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ORIGINAL PAPER

Exercise does not increase cyclooxygenase-2 myocardial levels in young or senescent hearts

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Abstract Increased myocardial cyclooxygenase-2 (COX-2) activity is essential for late phase ischemic preconditioning (IPC). Currently unknown is whether cardioprotection elicited by exercise also involves elevated myocardial COX-2 activity. This investigation tested whether aerobic exercise elevates myocardial COX-2 protein content or enzyme activity in young and senescent male Fisher 344 rats assigned to sedentary or cardioprotective endurance exercise treatments (3 consecutive days of treadmill exercise, 60 min/day @ ~70% VO_2max). Assay of cardiac COX-2 protein content, catalytic activity, and inducible nitric oxide synthase (iNOS) protein content reveal that exercise did not alter COX-2 activity (PGE_2, $p = 0.866$; PGF1α, $p = 0.796$) or protein levels ($p = 0.397$) within young or senescent hearts. In contrast, myocardial iNOS, an up-stream mediator of COX-2 expression, was over-expressed by an average of 37% in aged hearts ($p = 0.005$), though iNOS was not influenced by exercise. Findings reveal exercise does not elevate cardiac COX-2 activity and suggests that mechanisms responsible for cardioprotection differ between IPC and aerobic exercise.

Keywords Aging · Exercise · Prostaglandin · Myocardial · Physical activity · Senescence

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Introduction

In the United States and other industrialized countries, heart disease is the leading cause of death [1]. Coronary artery disease (CAD) is the most prevalent manifestation of heart disease resulting in myocardial death and dysfunction due to ischemia–reperfusion (IR) injury [1]. Further, the incidence of IR injury increases as a function of advancing age [1]. Developing strategic countermeasures to retard IR-induced cell death during an ischemic event is an ongoing research aim [3]. Two successful cardioprotective strategies to combat IR-injury include preconditioning via ischemic and exercise stimuli [2, 16, 17].

Ischemic preconditioning, a laboratory model of inducing a sub-lethal series of short duration (e.g., 2–5 min) ischemic episodes, results in a cardiac phenotype that is protected against subsequent IR injury [2]. Numerous studies reveal that ischemic preconditioning results in two windows of cardioprotection. An early window of protection occurs rapidly (a transient 2–4 h protective window) following ischemic preconditioning due to adenosine and bradykinin released during ischemia. In contrast, the second window appears ~24 h following the preconditioning treatment and provides a more robust cardioprotective response [2]. In

regard to the protein(s) responsible for late phase cardioprotection induced by ischemia, research indicates that over-expression of the enzyme cyclooxygenase (COX-2) is required to achieve preconditioning-induced cardioprotection [20, 23]. Indeed, when co-expressed with the inducible form of nitric oxide synthase (iNOS), COX-2 is thought to mediate cardioprotection through production of the prostanoids PGE_2 and $PGF_{1\alpha}$ [4, 21, 23]. Although the cellular mechanisms responsible for iNOS/COX-2-mediated cardioprotection remain a topic of debate, both iNOS and COX-2 could benefit the ischemic heart through the vasoactive properties of their respective products, NO, and PGE_2 and $PGF_{1\alpha}$, which can readily diffuse from myocardial to vascular tissue [14].

Similar to ischemic stimuli, as few as 1–3 days of moderate intensity ($\sim$0–70% VO_2max) aerobic exercise precondition the heart against IR injury [16, 17]. In contrast to ischemic preconditioning, however, the mechanism(s) responsible for the exercise-induced cardioprotective phenotype remain largely unknown. The fact that phenotypic similarities exist between ischemic-preconditioning and exercise-induced cardioprotection has led to speculation that common protective mechanisms exist for these two forms of cardioprotection [10].

Given the phenotypic parallels between ischemic and exercise preconditioning, it seems plausible that increased levels of COX-2 activity, and subsequent prostanoid production, may also be essential for exercise-induced cardioprotection. Accordingly, we tested the hypothesis that cardioprotective endurance exercise promotes increased myocardial COX-2 protein content and enzyme activity from heart tissue previously shown to be cardioprotected [18]. In light of the relationship between IR incidence and severity as a function of age, we chose to examine the effect of exercise on myocardial COX-2 content and activity in both young and senescent animals. Further, given that ischemic preconditioning is associated with a diminished cardioprotective adaptation in senescent animals [19], and exercise-mediated cardioprotection is not lost with age [18], the current study design takes on particular importance. For these experiments, we chose an established animal model for myocardial IR study (i.e., male Fisher 344 rats) and employed an exercise regimen that has been proven to promote cardioprotection in both young and senescent rats [18]. Our results reveal that exercise does not increase COX-2 enzyme activity or protein content within the hearts of either young or senescent animals. Therefore, our findings do not support the postulate that exercise-induced cardioprotection and ischemic preconditioning share over-expression of COX-2 as a common protective mechanism.

