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 F₂-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 \(600 \times g\) 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 Na₄P₂O₇, 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 \(\times g\) 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 \(\times g\) for 15 min at 4°C. The
supernatant was transferred and analysis was done on freshly prepared homogenates.
Total and oxidized glutathione were analysed using frozen muscle tissue which was homogenized in 20 μl of 5% (wt/vol) 5-sulfosalicylic acid (SSA)/mg tissue and centrifuged at 11,500 rpm for 5 min at 4°C. The supernatant was diluted 1:5.5 in ddH₂O. Triethanolamine was added to neutralise the solution to ensure optimal pH for the reaction. Separate aliquots of 100 μl were taken for total (tGSH) and oxidised (GSSG) glutathione measurement. The supernatant sample for the GSSG assay was derivatised in 16 μl of the following solution: 30.8% triethanolamine, 0.4% SSA, and 9% 2-vinylpyridine in ddH₂O (7).

**Plasma F₂-Isoprostanes**

As a marker of oxidative stress, F₂-Isoprostanes were extracted from plasma GC/MS/MS (6). Isoprostanes were extracted from plasma after saponification with methanolic NaOH. Samples were spiked with 8-iso-PGF2α-d4 (Cayman Chemicals, USA) as an internal standard, and incubated at 42°C for 60 min. Samples were then acidified to pH 3 with hydrochloric acid; hexane was then added and the sample was mixed for 10 min before centrifugation at 3000 × g. The supernatant was removed and the remaining solution extracted with ethyl acetate and dried under nitrogen. Samples were reconstituted with acetonitrile, transferred into vials with glass inserts and dried. The samples were then derivatised using 40 μl of a 10% pentafluorobenzylbromide/acetonitrile solution (v/v) and 20 μl of a 10% diisopropylethylamine/acetonitrile solution (v/v), and incubated at room temperature for 30 min. Samples were then dried again under nitrogen before 10 μl of pyridine and 20 μl of a Bis(trimethylsilyl)trifluoroacetamide/Trimethylchlorosilane solution (99:1) (Sigma, St. Louis, MO) were added, and the samples were incubated at 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°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°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).
Western Blot Analysis

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

Glutathione and Antioxidant Enzyme Activities

Total and GSSG glutathione concentrations were measured by modifying the method of Dudley (7). Absorbance was recorded on a plate reader (Fluostar Optima, BMG Labtech, Victoria, Australia). GPx activity was measured using a modified method for the Cobas Mira spectrophotometric analyser (Roche Diagnostics, Basel, Switzerland) (40). The method used to measure SOD2 activity was modified from Oyanagu (24). Protein concentration was determined using a BCA protein assay (Pierce, Rockford, IL) with BSA as the standard.
Data were checked for normality using the Shapiro-Wilk test. If the test was significant, data were log transformed and reanalyzed. Plasma \( F_2 \)-isoprostanes, and skeletal muscle tGSH, ratio of GSSG/tGSH, PGC-1\( \alpha \) mRNA, NRF-2 mRNA, phosphorylated p38 MAPK protein, CREB protein, GPx mRNA, GPx activity, SOD2 mRNA and SOD2 activity were all normally distributed. Skeletal muscle GSSG was normally distributed after log transformation.

An unpaired \( t \)-test was used to determine differences in time to fatigue between control exercise and DEM exercise groups. For all other data, a two-way ANOVA was used to determine an interaction between exercise and DEM treatment and if significant a Tukey’s post hoc was completed. If there was no interaction, where applicable, a main effect for exercise or DEM is provided. Animals sacrificed directly after exercise were used to determine plasma \( F_2 \)-isoprostanes, and skeletal muscle glutathione and cell signaling proteins (37). Markers of mitochondrial biogenesis and endogenous antioxidants were measured on animals euthanized 4 h after exercise (37). Significance was considered at \( P<0.05 \). Normalised data are presented as mean ± SE, and log 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 F₂-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
increase in PGC-1α gene expression compared to the control animals that were exercised (P<0.05). Exercise did not significantly increase NRF-2 gene expression (P=0.1; Figure 3B).

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

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

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

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

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

**Discussion**

This study is the first to investigate the effects of depleting glutathione and thus 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-buthathione-(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$_2$O$_2$ 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...
of oxidative stress, which confirmed the expected increase in ROS production within the
DEM groups.

