Redox-dependent increases in glutathione reductase and exercise preconditioning: role of NADPH oxidase and mitochondria

Chad R. Frasier1, Fatiha Moukdar1, Hetal D. Patel1, Ruben C. Sloan2, Luke M. Stewart1, Rick J. Alleman1, Justin D. La Favor2, and David A. Brown1*

1Department of Physiology, Brody 6N-98, 600 Mays Blvd, Brody School of Medicine, East Carolina University, Greenville, NC 27834, USA; and 2Department of Kinesiology, East Carolina University, Greenville, NC 27834, USA

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Aims
We have previously shown that exercise leads to sustainable cardioprotection through a mechanism involving improved glutathione replenishment. This study was conducted to determine if redox-dependent modifications in glutathione reductase (GR) were involved in exercise cardioprotection. Furthermore, we sought to determine if reactive oxygen species generated by NADPH oxidase and/or mitochondria during exercise were triggering events for GR modulations.

Methods and results
Rats were exercised for 10 consecutive days, after which isolated hearts were exposed to ischaemia/reperfusion (25 min/120 min). Exercise protected against infarction and arrhythmia, and preserved coronary flow. The GR inhibitor BCNU abolished the beneficial effects. GR activity was increased following exercise in a redox-dependent manner, with no change in GR protein levels. Because fluorescent labelling of GR protein thiols showed lower amounts of reduced thiols after exercise, we sought to determine the source of intracellular reactive oxygen species that may be activating GR. Subsets of animals were exercised immediately after treatment with either NADPH-oxidase inhibitors apocynin or Vas2870, or with mitoTEMPO or Bendavia, which reduce mitochondrial reactive oxygen species levels. The cardioprotective effects of exercise were abolished if animals exercised in the presence of NADPH oxidase inhibitors, in clear contrast to the mitochondrial reagents. These changes correlated with thiol-dependent modifications of GR.

Conclusion
Adaptive cardioprotective signalling is triggered by reactive oxygen species from NADPH oxidase, and leads to improved glutathione replenishment through redox-dependent modifications in GR.

Keywords
Cardioprotection • Glutathione • Bendavia • Exercise • Reactive oxygen species

1. Introduction
The burden of ischaemic heart disease continues to expand across the globe. Despite scores of treatments that decrease cardiac ischaemia/reperfusion injury in experimental models, none of these have translated to routine clinical practice. Exercise is one of the few preconditioning stimuli shown to provide sustainable cardioprotection in humans as well as experimental models. Despite the clear utility of exercise medicine, the underlying mechanisms are not completely understood, precluding the development of sustainable therapies that could benefit patients who cannot (or will not) adhere to an exercise prescription.

The cardiac glutathione redox couple is the largest capacity thiol buffer in the heart, and exerts a profound effect on myocardial viability. Oxidation of the glutathione couple promotes the opening of energy-dissipating channels/pores in heart mitochondria, and a number of studies by our group and others have shown that preservation of the glutathione couple correlates with protection against injury. We recently demonstrated that exercise augments glutathione replenishment and protects tissue against...
electromechanical dysfunction and cell death. This was associated with an increase in glutathione reductase (GR) activity, although the mechanism(s) responsible for up-regulating GR activity after repetitive exercise bouts are not known.

While an overload of reactive oxygen species (ROS) is associated with cellular injury, it is clear that ROS have a hormesis effect in many biological systems. Beneficial signalling triggered by reactive oxygen species is seen in several distinct preconditioning paradigms, implying that redox-dependent modifications of intracellular proteins and/or signalling cascades are centrally involved across models of cardioprotection. In previous exercise studies, ROS have been implicated in the protective signalling cascade, although the intracellular site(s) of ROS production, as well as the downstream targets of ROS-signalling, are not fully understood. Given that ROS are known to increase GR activity, we hypothesized that this mechanism was centrally involved in adaptive signalling with exercise.

This study was conducted with three major objectives: first, to confirm that exercise-induced cardioprotection is dependent on heightened GR activity. Secondly, to determine if increased GR activity following exercise was due to heightened GR protein levels and/or redox-dependent modifications of GR protein thiols. Finally, we used inhibitors known to influence ROS production by NADPH oxidase or mitochondria to determine the intracellular locus of ROS production during exercise that triggers cardioprotective cascades.

