Short-term exercise preserves myocardial glutathione and decreases arrhythmias after thiol oxidation and ischemia in isolated rat hearts

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Sudden cardiac death due to sustained ventricular arrhythmia is a significant cause of mortality after a myocardial infarction, especially in patients with underlying cardiovascular disease (31). Epidemiological evidence has indicated that humans who exercise (Ex) regularly are more prone to survive a myocardial infarction (32), likely due to a significantly lower incidence of sudden cardiac death among Ex-trained individuals (23). The cardioprotective effect of Ex training is also well documented in animal models, as Ex confers resistance against several different indexes of ischemia-reperfusion (I/R) injury, including infarction (for reviews, see Refs. 13 and 19), myocardial stunning (7, 11, 25, 30), and arrhythmia (for a review, see Ref. 6). With regard to the antiarrhythmic effects of Ex, several studies have noted that Ex evokes an antiarrhythmic phenotype characterized by a lower incidence of ventricular arrhythmia (6, 24, 26, 35) and an increased ventricular fibrillation (VF) threshold (33). Despite the clear association between Ex and resistance to arrhythmia, the cellular mechanisms have not been fully elucidated.

Although prompt reperfusion remains the best treatment of an ischemic event, the abrupt flow restoration is associated with a burst of ROS that is believed to be partly responsible for reperfusion injury (39, 50). This ROS burst may lead to fatal arrhythmias (for a review, see Ref. 14), and strategies that improve mitochondrial ROS scavenging have clear potential in mitigating electrical dysfunction. Ex has been shown to decrease ROS-mediated myocardial damage (41), but an explanation of how improved tolerance to an oxidative insult protects Ex-trained hearts against arrhythmia has not been provided.

Cardiac glutathione represents the largest capacity thiol buffer in the heart (36) and exerts a significant effect on mitochondrial function (3, 47). Recent data have implicated the glutathione redox couple as a “pivoting point” between ROS balance and mitochondrial dysfunction (3, 5, 28, 38). During conditions where ROS generation exceeds scavenging capacity (such as early reperfusion), ROS-mediated opening of energy-dissipating ion channels in the inner mitochondrial membrane leads to an instability in mitochondrial membrane potential (ΔΨm) (4). Oscillations in ΔΨm activate sarcolemmal ATP-sensitive K+ channels and can induce lability in the cardiac action potential (1, 4), a prime substrate for reentrant arrhythmia (14). Previous investigations have found that collapses in ΔΨm occur when the reduced-to-oxidized glutathione ratio (GSH/GSSG) reaches a “critical” level, and our previous work indicated that chemically oxidizing the cellular thiol pool induced ventricular arrhythmias under otherwise normoxic conditions (9). Pharmacological treatments that sustain GSH/GSSG have been shown to stabilize ΔΨm (3, 9, 20) and prevent arrhythmias (9), but whether a physiological stimulus such as Ex can reduce arrhythmia by maintaining GSH/GSSG is not known.

Given the important influence that glutathione can exert on cardiac electrical activity, we conducted this study to determine if the antiarrhythmic effects of short-term Ex were due to an improved tolerance to oxidative stress by the cardiac glutathione system. We hypothesized that 10 days of Ex would reduce arrhythmias evoked by either chemical oxidation of glutathione or I/R. We also postulated that isolated myocytes from Ex-trained animals would show diminished ROS emission and improved viability during cellular oxidative challenge.
and that this protective phenotype would be associated with a maintenance of the ROS-buffering capacity by the glutathione system.

**METHODS**

**Experimental animals.** Female Sprague-Dawley rats (150–250 g) were housed on a 12:12-h light-dark cycle with food and water provided ad libitum. All experiments were conducted in accordance with guidelines established by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 1996) and with prior approval from the Animal Care and Use Committee of East Carolina University.

