Altering the redox state of skeletal muscle by glutathione depletion increases the exercise-activation of PGC-1α

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Abstract

We investigated the relationship between mitochondrial biogenesis, cell signalling and antioxidant enzymes by depleting skeletal muscle glutathione with diethyl maleate (DEM) which resulted in a demonstrable increase in oxidative stress during exercise. Animals were divided into six groups: (1) sedentary control rats; (2) sedentary rats treated with DEM; (3) exercise control rats euthanized immediately after exercise; (4) exercise rats + DEM; (5) exercise control rats euthanized 4 h after exercise; and (6) exercise rats + DEM euthanized 4 h after exercise. Exercising animals ran on the treadmill at a 10% gradient at 20 m/min for the first 30 min. The speed was then increased every 10 min by 1.6 m/min until exhaustion. There was a reduction in total glutathione in the skeletal muscle of DEM treated animals compared to the control animals ($P<0.05$). Within the control group, total glutathione was higher in the sedentary group compared to after exercise ($P<0.05$). DEM treatment also significantly increased oxidative stress, as measured by increased plasma F_2-isoprostanes ($P<0.05$). Exercising animals given DEM showed a significantly greater increase in peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α) mRNA compared to the control animals that were exercised ($P<0.05$). This study provides novel evidence that by reducing the endogenous antioxidant glutathione in skeletal muscle and inducing oxidative stress through exercise, PGC-1α gene expression was augmented. These findings further highlight the important role of exercise induced oxidative stress in the regulation of mitochondrial biogenesis.

Keywords: reactive oxygen species; diethyl maleate; exercise; PGC-1α

Introduction

It is well documented that exhaustive exercise increases the production of reactive oxygen species (ROS) within skeletal muscle leading to oxidative stress (3, 4). It has been previously speculated that the increase in exercise-induced oxidative stress can reduce muscle performance (3). As such, antioxidants have been proposed to reduce exercise-induced oxidative stress and increase performance (41). Recently there have been a number of studies that suggests ROS are important cell signalling molecules, particularly for beneficial exercise-induced adaptations to skeletal muscle (9, 15). As a result, the use of antioxidant supplementation may result in a dampening of these positive adaptations initiated by ROS (10, 25). However, not all research supports this notion (12, 35, 45).

Mitochondrial biogenesis (synthesis) is one of the key processes involved in skeletal muscle adaptations to exercise. Peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α) is an important co-activator of this process and plays an intrinsic role in mitochondrial biogenesis (14, 27). PGC-1α activates a broad range of both nuclear and mitochondrial encoded genes including nuclear respiratory factor-1 (NRF-1), NRF-2, and mitochondrial transcription factor A (Tfam). Specifically, PGC-1α regulates NRF-1 and NRF-2, which in turn regulate Tfam (17). Acute exercise stimulates PGC-1α gene expression, which increases mitochondrial synthesis and adaptations (1, 2, 11, 42). Furthermore, upstream signalling pathways such as phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) and cAMP-response element binding protein (CREB) has been shown to activate PGC-1α (1, 16, 44).

A number of studies have attempted to elucidate mechanisms for the role of exercise-induced ROS in cell signalling and mitochondrial biogenesis. Experimental approaches have included inhibiting ROS production, either by enzymatic inhibitors such as the treatment of allopurinol, or through antioxidant supplementation (9, 12, 18, 37, 38). Some studies suggest that long-term antioxidant supplementation attenuates markers of mitochondrial biogenesis following endurance training (10, 23, 31). By contrast, other studies report that short-term antioxidant supplementation does not influence changes in markers of mitochondrial biogenesis after acute exercise (11, 12, 26, 37). Alternatively, allopurinol, a xanthine oxidase inhibitor, has been shown to hamper PGC-1α expression after acute exercise (9, 18). However, Wadley et al. recently found that allopurinol did not alter PGC-1α expression after acute exercise and endurance training (38). These data highlight that the role of ROS in skeletal muscle adaptations is still largely unclear.

We adopted an alternative approach to investigate the links between ROS, cell signalling and mitochondrial biogenesis following acute exercise. Specifically, we depleted skeletal muscle antioxidants using diethyl maleate (DEM) to increase oxidative stress during exercise, and measured the resultant changes in markers of mitochondrial biogenesis (PGC-1α and NRF-2), upstream signaling proteins (p38 MAPK and CREB) and endogenous antioxidants glutathione peroxidase (GPx-1) and superoxide dismutase 2 (SOD2). We hypothesized that reducing intracellular glutathione would increase ROS production during acute exercise, resulting in an increase in markers of mitochondrial biogenesis, signaling proteins, and antioxidant enzymes.

Materials and Methods

The University of Queensland Animal Ethics Committee approved this study in accordance with National Health and Medical Research Council guidelines.

Animals

Ten-week-old male Wistar rats (n=46) were purchased from the Central Animal Breeding House (The University of Queensland, Australia). They were housed two per cage for the duration of the study in a 12-hr light/dark cycle environment. Animals were fed on standard rat chow and tap water ad libitum.

Experimental Protocol

Animals were divided into six groups: (1) sedentary control rats (n=8); (2) sedentary rats treated with DEM (n=8); (3) exercise control rats euthanized immediately after exercise (n=8); (4) exercise rats + DEM (n=8); (5) exercise control rats euthanized 4 h after exercise (n=8); and (6) exercise rats + DEM euthanized 4 h after exercise (n=6). DEM rats were given an intraperitoneal injection of 3 mmol/kg body mass DEM dissolved in extra light olive oil and control animals were injected with the extra light olive oil 2 h prior to being sacrificed or exercised (8).

Rats were exercised on a modified treadmill divided into eight lanes separated by clear plastic enclosures. All animals were familiarised for 4 d to treadmill running prior to the start of the study, at a 10% gradient at 16-20 m/min for 30 min. Those rats that were willing to run were placed into the exercise groups. Previous research has shown that this selection process is considered appropriate, because health status and muscle physiology

properties do not differ between those rats willing to run or not (5). Approximately 72 h after familiarisation, the exercising rats ran on the treadmill at a 10% gradient at 20 m/min for the first 30 min. The speed was then increased every 10 min by 1.6 m/min until exhaustion. Exhaustion was defined as the inability of the animal to right itself when it was laid on its side. The time until exhaustion was recorded for each animal (9, 18). Animals were weighed and sacrificed with sodium pentobarbital (100 mg/kg i.p.). Under a surgical plane of anaesthesia, blood was taken by cardiac puncture, and placed on ice. Samples were centrifuged at 600 × g for 10 min, and plasma aliquots were stored at -80°C. Similar to previous studies depleting glutathione, red gastrocnemius skeletal muscle was excised, rapidly frozen in liquid nitrogen and stored at -80°C (32).

