Mol Cell Biochem (2015) 400:265–275
DOI 10.1007/s11010-014-2283-0

Glutathione depletion and acute exercise increase *O*-GlcNAc protein modification in rat skeletal muscle

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Received: 22 August 2014 / Accepted: 15 November 2014 / Published online: 23 November 2014

Abstract Post-translational modification of intracellular proteins with *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) profoundly affects protein structure, function, and metabolism. Although many skeletal muscle proteins are *O*-GlcNAcylated, the modification has not been extensively studied in this tissue, especially in the context of exercise. This study investigated the effects of glutathione depletion and acute exercise on *O*-GlcNAc protein modification in rat skeletal muscle. Diethyl maleate (DEM) was used to deplete intracellular glutathione and rats were subjected to a treadmill run. White gastrocnemius and soleus muscles were analyzed for glutathione status, *O*-GlcNAc and *O*-GlcNAc transferase (OGT) protein levels, and mRNA expression of OGT, *O*-GlcNAcase and glutamine:fructose-6-phosphate amidotransferase. DEM and exercise both reduced intracellular glutathione and increased *O*-GlcNAc. DEM upregulated OGT protein expression. The effects of the interventions were significant 4 h after exercise ($P < 0.05$). The changes in the mRNA levels of *O*-GlcNAc enzymes were different in the two muscles, potentially resulting from different rates of oxidative stress and metabolic demands between the muscle types. These findings indicate that oxidative environment promotes *O*-GlcNAcylation in skeletal muscle and suggest an interrelationship between cellular redox state and *O*-GlcNAc protein modification. This could represent one mechanism underlying cellular adaptation to oxidative stress and health benefits of exercise.

Keywords Diethyl maleate · Treadmill run · White gastrocnemius · Soleus · *O*-GlcNAc

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Introduction

During exercise, oxygen consumption rises dramatically, resulting in an increased generation of reactive oxygen species (ROS) within skeletal muscle [1]. This can disturb the cellular oxidant-antioxidant balance and lead to oxidative stress and oxidative damage. There is an increasing body of evidence that ROS are crucial for cellular adaptations to oxidative stress and serve multiple beneficial roles in cellular function and metabolism [2]. Exercise-produced reactive species have been associated with upregulation of the endogenous antioxidant system [3], induction of heat shock proteins (HSPs) [4], increased glucose uptake [5], and optimal contractile activity [6].

Mechanisms that promote stress tolerance and survival involve various energy and stress-sensing pathways. The hexosamine biosynthesis pathway (HBP) acts as a cellular stress and nutrient sensor and provides a substrate for post-translational modification of proteins [7]. *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) is a monosaccharide that binds to serine and threonine residues of intracellular proteins, altering their structure, function, and thereby metabolism. Glutamine:fructose-6-phosphate amidotransferase (GFAT) has been recognized as the rate-limiting enzyme of the HBP and two isoforms have been identified, GFAT1 and GFAT2, that differ in tissue distribution [8] and regulation by post-translational modifications such as phosphorylation [9]. Two highly conserved proteins, *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA), catalyze the attachment and removal of the sugar unit on and off proteins, respectively, and can form dynamic complexes with each other and other enzymes, providing a sensitive regulation of *O*-GlcNAc protein modification [10]. *O*-GlcNAcylation has been suggested to play roles in various processes, including the cellular response to stress [11]. *O*-GlcNAc binds to several transcription factors [12], HSPs [13] and antioxidant enzymes [14, 15], modulating their expression, stability, and function. Furthermore, it is involved in regulation of protein homeostasis [16], participates in cellular immune response [17] and DNA repair [18].

O-GlcNAc protein modification in skeletal muscle has been scarcely investigated, particularly in the context of exercise and exercise-induced oxidative stress. *O*-GlcNAcylated proteins are abundant in the muscle [19] and are involved in the processes such as insulin signaling [20] and muscle contraction [21]. Impaired *O*-GlcNAc cycling has been associated with insulin resistance [20] and muscle atrophy [22]. There have been reports demonstrating a link between *O*-GlcNAcylation and redox signaling, with several ROS-responsive enzymes recognized to be *O*-GlcNAc modified [12, 14, 23]. The interaction could represent one of the mechanisms underlying cellular adaptation to oxidative stress.

This study investigated the effects of glutathione depletion and acute exercise on protein *O*-GlcNAc modification in skeletal muscle. Rats were injected with diethyl maleate (DEM) to deplete intracellular glutathione and/or underwent a treadmill run to exhaustion. *O*-GlcNAc levels and OGT protein expression were measured in the white gastrocnemius and soleus muscles. mRNA levels of the enzymes OGT, OGA, GFAT1, and GFAT2 were also determined. It was hypothesized that DEM and exercise would induce oxidative stress in skeletal muscle and promote *O*-GlcNAcylation. This would be supported by a transient increase in the mRNA of GFAT isozymes and an upregulated expression of OGT.

Materials and methods

Materials

The antibodies used for Western blot analysis included anti-*O*-GlcNAc antibody (CTD110.6, Covance, Richmond, CA), anti-OGT antibody (SQ-17, Sigma, St. Louis, MO), anti-Tubulin (11H10, Cell Signaling, Danvers, MA), and alkaline phosphatase-conjugated antibodies (Sigma). *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc) was purchased from Toronto Research Chemicals Inc. (Toronto, ON) and CDP-star from Roche Applied Science (Penzberg, Germany). Total RNA was extracted using Sigma's TRI Reagent and reverse transcribed with the High Capacity cDNA Reverse Transcription Kit from Invitrogen (Life Technologies, Carlsbad, CA). The SYBR® Select Master Mix was obtained from Applied Biosystems (Life Technologies) and the qRT-PCR primers were purchased from Sigma.