**Ex protocol.** Rats were randomly assigned into one of two experimental groups: sedentary control (Sed) or short-term Ex. Ex was performed on a motorized treadmill similar to a well-established protocol (10). We chose an Ex protocol consisting of 10 days of running because our previous work indicated that this protocol evokes both a cardioprotective phenotype and skeletal muscle adaptation to Ex while minimizing the stress response in female rats (12). After 3 days of acclimation to the treadmill (15 m/min for 5, 10, and 15 min for days 1, 2, and 3, respectively), Ex animals received 10 consecutive days of treadmill running (6% grade) using the following protocol: 15 m/min for 15 min, 30 m/min for 30 min, and 15 m/min for 15 min (Fig. 1A).

Animals that did not run were placed back on the moving treadmill using prodding or a mild shock via the shock grid at the end of each running lane. Sed animals were handled and placed on the stationary treadmill for 5 min each day for 10 days.

**Experimental groups.** A total of 71 rats were used in the study. After 10 days of Ex (or handling control), experimental animals were used in one of the following six study arms: 1) whole heart diamide perfusion until the heart went into a sustained (>10 continuous seconds of) ventricular arrhythmia (*n* = 8 Sed and 8 Ex animals); 2) whole heart diamide perfusion for 39 min, the mean diamide perfusion time that evoked arrhythmia in Sed hearts (*n* = 8 for Ex animals only); 3) whole heart diamide perfusion for 30 min following a 20-min washout period (*n* = 7 Sed and 7 Ex animals); 4) whole heart I/R experiments (*n* = 7 Sed and 7 Ex animals); 5) whole heart perfusion for 15 min (with no diamide) for “untreated controls” (*n* = 6 Sed and 5 Ex animals); or 6) isolated cardiac myocyte experiments (*n* = 4 Sed and 4 Ex animals). A breakdown of the four whole heart treatment study arms is shown in Fig. 1B.

**Isolated heart perfusion.** Twenty-four hours after the last bout of Ex (or handling control), rats were anesthetized using a ketamine-xylazine mixture (90 mg/kg ketamine and 10 mg/kg xylazine ip). Upon the absence of animal response reflexes, hearts were removed via midline thoracotomy, placed briefly in 0.9% saline (4°C), and cannulated by the aorta on a modified Langendorff apparatus. Hearts were instrumented for the measurement of left ventricular (LV) function, coronary flow, and ECG as previously described (9, 37).

Briefly, hearts were retrograde perfused with gassed (95%O2-5%CO2) Krebs buffer containing (in mM) 118 NaCl, 24 NaHCO3, 4.8 KCl, 2 CaCl2, 1.2 MgSO4, 1.2 K2HPO4, and 10 glucose (37°C). The calcium content in the experimental buffers is on the high end of the physiological range, but normoxic hearts perfused under these experimental conditions retain function for at least 90 min with no loss of function (11), suggesting that calcium overload in our preparation (perfusion time: ~60 min) is minimal in nonstressed hearts. A latex balloon (Harvard Apparatus) was inserted through the mitral valve into the LV and inflated to a diastolic pressure of 4–7 mmHg for the measurement of LV pressures. ECG leads were placed in the bath for volume-conducted ECGs. Coronary flow was monitored throughout the protocol with a Transonic flow probe placed in series with the perfusion line proximal to the perfusion cannulas, and flow rates were normalized to heart wet weight. All measurements were recorded on a PowerLab System (AD Instruments) at a sampling rate of 1,000 Hz. Data were stored on a personal computer and subsequently analyzed using Chart 7.0 software (AD Instruments).

**Diamide experiments.** After instrumentation, 16 hearts (*n* = 8 Sed and 8 Ex hearts) were perfused for a 15-min equilibration period and baseline values were recorded, after which the buffer was switched to Krebs buffer with the addition of 200 µM diamide. The concentration of diamide used in this study has previously been shown to deplete the cardiac glutathione pool and induce cardiac arrhythmias (9). Diamide perfusion continued until the heart entered a sustained (>10 s) ventricular arrhythmia, at which time each heart was cut down and snap frozen in liquid nitrogen for subsequent biochemical analyses. An additional subset of Ex hearts (*n* = 8) received diamide perfusion for 39 min, and the tissue was then snap frozen. The 39-min time period was the average diamide perfusion time required for Sed hearts to go into an arrhythmia (also shown in Fig. 2), allowing us to directly compare biochemical differences in Sed versus Ex hearts along the same timescale.