Preparation of rat tissue

Total RNA was extracted from frozen muscle by use of the Micro-to-Midi Total RNA Purification System kit and DNase on-column digestion (Invitrogen, Carlsbad, CA) as previously described (35). For immunoblotting and mitochondrial enzyme activity, frozen muscle (10:1 buffer/mg muscle) was homogenised as previously described (35, 36) in freshly prepared ice-cold buffer (50 mM Tris at pH 7.5 containing 1 mM EDTA, 10% vol/vol glycerol, 1% vol/vol Triton X-100, 50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM DTT, 1 mM PMSF, and 5 µl/ml protease inhibitor cocktail (P8340; Sigma, St. Louis, MO)). Tissue lysates were incubated on ice for 20 min and then spun at 16,000 × *g* for 20 min at 4°C. The activities of antioxidant enzymes were measured using frozen muscle samples, which had been homogenised, on ice in 0.1 M sodium phosphate buffer ph 7.0 (10:1 buffer/mg muscle). Samples were spun at 16,000 × *g* for 15 min at 4ºC. The supernatant was transferred and analysis was done on freshly prepared homogenates.

145 Total and oxidized glutathione were analysed using frozen muscle tissue which was
146 homogenized in 20 µl of 5% (wt/vol) 5-sulfosalicylic acid (SSA)/mg tissue and
147 centrifuged at 11,500 rpm for 5 min at 4°C. The supernatant was diluted 1:5.5 in ddH_2O.
148 Triethanolamine was added to neutralise the solution to ensure optimal pH for the
149 reaction. Separate aliquots of 100 µl were taken for total (tGSH) and oxidised (GSSG)
150 glutathione measurement. The supernatant sample for the GSSG assay was derivatised in
151 16 µl of the following solution: 30.8% triethanolamine, 0.4% SSA, and 9% 2-
152 vinylpyridine in ddH2O (7).

153 ***Plasma F_2-Isoprostanes***

154 As a marker of oxidative stress, F_2-Isoprostanes were extracted from plasma GC/MS/MS
155 (6). Isoprostanes were extracted from plasma after saponification with methanolic NaOH.
156 Samples were spiked with 8-iso-PGF2α-d4 (Cayman Chemicals, USA) as an internal
157 standard, and incubated at 42°C for 60 min. Samples were then acidified to pH 3 with
158 hydrochloric acid; hexane was then added and the sample was mixed for 10 min before
159 centrifugation at 3000 × *g*. The supernatant was removed and the remaining solution
160 extracted with ethyl acetate and dried under nitrogen. Samples were reconstituted with
161 acetonitrile, transferred into vials with glass inserts and dried. The samples were then
162 derivatised using 40 µl of a 10% pentafluorobenzylbromide/acetonitrile solution (v/v)
163 and 20 µl of a 10% diisopropylethylamine/acetonitrile solution (v/v), and incubated at
164 room temperature for 30 min. Samples were then dried again under nitrogen before 10 µl
165 of pyridine and 20 µl of a Bis(trimethylsilyl)trifluoroacetamide/Trimethylchlorosilane
166 solution (99:1) (Sigma, St. Louis, MO) were added, and the samples were incubated at
167 45° for 20 min. Finally, hexane was added and 1 µl of the sample was injected for

analysis using gas chromatography mass spectrometry (Varian, Australia) in negative chemical ionization mode. The laboratory intra-assay coefficient of variation for this assay is 4.5%.

RT-PCR analysis

RNA concentration was determined by spectrophotometric analysis. First-strand cDNA was generated from 0.5 μg RNA using AMV Reverse Transcriptase (Promega, Madison, WI) (39). Following reverse transcription, the remaining RNA was degraded by treatment with RNase H (Invitrogen, Australia) for 20 min at 37^{o}C. The amount of single stranded DNA was then determined in each sample and compared against an oligonucleotide standard using OliGreen reagent (Invitrogen, Australia), which was incubated in the dark at 80^{o}C for 5 min prior to the measurement of fluorescence (30, 35). The primer sequences were obtained from gene sequences from GenBank: PGC-1α, AY237127; NRF2α, M74515; SOD2, NM_017051.2 and GPx-1, NM_030826.3 (Table 1).

Real-time PCR using SYBR Green chemistry was performed, using the sequence detector software (Rotor-Gene v6; Corbett Research, Sydney, Australia), as previously described (36). Samples were subjected to a heat dissociation protocol after the final cycle of PCR to ensure that only one product was detected. The mRNA of each gene was normalized to the cDNA content in each sample using the OliGreen assay as described above. This has previously been shown to be a robust and suitable method of normalization that avoids the many problems associated with "housekeeping genes" (22, 29, 30, 35).

189 ***Western Blot Analysis***

190 Lysates were solubilised in Laemmli sample buffer. Equal amounts of total protein,
191 determined by a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) with BSA
192 as the standard, were separated by SDS-PAGE and electrotransfer of proteins from the
193 gel to PVDF membranes. Blots were probed with anti-p38 rabbit polyclonal MAPK (Cell
194 Signalling, Hartsfordshire, England), and anti-phospho-CREB (Cell Signalling,
195 Hartsfordshire, England) antibodies. Binding was detected with IRDye 800-conjugated
196 anti-rabbit IgG (Rockland, Gilbertsville, PA) or IRDye 680-conjugated anti-mouse IgG
197 (Invitrogen, Carlsbad, CA) secondary antibodies. All data are expressed as integrated
198 intensity following infrared detection (Odyssey Imaging system; LI-COR Biosciences,
199 Lincoln, NE). For p38 MAPK signalling, membranes were then stripped (2% SDS (w/v)
200 in 25 mM glycine, pH 2.0) and re-probed with anti-phospho-p38 MAPK rabbit
201 polyclonal antibody (Cell Signalling, Hartsfordshire, England). As a loading control,
202 blots were then reprobed with anti-α-tubulin mouse monoclonal antibody (Sigma, St.
203 Louis, MO).

204 ***Glutathione and Antioxidant Enzyme Activities***

205 Total and GSSG glutathione concentrations were measured by modifying the method of
206 Dudley (7). Absorbance was recorded on a plate reader (Fluostar Optima, BMG Labtech,
207 Victoria, Australia). GPx activity was measured using a modified method for the Cobas
208 Mira spectrophotometric analyser (Roche Diagnostics, Basel, Switzerland) (40). The
209 method used to measure SOD2 activity was modified from Oyanagu (24). Protein
210 concentration was determined using a BCA protein assay (Pierce, Rockford, IL) with
211 BSA as the standard.