2. Methods

2.1 Animals

All experiments were conducted in accordance with guidelines established by the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996), and with prior approval by East Carolina University’s Animal Care and Use Committee (Internal Animal Use Protocol #Q279). Female Sprague-Dawley rats (150–250 g) were housed on a 12:12 h light–dark cycle with free access to food and water. A total of 114 animals were used in this study.

2.2 Study overview

The study was conducted using the research design depicted in Figure 1. A total of three animals were excluded because they received additional ketamine/xylazine anaesthesia ‘boosters’ before reflexes were absent, which is known to influence the extent of injury. Three animals were excluded from the study due to problems establishing coronary flow ex vivo.

2.3 Exercise protocol

Rats were exercised daily as described previously. Briefly, rats were given a 3-day acclimation period on the treadmill. Exercised (Ex) animals then received 10 consecutive days of treadmill running for 60 min per day at 15 m/min for 15 min, 30 m/min for 30 min, and 15 m/min for 15 min. Animals in the sedentary (Sed) group were placed on the non-moving treadmill each day as handling controls.

2.4 Inhibition of ROS-mediated signalling during exercise

To test our hypothesis that ROS produced during exercise may trigger cardioprotection, we administered various blockers of ROS production prior to exercise. To block the NADPH oxidase, we used apocynin or Vas2870. To block mitochondria-dependent ROS signalling, we used mitoTEMPO30–32 or Bendavia. For the establishment of our control groups, a subset of rats was randomly assigned to receive a daily injection (i.p.) of 0.9% saline (n = 14 Sed and 12 Ex). To blunt ROS-mediated signalling during exercise, rats were randomly assigned to receive either 1.5 mg/kg Bendavia (n = 7), 0.7 mg/kg mitoTEMPO (n = 7), 5 mg/kg apocynin (n = 12), or 2 mg/kg Vas2870 (n = 5) 10 min prior to each bout of exercise. A separate subset of Sed animals was given Bendavia or apocynin prior to handling control for 10 days (n = 7 for Bendavia and 8 for apocynin).

2.5 Isolated heart experiments

Twenty-four hours after the last bout of exercise, rats were injected with ketamine/xylazine (90 mg/kg ketamine, 10 mg/kg xylazine, i.p.). Upon the absence of animal eye-blink, toe-pinch, and righting reflexes, hearts were excised and retrograde perfused on the cannula of a modified Langendorff apparatus. Electromechanical function and coronary flow were monitored continuously using our established protocols. Following a 15 min baseline period global, no-flow, ischaemia was induced for 25 min. After 25 min, the static buffer was drained from the perfusion lines and flow was re-established for 2 h. Immediately following reperfusion, infarct size and arrhythmia scores were determined as described previously. The incidence of ventricular fibrillation (VF) and fatal arrhythmias was also noted for each group.

A separate subgroup of hearts was perfused under normoxic conditions for 10 min before being snap frozen for subsequent biochemical analysis (n = 4/group for Sed, Ex, and Ex + Apocynin).

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/98/1/47/313200)
2.6 GR inhibition

Another subset of hearts was perfused with BCNU (carmustine) to inhibit GR activity. Following a 10 min baseline period, the buffer was switched to a Kreb’s buffer with 150 μM BCNU for 5 min before global ischaemia induction (n = 6–7/group for Sed, Sed + BCNU, Ex, and Ex + BCNU) and continued throughout the reperfusion period. This concentration of BCNU has been previously shown to adequately inhibit GR activity.14,39

2.7 Myocardial glutathione content and GR/peroxidase activity

Glutathione content was measured as described previously.12,13 To assess the amount of glutathione oxidation that occurs during exercise, animals were anaesthetized and left ventricles snap frozen (n = 4/group for Sed, Ex, and Ex + Apo).

Glutathione reductase and glutathione peroxidase activity was measured in tissue homogenates as described previously.11 To determine the redox sensitivity of GR within cardiac tissue a subset of experiments were carried out in which tissue homogenates were incubated with DTT (2 mM) or diamide (200 μM) at 4°C for 20 min prior to testing the activity.

2.8 GR protein content and thiol redox state

For GR protein content, left-ventricular homogenates (from separate samples that did not undergo ischaemia/reperfusion) were subjected to SDS–PAGE and subsequent transfer to a nitrocellulose membrane. Membranes were blocked for 1 h at room temperature with Tris-buffered saline containing 0.1% Tween and 4% bovine serum albumin. Following blocking, the membrane was incubated with an antibody for GR (1:1000; Santa Cruz) or GAPDH (1:2000; Sigma) overnight at 4°C. The membrane was then washed and incubated with the appropriate IR-Dye-conjugated secondary antibody (LiCor Biosciences). Membranes were scanned and quantified using the Odyssey Infrared Imaging system (LiCor Biosciences).