To determine if Ex improved the ability of hearts to recover after diamide treatment, 14 hearts (*n* = 7 hearts/group) were perfused with diamide for 30 min. After the 30-min diamide perfusion, the buffer was switched back to the diamide-free perfusion buffer for a 20-min washout period. Immediately after the end of the washout, the LV was isolated and frozen in liquid nitrogen for biochemical analysis.

**I/R experiments.** A subset of 14 hearts (*n* = 7 Sed and 7 Ex hearts) was subjected to 30 min of global no-flow ischemia by stopping flow to the heart. After 30 min of ischemia, the static buffer was drained from the perfusion lines, and flow was restored for 30 min. As with the other groups, the LV was isolated and frozen at the end of reperfusion for biochemical analysis.

**Arrhythmia assessment.** Arrhythmias were scored in accordance with the Lambeth conventions (45). We used two different arrhythmia scoring systems in the study, described as *arrhythmia score system A* and *B* herein. Typical arrhythmia scoring systems increase the score with increasing duration of ventricular tachycardia (VT)/VF (18), and the results are skewed for hearts that experience longer durations of VT/VF, which may have limited clinical relevance as VF rarely reverts spontaneously in hearts from larger animals. Scores using *arrhythmia score system A* were calculated as follows: 0 = 0–49 premature ventricular beats, 1 = >50 premature beats, 2 = at least one episode of VT (regardless of duration), 3 = at least one episode of VF (regardless of duration), and 4 = fatal (nonreverting) arrhythmia.

**Arrhythmia scoring system B** has previously been used by us (9, 37) and others (18). Arrhythmia scores using *arrhythmia scoring system B* were calculated as follows: 0 = 0–49 premature ventricular beats, 1 = 50–499 premature ventricular beats, 2 = >500 premature ventricular
beats and/or 1 episode of spontaneously reverting VT or VF that was <30 s in total duration, 3 = >1 episode of reverting VT/VF that was <60 s in total duration, 4 = >1 episode of reverting VT/VF that was 61–119 s in total duration, 5 = VT/VF of >119 s in combined duration, 6 = fatal (nonreverting) VT/VF that began >15 min into treatment, 7 = fatal VT/VF that began between 4 and 15 min into treatment, 8 = fatal VT/VF that began between 1 and 4 min into treatment, and 9 = fatal VT/VF that began within the first 59 s of treatment.

Cardiac myocyte isolation. Hearts from Sed and Ex animals (n = 4 hearts/group) were excised after anesthesia as described above and promptly placed on a retrograde perfusion cannula. Isolated cardiac myocytes were prepared using methods similar to those previously described (8). Briefly, hearts received 5 min of perfusion with Ca²⁺-free Tyrode solution containing (in mM) 140 NaCl, 10 HEPES, 5 KCl, 1 MgCl₂, and 10 glucose (pH 7.4, 37°C). The solution was then switched to a digestion buffer consisting of Tyrode solution plus 25 µg/ml Liberase DH (Roche) and 20 µM CaCl₂ for 22–26 min. The heart was cut down, and the LV minced in a digestion dish (37°C) dissection dish. Chunks were gently aspirated with pipettes of increasing resistance (25-, 10-, and 5-ml serological pipettes) for 5 min. The cell suspension solution was filtered through 0.25-mesh m m mesh and allowed to gravity precipitate for 12 min. After gravity precipitation, cells were exposed to increasing amounts of calcium in Tyrode solution for 25–26 min. The cell suspension solution was filtered through 0.25-µm mesh and allowed to gravity precipitate for 12 min. After gravity precipitation, cells were exposed to increasing amounts of calcium in Tyrode solution (50, 100, 200, and 400 µM and 1 mM), each followed by a 12 min of gravity precipitation, before being put into DMEM with 10% FBS. Once in DMEM, cells were incubated (37°C, 5% CO₂-air) for 72 h to prove clarity.