Methods

Animals and experimental design

The experimental protocol was approved by the University of Florida Animal Care and Use Committee and followed guidelines established by the American Physiological Society for the ethical use of animals in research. Young (6 months old) and senescent (24–26 months old) male Fisher 344 rats were used in this study (n = 8–9/treatment group). Young and senescent rats were randomly assigned to one of two experimental groups: (1) control-sedentary; or (2) exercise training. Animal treatment groups were abbreviated as follows: Y Sed = Young Sedentary; Y Ex = Young Exercised; O Sed = Old Sedentary; O Ex = Old Exercised. During the experimental period, all animals were housed on a 12:12-h light–dark cycle and provided food (AIN93 diet) and water ad libitum.

Cardioprotective exercise training protocol

We previously demonstrated that both long-term (weeks–months) and short-term (3–5 days) exercise are equally effective stimuli for exercise-induced cardioprotection against IR injury [7, 8]. Moreover, the time course to elicit cardioprotection with short-term exercise is similar to the time course for COX-2-dependent cardioprotection in "late phase" ischemic preconditioning models [2, 8, 15]. Therefore, we chose a short-term exercise training protocol in the current experiments. Animals assigned to the exercise group were habituated to the treadmill for 10 consecutive days. This habituation period involved a gradual increase in running time beginning with 5 min per day and ending with 50 min per day). Following 2 days of rest, the exercise habituated animals performed 3 consecutive days of treadmill exercise for 60 min/day at 30 m/min, 0% grade (young animals), and 20 m/min, 0% grade (senescent animals) [estimated work rate of $\sim$70% maximum oxygen consumption (VO_2max) for both young and old animals] [6, 13]. Hearts were excised 24 h following the final exercise bout for subsequent biochemical assay.

Tissue preparation

At the conclusion of the experimental period, animals were anesthetized with 60 mg/kg sodium pentobarbital. Once a surgical plane of anesthesia was reached, the hearts were rapidly removed. Portions of left ventricular free wall were sectioned into vertical strips cut from base to apex, loaded into freezer vials, and quickly frozen in liquid nitrogen for storage at -80°C until subsequent biochemical analysis could be performed.

Biochemical analysis of endogenous COX-2 enzyme activity

To assess the effect of exercise training on myocardial COX-2 capacity, ventricular samples from both groups were homogenized in cold 100 mM phosphate buffer with 0.5% bovine serum albumin (pH 7.4). Homogenates were centrifuged at 20,000*g* for 10 min at 4°C. The resulting supernatant was used to determine COX-2 enzyme activity and protein content of the homogenate [5]. Myocardial COX-2 activity was assessed via PGE_2 and $PGF_{1\alpha}$ enzyme immunoassay (EIA) kits purchased from Cayman Chemical (Ann Arbor, MI).

Western blot analysis of myocardial COX-2 and iNOS protein content

Myocardial COX-2 and iNOS protein levels were evaluated via western blot. Briefly, 90 μg of myocardial protein/sample were separated using standard SDS-PAGE techniques on a 7.5% polyacrylamide gel. Following electrophoresis, proteins were transferred to PVDF membranes and exposed to a rabbit polyclonal primary antibody for COX-2 (AbCam, Cambridge, MA, USA) and anti-rabbit IgG–HRP-conjugated secondary antibody (Amersham, Piscataway, NJ, USA) for chemiluminescence detection. Membranes were then re-probed for iNOS using a monoclonal antibody (AbCam) and anti-mouse IgG–HRP-conjugated secondary (AbCam). Western blots were analyzed using a Kodak digital imaging device (Rochester, NY, USA), and COX-2 and iNOS protein levels in hearts from exercised animals were expressed as a percent of the sedentary (control) animals.

Data analysis

Data are presented as mean ± standard error of the mean unless otherwise stated. A one-way ANOVA was performed to assess group differences for all dependent measures. A Tukey post hoc was performed when appropriate. Significance was established a priori at $p < 0.05$.