Animals

The experiments were approved by The University of Queensland Animal Ethics Committee in accordance with National Health and Medical Research Council guidelines. Ten-week-old male Wistar rats (Central Animal Breeding House, The University of Queensland, Australia) were housed two per cage, maintained on a 12-h light/dark cycle and had ad libitum access to standard rat chow and tap water. Animals were divided into six groups: (1) sedentary (C; $n = 8$), (2) sedentary + DEM (C_d; $n = 8$), (3) exercise (E; $n = 8$), (4) exercise + DEM (E_d; $n = 8$), (5) exercise + recovery (R; $n = 8$), and (6) exercise + recovery + DEM (R_d; $n = 5$). DEM rats were given an intraperitoneal injection of 3 mmol/kg body weight 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.

Treadmill exercise

Rats were exercised on a modified treadmill divided into 8 lanes separated by clear plastic enclosures. All animals were familiarized for 4 days to treadmill running prior to the start of the study, at a 10 % uphill gradient at 1–1.2 km/h for 30 min. The rats that were more willing to run were placed into the exercise groups. According to the previous research, this selection process is considered appropriate, because health status and muscle physiology properties do not differ between those rats willing to run or not [24]. Rats ran up a 10 % grade at 1.2 km/h for the first 30 min, the speed was then increased every 10 min by 0.1 km/h until exhaustion. Exhaustion was defined as the inability of the

animal to right itself when being laid on its side [25], with time until exhaustion recorded for each animal.

Tissue collection

Animals were weighed and sacrificed via intraperitoneal injection of sodium pentobarbital (100 mg/kg), directly after exercise or 4 h after exercise. Under a surgical plane of anesthesia, blood was taken via cardiac puncture and placed on ice. Blood samples were centrifuged at 600×*g* for 10 min and plasma aliquots were stored at −80 °C until analyzed. The white gastrocnemius and soleus muscles were excised, snap frozen in liquid nitrogen and stored at −80 °C.

Plasma isoprostanes

The levels of F(2)-isoprostanes in rat plasma samples were analyzed with gas chromatography–tandem mass spectrometry (GC–MS–MS), using a modified method adopted from Taylor et al. [26]. The isoprostanes were extracted from serum after saponification with methanolic NaOH. The samples were spiked with an internal standard 8-iso-PGF2α-d4 (Cayman Chemical Co., Ann Arbor, MI) and incubated at 42 °C for 60 min. The samples were then acidified with hydrochloric acid. After adding hexane, the samples were mixed for 10 min before centrifugation at 3,000×*g* for 10 min. The supernatant was removed and the remaining solution was extracted with ethyl acetate and dried under nitrogen. The samples were reconstituted with acetonitrile, transferred into vials with silanized glass inserts and dried. This was followed by derivatization with 40 µl of 10 % (v/v) pentafluorobenzyl bromide/acetonitrile solution and 20 µl of 10 % (v/v) diisopropylethylamine/acetonitrile solution and a 30 min incubation at room temperature. After the samples were dried under nitrogen, 10 µl of pyridine and 20 µl of a Bis(trimethylsilyl)trifluoroacetamide/Trimethylchlorosilane solution (99:1) (Sigma) were added, followed by incubation at 45 °C for 20 min. Finally, 60 µl of hexane was added and 1 µl of the sample was injected for analysis with GC–MS–MS (Varian, Palo Alto, CA) in negative chemical ionization mode.

Total and oxidized glutathione levels

Total (tGSH) and oxidized (GSSG) glutathione were measured by modifying the method of Dudley et al. [27]. Frozen muscle tissue 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 ddH_2O. Triethanolamine (4 M) was added to neutralize the solution to ensure optimal pH for the reaction. Separate aliquots of 100 µl were taken for tGSH and GSSG determination. 125 µl of 0.3 mM NADPH, 16 µl of 6.0 mM 5,5′-dithiobis(2-nitrobenzoic acid), and 48 µl of either sample, standard, or 5 % SSA were pipetted in a plate. The reaction mixture was incubated at 37 °C for 4 min. Next, 8 µl of glutathione reductase enzyme (1.0 U/100 µl) was added and the reaction was monitored at 405 nm every 5 min for a period of 25 min. Absorbance was recorded on a plate reader (Fluostar Optima, BMG Labtech, Victoria, Australia). Total glutathione in each sample was determined from a calibration curve produced using known glutathione standards. The supernatant sample for the GSSG assay was derivatized in 16 µl of the following solution: 30.8 % triethanolamine, 0.4 % SSA, and 9 % 2-vinylpyridine in ddH2O. The mixture was incubated for 10 min at room temperature (on a rotator). To quantify the amount of GSSG, known standards of oxidized glutathione were derivatized and assayed. The samples were then analyzed as for total glutathione above.

Immunoblotting

Muscle tissue was weighed and pulverized under liquid nitrogen and homogenized in ice-cold Western Extraction Buffer containing: RIPA Buffer pH 7.5 (50 mM Tris, 1 mM EDTA, 10 % (v/v) glycerol, 1 % (v/v) Triton X-100), 50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM PMSF, 1 mM DTT, 0.5 % (v/v) Protease Inhibitor Cocktail (Sigma), and 40 µM PUGNAc (Toronto Research Chemicals Inc.). Protein concentration was determined with Pierce® BCA Protein Assay Kit (Pierce, Rockford, IL). The samples were added Sample Reducing Buffer pH 6.8 (0.5 M Tris, 6 % (v/v) SDS, 30 % (v/v) glycerol, bromophenol blue and 30 mM DTT), boiled for 5 min and stored at −80 °C for further analysis. Forty microgram of proteins was separated by SDS-PAGE and transferred to PVDF membranes. The membranes were probed with mouse monoclonal anti-*O*-GlcNAc antibody (CTD110.6, Covance; 1:800) or rabbit monoclonal anti-OGT antibody (SQ-17, Sigma; 1:700) overnight at 4 °C, followed by incubation with appropriate alkaline phosphatase-labeled secondary antibodies for 1 h at room temperature diluted 1:1,800. Tubulin (11H10, Cell Signaling; 1:700) was used as a protein loading control. Immunoblots were developed with chemiluminescence (CDP-Star, Roche), exposed to X-ray film and densitometry analyzed using Scion Image Beta 4.02 software (Scion Corporation, Frederick, MD). Densitometric values for *O*-GlcNAc and OGT bands were normalized against corresponding tubulin values and the ratios were used in statistical analysis.