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Cellular ROS fluorescence measurements. At the time of each experiment, isolated myocytes were placed in Tyrode solution containing 1.8 mM CaCl₂ and cellular ROS production was measured with the fluorescent probe 5-(6)-chloromethyl-2,7-dichlorofluorescein diacetate (CM-DCF; Invitrogen). CM-DCF fluorescence increases in proportion with cellular ROS production, specifically production of H₂O₂ and hydroxyl radical, but not superoxide (44). Ventricular cardiomyocytes from Sed and Ex animals were loaded with 500 nM CM-DCF for 10 min and placed in a heated (37°C) flow-through perfusion chamber (Warner Instruments) housed on the stage of an inverted fluorescent microscope (Leica). CM-DCF fluorescence was evoked using light from a metal-halide lamp filtered to an excitation wavelength of 472 nm (bandpass filter width: 30 nm), and emission was collected at 520 nm (bandpass filter width: 36 nm). Emitted light was captured with a charge-couple device camera, and images were acquired on a personal computer. To avoid photobleaching of the probe, the sampling rate was set at 1-min intervals. Our preliminary data indicated that this sampling rate and fluorophore concentration led to stable recordings in normoxic (nonstressed) myocytes for up to 50 min (data not shown). After 5 min of baseline imaging, the solution was switched to Tyrode solution plus 200 μM diamide. Fluorescence was monitored every minute for 40 min or until cell death occurred, whichever came first.

Changes in fluorescence intensity were quantified for each time point by subtracting the cell fluorescence (obtained via a region of interest drawn around the cell perimeter) from background fluorescence (obtained via a region of interest in an area adjacent to each myocyte). To account for unequal fluorophore loading across cells, each cellular fluorescence trace was normalized to baseline fluorescence intensity (Fₒ; before diamide) for each cell. As expected, diamide treatment led to an increase in the CM-DCF signal over time, rapidly increasing after ~15 min of diamide treatment. Because some of our myocytes died during diamide treatment (as shown in Fig. 6D), we were not able to plot the average fluorescence intensity increase over the time of treatment (as cell death precludes accurate fluorescence measurements). Therefore, we quantified an “inflection point,” when the fluorescence signal significantly increased from baseline. We defined the inflection point as the time point when the fluorescence slope (using a linear fit) increased more than three times from the mean slope during the first 5 min of treatment. At the beginning and end of each experiment, a bright-field image of each cardiac myocyte was obtained using differential interference contrast, and these images are presented along with fluorescence traces for improved clarity.

Myocardial glutathione, glutathione peroxidase, and glutathione reductase. Myocardial glutathione was assessed as previously described (9). Briefly, total glutathione and GSSG were determined using a commercially available kit (Oxis). Final concentrations were normalized to protein content using a BCA protein assay (Thermo Scientific).

For the determination of glutathione peroxidase (GPx) and glutathione reductase (GR) activities, 50–70 mg of frozen powdered tissue were homogenized in the presence of 0.3 mM 1-methyl-2-vinylpyridinium trifluoromethanesulfonate, and samples were diluted in double-distilled H₂O so that 1.5 mg protein/sample was loaded into a 96-well plate. GR was measured similar to the method of Carlberg and Mannervik (16). Briefly, samples were loaded in triplicate on a 96-well plate containing 1 mM GSSG and 0.5 mM NADPH in Tris-EDTA-EGTA (TEE) buffer (10 mM Tris, 1 mM EDTA, and 1 mM EGTA; pH 7.4). Absorbance at 340 nm was measured over 5 min, and the rate of oxidation for 1 μM NADPH/min is equivalent to 1 μU of GR activity. A similar method was used for the measurement of GPx as previously described (40). Samples were loaded into 96-well plate along with 1 mM GSH, 100 μM/ml GR, 0.5 mM NADPH, and t-butyl hydroperoxide in TEE buffer. Absorbance at 340

Fig. 2. Ex delays the onset of ventricular arrhythmias during sustained thiol oxidation. A: representative left ventricular developed pressure (LVDP; black trace) and volume-conducted ECG (gray trace) recordings from a Sed animal ~39 min into diamide treatment. The transition to ventricular arrhythmia and subsequent loss of pump function is denoted by the arrow. B: representative trace from an Ex animal during the same duration of diamide treatment as the Sed trace. C: mean time to the onset of ventricular arrhythmia after sustained diamide treatment. Values are means ± SE; n = 8 animals/group. *P < 0.05 vs. the Sed group.