Detection of free thiol (SH) groups on GR was done through free thiol labelling with IR-Dye 800CW-Maleimide as described previously,39 followed by GR immunoprecipitation. Briefly, left ventricle tissues were homogenized in buffer (0.1 M Na2HPO4, 0.15 M NaCl, and 0.1% Tween; pH 7.5) containing protease inhibitor cocktail and 100 mM of IR-Dye 800CW-Maleimide. Five hundred micrograms of total labelled proteins were incubated with 2 μg GR antibody and 40 μL of Protein A/G PLUS-Agarose beads slurry (Santa Cruz), overnight at 4°C to immunoprecipitate GR. The immunoprecipitated proteins were eluted with Laemmli loading buffer, underwent SDS–PAGE, and gels were scanned and quantified as described earlier. As positive controls, separate LV samples were incubated with either 5 mM H2O2 (oxidizes thiols) or 5 mM diithiothreitol (DTT; reduces thiols) and underwent the labelling with the IR-Dye 800CW-Maleimide (after removing excess DTT and H2O2 over Zeba desalting columns). The samples were then subjected to SDS–PAGE as described earlier. Thiol redox state of GR (as assessed with maleimide) was normalized to total GR content by dividing the intensity of the bands following the maleimide labelling by the band intensity of the same samples run in a separate western blot and probed for total GR (GR-maleimide/Total GR for the same samples).

2.9 Statistical analysis

All data are presented as mean ± s.e.m. Data in Table 1 were pooled as a function of exercise group since there were no effects of the injected compound on any variables. Infarct size, arrhythmia scores, mean coronary flow, tissue glutathione, Gpx activity, GR activity, GR content, and GR redox status were analysed with an ANOVA followed by Newman–Keuls post-hoc test. Coronary flow data in Figure 2D were analysed with repeated measures ANOVA. Between-group comparisons for the incidence of VF and fatal arrhythmias were made using a χ² test. For all comparisons, statistical significance was determined when P < 0.05.

3. Results

3.1 Animal morphology and baseline characteristics

Rat morphological data are presented in Table 1. No differences were observed in body weight between groups. Consistent with our previous studies,13,41 no indices of stress (as assessed by splenic atrophy or adrenal hypertrophy) were seen following the exercise regimen. Ten days of exercise was sufficient to induce cardiac hypertrophy in our Ex animals (P < 0.05), in agreement with previous findings from our group.13

3.2 Inhibition of GR abolishes exercise cardioprotection

The effects of exercise and GR inhibition on infarction and arrhythmia are presented in Figure 2. Ex animals had significantly lower levels of myocardial infarction when compared with Sed controls (P < 0.05; Figure 2A). Perfusion with BCNU abolished the protective effects of Ex from myocardial infarction (P < 0.05), but had no significant effect on the Sed group.

The severity of ventricular arrhythmias during early reperfusion was significantly reduced in our Ex group (Figure 2B). Ex also decreased the incidence of VF (67 vs. 17% for Sed and Ex, respectively; P < 0.05). Perfusion with BCNU also abolished the protection of exercise against the severity of arrhythmias (Figure 2B; P < 0.05 vs. Ex) and incidence of VF (83% for Ex + BCNU; P < 0.05 vs. Ex). There was no significant effect of BCNU on the incidence or severity of arrhythmias in the Sed group.

Exercise also led to a significant improvement in coronary flow during reperfusion. Sustained coronary flow in the exercise group was apparent within the first 15 min of reperfusion (Figure 2C), and was sustained throughout the reperfusion period (for clarity, the first hour of reperfusion is depicted in Figure 2D). Improved coronary flow rates with exercise were abolished with BCNU treatment.

3.3 Reactive oxygen species signalling in exercise-induced preconditioning

We then determined if the location of ROS production during a bout of exercise plays a role in the cardioprotective phenotype (Figure 3). Ex animals treated with 0.9% NaCl had a significant reduction in infarct size compared with Sed counterparts (P < 0.05). Treatment
**Figure 2** Effects of exercise and glutathione reductase inhibition (with BCNU) on cardiac ischaemia/reperfusion injury. (A) Infarct sizes, expressed as a % of the area-at-risk (AAR) in sedentary (Sed) and exercised (Ex) hearts. (B) Arrhythmia scores for hearts in the study. (C) Mean rates of myocardial coronary flow in early reperfusion (15 min after initiation of reperfusion). (D) Coronary flow rates (taken in 30 s epochs) for the first hour of reperfusion. All data are presented as mean ± s.e.m. *P < 0.05 vs. Sed untreated; #P < 0.05 vs. Ex untreated; n’s are 6–7 per group.