Real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis

RNA extraction

Total RNA was extracted from frozen muscle tissue using TRI Reagent (Sigma). 20–30 mg of muscle tissue was homogenized in 1 ml of TRI Reagent in a polytron homogenizer and centrifuged at 12,000×*g* for 10 min at 4 °C. Supernatant was added 0.2 ml of chloroform, vortexed, and incubated for 3 min at room temperature. After centrifuging for 15 min at 12,000×*g* at 4 °C, the upper aqueous phase containing RNA was transferred into fresh tubes. 0.5 ml of isopropanol and 1.5 µl of glycogen were added, samples were mixed, incubated at room temperature for 10 min, and centrifuged at 12,000×*g* for 10 min at 4 °C. The supernatant was removed and the RNA pellet was washed in 1 ml of 75 % (v/v) ethanol. The samples were vortexed and centrifuged at 7,500×*g* for 5 min at 4 °C. The pellets were then dried and dissolved in 30 µl RNA-free water and stored at −80 °C until later analysis. RNA concentration was determined spectrophotometrically (UV/VIS spectrophotometer) and $A_{260/280}$ ratio was calculated to assess RNA purity. Gel agarose electrophoresis was used to check RNA integrity.

qRT-PCR

Following total RNA concentration determination, 500 ng of total RNA was reverse transcribed to synthesize cDNA, using High Capacity cDNA Reverse Transcription Kit (Life Technologies). For each sample, a reverse transcription reaction mixture (20 µl total) was prepared containing 500 ng of total RNA diluted with 4.2 µl RNase-free water, 2 µl 10× reverse transcription buffer, 0.8 µl 25× deoxynucleoside triphosphate (dNTP) mixture (dATP, dCTP, dGTP, dTTP, $MgCl_2$, RNase inhibitor, oligo$(dT)_{15}$ primer), and 1 µl of reverse transcriptase enzyme. The mixture was treated at 25 °C for 10 min, followed by 120 min at 37 °C, then heated to 85 °C for 5 min and quick chilled to 4 °C on ice, yielding the cDNA product. 180 µl of RNase-free water was then added and the solution was frozen at −80 °C until qRT-PCR analysis was performed. The pre-designed primers for OGT, OGA, GFAT1, and GFAT2 genes were purchased from Sigma. Primer sequences for GAPDH genes were obtained from literature [28]. GAPDH was used as an internal reference for detecting relative change in the quantity of target mRNA. Melting curves were performed to ensure that prominent PCR product was being produced by each primer in the absence of primer dimmers. The primer sequences are presented in Table 1.

Statistical analysis

Values are presented as mean ± SEM. Student's *t* test was used to determine the differences in time to fatigue between non-treated and DEM-treated exercised rats. All other data were analyzed using two-way ANOVA with Tukey's post hoc analysis where appropriate. Data that were not normally distributed and/or of unequal variance underwent logarithmic transformations, and the subsequent analyses were performed on the transformed data. Statistical significance was established at $P < 0.05$.

Results

Time to fatigue, plasma isoprostanes and skeletal muscle total and oxidized glutathione

Time to fatigue was significantly reduced in DEM-treated rats (51 ± 5 min) compared to controls (68 ± 5 min) ($P < 0.05$). Plasma isoprostane levels were significantly increased with DEM in sedentary rats ($P < 0.05$) and returned to baseline 4 h after exercise (data not shown). Non-treated animals showed significantly higher isoprostane levels immediately after exercise when compared to the measures 4 h after exercise ($P < 0.05$). Furthermore, DEM-treated rats had significantly lower levels of tGSH compared to non-treated animals in both muscles 4 h after exercise and directly after exercise in the white gastrocnemius ($P < 0.05$; Fig. 1). In addition, non-treated rats showed significantly reduced tGSH levels in the white gastrocnemius 4 h after exercise, relative to sedentary controls ($P < 0.05$). No significant differences between groups were detected in this muscle when comparing

Table 1 Primer sequences used to probe genes of interest

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	GenBank Accession No.
OGT	AAAGCATTAGAGGTCTTCCC	CATCTCCTTTAAAGTGTTTCCC	NM_017107
OGA	AATTGCAAAGGGAAAGACTC	CTCTTGATATCCCACACATAG	NM_131904
GFPT1 (GFAT1)	GGCAAAGACAAGAAAGGAAG	CAGAAAGATGACACGATTGG	NM_001005879
GFPT2 (GFAT2)	AAAGAGTCATTCAGCAGTTG	CATTCTCAATAGTGCATGTCC	NM_001002819
GAPDH	TGGTCCAGGGTTTCTTACT	ATTCTTCCACCTTTGATGC	NM_001001303