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nm was measured over 5 min, and the rate of NADPH oxidation per minute was used to calculate GPx activity.

Statistical analysis. All data are expressed as means ± SE. Comparisons for dichotomous variables between Sed and Ex animals were done using a Student’s t-test. Arrhythmia scores and glutathione content were analyzed with two-way ANOVA (group × treatment) followed by Newman-Keuls post hoc tests. Between-group comparisons for incidence of VF and fatal arrhythmias were made using a χ²-test. Analysis of LV developed pressure (LVDP) was determined via a two-factor (group × time) ANOVA with repeated measures (time). Analysis of the Kaplan-Meier survival curve was done with a Mantel-Cox test. For all comparisons, statistical significance was determined by P values of <0.05.

RESULTS

Animal characteristics after Ex. Morphological data from animals in the study are shown in Table 1. Consistent with our previous work (12), our short-term Ex protocol did not lead to significant differences in body, adrenal, or spleen weights. We observed significant hypertrophy of the heart after 10 days of Ex. Baseline hemodynamic values are shown in Table 2, and we found no differences in cardiac function between Sed and Ex hearts during baseline recordings.

Incidence of ventricular arrhythmia during myocardial I/R. The time to the onset of arrhythmia (>10 s in duration) during myocardial treatment is shown in Fig. 2. Ex hearts had a delayed onset of ventricular arrhythmia compared with their Sed counterparts (P < 0.05; Fig. 2). The mean time for Sed animals to enter an arrhythmia was 39 min, and this duration of myocardial treatment served as the foundation for a second set of Ex hearts that were perfused for this amount of time and then frozen for subsequent biochemical experiments.

The extent and severity of cardiac arrhythmias for hearts exposed to a shorter diamide treatment (30 min) followed by a washout period (20 min) are shown in Fig. 3. During the shortened diamide treatment, the incidence and severity of arrhythmia were not significantly different between Sed and Ex hearts. However, during the washout period, hearts from the Ex group had a significantly lower incidence of arrhythmia, as reflected by lower arrhythmia scores (using two different scoring systems; Fig. 3, A and B), significantly lower incidence of hearts that had an episode of VF (P < 0.05; Fig. 3C), and lower incidence of nonreverting (fatal) arrhythmia at the end of the protocol (P < 0.05; Fig. 3D).

Incidence of arrhythmia after global I/R. Reperfusion arrhythmias in hearts exposed to 30 min of global I/R are shown in Fig. 4. During reperfusion, hearts from Ex animals had a significantly lower arrhythmia scores (P < 0.05; Fig. 4, A and B). Ex also significantly decreased the number of hearts that experienced an episode of VF (P < 0.05; Fig. 4C). The incidence of fatal arrhythmias was not significantly different between Sed and Ex animals during reperfusion, although there was a strong statistical trend (P = 0.06; Fig. 4D).

LV function. LVDP data during reversible diamide treatment are shown in Fig. 5. Diamide treatment induced a steady decline in the pressure developed in the LV. This decrease in LVDP was significantly attenuated in the Ex group (P < 0.05). Developed pressure rebounded during the washout period in the Ex group and was significantly greater than the Sed group starting 11.5 min into the washout period and lasting throughout the remainder of the protocol (P < 0.05; Fig. 5).

Hearts exposed to I/R displayed no statistically significant differences in the recovery of LV function between Sed and Ex animals (LVDP at the end of reperfusion was 18 ± 3 and 27 ± 4 mmHg for Sed and Ex animals, respectively, P = 0.14).