Results

Animal characteristics

Physical characteristics for young and old animals are presented in Table 1. As expected, old animals had a higher body mass and heart mass compared to young animals. Although our exercise program did not result in body weight differences in the young animals, exercise promoted a lower body weight in the O Ex animals compared to O Sed.

Biochemical analysis of COX-2 activity and protein content

Since exposure of the heart to an IR insult may promote over-expression of both iNOS and COX-2, we examined unstressed hearts from exercised and sedentary animals. Nonetheless, we have previously shown that the exercise training protocol used in these experiments results in cardioprotection against IR injury in both young and old F-344 rats exposed to an identical exercise regimen [18].

COX-2 protein content in hearts from young and old animals were present in low levels. Cardioprotective exercise training did not increase myocardial levels of COX-2 protein ($p = 0.397$) in the hearts of either young or old animals (Fig. 1a, b). Similarly, ventricular levels of iNOS, the up-stream mediator for COX-2, were detectable in low levels. In contrast to COX-2, significant differences in cardiac iNOS protein content were observed as a function of age ($p = 0.005$), but not exercise exposure (Fig. 2).

Myocardial COX-2 activity, measured by cardiac levels of PGE_2 and $PGF_{1\alpha}$, were determined to account for potential increases in COX-2 activity independent of expression. Results indicate that exercise training did not

Table 1 Experimental group size and animal body/heart weights

Group	Number	Body weight (g)	Heart weight (g)	Heart/body weight ratio (mg/g)
Y Sed	9	307 ± 7.4*#	0.96 ± 0.02*#	3.12 ± 0.07
Y Ex	8	302 ± 4.4*#	0.95 ± 0.02*#	3.17 ± 0.10
O Sed	8	419 ± 10.1#	1.28 ± 0.03	3.07 ± 0.12
O Ex	9	389 ± 3.8*	1.27 ± 0.02	3.27 ± 0.05

Values are means ± SE

Y Sed Young sedentary, *Y Ex* young exercised, *O Sed* old sedentary, *O Ex* old exercised

*Significantly different from O Sed, $P < 0.05$

#Significantly different from O Ex, $P < 0.05$

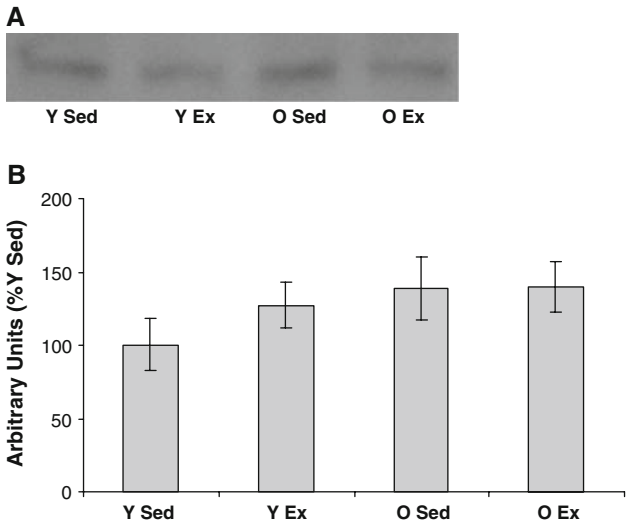

Fig. 1 Myocardial COX-2 protein content. **a** Representative western blot image of COX-2 in the exercised and sedentary myocardium of young and old animals. **b** Semi-quantitative analysis of COX-2 protein content integrated from multiple western blots. Significant differences were not observed between groups; $p > 0.05$. Data represent means ± SEM. *Y Sed* Young sedentary, *Y Ex* young exercised, *O Sed* old sedentary, *O Ex* old exercised