212 ***Statistical Analyses***

213 Data were checked for normality using the Shapiro-Wilk test. If the test was significant,
214 data were log transformed and reanalyzed. Plasma F_2-isoprostanes, and skeletal muscle
215 tGSH, ratio of GSSG/tGSH, PGC-1α mRNA, NRF-2 mRNA, phosphorylated p38 MAPK
216 protein, CREB protein, GPx mRNA, GPx activity, SOD2 mRNA and SOD2 activity were
217 all normally distributed. Skeletal muscle GSSG was normally distributed after log
218 transformation.

219

220 An unpaired *t*-test was used to determine differences in time to fatigue between control
221 exercise and DEM exercise groups. For all other data, a two-way ANOVA was used to
222 determine an interaction between exercise and DEM treatment and if significant a
223 Tukey's post hoc was completed. If there was no interaction, where applicable, a main
224 effect for exercise or DEM is provided. Animals sacrificed directly after exercise were
225 used to determine plasma F_2-isoprostanes, and skeletal muscle glutathione and cell
226 signaling proteins (37). Markers of mitochondrial biogenesis and endogenous
227 antioxidants were measured on animals euthanized 4 h after exercise (37). Significance
228 was considered at $P<0.05$. Normalised data are presented as mean ± SE, and log
229 transformed data as geometric mean ± 95% confidence intervals.

Results

Effects of DEM and exercise on markers of skeletal muscle ROS directly after acute exercise

Time to fatigue was significantly decreased as a result of DEM treatment ($P<0.05$; 68 ± 5 mins exercise control (n=16) versus 51 ± 5 mins exercise DEM (n=14)).

For tGSH levels, there was a significant interaction between DEM treatment and exercise ($P<0.05$; Figure 1A). In the control (untreated) rats, acute exercise significantly decreased skeletal muscle tGSH levels ($P<0.05$; Figure 1A). DEM treatment significantly reduced skeletal muscle tGSH levels compared to untreated rats ($P<0.05$, Figure 1A), with exhaustive exercise not reducing these levels any further (Fig 1A). In the control group, exercise resulted in a greater GSSG/tGSH ratio compared to the sedentary group ($P<0.05$), yet in the DEM treated animals, GSSG/tGSH ratio did not differ between the exercise and sedentary animals ($P<0.05$ interaction between exercise and DEM; Figure 1C). There were no differences in GSSG between groups ($P>0.05$; Figure 1B). DEM treatment significantly increased oxidative stress, as measured by changes in plasma F_2-isoprostanes ($P<0.05$ main effect for DEM; Figure 2).

Effect of DEM and exercise on mitochondrial biogenesis markers 4 h after acute exercise

Four hours after exercise, PGC-1α gene expression was significantly increased in both control and DEM treated animals ($P<0.05$ interaction between exercise and DEM; Figure 3A). Furthermore, exercising animals treated with DEM showed a significantly greater

252 increase in PGC-1α gene expression compared to the control animals that were exercised
253 ($P<0.05$). Exercise did not significantly increase NRF-2 gene expression ($P=0.1$; Figure
254 3B).

255 ***Effect of DEM and exercise on exercise-induced mitochondrial biogenesis signaling***
256 ***directly after exercise***

257 Phosphorylation of p38 MAPK was significantly increased after exercise in both the
258 control and DEM treatment groups ($P<0.05$ main effect for exercise; Figure 4A). DEM
259 tended to increase the phosphorylation of p38 MAPK ($P=0.06$; Fig 4A). Phosphorylated
260 CREB was not altered as a result of exercise or DEM treatment (Figure 4B).

261 ***Effect of DEM on endogenous antioxidants 4 h after acute exercise***

262 DEM treatment prevented the increase in GPx-1 mRNA observed in the control
263 (untreated) group following acute exercise ($P<0.05$, Fig 5A). In animals treated with
264 DEM, there was a significant reduction in GPx activity levels in the sedentary and
265 exercise groups ($P<0.05$ main effect for DEM; Figure 5B).

266

267 There was a trend for exercise to increase SOD2 gene expression ($P=0.08$; Figure 5C).
268 SOD2 activity was reduced in the control exercise and DEM exercised animals ($P<0.05$
269 main effect of exercise; Figure 5D). In addition, in the DEM groups SOD2 activity was
270 reduced ($P<0.05$ main effect for DEM; Figure 5D).

271 **Discussion**

272 This study is the first to investigate the effects of depleting glutathione and thus
273 increasing oxidative stress on skeletal muscle markers of mitochondrial biogenesis,

upstream signaling proteins and endogenous antioxidants following an acute exercise bout. Consistent with our hypothesis, DEM decreased tGSH and GSSG/tGSH. Furthermore, DEM treatment significantly increased oxidative stress during exhaustive exercise as measured by increased plasma isoprostanes. Our main finding was that DEM treatment significantly augmented PGC-1α mRNA following exhaustive exercise. In addition, both DEM and exhaustive exercise significantly lowered GPx-1 gene expression. DEM also lowered total GPx-1 and SOD2 activity in both sedentary and exercise groups. DEM treatment also tended to augment the increased phosphorylation of p38 MAPK following exhaustive exercise.

DEM treatment decreased skeletal muscle tGSH by approximately 52% which has been previously demonstrated in similar exercise studies using $_{L}$-butathione-(S,R)-sulfoximine (BSO), which also reduces glutathione biosynthesis (20, 32). The decrease in skeletal muscle tGSH post-exercise in the control group suggests that exercise alone depleted skeletal muscle glutathione, and is the result of tGSH responding to the oxidative challenge induced by exercise (21). Consistent with previous rodent treadmill studies, skeletal muscle GSSG/tGSH ratio was significantly increased in the control animals post-exercise, which is indicative of oxidative stress (37, 38). Wadley et al. have previously found that GSSG/tGSH as an indirect marker of ROS production during exercise is more sensitive than other ROS measures, such as protein carbonyls and H_2O_2 levels (37). However, in this instance these markers are not appropriate to determine the increase in ROS in DEM-treated animals, because DEM reduced both tGSH and GSSG/tGSH. Accordingly, we measured plasma F_2-isoprostane concentration as an alternative marker