Cellular ROS production. Cellular ROS (specifically H₂O₂ and hydroxyl radical) production during diamide treatment was monitored in isolated ventricular myocytes with the fluorophore CM-DCF, and representative images and traces are shown in Fig. 6. A and B. The black and white images represent bright-field images of the myocyte at the beginning (left) and end (right) of each experiment. The sudden increase in CM-DCF fluorescence after ∼15 min of diamide has been previously observed in isolated cardiac myocytes (3) and is consistent with the concept of mitochondrial criticality (for a review, see Ref. 2). This steep rise in H₂O₂ emission that preceded myocyte death was quantified using an inflection point, and the time to inflection was significantly delayed in Ex animals (P < 0.05; Fig. 6C). As diamide exposure led to cell death in some cells (as shown in the representative Sed image in Fig. 6A), we plotted a Kaplan-Meier survival curve for myocytes in the study (Fig. 6D). Myocytes from Ex animals displayed both a delay in the onset of cell death as well as a decrease in the total number of cells that died after diamide treatment (P = 0.03).

Myocardial glutathione, GPx, and GR. Myocardial glutathione content data are shown in Fig. 7. At baseline, there were no significant differences between Sed and Ex in total glutathione, GSSG, or GSH/GSSG (Fig. 7, A–C, respectively). Total glutathione in the Sed group decreased significantly in the washout and reperfusion groups but did not significantly change in the Ex group (P < 0.05; Fig. 7A). GSSG levels increased significantly in the Ex group at the time of arrhythmia onset, and this rise in GSSG was significantly lower at the 39-min time point in our Ex group (Fig. 7B). Although GSSG was elevated in Sed hearts at the time of arrhythmia onset, this was not statistically different from baseline (by ANOVA).

In the Sed and Ex groups that were allowed to enter a sustained arrhythmia, perfusion with diamide led to a signifi-
cant decrease in GSH/GSSG. In the Ex group perfused with diamide for only 39 min (the mean time for Sed hearts to transition to arrhythmia), there was also a significant decline in GSH/GSSG from baseline. However, this group had a significantly higher GSH/GSSG than both Sed and Ex hearts during the onset of arrhythmia (Fig. 7C). GSH/GSSG declined significantly after washout in Sed hearts perfused with diamide for 30 min but was not significantly decreased in the Ex group (Fig. 7C). At the end of I/R, Sed hearts also showed a decline in GSH/GSSG, which remained unaltered from baseline in the Ex group (Fig. 7C).

GR activities from hearts that received irreversible diamide perfusion are shown in Fig. 8. In Sed and Ex hearts perfused until a ventricular arrhythmia occurred, there were no significant differences in GR. Ex hearts perfused for 39 min showed a significant preservation in GR activity versus both Sed and Ex groups perfused until arrhythmia ($P < 0.05$; Fig. 8).

GR and GPx activities from untreated hearts are shown in Fig. 9. There were no differences in the activity of myocardial GPx between Sed and Ex groups. On the other hand, GR activity was significantly greater in the hearts of Ex animals than in Sed animals ($P < 0.05$; Fig. 9).

DISCUSSION

Cardiac glutathione levels, specifically GSH/GSSG, influence the susceptibility of the heart to electrical dysfunction and arrhythmia (3, 9, 14). This study was conducted to determine if short-term Ex protects hearts against arrhythmias by preserving...
GSH/GSSG during conditions of oxidative stress. To the best of our knowledge, several aspects of this work represent novel findings. First, we demonstrated that Ex confers an antiarrhythmic phenotype in isolated female hearts exposed to two different oxidative challenges. Second, we showed that the time course for the onset of arrhythmia is consistent with a substantial drop of myocardial GSH/GSSG. Ex led to preservation of GSH/GSSG across experimental models, yet Ex hearts also transitioned to ventricular arrhythmia when GSH/GSSG fell substantially. Third, the cardioprotective phenotype observed in intact hearts was also seen in isolated ventricular myocytes. Cardiomyocytes from Ex animals displayed slower rises in cellular H₂O₂ levels and a lower incidence of cell death during thiol oxidation compared with Sed cells. Finally, Ex upregulated GR activity, indicating that Ex-induced improvements in GSH replenishment contribute to improved GSH/GSSG maintenance.