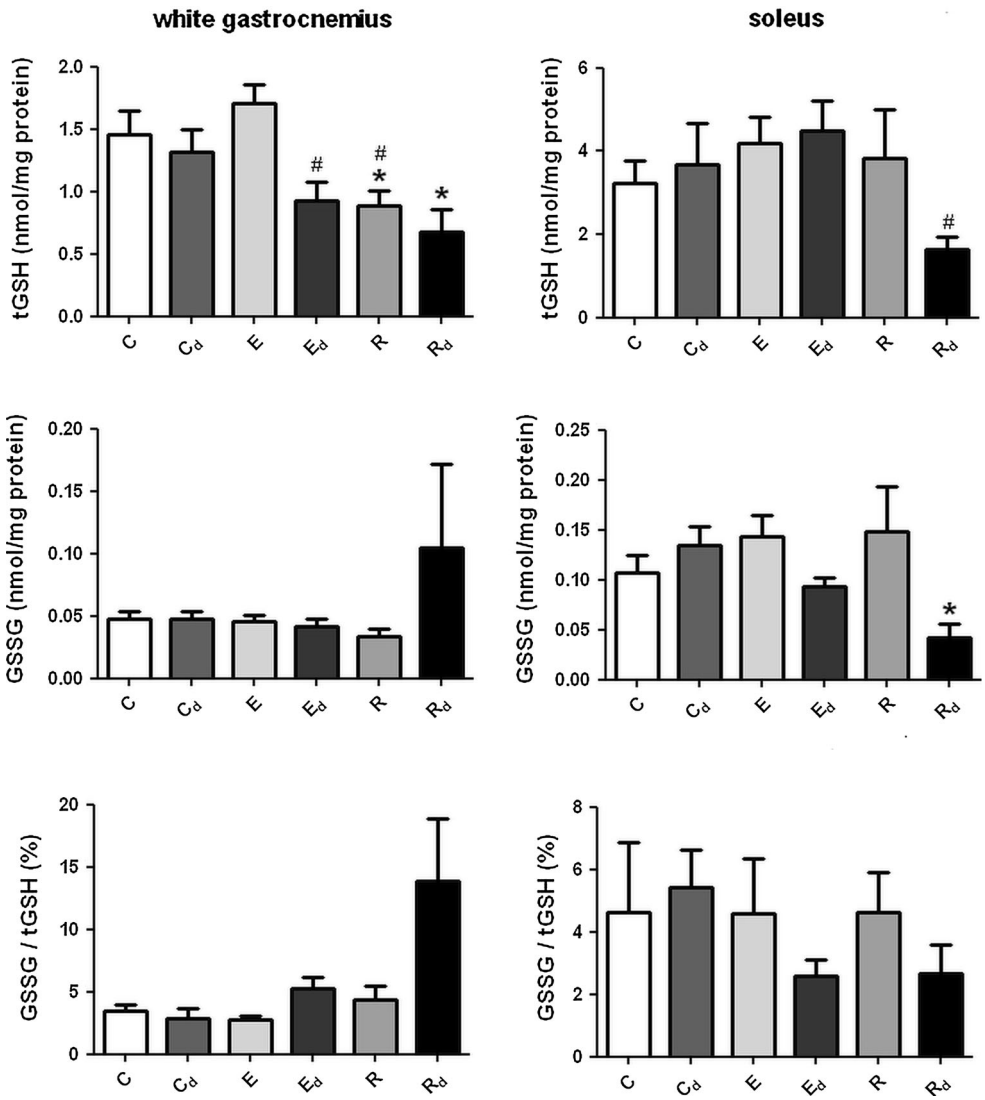

Fig. 1 Total glutathione (tGSH), oxidized glutathione (GSSG), and GSSG/tGSH ratio in white gastrocnemius and soleus muscles. *C* control ($n = 8$), C_d control + DEM ($n = 8$), *E* exercise (n = 8), E_d exercise + DEM ($n = 8$), *R* exercise + recovery ($n = 8$), R_d exercise + recovery + DEM ($n = 5$). Values are presented as mean ± SEM. $^{*}P < 0.05$ vs relevant control group (*C* or C_d), $^{\#}P < 0.05$ vs relevant exercise group (*E* or E_d)

GSSG levels ($P > 0.05$), there was a trend for an increased GSSG/tGSH ratio in DEM-treated rats 4 h after exercise ($P = 0.083$). In the soleus of DEM-treated animals, the decrease in tGSH 4 h post-exercise was accompanied by reduced GSSG levels ($P < 0.05$).

O-GlcNAcylation and OGT protein expression

DEM increased *O*-GlcNAc levels, which was significant in the control group in the white gastrocnemius and 4 h after exercise in both muscles ($P < 0.05$; Fig. 2B). Moreover, exercise itself promoted *O*-GlcNAcylation, as indicated by higher *O*-GlcNAc levels in the white gastrocnemius of non-treated animals sacrificed immediately or 4 h post-exercise, relative to sedentary controls ($P < 0.05$). *O*-GlcNAc levels of individual proteins (Fig. 2A: bands a, b, c, d, e, f) were determined and the results were correlative with the changes in overall *O*-GlcNAcylation (data not shown). DEM treatment also increased OGT protein expression in both muscles, this was significant 4 h after exercise ($P < 0.05$; Fig. 2C).

mRNA expression of the *O*-GlcNAc cycling enzymes

In the white gastrocnemius, DEM blunted the exercise-induced increase in OGT mRNA; there was a trend ($P = 0.067$) for reduced mRNA levels after DEM treatment when comparing the two groups of rats sacrificed immediately after exercise (Fig. 3). In contrast, the soleus muscle showed a decrease in OGT mRNA expression with exercise, which was significant 4 h post-exercise ($P < 0.05$). The mRNA levels were further reduced with DEM treatment in the rats sacrificed immediately after exercise ($P < 0.05$), while 4 h after exercise DEM indicated a trend for upregulating OGT mRNA expression ($P = 0.052$). Similarly, exercise promoted OGA mRNA