297 of oxidative stress, which confirmed the expected increase in ROS production within the
298 DEM groups.
299
300 A major finding of this study was that DEM treatment diminished the glutathione pool,
301 resulting in inability of skeletal muscle to limit oxidative stress, and therefore
302 augmented PGC-1α gene expression following exhaustive exercise. Recent *in vitro*
303 evidence suggests that increases in PGC-1α promoter activity and gene expression are
304 mitigated by increased hydrogen peroxide levels in skeletal muscle (15, 33, 34). Given
305 that one of the major roles of glutathione system is to reduce hydrogen peroxide to water,
306 and we diminished the glutathione pool, it is possible this resulted in an increase in
307 hydrogen peroxide, thus a potential pathway for the changes seen in PGC-1α gene
308 expression. Although there was a trend for exercise to increase NRF-2 gene expression,
309 DEM treatment did not affect this gene. It is possible that NRF-2 gene expression is not
310 sensitive to increases in ROS following treatment with DEM.
311
312 The signaling proteins, p38 and CREB, are involved in the regulation of PGC-1α gene
313 expression (1, 43). Consistent with our previous studies, acute exercise significantly
314 increased p38 MAPK phosphorylation (37, 38). Indeed, an increase in phosphorylation of
315 p38 MAPK, followed by the exercise-induced increase in PGC-1α gene expression is also
316 similar to that reported by Akimoto et al., providing further support of the functional role
317 of p38 in skeletal muscle adaptations (1). Skeletal muscle phosphorylation of p38 MAPK
318 also tended (P=0.06) to be higher with DEM-treatment. It appears that oxidative stress
319 resulting from DEM treatment may have increased p38 MAPK activation. Indeed, this is

supported by previous evidence which has demonstrated acute exercise increased phosphorylated p38 (37, 38). Alternatively, it does not appear that CREB is activated by oxidative stress, which did not change significantly.

In vitro evidence suggests that PGC-1α is a powerful inducer of antioxidants GPx and SOD2. When PGC-1α is gene silenced, there are concurrent decreases in the gene expression of these antioxidants (34). Indeed, as exercise increased PGC-1α gene expression in the controls animals within this study, similar increases were also seen in GPx-1 and a tendency for reduced SOD2 gene expression. These increases have been previously reported in skeletal muscle after acute exercise (37). DEM-treatment decreased both GPx-1 gene expression and GPx activity. Most likely this is the result of the significant reduction in tGSH in the DEM animals. Although we did not expect to see any changes in GPx activity following acute exercise, this measure was used to demonstrate how DEM influences different components of the glutathione system. In the control group, SOD2 activity was reduced after exercise. Several studies have reported no alteration in SOD2 activity after acute exercise (13, 28). Nevertheless, it remains unclear why exercise does not alter SOD2 activity. In addition, DEM decreased SOD2 activity in both the sedentary and exercise groups. We speculate that this overall decrease indicates that SOD2 activity is dependent on glutathione status at rest compared to exercise.

DEM significantly decreased time to fatigue by 25%, confirming the findings of others that reported impaired skeletal muscle function with oxidative stress (19, 32). Both DEM and BSO treatment have previously resulted in reductions in swimming distance (19) and

time to fatigue (32), respectively. One limitation of the present study was the exercise to exhaustion protocol, where the reduced time to fatigue in the exercise DEM group would have resulted in reduced energy expenditure compared to the exercise controls, and therefore the possibility of reduced exercise-induced adaptations. Nevertheless, irrespective of the energy expenditure and shorter running time, PGC-1α gene expression following exercise was actually higher in the DEM group. In addition we also chose to use the DEM as a glutathione inhibitor, however, we suspect similar results would still be yielded using the alternative glutathione inhibitor BSO or the SOD inhibitor, diethyldithiocarbamate. Further studies could explore these alternative inhibitors to confirm findings reported here.

This study has demonstrated novel evidence that by reducing endogenous antioxidant glutathione content, there was impaired capacity for skeletal muscle to neutralize oxidative stress during exercise, resulting in greater PGC-1α gene expression. Therefore, providing *in vivo* evidence for the important role of oxidative stress plays in the regulation of mitochondrial biogenesis.

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363 **Disclosures**

364 The authors have no conflicts of interest to declare.

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366 **References**

367 1. **Akimoto T, Pohnert SC, Li P, Zhang M, Gumbs C, Rosenberg PB, Williams**
368 **RS, and Yan Z.** Exercise stimulates Pgc-1alpha transcription in skeletal muscle through
369 activation of the p38 MAPK pathway. *J Biol Chem* 280: 19587-19593, 2005.

370 2. **Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, Kelly DP, and**
371 **Holloszy JO.** Adaptations of skeletal muscle to exercise: rapid increase in the
372 transcriptional coactivator PGC-1. *FASEB J* 16: 1879-1886, 2002.

373 3. **Bailey DM, Davies B, Young IS, Jackson MJ, Davison GW, Isaacson R, and**
374 **Richardson RS.** EPR spectroscopic detection of free radical outflow from an isolated
375 muscle bed in exercising humans. *J Appl Physiol* 94: 1714-1718, 2003.

376 4. **Bailey DM, Young IS, McEneny J, Lawrenson L, Kim J, Barden J, and**
377 **Richardson RS.** Regulation of free radical outflow from an isolated muscle bed in
378 exercising humans. *Am J Physiol Heart Circ Physiol* 287: H1689-1699, 2004.

379 5. **Bedford TG, Tipton CM, Wilson NC, Oppliger RA, and Gisolfi CV.**
380 Maximum oxygen consumption of rats and its changes with various experimental
381 procedures. *J Appl Physiol* 47: 1278-1283, 1979.

382 6. **Briskey DR, Wilson GR, Fassett RG, and Coombes JS.** Optimized method for
383 quantification of total F-isoprostanes using gas chromatography-tandem mass
384 spectrometry. *J Pharm Biomed Anal* 90C: 161-166, 2013.

385 7. **Dudley RW, Khairallah M, Mohammed S, Lands L, Des Rosiers C, and**
386 **Petrof BJ.** Dynamic responses of the glutathione system to acute oxidative stress in
387 dystrophic mouse (mdx) muscles. *Am J Physiol Regul Integr Comp Physiol* 291: R704-
388 710, 2006.

8. **Gerard-Monnier D, Fougeat S, and Chaudiere J.** Glutathione and cysteine depletion in rats and mice following acute intoxication with diethylmaleate. *Biochem Pharmacol* 43: 451-456, 1992.

9. **Gomez-Cabrera MC, Borras C, Pallardo FV, Sastre J, Ji LL, and Vina J.** Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats. *J Physiol* 567: 113-120, 2005.

10. **Gomez-Cabrera MC, Domenech E, Romagnoli M, Arduini A, Borras C, Pallardo FV, Sastre J, and Vina J.** Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. *Am J Clin Nutr* 87: 142-149, 2008.