**Figure 3** Effects of selective reactive oxygen species inhibition during exercise on cardiac ischaemia/reperfusion injury. Immediately prior to exercise, rats were treated with 0.9% NaCl (controls), with compounds to inhibit mitochondrial ROS-mediated signalling (Bendavia or mitoTEMPO), or with compounds to inhibit NADPH-oxidase activity (apocynin or Vas2870). Infarct sizes were assessed 24 h after the last exercise bout. N’s were 5–8 per group. *P < 0.05, vs. Sed (ANOVA).
with Bendavia or mitoTEMPO did not influence the reduction in infarct size in our Ex group. Treatment with NADPH Oxidase inhibitors apocynin and Vas2870 abolished the protective effects of exercise ($P < 0.05$ vs. Ex + 0.9% NaCl). Sed animals treated for 10 days with Bendavia displayed a reduction of infarct size from 56 $\pm$ 3 to 41 $\pm$ 3% of the area-at-risk ($P < 0.05$), while treatment with apocynin had no effect on infarct size (infarct size was 55 $\pm$ 3% of area-at-risk; $P > 0.05$ vs. untreated Sed).

### 3.4 Acute exercise leads to glutathione oxidation and activation of GR

The effects of exercise on GSH/GSSG and GR activity are presented in Figure 4. The GSH/GSSG ratio was significantly oxidized immediately following exercise, which was blunted with the NADPH oxidase inhibitor apocynin ($P < 0.05$). Total glutathione (GSH$_{t}$) and oxidized glutathione (GSSG) were not significantly altered immediately after exercise, although there were statistical trends for both variables with the ANOVA employed ($P = 0.11$ for both GSH$_{t}$ and GSSG).

Ex animals had significantly higher GR activity both immediately after exercise, as well as 24 h later, and in both cases this was abolished with apocynin (Figure 4D). GR activity was also very sensitive to thiol redox modification. Across groups, GR activity increased when samples were treated with diamide, a thiol oxidant, and conversely GR activity went down when samples were treated with the thiol reductant DTT (Figure 4D; $P < 0.05$ for main effect of thiol state, 2-way ANOVA). Activities of glutathione peroxidase were not different between Sed and Ex hearts ($11.9 \pm 0.7$ and $11.4 \pm 0.3$ U/g protein; $P > 0.05$).

![Figure 4](https://academic.oup.com/cardiovascres/article-abstract/98/1/47/313200) Glutathione and glutathione reductase activities for hearts in the study. (A) Myocardial glutathione levels immediately after exercise. Exercise resulted in a significant reduction in the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Inhibition of NADPH oxidase activity during exercise with apocynin (5 mg/kg) attenuated the drop in GSH/GSSG with exercise, suggesting that NADPH oxidase is a source of reactive oxygen species during exercise. (B) Total myocardial glutathione levels (GSH$_{t}$) immediately after exercise. (C) Oxidized glutathione (GSSG) immediately after exercise. (D) Myocardial glutathione reductase activity for hearts in the study. *$P < 0.05$ vs. respective Sed group (ANOVA followed by Newman–Keuls post-hoc test); **$P < 0.05$ vs. Ex (ANOVA followed by Newman–Keuls post-hoc test); ***$P < 0.05$ (Group main effect of thiol treatment; 2-way ANOVA).
3.5 GR thiol redox state and protein expression

We then determined if the increased activity of GR 24 h after exercise (i.e. at the timepoint when cardioprotection was observed), was due to redox-dependent modulation of GR protein thiols. Using fluorescent maleimide labelling, free thiols on GR from Ex animals were significantly lower than Sed counterparts ($P < 0.05$; Figure 5A and B). This decrease was abolished if animals exercised in the presence of apocynin. No difference was seen in the expression of GR protein between any of the groups ($P > 0.05$; Figure 5C and D), using GAPDH as a loading control, suggesting that the increased activity we observed was likely due to post-translational modification.

