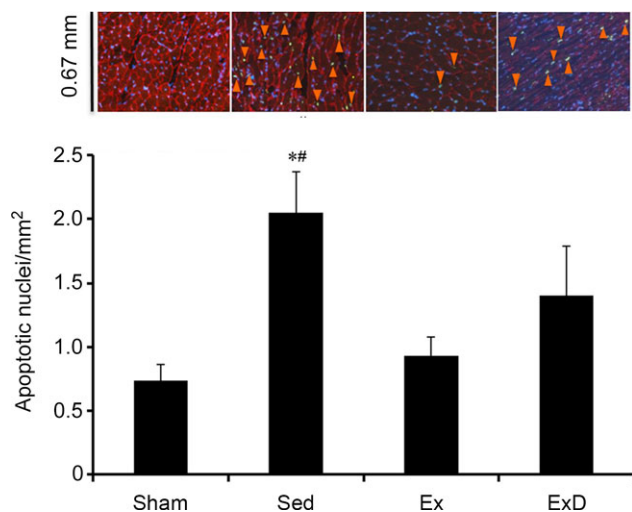


Figure 3. TUNEL assay, showing apoptotic nuclei per square millimetre of tissue area

Data represent group means \pm SEM. Sham, $n = 8$; Sed, $n = 9$; Ex, $n = 10$; and ExD, $n = 10$. * $P \leq 0.05$ different from Sham; # $P \leq 0.05$ different from Ex.

they serve as a reservoir for enkephalin release (van den Brink *et al.* 2003), enabling the ability of the myocardium to alter the type of enkephalin released according to different physiological stimuli. This reasoning is in line with the finding that the type of enkephalin in greatest circulating concentration is influenced by cardiac events (Schultz & Gross, 2001; van den Brink *et al.* 2003). For example, following ischaemia, an increase in Leu-enkephalin is detected in the coronary sinus (van den Brink *et al.* 2003). Although we cannot draw conclusions based upon the present data, we hypothesize that exercise increases the ability of the myocardium to release Leu-enkephalin in response to a stressor, such as IR, thereby mediating cardioprotection in an autocrine/paracrine manner.

We did not observe a significant rise in plasma enkephalin following exercise. Minute amounts of endogenous enkephalin may mediate cardioprotection in the minutes following exercise, at levels that are not readily detectable from circulating serum samples. However, given the short half-life of this compound and the modest increase observed during moderate-intensity exercise in humans elsewhere, the present finding is not necessarily surprising (Kraemer *et al.* 1985; Handa *et al.* 1991). Furthermore, the lack of an alteration in circulating enkephalin may support our previously proposed hypothesis, where the myocardium orchestrates release of Leu-enkephalin or other opioid compounds in response to physiological stress, such as IR. However, in a

recent investigation by Michelsen *et al.* (2012), dialysates prepared from exercising humans were administered to rabbits before *ex vivo* ischaemia–reperfusion. Their results demonstrated that the cardioprotective effect of exercise is mediated, at least in part, by a humoral factor, and the protection was diminished by administration of a non-specific δ -opioid antagonist (Michelsen *et al.* 2012). Neither administration of a non-selective opioid antagonist in their study nor the δ -opioid specific antagonist used in our study resulted in complete abolishment of exercise-induced protection against tissue necrosis (Michelsen *et al.* 2012); therefore, it is possible that protection is conferred by multiple receptor subtypes and compounds from within the heart as well as being delivered via circulation. Further study using reductive approaches and cell isolates are needed to resolve these unknowns.

Study limitations

A short-term exercise protocol was used in the present study to investigate the cardioprotective effect of exercise while avoiding confounding effects of cardiac remodelling. Whether a long-term exercise protocol would also elicit a δ -opioid receptor-mediated cardioprotective effect against tissue necrosis is unknown. However, trained men have elevated resting and postexercise levels of plasma enkephalin compared with untrained