A major finding of this study was that DEM treatment diminished the glutathione pool,
resulting in inability of skeletal muscle to limit oxidative stress, and therefore
augmented PGC-1α gene expression following exhaustive exercise. Recent in vitro
evidence suggests that increases in PGC-1α promoter activity and gene expression are
mitigated by increased hydrogen peroxide levels in skeletal muscle (15, 33, 34). Given
that one of the major roles of glutathione system is to reduce hydrogen peroxide to water,
and we diminished the glutathione pool, it is possible this resulted in an increase in
hydrogen peroxide, thus a potential pathway for the changes seen in PGC-1α gene
expression. Although there was a trend for exercise to increase NRF-2 gene expression,
DEM treatment did not affect this gene. It is possible that NRF-2 gene expression is not
sensitive to increases in ROS following treatment with DEM.

The signaling proteins, p38 and CREB, are involved in the regulation of PGC-1α gene
expression (1, 43). Consistent with our previous studies, acute exercise significantly
increased p38 MAPK phosphorylation (37, 38). Indeed, an increase in phosphorylation of
p38 MAPK, followed by the exercise-induced increase in PGC-1α gene expression is also
similar to that reported by Akimoto et al., providing further support of the functional role
of p38 in skeletal muscle adaptations (1). Skeletal muscle phosphorylation of p38 MAPK
also tended (P=0.06) to be higher with DEM-treatment. It appears that oxidative stress
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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Disclosures

The authors have no conflicts of interest to declare.
1. Akimoto T, Pohnert SC, Li P, Zhang M, Gumbs C, Rosenberg PB, Williams RS, and Yan Z. Exercise stimulates Pgc-1alpha transcription in skeletal muscle through activation of the p38 MAPK pathway. *J Biol Chem* 280: 19587-19593, 2005.

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

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

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

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

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

7. Dudley RW, Khairallah M, Mohammed S, Lands L, Des Rosiers C, and Petrof BJ. Dynamic responses of the glutathione system to acute oxidative stress in dystrophic mouse (mdx) muscles. *Am J Physiol Regul Integr Comp Physiol* 291: R704-710, 2006.
8. **Gerard-Monnier D, Fouveat 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.
15. **Irrcher I, Ljubicic V, and Hood DA.** Interactions between ROS and AMP kinase activity in the regulation of PGC-1alpha transcription in skeletal muscle cells. *Am J Physiol Cell Physiol* 296: C116-123, 2009.

16. **Irrcher I, Ljubicic V, Kirwan AF, and Hood DA.** AMP-activated protein kinase-regulated activation of the PGC-1alpha promoter in skeletal muscle cells. *PLoS One* 3: e3614, 2008.

17. **Joseph AM, Pilegaard H, Litvintsev A, Leick L, and Hood DA.** Control of gene expression and mitochondrial biogenesis in the muscular adaptation to endurance exercise. *Essays Biochem* 42: 13-29, 2006.

18. **Kang C, O'Moore KM, Dickman JR, and Ji LL.** Exercise activation of muscle peroxisome proliferator-activated receptor-gamma coactivator-1alpha signaling is redox sensitive. *Free Radic Biol Med*, 2009.

19. **Kramer K, Dijkstra H, and Bast A.** Control of physical exercise of rats in a swimming basin. *Physiol Behav* 53: 271-276, 1993.

20. **Leeuwenburgh C and Ji LL.** Glutathione depletion in rested and exercised mice: biochemical consequence and adaptation. *Arch Biochem Biophys* 316: 941-949, 1995.

21. **Lew H, Pyke S, and Quintanilha A.** Changes in the glutathione status of plasma, liver and muscle following exhaustive exercise in rats. *FEBS Lett* 185: 262-266, 1985.

22. **Lundby C, Nordsborg N, Kusuhara K, Kristensen KM, Neufner PD, and Pilegaard H.** Gene expression in human skeletal muscle: alternative normalization 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
30. **Rhinn H, Scherman D, and Escriou V.** One-step quantification of single-stranded DNA in the presence of RNA using Oligreen in a real-time polymerase chain reaction thermocycler. *Anal Biochem* 372: 116-118, 2008.

31. **Ristow M, Zarse K, Oberbach A, Kloting N, Birringer M, Kiehntopf M, Stumvoll M, Kahn CR, and Bluher M.** Antioxidants prevent health-promoting effects of physical exercise in humans. *Proc Natl Acad Sci U S A* 106: 8665-8670, 2009.