4. Discussion

In this study, we tested the hypothesis that redox-dependent increases in GR activity contributes to exercise preconditioning. We sought to determine if reactive oxygen species production (ROS) by NADPH oxidase and/or heart mitochondria during exercise triggered the adaptive signalling. To the best of our knowledge, several aspects of this work represent novel findings. First, this work is the first to show that inhibition of GR abolishes exercise-induced protection against infarction and arrhythmia. Inhibiting GR also abrogated the beneficial effects of exercise on coronary flow during reperfusion.

Secondly, increased cardiac GR activity following exercise is due to redox-dependent modulation of GR protein free thiols, and not due to an increase in GR protein expression. Thirdly, the source of ROS that ‘triggers’ the increase in GR activity is NADPH oxidase, and does not appear to be mitochondrial in origin. Taken together, this study enhances our mechanistic understanding of endogenous cardioprotective signalling in the heart, from the ‘triggering’ events to the end-effector.

4.1 Cardiac glutathione and ischaemia/reperfusion injury

Although the aetiology of cardiac ischaemia/reperfusion injury is multifactorial and varies as a function of time, ROS are significant contributors to necrotic and apoptotic cell death (reviewed in Murphy and Steenbergen). Among endogenous ROS-scavenging mechanisms, the glutathione redox couple is the largest capacity thiol buffer in the heart, and oxidation of glutathione is commonly observed in ischaemia/reperfusion. Reductions in the reduced/oxidized glutathione ratio (GSH/GSSG) are known to collapse mitochondrial energetics and lead to electromechanical dysfunction and cell death. Interventions that sustain GSH/GSSG protect the heart by maintaining the bioenergetic integrity of the cell.

With regards to exercise preconditioning in particular, several different ROS-scavenging systems are enhanced after exercise. Increased superoxide dismutase (specifically the manganese isoform associated

Figure 5 Redox-dependent modification and total content of glutathione reductase from hearts in the study. Free (reactive) thiols were labelled with fluorescent maleimide, followed by glutathione reductase immunoprecipitation. (A) Representative blots from hearts in the study, as well as blots following treatment with HO2 and dithiothreitol. (B) Quantification of the thiol redox state of glutathione reductase, normalized to total glutathione reductase content from the same samples. All bands are at 50 kDa. *$P < 0.05$ vs. control (ANOVA). (C) Western blots for total glutathione reductase content, normalized to GAPDH on the same gel. (D) Quantification of total glutathione reductase divided by GAPDH. There were no differences in the protein content of glutathione reductase between any of the groups in the study.
with mitochondria) is commonly observed after exercise (reviewed in Brown and Moore\textsuperscript{69} and Powers et al.\textsuperscript{70}), but there is less support for heightened enzymatic $\text{H}_2\text{O}_2$-scavenging following exercise. Specifically, most studies find that basal activities of catalase, and thioredoxin are not changed after exercise (reviewed in Frazier et al.\textsuperscript{51}). Furthermore, we found no change in glutathione peroxidase activity following exercise in this study, consistent with a number of previous investigations.\textsuperscript{10,13,32–54} We very recently found that total cardiac glutathione is not altered after exercise in our model,\textsuperscript{13} which was also observed by others using female rats and treadmill running.\textsuperscript{57}

While $\text{H}_2\text{O}_2$-scavenging capacity is not influenced by exercise, we (Frazier et al.\textsuperscript{13} and herein) and others\textsuperscript{57,58} have shown that increased GR is observed in the heart after repetitive (>5 days) exercise. This heightened GR activity improves the heart's ability to replenish glutathione, and helps maintain favourable GSH/GSSG in the face of oxidative stress.\textsuperscript{13} Since there is little de novo synthesis of GSH in the heart\textsuperscript{69} the ability to replenish GSH keeps the total cellular glutathione pool high, as GSSG displays high cell permeability and is released from tissue during oxidative stress.\textsuperscript{50,61} The observation in this study that inhibition of GR abolishes the cardioprotection by exercise is the first to confirm the importance of GR in exercise preconditioning. An interesting area for subsequent investigation will be to ascertain spatial changes in GSH/GSSG. Specifically, future studies examining the replenishment of GSH/GSSG in cytosolic vs. mitochondrial fractions will advance our mechanistic understanding of how augmented GR enhances cardioprotection. In light of the protection was observed with exercise, we then sought to identify the mechanism whereby exercise enhanced GR activity.