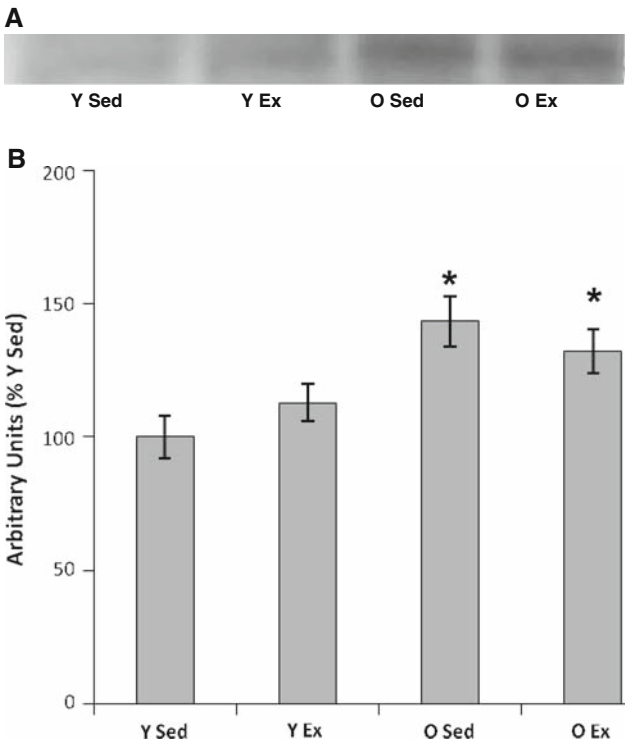

Fig. 2 Myocardial iNOS protein content **a** Representative Western blot image of iNOS in the exercised and sedentary myocardium of young and old animals. **b** Semi-quantitative analysis of iNOS protein content integrated from multiple western blots. *Denotes significant differences from Y Sed; $p = 0.005$. Data represent means ± SEM. *Y Sed* Young sedentary, *Y Ex* young exercised, *O Sed* old sedentary, *O Ex* old exercised

alter myocardial COX-2 catalytic activity ($p = 0.866$ for PGE_2, $p = 0.796$ for $PGF_{1\alpha}$) in the heart of either young or old animals (Fig. 3a, b). Collectively, these data demonstrate that exercise training does not promote increased myocardial levels of COX-2 protein or catalytic activity.

Discussion

Although it is clear that aerobic exercise results in cardioprotection, the mechanisms responsible for exercise-induced resistance to IR injury are not fully understood. To this end, we tested the hypothesis that exercise promotes increased myocardial COX-2 protein content and enzyme activity in young and senescent animals shown previously to be cardioprotected against IR-mediated cell death [18]. This hypothesis was formulated from evidence indicating: (1) ischemic and exercise preconditioning elicit cardioprotection against IR insults [2, 16, 17]; (2), the time course for exercise and late phase ischemic preconditioning are similar [2, 16, 17]; and (3) over-expression of myocardial COX-2 is an essential component of late phase ischemic preconditioning. Findings from the current study indicate that exercise training does not elevate myocardial levels of COX-2 in young or senescent animals. We conclude that increased COX-2 activity in the heart is not a mediator of cardioprotection induced by a short duration exercise.

COX-2 mediated cardioprotection

COX-2 is the rate-limiting enzyme for prostaglandin production via the arachidonic acid pathway [4]. Previous ischemic preconditioning research indicates that elevated COX-2 enzyme activity, demonstrated by elevations in COX-2-generated prostanoids including PGE_2 and $PGF_{1\alpha}$, is essential for late phase cardioprotection [4, 9, 20, 21]. While the salutary effects of increased prostanoids on the myocardium are not fully understood, potential mechanisms include activation of the mitochondrial K_{ATP} channels and improved coronary vascular compliance [4]. In the healthy unstressed heart, constitutive COX-2 levels are extremely low. However, COX-2 protein up-regulation is an obligatory cellular defense mechanism in response to stress cytokines, hypoxia, and ischemia [4]. In this regard, numerous factors associated with ischemic preconditioning have been shown to promote COX-2 expression including increases in protein kinase-c (PKCε), protein tyrosine kinases (PTK), janus kinase (JAK), nuclear factor kappa B (NF-κB), and signal transducer activator of transcription (STAT) 1, 3 [4]. These redundant signaling factors for COX-2 expression ensure elevated COX-2 activity in the post ischemic myocardium and implicate COX-2 as an

