Effect of N-acetylcysteine infusion on exercise-induced modulation of insulin sensitivity and signaling pathways in human skeletal muscle

Adam J. Trevin,1,2 Leonidas S. Lundell,3* Ben D. Perry,1,2* Kim Vikhe Patil,3 Alexander V. Chibalin,4 Itamar Levinger,1,2 Leon R. McQuade,5 and Nigel K. Stepto1,2

1College of Sport and Exercise Science and 2Institute of Sport, Exercise and Active Living, Victoria University, Melbourne, Victoria, Australia; Departments of 3Physiology and Pharmacology and 4Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden; and 5Australian Proteome Analysis Facility, Macquarie University, Sydney, New South Wales, Australia

Submitted 22 December 2014; accepted in final form 16 June 2015

Trevin AJ, Lundell LS, Perry BD, Patil KV, Chibalin AV, Levinger I, McQuade LR, Stepto NK. Effect of N-acetylcysteine infusion on exercise-induced modulation of insulin sensitivity and signaling pathways in human skeletal muscle. Am J Physiol Endocrinol Metab 309: E388–E397, 2015. First published June 23, 2015; doi:10.1152/ajpendo.00605.2014.—Reactive oxygen species (ROS) produced in skeletal muscle may play a role in potentiating the beneficial responses to exercise; however, the effects of exercise-induced ROS on insulin action and protein signaling in humans has not been fully elucidated. Seven healthy, recreationally active participants volunteered for this double-blind, randomized, repeated-measures crossover study. Exercise was undertaken with infusion of saline (CON) or the antioxidant N-acetylcysteine (NAC) to attenuate ROS. Participants performed two 1-h cycling exercise sessions 7–14 days apart, 55 min at 65% V̇O₂peak plus 5 min at 85% V̇O₂peak, followed 3 h later by a 2-h hyperinsulinemic euglycemic clamp, a gold-standard measure of Sport and Exercise Science, Victoria University, P. O. Box 14428, Melbourne, VIC, Australia 8001 (e-mail: Nigel.Stepto@vu.edu.au).

Skeletal muscle is responsible for up to 80% of whole body insulin-stimulated glucose uptake (6). Exercise increases glucose uptake both independently and synergistically with insulin. Contraction-induced glucose uptake is thought to be mediated by AMP-activated protein kinase (AMPk) and occurs during and up to ~1–2 h postexercise. Exercise also increases insulin sensitivity (IS) over extended periods for up to 48 h (31), which is thought to be mediated by increased phosphorylation of two GTPase activating proteins, AS160 (TBC1D4) (9) and to some extent TBC1D1 (17). Both contraction- and exercise-induced potentiation of IS lead to increased GLUT4 translocation to the sarcolemma and increased glucose uptake (22). The precise mechanisms of this process are yet to be fully elucidated, although ROS may be involved via posttranslational modifications to proteins that regulate these pathways (20, 47).

In humans, a seminal study by Ristow et al. (37) showed that antioxidant supplementation in the form of vitamins C and E reduced the exercise training-induced enhancement of glucose infusion rates after both long- (4 wk) and short-term (3 days) exercise training. Although investigations using antioxidant infusion during exercise found no differences in rates of contraction-mediated glucose uptake in humans (28) or during in situ contraction in rats (27), a study by Loh et al. (20) used glutathione peroxidase knockout (Gpx1−/−) mice (which produce elevated basal levels of ROS) to mechanistically link ROS to the potentiation of IS after exercise. Interestingly, IS in the Gpx1−/− mice was not different immediately postexercise but was enhanced 1 hr postexercise compared with WT mice. This finding also coincided with greater Akt Ser173 phosphorylation 1 h postexercise, suggesting that ROS plays a role in the postexercise enhancement of IS via these downstream targets in the insulin-signaling pathway. Despite this, the role of exercise-induced ROS on the postexercise enhancement of IS has not been investigated previously in humans using the hyperinsulinemic euglycemic clamp, a gold-standard measure of IS.

Furthermore, given that the Akt/mTOR pathway can interact (40) and play a dual role in both insulin signaling and other protein synthesis regulation-signaling pathways (23), it is possible that ROS may also be involved in the regulation of the latter (41). Indeed, supplementation with the antioxidant N-acetylcysteine (NAC) hampered phosphorylation of the protein synthesis regulator mTOR, p70S6K protein kinase and its downstream target ribosomal protein S6 after intense eccentric exercise; insulin sensitivity; signaling; reactive oxygen species

EXERCISE INDUCES A CASCADE OF SIGNALING EVENTS WITHIN METABOLIC, INSULIN, CELL STRESS, GROWTH, AND TRANSCRIPTION PATHWAYS IN SKELETAL MUSCLE. IMPORTANTLY, THESE SIGNALING EVENTS OCCUR PRIMARILY VIA PROTEIN PHOSPHORYLATION UNDER THE CONTROL OF KINASE AND PHOSPHATASE ENZYMES, MANY OF WHICH HAVE BEEN SHOWN TO BE SENSITIVE TO REACTIVE OXYGEN SPECIES (ROS) (8). ALTHOUGH CHRONICALLY LEVELS OF ROS (I.E., OXIDATIVE STRESS) HAVE BEEN ASSOCIATED WITH CELLULAR DAMAGE AND VARIOUS DISEASES (13), TRANSIENT AND SPATIOTEMPORAL ROS PRODUCTION IN SKELETAL MUSCLE HAS BEEN SHOWN TO BE AN INTEGRAL PART OF NORMAL SIGNALING CASCADES (34, 42, 57).