**Ex-induced protection against arrhythmia.** Our observation that Ex reduced fatal ventricular arrhythmias confirms findings in both a human epidemiological study (23) and previous reports using animal models (24, 26, 33, 35). Using an isolated heart model, we found that Ex reduced the incidence and severity of VT/VF either after overwhelming cellular antioxidant defenses with the thiol oxidant diamide or after global I/R. These findings are in agreement with other studies where Ex protected against arrhythmias induced by in vivo I/R (24, 26, 35) or ectopic ventricular pacing protocols (33). Using the isolated female rat heart, we were able to corroborate earlier work (in male animals) (33) showing that Ex induces intrinsic adaptations in the heart that protect against arrhythmia. The Ex-induced decrease in arrhythmias allowed for better recovery of cardiac function, also consistent with previous I/R studies (7, 11, 30, 49).

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**Fig. 5.** LVDP over the course of the protocol for Sed (black circles) and Ex (gray circles) groups. Hearts were perfused with 200 μM diamide for 30 min followed by a 20-min washout. Values are means ± SE; n = 7 animals/group. *P < 0.05 for main effect; **P < 0.05 for repeated measures (time).

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**Fig. 6.** Ex delays the increase in ROS fluorescence and decreases cell death in isolated ventricular myocytes exposed to oxidative stress. **A:** representative images over time for Sed and Ex myocytes. Black and white pictures represent bright-field images of myocytes at the beginning and end of each experiment, and fluorescence traces in between are fluorescence images from cells loaded with the ROS sensor 5-(6)-chloromethyl-2,7-dichlorofluorescein diacetate (CM-DCF). **B:** representative CM-DCF traces from Sed (black line) and Ex (gray line) myocytes. **C:** average time to inflection for both groups. **D:** survival curve for Sed (black) and Ex (gray) groups. Values are means ± SE; n = 18 myocytes (from 4 animals) for the Sed group and 25 myocytes (from 4 animals) for the Ex group. P < 0.05 for Sed vs. Ex myocyte survival.
Consistent with the cardioprotection observed in the intact organ, cellular H$_2$O$_2$ buffering and resistance to cell death were significantly improved in isolated Ex myocytes during thiol oxidation. During oxidative stress, there is a sharp increase in ROS emission observed when a critical level of mitochondrial ROS is reached [“mitochondrial criticality” (2)]. In Sed myocytes, we observed a spike in cellular ROS that immediately preceded necrotic cell death, occurring ~15 min after diamide treatment began. The time until the ROS surge was significantly prolonged in myocytes from Ex animals, and, to the best of our knowledge, this is the first direct demonstration that ROS-induced cell death is delayed in isolated ventricular myocytes from Ex animals undergoing oxidant challenge.

Cardiac glutathione and arrhythmias. Glutathione is the largest capacity thiol buffer in the heart (36), and uncompensated oxidation of GSH to GSSG can collapse mitochondrial energetics, activate sarcolemmal K$^+$ currents, and scale to induce catastrophic ventricular arrhythmias (for reviews, see Refs. 2 and 14). In this study, we used two different models of glutathione oxidation to evoke arrhythmia: 1) chemical oxidation of the glutathione pool with diamide and 2) I/R. As expected, each intervention reduced both total glutathione and GSH/GSSG in the myocardium (see Fig. 7). These findings are in agreement with previous studies where I/R depleted cardiac glutathione (29) and lowered GSH/GSSG (17, 34, 42, 46).