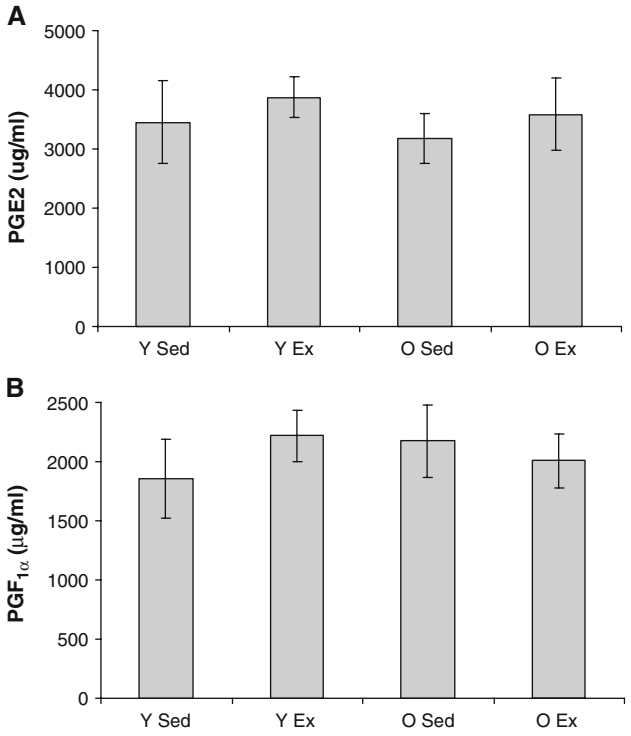

Fig. 3 Analysis of myocardial COX-2 generated prostanoids. **a** EIA analysis of COX-2-generated PGE_2 in the exercised and sedentary myocardium of young and old animals. Significant differences were not observed between groups; $p > 0.05$. **b** EIA analysis of COX-2-generated $PGF_{1\alpha}$ in the exercised and sedentary myocardium of young and old animals. Significant differences were not observed between groups; $p > 0.05$. Data represent means ± SEM. *Y Sed* Young sedentary, *Y Ex* young exercised, *O Sed* old sedentary, *O Ex* old exercised

important cytoprotective mediator [4]. Further, previous ischemic preconditioning research indicates that iNOS up-regulation must precede COX-2 increases [23].

Findings from the current study demonstrate that cardiac iNOS levels exhibited modest, but significant, increases in senescent hearts, while short duration exercise did not influence iNOS expression. Recent findings by Lawler et al. [12] found cardiac iNOS levels in sedentary aged hearts to be 6-fold higher than young counterparts, while 12 weeks of exercise training abolished this age-dependent increase in cardiac iNOS. In aggregate, we conclude that neither short nor long duration exercise elevates cardiac iNOS as part of the preconditioning stimulus. This conclusion supports the notion that exercise is a sustainable "stress without distress" stimulus [11]. In contrast, the inflammatory mediators iNOS and COX-2 are integral to ischemic preconditioning suggesting that the initial ischemic stimulus is distressing to the myocardium. In support, inflammatory responses to ischemic stress are not biologically intended to promoting long-term survival [22]. This conclusion supports the observation that exercise-induced cardioprotection is maintained with senescence, while the protective stimulus of ischemic preconditioning is lost as a function of increasing age [18, 19].

The search for mechanisms of exercise-induced cardioprotection

The quest to discover mechanisms of exercise-induced cardioprotection has produced a growing list of potential cardioprotective mediators. Our approach to investigate mechanisms of exercise-induced cardioprotection test each potential mediator as a rival hypothesis. To this end, the current study reveals that myocardial COX-2 protein activity is not elevated by short duration exercise. As such, COX-2 can be added to a growing list of disproved protective mechanisms that are not essential cardioprotective mediators of short duration exercise against IR injury. Hence, by elimination of a rival hypothesis, the current study advances our knowledge of exercise-induced cardioprotection against IR injury. Moreover, that COX-2 is not responsible for cardioprotection in exercised reveals important mechanistic differences between exercise training and ischemic preconditioning. Future work is needed to better understand the unique cardioprotective stimulus of short duration exercise.

Conclusions

The impetus for this study evolved from the knowledge that myocardial COX-2 activity is essential for the late phase ischemic preconditioning [3]. We hypothesized that exercise would promote increased cardiac COX-2 activity and that cardioprotection elicited by exercise and ischemic preconditioning may share a common mechanism of increased myocardial COX-2 activity. Our results from tissues previously shown to be cardioprotected [18] do not support this hypothesis, and these data provide the first evidence that myocardial COX-2 expression is not increased in young or senescent animals exposed to short duration exercise training. The observation that exercise and ischemic preconditioning may involve unique mechanisms of cardioprotection is an important finding and suggests the existence of multiple independent cardioprotective mediators against IR. Moreover, disparate cardioprotective mechanisms between exercise and ischemic preconditioning may help explain why exercise confers cardioprotection in senescent hearts while ischemic preconditioning does not [18, 19].

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