32. **Sen CK, Atalay M, and Hanninen O.** Exercise-induced oxidative stress: glutathione supplementation and deficiency. *J Appl Physiol* 77: 2177-2187, 1994.

33. **Silveira LR, Pilegaard H, Kusuhara K, Curi R, and Hellsten Y.** The contraction induced increase in gene expression of peroxisome proliferator-activated receptor (PPAR)-gamma coactivator 1alpha (PGC-1alpha), mitochondrial uncoupling protein 3 (UCP3) and hexokinase II (HKII) in primary rat skeletal muscle cells is dependent on reactive oxygen species. *Biochim Biophys Acta* 1763: 969-976, 2006.

34. **St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jager S, Handschin C, Zheng K, Lin J, Yang W, Simon DK, Bachoo R, and Spiegelman BM.** Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* 127: 397-408, 2006.

35. **Strobel NA, Peake JM, Matsumoto A, Marsh SA, Coombes JS, and Wadley GD.** Antioxidant supplementation reduces skeletal muscle mitochondrial biogenesis. *Med 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.
Table 1: Primers for mRNA analyses

| Gene  | Forward Primer (5′-3′)         | Reverse Primer (5′-3′)         |
|-------|--------------------------------|--------------------------------|
| PGC-1α| ACCCACAGGATCAGAACAACC          | GACAAATGCTCTTTGCTTTATTC       |
| NRF2α | CTCGGAGCAGGTGACGAG             | TGGACCAGCGTATAGGATCA           |
| SOD2  | TGGACAAACCTGAGCCCTAA           | GACCCAAAGTCACGTGTTGATA        |
| GPx-1 | CGACATCGAAACCGATATAGA          | ATGCCTTAGGGTGTGGTACTAGG       |
Figure 1: Effects of DEM and exercise on levels of total glutathione (tGSH) (A), oxidised glutathione (GSSG) (B) and the GSSG/tGSH ratio (C). Animals in the post-exercise groups were sacrificed directly after exercise. Values are mean ± SE for tGSH, GSSG/tGSH ratio and geometric mean (95% CI) for GSSG (n=5-8 for all groups). ‡ P<0.05 interaction between exercise and DEM.

Figure 2: Effects of DEM and exercise on concentration of plasma F₂-isoprostanes. Animals in the post-exercise groups were sacrificed directly after exercise. Values are mean ± SE (n=6-8 for all groups). +P<0.05 main effect for DEM.

Figure 3: Effects of DEM and exercise on PGC-1α (A) and NRF-2 (B) gene expression. Animals in the exercise-recovery groups were sacrificed 4 h after exercise. Values are mean ± SE (n=6-8 for all). ‡ P<0.05 interaction between exercise and DEM.

Figure 4: Effects of DEM and exercise on protein content of phosphorylated p38 (A) and phosphorylated CREB (B). Animals in the post-exercise groups were sacrificed directly after exercise. Values are mean ± SE (n=8 for all groups). Boxes: Western blot showing representative results from 1 rat/group with boxes around blots indicating blots were obtained from different parts of the same membrane. *P<0.05 main effect for exercise.

Figure 5: Effects of DEM and exercise on changes in gene expression and enzyme activity of GPx-1 (A and B) and SOD2 (C and D). Animals in the exercise-recovery 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.
Control DEM

Isoprostanes (pg/ml)

Sedentary
Exercise

Control
DEM

+
A

![Graph showing PGC-1α mRNA normalised to cDNA for Control and DEM groups.](image)

B

![Graph showing NRF-2 mRNA normalised to cDNA for Sedentary and ExRecovery groups.](image)
**A**

- **p-p38**
- **Total p38**

|          | Control | DEM |
|----------|---------|-----|
| p-p38    |         |     |
| Total p38|         |     |

* Denotes significant difference.

**B**

- **p-CREB**
- **Tubulin**

|          | Sedentary | Exercise |
|----------|-----------|----------|
| p-CREB   |           |          |
| Tubulin  |           |          |

Bar graphs showing normalized protein levels for p-p38 and p-CREB under control and DEM conditions.
A

GPx mRNA
normalised to cDNA

Gp x Activity (U/mg protein)

Control DEM

‡

B

Sedentary

ExRecovery

Control DEM

C

SOD2 mRNA
normalised to cDNA

Control DEM

D

SOD2 Activity (U/mg protein)

Control DEM