We examined the time course for the transition to arrhythmia and cardiac glutathione levels. The transition to arrhythmia occurred in both Sed and Ex hearts when myocardial GSH/GSSG dropped significantly (ratios were below ~30). Although there may be variability across animal species, it is interesting to note that a GSH/GSSG of <50 has been implicated as a threshold for the opening of the mitochondrial permeability transition pore (3), which induces necrotic and apoptotic cell death (22). This GSH/GSSG drop, transition to arrhythmia, and onset of cell death were all delayed in hearts from Ex animals. Our observation that GSH/GSSG remained above 50 in each of the Ex groups that displayed resistance to arrhythmia supports the general idea that maintaining GSH/GSSG above a critical level is obligatory to maintain cardiac electromechanical function. This is also in line with a previous study (27) indicating that Ex elicits a phenotype characterized by reduced opening of the mitochondrial permeability transition pore.

The Ex-induced preservation of GSH/GSSG was likely due to an increased activity of GR, which has been previously reported in female hearts after Ex (47). As the enzyme responsible for reducing GSSG back to GSH, these data suggest that it is not the ROS-scavenging ability of the glutathione system that is protective after Ex (as there is no increase in GPx activity; see Fig. 9) but rather the ability of the heart to replenish GSH. Given that the de novo synthesis of glutathione is very low in the heart (21) and that GSSG is released from the heart under conditions of oxidative stress (43), the importance of GSH replenishment appears to be obligatory for the heart’s ability to withstand sustained oxidative challenge.
Our finding that Ex improves the ROS-scavenging ability of the heart through improved GSH replenishment compliments several studies examining other ROS-scavenging mechanisms within the myocardium. Using antisense oligonucleotides, Hamilton et al. (24) showed a role for MnSOD in Ex-induced protection against arrhythmia, and a recent study by Quindry et al. (35) also showed upregulated MnSOD activity after short-term Ex training that was associated with the antiarrhythmic phenotype. In both of these studies, there was no clear Ex-induced upregulation in either GPx or catalase, the major routes for the conversion of H$_2$O$_2$ to water. Given that the product of enzymatic superoxide dismutation is H$_2$O$_2$, the lack of heightened capacity to break down H$_2$O$_2$ (augmented by upregulated MnSOD) left several unanswered questions. In this study, we demonstrate that H$_2$O$_2$ production is delayed in myocytes from Ex animals, leading us to speculate that the improved capacity of the Ex heart to scavenge H$_2$O$_2$ lies not in improved scavenging by GPx (seen the present study and also Refs. 24 and 35) but by augmented replenishment of GSH during oxidant challenge through GR. Interestingly, another study (48) has shown that GR activity also increased (in the liver) proportionally with the amount of Ex.

**Limitations.** This study used 10 days of Ex to elicit a cardioprotective phenotype. Our previous work showed that 10 days of Ex in female rats evoked some early adaptations to training (such as increased skeletal citrate synthase activity) while minimizing markers of systemic stress (12). This model of Ex may be independent of changes seen with classical “Ex training,” where the Ex stimulus is carried out for many months and more robust training adaptations (such as resting bradycardia) are observed. Another study limitation is that we collected the ECG signal from leads placed directly into the bath surrounding the heart. The clarity of these “volume-conducted” ECG signal amplitudes is generally more variable than typical surface lead ECGs. We also used global ischemia, which is a widely used experimental model to evoke ventricular arrhythmias but is not as clinically relevant to acute myocardial infarction as regional ischemia models. Finally, we used 2.0 mM CaCl$_2$ in our heart perfusion buffers, and this Ca$^{2+}$ concentration may be supraphysiological.

**Conclusions.** In this study, Ex induced intrinsic changes to the female heart that reduced the susceptibility to arrhythmias during two distinct oxidative challenges. This cardioprotection was observed in intact hearts and isolated cardiac myocytes and involved an augmented ROS buffering capacity after Ex. Maintaining a GSH environment through heightened GR activity appears to be involved in the Ex-induced protection against arrhythmia. Our findings contribute to a growing body of literature describing the cardioprotective effects of Ex, a very inexpensive and widely available preventative strategy. Future studies examining pharmacological strategies designed to improve glutathione replenishment have enormous potential as prophylactic therapies seeking to abrogate fatal ventricular arrhythmias.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**

EXERCISE AND ARRHYTHMIA