11. **Hellsten Y, Nielsen JJ, Lykkesfeldt J, Bruhn M, Silveira L, Pilegaard H, and Bangsbo J.** Antioxidant supplementation enhances the exercise-induced increase in mitochondrial uncoupling protein 3 and endothelial nitric oxide synthase mRNA content in human skeletal muscle. *Free Radic Biol Med* 43: 353-361, 2007.

12. **Higashida K, Kim SH, Higuchi M, Holloszy JO, and Han DH.** Normal adaptations to exercise despite protection against oxidative stress. *Am J Physiol Endocrinol Metab* 301: E779-784, 2011.

13. **Hollander J, Fiebig R, Gore M, Ookawara T, Ohno H, and Ji LL.** Superoxide dismutase gene expression is activated by a single bout of exercise in rat skeletal muscle. *Pflugers Arch* 442: 426-434, 2001.

14. **Hood DA.** Mechanisms of exercise-induced mitochondrial biogenesis in skeletal muscle. *Appl Physiol Nutr Metab* 34: 465-472, 2009.

411 15. **Irrcher I, Ljubicic V, and Hood DA.** Interactions between ROS and AMP
412 kinase activity in the regulation of PGC-1alpha transcription in skeletal muscle cells. *Am*
413 *J Physiol Cell Physiol* 296: C116-123, 2009.
414 16. **Irrcher I, Ljubicic V, Kirwan AF, and Hood DA.** AMP-activated protein
415 kinase-regulated activation of the PGC-1alpha promoter in skeletal muscle cells. *PLoS*
416 *One* 3: e3614, 2008.
417 17. **Joseph AM, Pilegaard H, Litvintsev A, Leick L, and Hood DA.** Control of
418 gene expression and mitochondrial biogenesis in the muscular adaptation to endurance
419 exercise. *Essays Biochem* 42: 13-29, 2006.
420 18. **Kang C, O'Moore KM, Dickman JR, and Ji LL.** Exercise activation of muscle
421 peroxisome proliferator-activated receptor-gamma coactivator-1alpha signaling is redox
422 sensitive. *Free Radic Biol Med*, 2009.
423 19. **Kramer K, Dijkstra H, and Bast A.** Control of physical exercise of rats in a
424 swimming basin. *Physiol Behav* 53: 271-276, 1993.
425 20. **Leeuwenburgh C and Ji LL.** Glutathione depletion in rested and exercised mice:
426 biochemical consequence and adaptation. *Arch Biochem Biophys* 316: 941-949, 1995.
427 21. **Lew H, Pyke S, and Quintanilha A.** Changes in the glutathione status of plasma,
428 liver and muscle following exhaustive exercise in rats. *FEBS Lett* 185: 262-266, 1985.
429 22. **Lundby C, Nordsborg N, Kusuhara K, Kristensen KM, Neufer PD, and**
430 **Pilegaard H.** Gene expression in human skeletal muscle: alternative normalization
431 method and effect of repeated biopsies. *Eur J Appl Physiol* 95: 351-360, 2005.

23. **Meier P, Renga M, Hoppeler H, and Baum O.** The impact of antioxidant supplements and endurance exercise on genes of the carbohydrate and lipid metabolism in skeletal muscle of mice. *Cell Biochem Funct* 31: 51-59, 2013.

24. **Oyanagui Y.** Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. *Anal Biochem* 142: 290-296, 1984.

25. **Paulsen G, Cumming KT, Holden G, Hallen J, Ronnestad BR, Sveen O, Skaug A, Paur I, Bastani NE, Ostgaard HN, Buer C, Midttun M, Freuchen F, Wiig H, Ulseth ET, Garthe I, Blomhoff R, Benestad HB, and Raastad T.** Vitamin C and E supplementation hampers cellular adaptation to endurance training in humans: a double-blind, randomised, controlled trial. *J Physiol* 592: 1887-1901, 2014.

26. **Petersen AC, McKenna MJ, Medved I, Murphy KT, Brown MJ, Della Gatta P, and Cameron-Smith D.** Infusion with the antioxidant N-acetylcysteine attenuates early adaptive responses to exercise in human skeletal muscle. *Acta Physiol (Oxf)* 204: 382-392, 2012.

27. **Pilegaard H, Saltin B, and Neufer PD.** Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. *J Physiol* 546: 851-858, 2003.

28. **Pimenta Ada S, Lambertucci RH, Gorjao R, Silveira Ldos R, and Curi R.** Effect of a single session of electrical stimulation on activity and expression of citrate synthase and antioxidant enzymes in rat soleus muscle. *Eur J Appl Physiol* 102: 119-126, 2007.

29. **Rhinn H, Marchand-Leroux C, Croci N, Plotkine M, Scherman D, and Escriou V.** Housekeeping while brain's storming Validation of normalizing factors for

455 gene expression studies in a murine model of traumatic brain injury. *BMC Mol Biol* 9: 62,
456 2008.
457 30. **Rhinn H, Scherman D, and Escriou V.** One-step quantification of single-
458 stranded DNA in the presence of RNA using Oligreen in a real-time polymerase chain
459 reaction thermocycler. *Anal Biochem* 372: 116-118, 2008.
460 31. **Ristow M, Zarse K, Oberbach A, Kloting N, Birringer M, Kiehntopf M,**
461 **Stumvoll M, Kahn CR, and Bluher M.** Antioxidants prevent health-promoting effects
462 of physical exercise in humans. *Proc Natl Acad Sci U S A* 106: 8665-8670, 2009.
463 32. **Sen CK, Atalay M, and Hanninen O.** Exercise-induced oxidative stress:
464 glutathione supplementation and deficiency. *J Appl Physiol* 77: 2177-2187, 1994.
465 33. **Silveira LR, Pilegaard H, Kusuhara K, Curi R, and Hellsten Y.** The
466 contraction induced increase in gene expression of peroxisome proliferator-activated
467 receptor (PPAR)-gamma coactivator 1alpha (PGC-1alpha), mitochondrial uncoupling
468 protein 3 (UCP3) and hexokinase II (HKII) in primary rat skeletal muscle cells is
469 dependent on reactive oxygen species. *Biochim Biophys Acta* 1763: 969-976, 2006.
470 34. **St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jager S, Handschin C,**
471 **Zheng K, Lin J, Yang W, Simon DK, Bachoo R, and Spiegelman BM.** Suppression of
472 reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators.
473 *Cell* 127: 397-408, 2006.
474 35. **Strobel NA, Peake JM, Matsumoto A, Marsh SA, Coombes JS, and Wadley**
475 **GD.** Antioxidant supplementation reduces skeletal muscle mitochondrial biogenesis. *Med*
476 *Sci Sports Exerc* 43: 1017-1024, 2011.