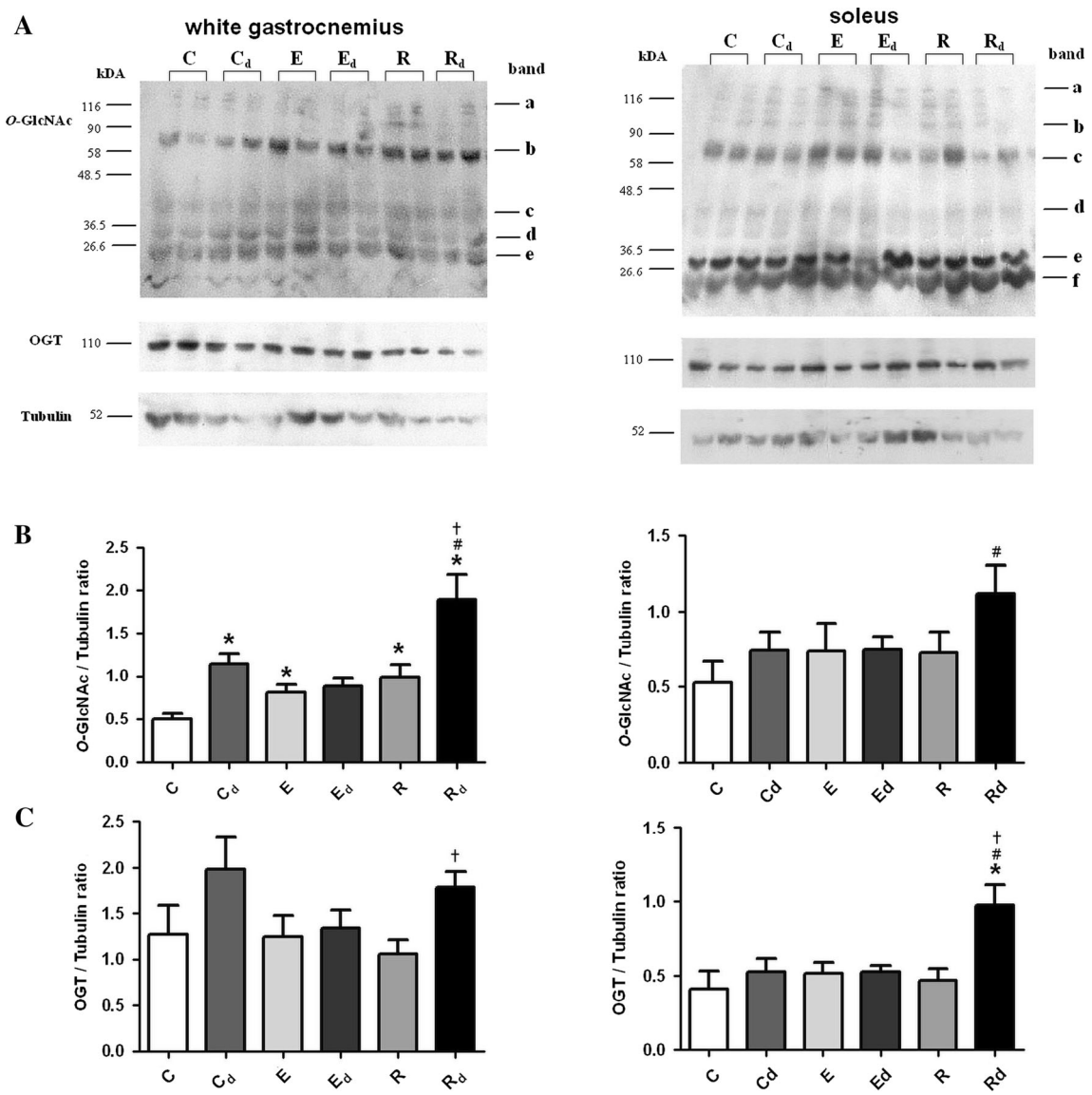

Fig. 2 Immunoblots of *O*-GlcNAc-modified proteins and OGT enzyme (**A**) in white gastrocnemius and soleus muscles. Mean densitometric data for general *O*-GlcNAc levels (**B**) and OGT protein expression (**C**) normalized to tubulin. *C* control ($n = 8$), C_d control + DEM ($n = 8$), *E* exercise ($n = 8$), E_d exercise + DEM ($n = 8$), *R* exercise + recovery ($n = 8$), R_d exercise + recovery + DEM ($n = 5$). Values are presented as mean ± SEM. $^{*}P < 0.05$ versus relevant control group (*C* or C_d), $^{\#}P < 0.05$ versus relevant exercise group (E or Ed), $^{\dagger}P < 0.05$ versus *R*

expression in the white gastrocnemius and had an inhibitory effect in the soleus, this was significant 4 h post-exercise ($P < 0.05$). The two muscles also showed an opposite response of OGA gene expression to DEM treatment. In the white gastrocnemius, DEM increased OGA mRNA levels immediately after exercise ($P < 0.05$), but reduced it to baseline levels after the 4 h recovery. This was reversed in the soleus, where the mRNA levels were reduced in DEM-treated rats immediately after the exercise bout ($P < 0.05$) and restored to baseline levels 4 h post-exercise. The isozymes GFAT1 and GFAT2 demonstrated a different pattern of changes in their mRNA expression after DEM administration and the exercise bout, which was particularly evident in the soleus muscle. Immediately after exercise, DEM blunted the exercise-induced increase in GFAT1 mRNA in the white gastrocnemius (trend, $P = 0.067$) and had a significant ($P < 0.05$) inhibitory effect in the soleus. mRNA expression of GFAT2 significantly increased immediately after exercise in both muscles ($P < 0.05$), but returned to baseline after 4 h. DEM significantly reduced GFAT2 mRNA levels 4 h post-exercise in the white gastrocnemius and blunted the exercise-induced increase in the soleus ($P < 0.05$).