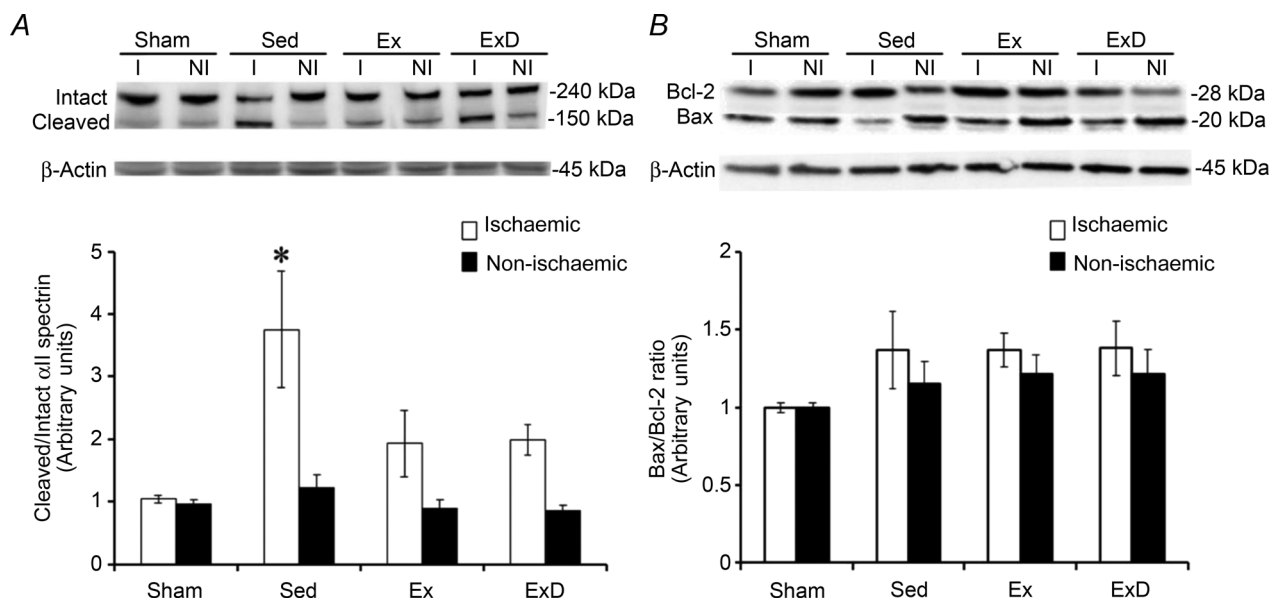


Figure 4. Western blot of apoptotic markers

A, cleaved/intact α II spectrin ratio. *B*, Bax/Bcl-2 ratio. Open bars represent ischaemic tissue ('I', non-perfused area at risk); filled bars represent non-ischaemic (perfused) tissue ('NI'). Values are normalized to β -actin and expressed relative to Sham. Data represent groups means \pm SEM. Sham, $n = 8$; Sed, $n = 9$; Ex, $n = 10$; and ExD, $n = 10$. * $P < 0.05$ significantly different from Sham group.

counterparts. Moreover, enkephalin release in response to chronic exercise is maintained and/or accentuated, while the response to non-exercise chronic stress models (e.g. restraint) is diminished by exposure day 7 (Pierzchala & Van Loon, 1990). Given these findings relative to our 7 day combined habituation and exercise protocol, there is cause to reject the notion that psychological stress due to forced treadmill running confounded the cardioprotective effects of exercise (Brown *et al.* 2007). Finally, experiments were performed to determine the effect of naltrindole in sedentary animals (Sed *versus* SedD) for only the key dependent measures of percentage infarct and TUNEL assay. Incorporation of a SedD control group in all analyses would have strengthened the present study, a practice that is recommended for potential follow-up investigations.

Conclusions

We provide novel evidence, which suggests that the δ -opioid receptor may be partly responsible for exercise-induced cardioprotection against necrotic tissue death following an ischaemia–reperfusion challenge. The known cardioprotective properties of opioid compounds, in combination with the sustainable protection afforded by exercise, make this area of research extremely important for the development of future therapies against IR injury. Furthermore, the possibility exists that other opioid receptor subtypes and peptides participate in exercise preconditioning. Characterization of the exercise effect on potentially cardioprotective opioid compounds in addition to receptor expression and activity may eventually prove beneficial with regard to prescription and dosage of pharmacological opioid compounds to patients undergoing cardiovascular procedures.

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Additional information

Competing interests

None declared.

Author contributions

All authors approved the final version of the manuscript. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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