36. **Wadley GD and McConell GK.** Effect of nitric oxide synthase inhibition on mitochondrial biogenesis in rat skeletal muscle. *J Appl Physiol* 102: 314-320, 2007.

37. **Wadley GD and McConell GK.** High-dose antioxidant vitamin C supplementation does not prevent acute exercise-induced increases in markers of skeletal muscle mitochondrial biogenesis in rats. *J Appl Physiol* 108: 1719-1726, 2010.

38. **Wadley GD, Nicolas MA, Hiam DS, and McConell GK.** Xanthine oxidase inhibition attenuates skeletal muscle signaling following acute exercise but does not impair mitochondrial adaptations to endurance training. *Am J Physiol Endocrinol Metab* 304: E853-862, 2013.

39. **Wadley GD, Tunstall RJ, Sanigorski A, Collier GR, Hargreaves M, and Cameron-Smith D.** Differential effects of exercise on insulin-signaling gene expression in human skeletal muscle. *J Appl Physiol* 90: 436-440, 2001.

40. **Wheeler CR, Salzman JA, Elsayed NM, Omaye ST, and Korte DW, Jr.** Automated assays for superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activity. *Anal Biochem* 184: 193-199, 1990.

41. **Williams SL, Strobel NA, Lexis LA, and Coombes JS.** Antioxidant requirements of endurance athletes: implications for health. *Nutr Rev* 64: 93-108, 2006.

42. **Wright DC, Han DH, Garcia-Roves PM, Geiger PC, Jones TE, and Holloszy JO.** Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1alpha expression. *J Biol Chem* 282: 194-199, 2007.

43. **Wu H, Kanatous SB, Thurmond FA, Gallardo T, Isotani E, Bassel-Duby R, and Williams RS.** Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. *Science* 296: 349-352, 2002.

44. **Wu Z, Huang X, Feng Y, Handschin C, Gullicksen PS, Bare O, Labow M, Spiegelman B, and Stevenson SC.** Transducer of regulated CREB-binding proteins (TORCs) induce PGC-1alpha transcription and mitochondrial biogenesis in muscle cells. *Proc Natl Acad Sci U S A* 103: 14379-14384, 2006.

45. **Yfanti C, Akerstrom T, Nielsen S, Nielsen AR, Mounier R, Mortensen OH, Lykkesfeldt J, Rose AJ, Fischer CP, and Pedersen BK.** Antioxidant supplementation does not alter endurance training adaptation. *Med Sci Sports Exerc* 42: 1388-1395, 2010.

510 Table 1: Primers for mRNA analyses

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
PGC-1α	ACCCACAGGATCAGAACAAACC	GACAAATGCTCTTTGCTTTATTC
NRF2α	CTCGGAGCAGGTGACGAG	TGGACCAGCGTATAGGATCA
SOD2	TGGACAAACCTGAGCCCTAA	GACCCAAAGTCACGCTTGATA
GPx-1	CGACATCGAACCCGATATAGA	ATGCCTTAGGGGTTGCTAGG