* L. S. Lundell and B. D. Perry contributed equally to this study.

Address for reprint requests and other correspondence: N. K. Stepto, College of Sport and Exercise Science, Victoria University, P. O. Box 14428, Melbourne, VIC, Australia 8001 (e-mail: Nigel.Stepto@vu.edu.au).

Copyright © 2015 the American Physiological Society. http://www.ajpendo.org
exercise (30). Together these data suggest that ROS plays a role in postexercise cell stress and protein synthesis responses.

We hypothesized that infusion of an antioxidant during exercise would attenuate insulin-stimulated glucose uptake via canonical signaling pathways in human skeletal muscle. Here, we present data on the in vivo role of exercise-induced ROS in potentiating postexercise IS. We infused the sulphur-containing antioxidant compound NAC in one session and saline placebo in another session of aerobic exercise in young, healthy, and recreationally active individuals, followed 3 h later by a hyperinsulinemic euglycemic clamp to determine IS. We also provide data to further elucidate the in vivo role of ROS on the Akt/mTOR and MAPK signaling pathways.

**MATERIALS AND METHODS**

**Participants**

Seven young, healthy participants (6 males, 1 female) volunteered to participate in this study after giving their written, informed consent (means ± SD; age 22.1 ± 3.2 yr, height 1.8 ± 0.1 m, weight 81.1 ± 14.1 kg, BMI 24.8 ± 3.0 kg/m², VO₂peak 50.6 ± 4.0 ml·kg⁻¹·min⁻¹). All volunteers were prescreened via a health assessment questionnaire. This study was approved by the Victoria University Human Research Ethics Committee and Karolinska Institute Ethics Review Board. The study was deemed to have met the National Health and Medical Research Council guidelines and Helsinki declaration for ethical research in humans.

**Experimental Design**

Participants visited the Victoria University exercise physiology laboratory on three occasions. The initial visit included screening, a graded exercise test to determine VO₂peak, and subsequent exercise workloads followed by familiarization to the experimental exercise protocol. This was followed by two separate experimental trials in which participants arrived at the laboratory between 0700 and 0800 in a overnight-fasted state then during a 1-h exercise bout and received an infusion of NAC in one trial and saline placebo control (CON) during the other trial. This was followed by a 3-h recovery period and then a 2-h hyperinsulinemic euglycemic insulin clamp. Venous blood was sampled, and four muscle biopsies were taken in each session: at baseline (BASE), immediately postexercise (EX), 3 h postexercise recovery/before insulin clamp (REC), and postinsulin clamp (PI), as shown in Fig. 1. The two experimental trials were separated by ≥7 days but no more than 14 days apart, except for the female participant, who performed the trials 28 days apart and during the follicular phase of the menstrual cycle to control for ovulatory fluctuations. The study was conducted using a double-blind, randomized, and repeated-measures crossover design (Fig. 1). Participants and investigators were blinded to the treatment. A laboratory technician oversaw the trial order randomization and was not otherwise involved in the study. By necessity, the medical doctor who performed the NAC or saline-placebo infusion was aware of the treatment during the trials.

**Graded Exercise Test**

All exercise was performed on an electrically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands). Graded exercise tests were conducted to determine VO₂peak using a previously described protocol (2). Briefly, the test started at 100 W (50 W for female) and increased initially by 50 and 25 W thereafter every 2.5 min. VO₂peak was determined at the point of volitional fatigue. Throughout the VO₂peak and during periods in the trials, expired volume was measured using a turbine flowmeter (KL Engineering, Sunnyvale, CA), and mixed expired O₂ and CO₂ contents were analyzed by gas analyzers (S-3A O₂ and CD-3A CO₂; Applied Electrochemistry) interfaced with Turbofit software (Vacumetrics) and calibrated with known gas concentrations (BOC). The turbine flowmeter was calibrated using a 3-liter Hans Rudolph syringe. As a precaution to ensure participant safety, heart rate was measured continuously during and after the trial by 12-lead ECG (Model X-Scribe Stress Test 114 System; Mortara Instrument, Milwaukee, WI).

**Dietary and Exercise Control**

To standardize preexperimental muscle glycogen content, the day before each trial participants were provided with a food parcel (14 MJ, 80% carbohydrates) and instructed to abstain from alcohol, exercise,
and caffeine (15, 52). Any diet inconsistencies occurring prior to the first trial were recorded and replicated for the second trial.