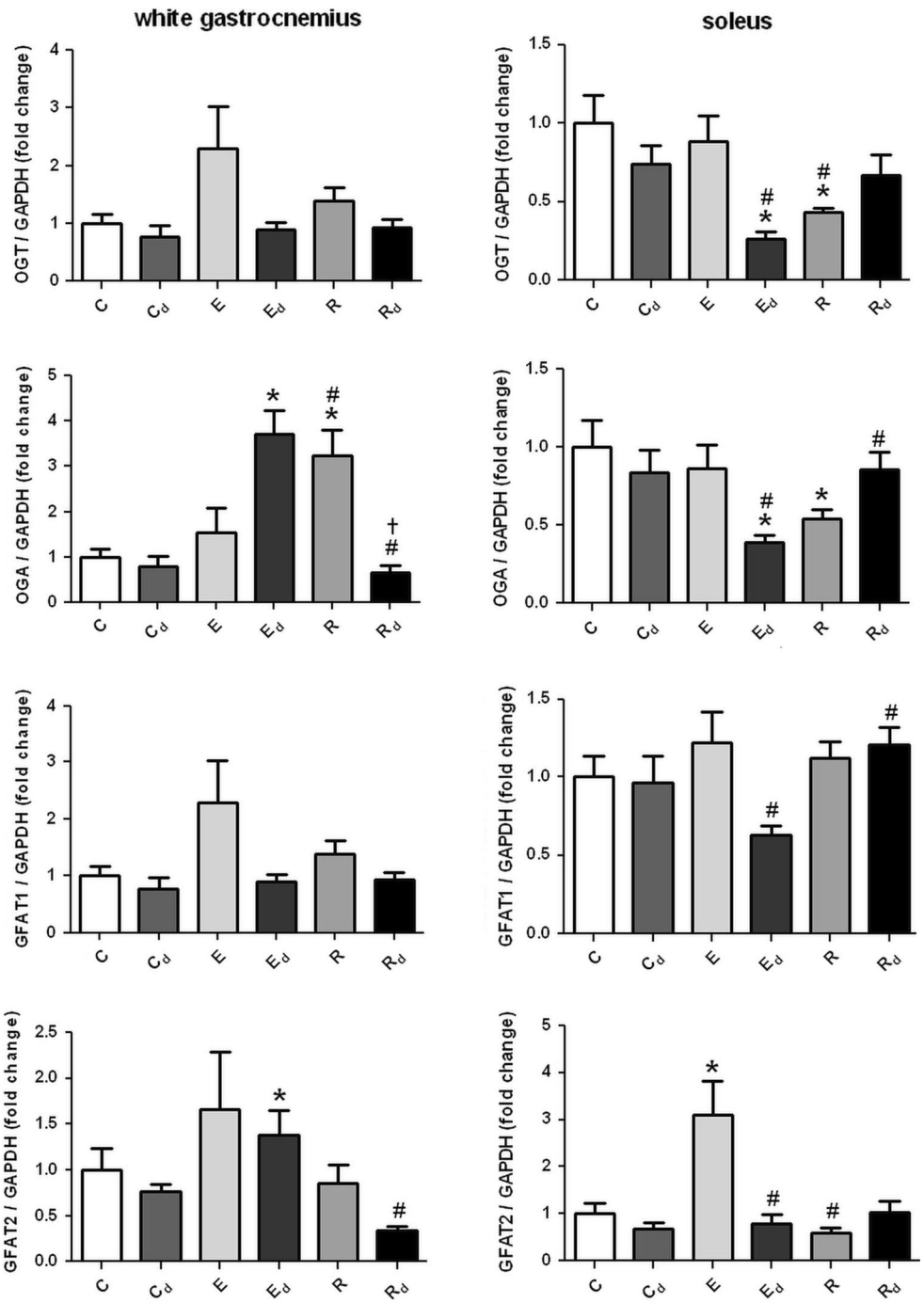

Fig. 3 OGT, OGA, GFAT1, and GFAT2 mRNA expression in white gastrocnemius and soleus muscles. Fold change values (relative to control group C) were normalized to GAPDH. *C* control ($n = 8$), C_d control + DEM ($n = 8$), *E* exercise ($n = 8$), E_d exercise + DEM ($n = 8$), *R* exercise + recovery ($n = 8$), R_d exercise + recovery + DEM ($n = 5$). Values are presented as mean ± SEM. $^{*}P < 0.05$ versus relevant control group (C or C_d), $^{\#}P < 0.05$ vs relevant exercise group (E or Ed), $^{\dagger}P < 0.05$ vs *R*

Discussion

This study investigated the effects of glutathione depletion and acute exercise on *O*-GlcNAc modification of skeletal muscle proteins. Protein *O*-GlcNAcylation increased significantly after the treadmill run and DEM administration. The protein and gene expression changes of the *O*-GlcNAc cycling enzymes were different in the white gastrocnemius and soleus muscles, likely caused by the differences in oxidative and glucose metabolism between the two muscle types. These findings demonstrate that stressors such as glutathione depletion and acute exercise affect the process of *O*-GlcNAcylation in skeletal muscle and promote this post-translational modification.