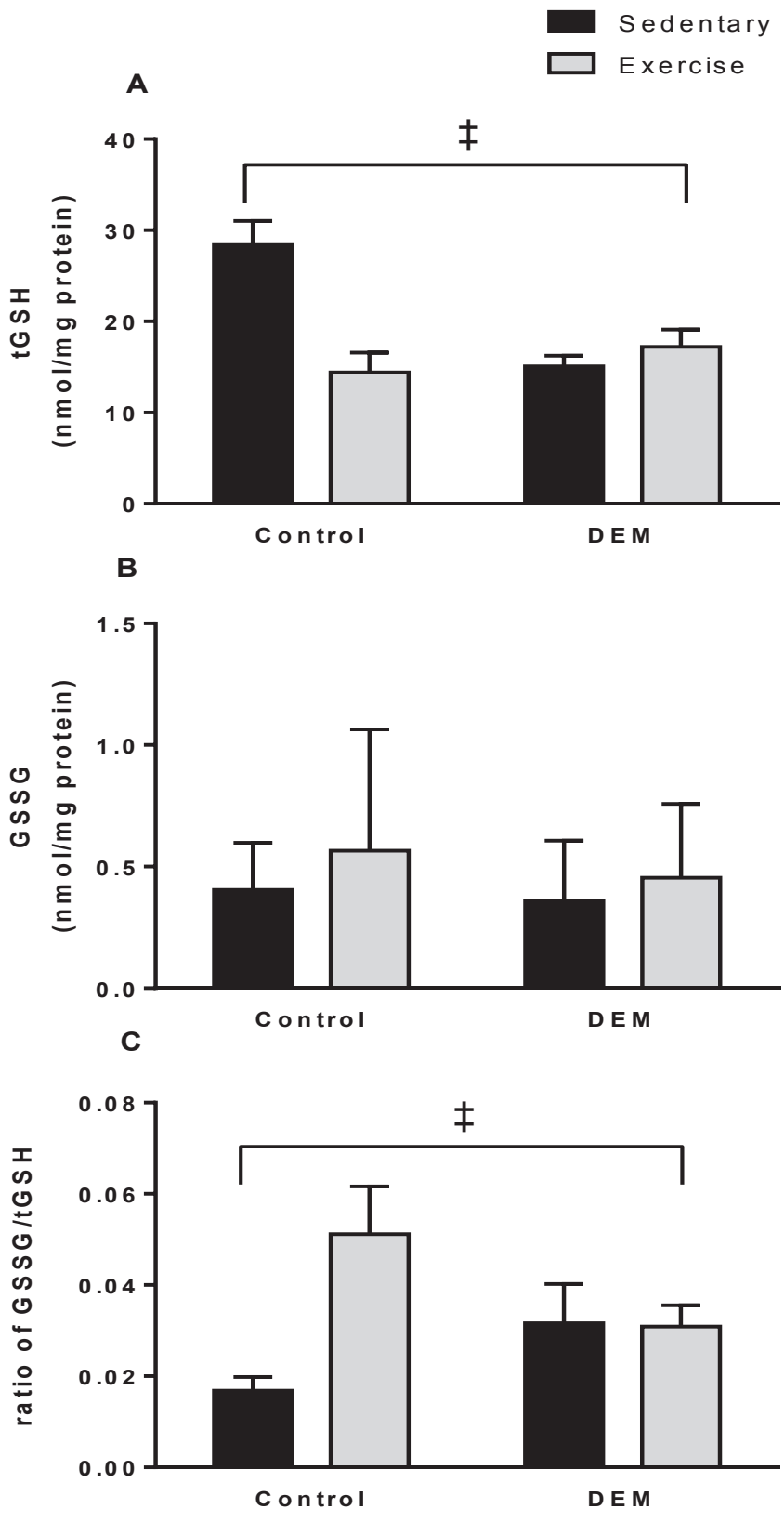
511

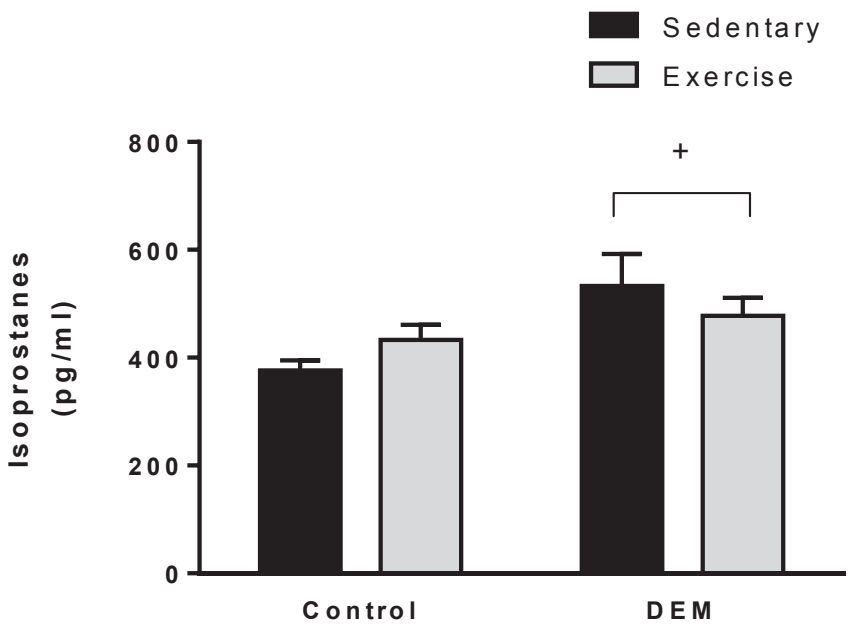
512

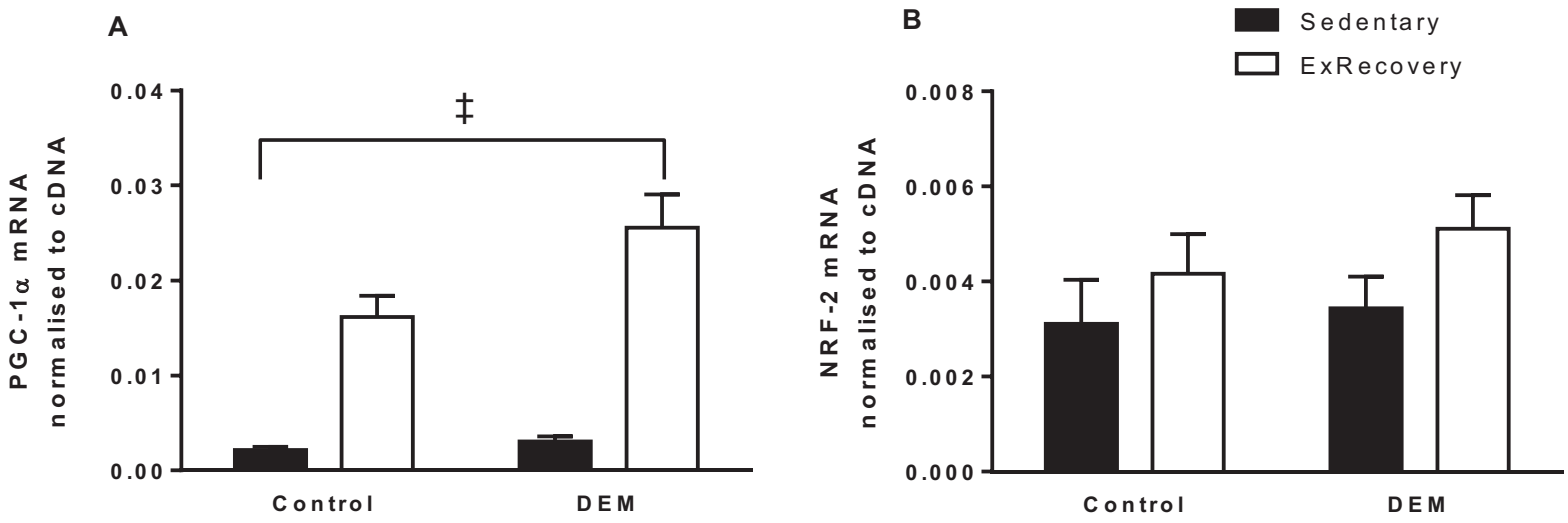
513 Figure 1: Effects of DEM and exercise on levels of total glutathione (tGSH) (A), oxidised
514 glutathione (GSSG) (B) and the GSSG/tGSH ratio (C). Animals in the post-exercise
515 groups were sacrificed directly after exercise. Values are mean ± SE for tGSH,
516 GSSG/tGSH ratio and geometric mean (95% CI) for GSSG (n=5-8 for all groups).
517 ‡ $P<0.05$ interaction between exercise and DEM.
518
519 Figure 2: Effects of DEM and exercise on concentration of plasma F_2-isoprostanes.
520 Animals in the post-exercise groups were sacrificed directly after exercise. Values are
521 mean ± SE (n=6-8 for all groups). +$P<0.05$ main effect for DEM.
522
523 Figure 3: Effects of DEM and exercise on PGC-1α (A) and NRF-2 (B) gene expression.
524 Animals in the exercise-recovery groups were sacrificed 4 h after exercise. Values are
525 mean ± SE (n=6-8 for all). ‡ $P<0.05$ interaction between exercise and DEM
526
527 Figure 4: Effects of DEM and exercise on protein content of phosphorylated p38 (A) and
528 phosphorylated CREB (B). Animals in the post-exercise groups were sacrificed directly
529 after exercise. Values are mean ± SE (n=8 for all groups). Boxes: Western blot showing
530 representative results from 1 rat/group with boxes around blots indicating blots were
531 obtained from different parts of the same membrane. *$P<0.05$ main effect for exercise.
532
533 Figure 5: Effects of DEM and exercise on changes in gene expression and enzyme
534 activity of GPx-1 (A and B) and SOD2 (C and D). Animals in the exercise-recovery
535 groups were sacrificed 4 h after exercise. Values are mean ± SE (n=6-8 for all groups). ‡