### 4.2 Adaptive signalling during exercise is triggered by ROS

Although commonly attributed with cellular injury, small amounts of ROS are integral to cardioprotective signalling across models.\textsuperscript{16–18,26,62–68} With exercise preconditioning, several studies have shown that ROS-scavenging during exercise abrogates the beneficial effects against infarction\textsuperscript{13,69} and LV dysfunction,\textsuperscript{19} although the site of ROS production during exercise was not ascertained with the scavenger used in these studies (mercaptopyrroionyl glyoxime). The ROS produced by exercise is reflected by an acute reduction in GSH/GSSG immediately after exercise\textsuperscript{69} (Figure 4), which is commonly seen to recover within 24 h,\textsuperscript{13,69} when cardioprotection is observed. In the present work, we found that inhibition of NADPH oxidase with either apocynin or Vas2870 during exercise abolished exercise-induced reductions in infarct size, corroborating previous findings in dogs by Sanchez et al.\textsuperscript{20} Our finding that Vas2870 treatment was similar to apocynin is important in light of recent studies questioning the specificity of apocynin as a NADPH oxidase blocker.\textsuperscript{70,71}

This study extends our understanding regarding the locus of ROS production during exercise, as the mitochondria-targeting compounds Bendavia and mitoTEMPO did not diminish the cardioprotection seen after exercise. Ten consecutive days of Bendavia treatment protected sedentary hearts against injury, corroborating previous studies where this compound was administered acutely.\textsuperscript{33,34,72}

### 4.3 Redox-dependent alterations in GR

In our study, exercise evoked redox-dependent changes in GR activity in the absence of increased GR protein expression. Following exercise, GR activity increased, concomitant with an observed decrease in GSH/GSSG. These data are in agreement with a number of studies that show an inverse relationship between GR activity and reduced glutathione. Specifically, GR activity is inhibited with high concentrations of GSH,\textsuperscript{22,23} as well as thiol reductants,\textsuperscript{24} (Figure 4). Conversely, oxidative shifts in the intracellular redox environment are known to activate GR activity,\textsuperscript{24} and we also observed increased GR activity across experimental groups treated with the thiol oxidant DTT. Our study is the first to show bioavailability of free $-\text{SH}$ groups on GR decreases after exercise, resulting in increased GR activity and reduction in ischaemia/reperfusion injury. These data are in general agreement with previous studies showing oxidation of intracellular protein thiols as key events underlying exercise cardioprotection.\textsuperscript{20} An interesting area for future research will be to ascertain the exact nature, time-course, and reversibility of the GR post-translational modification(s) responsible for enhanced GR activity.

### 4.4 Physiological role for NADPH oxidase-generated ROS

Our findings implicate ROS generated by NADPH oxidase, and not by heart mitochondria, as the culprit triggering exercise preconditioning. ROS production by NADPH oxidase is commonly associated with pathological states in the heart (reviewed in Murdoch et al.\textsuperscript{73}), but findings of this study as well as other groups\textsuperscript{57,74} suggest that transient activation of NADPH promotes cardioprotective phenotypes. The major isoforms of NADPH oxidase in the heart are NOX2 and NOX4.\textsuperscript{73,75} Although Vas2870 is a pan-NOX inhibitor,\textsuperscript{76} apocynin inhibits NOX2 by interfering with the association of cytosolic p$47^{\text{phox}}$ subunits with the membrane-bound complex.\textsuperscript{77} As NOX4 has no p$47^{\text{phox}}$, it is tempting to speculate that our exercise-induced cardioprotection was triggered specifically by NOX2, and not by NOX4. While we did not investigate the expression/trafficking of NOX2 subunits in our study, previous work by Donoso’s group found that p$47^{\text{phox}}$ (as well as rac1 subunit) protein expression into membrane fractions was higher after exercise,\textsuperscript{20} and led to greater superoxide production (which was inhibited if animals exercised in the presence of apocynin). Consistent with the idea that transient NOX2 activation is involved in endogenous cardioprotective signalling, NOX2-deficient mice have been shown to be insensitive to ischaemic preconditioning.\textsuperscript{74} Since physiological stretch of the heart has been shown to activate ROS production by NOX2,\textsuperscript{78} one could envision a mechanism of protection where the positive inotropic effects of exercise stretches the myocardium, activating ROS through a NOX-dependent pathway and promoting cardioprotection by altering the thiol redox state of key intracellular proteins (such as GR). Clearly, further research is warranted to substantiate this line of thinking. The advent of new genetic knockout models for subunits of NADPH-oxidase and GR (Gr1a1Neu) will surely improve our understanding of this protective signalling pathway, with the ultimate hope that new and sustainable cardioprotective strategies may reduce the burden of ischaemic heart disease by exploiting these pathways.

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