DEM depletes intracellular glutathione [29] and this was confirmed in our study where DEM administration significantly decreased total glutathione levels in rat skeletal muscle. DEM-treated rats exhibited a trend for higher

GSSG/tGSH ratio post-exercise in the white gastrocnemius, whereas reduced GSSG levels were found in the soleus, possibly due to less available glutathione in the cells to be oxidized. The changes were most significant 6 h after the animals were injected DEM (4 h post-exercise), indicating a time effect of DEM to reduce glutathione levels. Furthermore, plasma isoprostane levels increased significantly in the DEM-treated rats. These measures were considered indicative of oxidative stress. DEM administration also significantly reduced time to fatigue in the rats subjected to a treadmill run, which was consistent with the findings of Sen et al. [30]. This demonstrates an important role of endogenous glutathione in the alleviation of exercise-induced oxidative stress and as a determinant of exercise performance.

Non-treated exercised rats also exhibited elevated plasma isoprostanes. In addition, exercise decreased total glutathione levels in the white gastrocnemius, but had no effect in the soleus. Increased ROS formation is characteristic of exercise [1] and was likely responsible for the lower tGSH and elevated GSSG content observed in the muscle. Our findings are not unexpected as the two muscles differ in fiber type composition: white gastrocnemius consists mainly of type 2 glycolytic fibers, while type 1 oxidative fibers are predominant in the soleus [31]. A differential response to oxidative stress between muscle fiber types was reported in rats after exposure to hypobaric hypoxia; significantly higher levels of oxidative stress and lower glutathione content were found in the gastrocnemius muscle in comparison to the soleus [32]. In treadmill-trained dogs, regular exercise elevated total glutathione levels and glutathione peroxidase activity most significantly in the red gastrocnemius muscle, which is predominantly oxidative by composition, while extensor radialis and triceps muscles showed higher glutathione reductase activity [33]. As tGSH (and GSSG) levels were generally higher in the soleus compared to the white gastrocnemius muscle (Fig. 1), we propose that the more intense oxidative metabolism in the slow-twitch soleus could reflect an upregulated antioxidant defense in this muscle and a glutathione system less sensitive to oxidative stress. Indeed, information about the ROS levels in skeletal muscle tissue would deliver a clearer picture of the oxidative stress occurring within the muscle.

The increase in *O*-GlcNAc levels with DEM treatment and exercise was most significant 4 h post-exercise, indicating an interaction and time effect of the two interventions. Similarly, DEM-induced upregulation of OGT protein expression was evident 4 h after the exercise and could partly explain the increase in *O*-GlcNAcylation. Although OGT has been shown to respond to stress [11, 34], in this study, the exercise-induced increase in oxidants in skeletal muscle might have been insignificant or counteracted by the cellular antioxidants and did not lead to the changes in OGT protein expression. Furthermore, *O*-GlcNAcylation is a dynamic modification; however, time is required for the changes to occur, especially at the protein level, as seen with the absence of detectable changes in the skeletal muscle of animals sacrificed immediately post-exercise. In a study by Zachara et al. [11], *O*-GlcNAc levels were significantly elevated immediately after the heat shock and continued to rise for 9 h, returning to normal by 48 h. In neonatal cardiomyocytes, glucose deprivation also increased *O*-GlcNAc levels in a time-dependent manner; the increase in *O*-GlcNAc was apparent within 3 h after the treatment, significant by 6 h and reached maximal levels by 12 h [35]. OGT and OGA mRNA expression increased, whereas OGA protein levels decreased and there was no change in OGT protein expression. Similar studies showed an increase in *O*-GlcNAc levels with glucose deprivation, while the changes in expression and activity of *O*-GlcNAc enzymes varied between different cell lines, suggesting the response is cell-type dependent [36, 37]. A rapid augmentation of *O*-GlcNAcylation with heat stress was reported, but there were no significant changes in the mRNA expression and activity of OGT, OGA, and the levels of UDP-GlcNAc [38]. It was proposed that elevated *O*-GlcNAc levels could be explained by an increased accessibility of OGT to its targets, caused by conformational changes of heat-denatured proteins. Taken together, various factors can influence protein *O*-GlcNAc levels and without further analyses we cannot ascertain the mechanisms underlying the changes or lack there of observed in our study.

The absence of the exercise effect on OGT protein levels in the white gastrocnemius was consistent with no changes in the mRNA expression. In the soleus, exercise reduced the gene expression of OGT, but there were no changes at the protein level, possibly due to a time delay of protein translation. DEM inhibited OGT mRNA expression immediately after exercise in both muscles, but showed a trend for a positive regulatory effect in the soleus 4 h post-exercise. However, 4 h after exercise, OGT mRNA content did not differ from baseline levels, yet OGT protein levels were found to be increased. Plasma levels of isoprostanes in DEM-treated rats were found to revert to baseline 4 h after the treadmill run, which could mean a lack of stimulus for the upregulation of gene expression. Furthermore, OGT responds to the changes in HBP flux and substrate availability [36, 39], is a subject of post-translational modifications, including *O*-GlcNAcylation and phosphorylation [40], and interacts with a number of other enzymes [12, 34]. DEM-induced stress could interfere with these processes and lead to the changes in protein expression and its distribution across cellular compartments, resulting in the change of the measured OGT protein content. When

assessing mRNA expression of OGA, a combination of exercise and DEM was associated with significantly increased levels in white gastrocnemius. Exercise was found to upregulate OGA mRNA expression independently of DEM, whereas DEM increased the mRNA levels immediately after the run, but reduced it to baseline after the 4 h recovery. The opposite was observed in the soleus, where DEM-treated rats exhibited attenuated OGA mRNA expression immediately post-exercise and exercise alone reduced the mRNA levels. DEM increased OGA mRNA back to baseline after 4 h of recovery. Contracting skeletal muscle is a major site of glucose metabolism [41] and ROS formation [42]. The differences in oxidative and glycolytic capacity of the soleus slow-twitch oxidative fibers and fast-twitch glycolytic fibers of the white gastrocnemius [31] may explain the disparate responses of *O*-GlcNAc enzymes in the two muscles. Our findings are significant and warrant further research to elucidate *O*-GlcNAc protein modification across different muscle types.