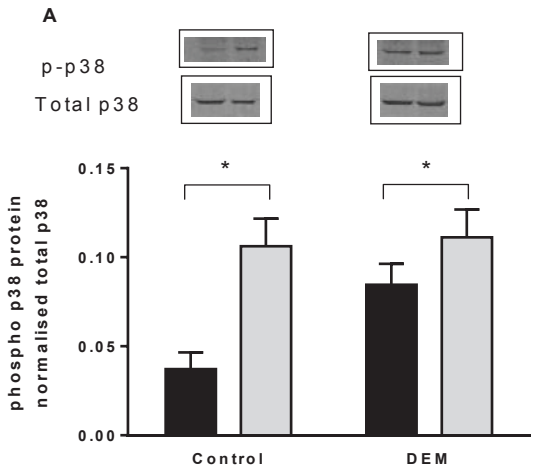
536 P<0.05 interaction between exercise and DEM; *P<0.05 main effect for exercise;

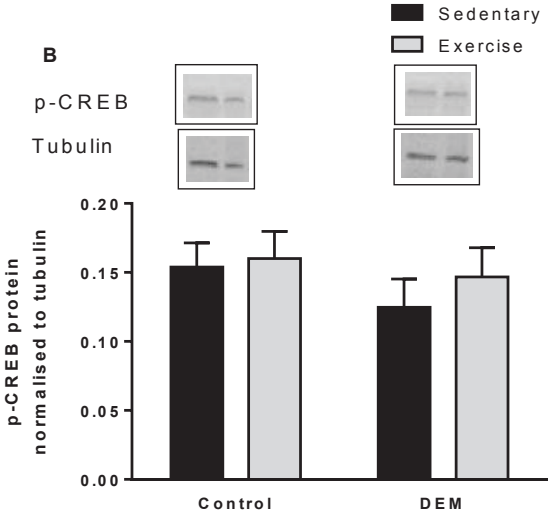
537 +P<0.05 main effect for DEM.

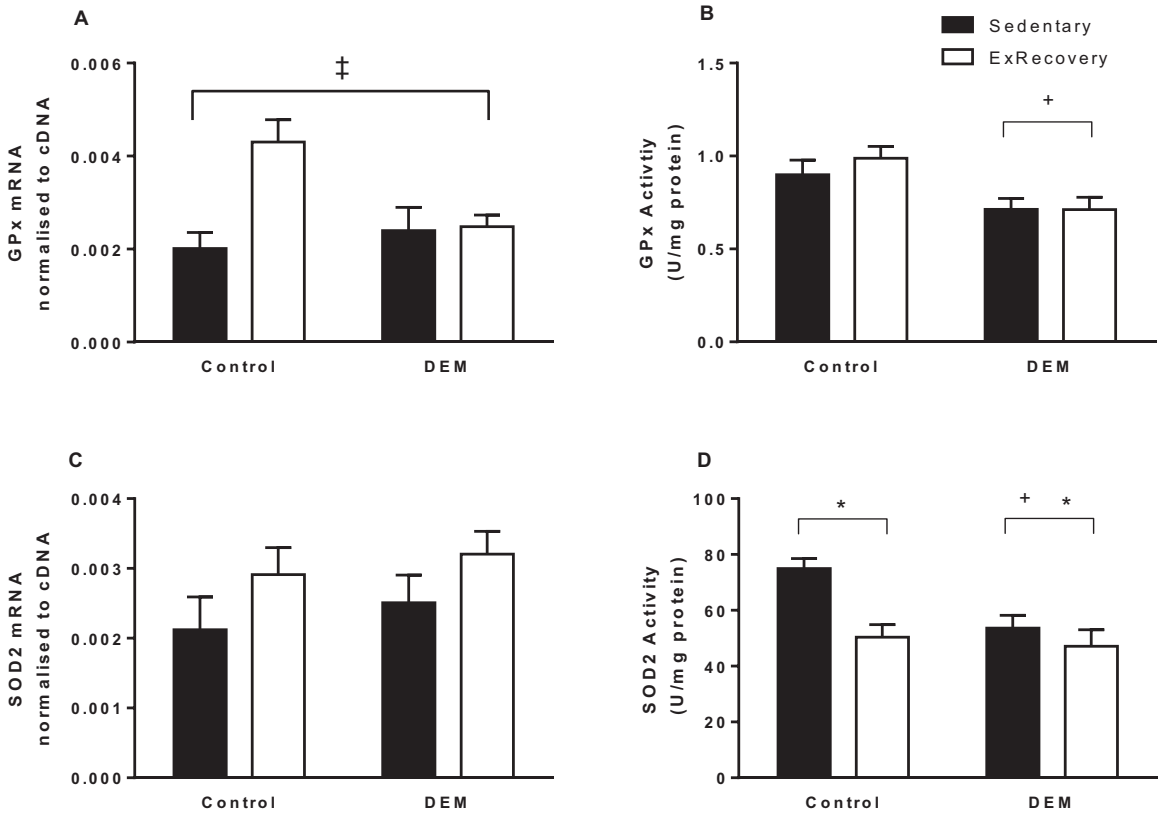

Sedentary
Exercise
A
‡
40
30
20
10
0
tGSH
(nmol/mg protein)
Control
DEM
B
1.5
1.0
0.5
0.0
GSSG
(nmol/mg protein)
Control
DEM
C
‡
0.08
0.06
0.04
0.02
0.00
ratio of GSSG/tGSH
Control
DEM


Sedentary
Exercise
+
800
600
400
200
0
Isoprostanes (pg/ml)
Control
DEM


A
PGC-1α mRNA normalised to cDNA
0.04
0.03
0.02
0.01
0.00
‡
Control
DEM
B
Sedentary
ExRecovery
NRF-2 mRNA normalised to cDNA
0.008
0.006
0.004
0.002
0.000
Control
DEM


A
p-p38
Total p38
phospho p38 protein normalised total p38
0.15
0.10
0.05
0.00
*
*
Control
DEM


Sedentary
Exercise
B
p-CREB
Tubulin
p-CREB protein normalised to tubulin
0.20
0.15
0.10
0.05
0.00
Control
DEM


A
GPx mRNA normalised to cDNA
0.006
0.004
0.002
0.000
‡
Control
DEM
B
Sedentary
ExRecovery
GPx Activtiy (U/mg protein)
1.5
1.0
0.5
0.0
+
Control
DEM
C
SOD2 mRNA normalised to cDNA
0.004
0.003
0.002
0.001
0.000
Control
DEM
D
SOD2 Activity (U/mg protein)
100
80
60
40
20
0
*
+ *
Control
DEM