Synthesis of UDP-GlcNAc, the substrate for *O*-GlcNAc, is controlled by GFAT, the rate-limiting enzyme of HBP. GFAT1 mRNA expression increased immediately after exercise in the white gastrocnemius, but the change was transient and not significant. However, GFAT2 mRNA levels increased with exercise in both muscles, but returned to baseline after the 4 h recovery. This is an intriguing finding and is consistent with the findings of Young and colleagues [43], who reported increased GFAT2 but not GFAT1 mRNA with increased workload in the cardiac muscle. The GFAT gene expression and enzyme activity have been demonstrated to be upregulated in the hyperglycemic environment, which correlated with increased oxidative stress levels [44]. DEM blunted the exercise-induced increase in GFAT1 mRNA in the white gastrocnemius and reduced the mRNA expression in the soleus immediately after exercise, but these changes were only transient. DEM treatment downregulated GFAT2 expression in the white gastrocnemius, while this was a transient effect in the soleus, where a trend for elevated GFAT2 levels was found in DEM-treated rats 4 h post-exercise. One possible explanation for the fluctuations in GFAT expression is that oxidative stress induced by exercise and DEM stimulated the HBP and promoted UDP-GlcNAc synthesis, ultimately resulting in the feedback inhibition of the pathway via suppression of GFAT. Evaluation of the HBP flux and enzyme activity would need to be performed in order to confirm these predictions. Furthermore, diverse responses of the two GFAT isoforms to DEM and exercise can be partly explained by the differences in their tissue distribution, with GFAT1 being ubiquitous and GFAT2 predominating in the central nervous system [8]. The inhibition of GFAT activity with UDP-GlcNAc has been shown to vary between the two isoforms and phosphorylation at a similar site in the two enzymes attenuated GFAT1 activity, but increased the activity of GFAT2 [9]. Isoform-specific inhibitors and gene deletion would provide further insight into the roles of GFAT isozymes in *O*-GlcNAc modification of skeletal muscle proteins and its response to the conditions used in this study.

There has been a limited investigation into the role of *O*-GlcNAc in the exercising skeletal muscle, with only three published studies to date [45–47]. These studies reported increased UDP-GlcNAc but no effect on GFAT activity after acute swimming exercise in rat skeletal muscle [45] and decreased *O*-GlcNAc protein modification in human skeletal muscle following 60 days of bed rest that was restored by exercise [46]. A recent study found no effect of a yearlong power training on OGT and OGA mRNA expression in skeletal muscle of postmenopausal women [47]. In response to exercise training, however, *O*-GlcNAc and OGT protein decreased in the heart of non-diabetic mice [48] and attenuated *O*-GlcNAcylation was accompanied by an upregulation of OGA expression and activity in the heart of diabetic mice [49]. Another study showed cytosolic *O*-GlcNAc levels in mouse hearts decreased after a 15-min treadmill run, but there were no changes after a 30 min run [50]. It should be noted that cell type, different exercise protocols, and (patho)physiological conditions in these studies contributed to the discrepant findings and have to be considered when comparing the results. Nonetheless, these reports, together with our findings, demonstrate that *O*-GlcNAc protein modification responds to muscle contractile activity and may reflect the oxidative and metabolic changes induced by exercise, warranting further investigation.

The primary focus of our study was to determine *O*-GlcNAc levels on skeletal muscle proteins and the response of this modification to glutathione depletion and exercise. Indeed, the future studies will have to investigate whether the observed augmentation in *O*-GlcNAc protein modification influences the homeostasis of skeletal muscle proteins, muscle contractile activity, and resistance to oxidative damage. Unfortunately, we were not able to perform additional experiments due to the limited muscle tissue availability. Furthermore, it has to be noted the treadmill run the rats were subjected to might have induced a certain level of stress, despite the animals being familiarized with the treadmill and the exercise groups chosen due to the greater willingness to run than their sedentary controls. This protocol appeared to be the most sensible to investigate the effects of acute exercise, but its contribution to the overall stress levels and *O*-GlcNAc modification cannot be excluded. Evaluation of the protein expression of *O*-GlcNAc enzymes other than OGT and measurement of the enzyme activities would provide further insight into the mechanisms of *O*-GlcNAc protein modification in skeletal

muscle. In addition, ROS measures in skeletal muscle and HBP metabolite profiling could deliver valuable information about the oxidative stress and glucose metabolism in this tissue during and after the interventions.

In summary, glutathione depletion and acute exercise increased *O*-GlcNAc protein modification in skeletal muscle. Skeletal muscles, the largest organ grouping in human body, are critically involved in glucose and protein metabolism, have demonstrated an endocrine function and are the major site of ROS production during exercise [51, 52]. Therefore, as increased skeletal muscle mass and improved cellular function lead to beneficial systemic metabolism, the link between *O*-GlcNAc and cellular redox state could be of significant importance for skeletal muscle physiology, particularly in the context of beneficial effects of exercise.

Acknowledgments We thank Gary Wilson for his technical assistance. This work was supported by the School of Human Movement Studies, The University of Queensland (Tina-Tinkara Peternelj, Natalie Strobel, Aya Matsumoto, David Briskey and Jeff Coombes), Institute for Health and Social Science Research, Central Queensland University (Vincent Dalbo and Patrick Tucker), and the College of Pharmacy, Washington State University (Susan Marsh).

Conflict of Interest There have been no conflicts of interest and sources of funding for all authors involved in the preparation of this manuscript.

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