**Exercise Protocol**

Participants performed a 55-min bout of cycling exercise at a workload corresponding to 65% of their \( \text{VO}_{2\text{peak}} \). Following this, the workload was increased to that which corresponded to 85% of their \( \text{VO}_{2\text{peak}} \) for the final 5 min to maximize the physiological demands of the exercise session.

**NAC Infusion and Plasma Content**

NAC (Parvolex; Faulding Pharmaceuticals, Melbourne, Australia) was infused intravenously with an initial loading dose of 62.5 mg·kg\(^{-1}\)·h\(^{-1}\) for the first 15 min, followed by a constant infusion of 25 mg·kg\(^{-1}\)·h\(^{-1}\) for the next 80 min using a syringe pump (Graseby 3400; Graseby Medical). This is a protocol similar to those used in previous human NAC studies (25, 26), and the human pharmacokinetics of NAC at rest and during exercise have been well characterized previously, whereby whole body total NAC clearance was \( \sim 160 \) ml·kg\(^{-1}\)·h\(^{-1}\) during exercise (5). Plasma NAC concentration was later analyzed by reversed-phase ultra-high-performance liquid chromatography, as described previously (50).

**Muscle Biopsy Sampling**

Muscle samples were obtained from the middle third of the vastus lateralis muscle using the percutaneous needle biopsy technique (19). After injection of a local anesthetic into the skin and fascia (1% Xylocaine; Astra Zeneca), a small incision was made and a muscle sample taken (~100–200 mg) using a Bergström biopsy needle with suction. Each biopsy was taken from a separate incision 1–2 cm distal from the previous biopsy. Muscle samples were washed free of blood and dissected of any other tissue and then immediately frozen in liquid nitrogen.

**Hyperinsulinemic Euglycemic Clamp**

IS was determined using a hyperinsulinemic euglycemic insulin clamp, as described previously (16, 19). Briefly, 3 h after the exercise bout, insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was infused (initial bolus 9 mU/kg and then continuously at 40 mU·m\(^{-2}\)·min\(^{-1}\)) for ~120 min, with plasma glucose maintained at ~5 mmol/l, using variable infusion rates of 25% vol/vol glucose. Blood glucose concentration was assessed every 5 min using a glucose analyzer (YSI 2300 STAT Plus Glucose & Lactate Analyzer). Glucose infusion rates were calculated during steady state, defined as the last 30 min of the insulin-stimulated period and expressed as glucose (milligrams) per body surface area (square meter) per minute. IS was expressed as an M-I index, where mean glucose infusion rate (I, mg/kg·h\(^{-1}\)) over the final 30 min of the insulin clamp was divided by the mean plasma insulin concentration (mU/l), as described previously (15). Plasma insulin concentration was determined using a 96-well ELISA insulin kit (Dako) according to the manufacturer’s protocol. Briefly, duplicates of 25-μl plasma samples and standards were combined with a conjugate diluent. A conjugate concentrate mixture was then added to each well, and the plate was shaken for 1 h. Well contents were removed then washed three times using supplied wash buffer. After washing, substrate solution was added and placed in a shaker for 10 min. A stop solution was then added before determining absorbance at 460 nm using a microplate reader (iMark; Bio-Rad, Richmond, CA).

**Western Blot Analysis**

Freeze-dried muscle samples were crushed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM NaF, and 1 mM Na₃VO₄ at pH 7.4), with protease inhibitors; Roche Diagnostics, Mannheim, Germany), rotated for 1 h at 4°C, and centrifuged at 12,000 g for 10 min. Protein concentration of the resulting supernatant was determined using a commercially available BCA kit (Thermo Scientific, Waltham, MA), as described previously (2). Aliquots of the muscle lysates were diluted with Laemmli (18). Sample buffer heated at 56°C for 20 min and 15 μg of total protein/sample were separated by SDS-PAGE in Criterion XT Bis-Tris precast gels (Bio-Rad). Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA). Membranes were blocked in 10 mM Tris, 100 mM NaCl, and 0.02% Tween-20 (TBST) containing 5% nonfat milk for 1 h at room temperature, washed three times in TBST, and incubated with primary antibody overnight at 4°C. After incubation membranes were washed three times with TBST and probed with appropriate horseradish peroxidase-conjugated secondary antibody (Bio-Rad) according to standard procedures. Proteins were visualized by enhanced chemiluminescence detection (Amersham, Arlington, IL), following the manufacturer’s instructions, and then quantified using software (Image Lab version 5.1; Bio Rad). All blots were normalized to their respective total protein loaded to each lane determined via Ponceau staining of the membrane (38).

**Antibodies**

To determine protein expression and phosphorylation, the following primary antibodies were used: acetyl-CoA carboxylase (p-ACC Ser\(^{134}\), no. 3661; Cell Signaling Technology), Akt [total Akt (no. 9272), p-Akt Thr\(^{308}\) (no. 4056), and Akt Ser\(^{473}\) (no. 9271); Cell Signaling Technology], AMPK (p-AMPKα Thr\(^{172}\), no. 2531; Cell Signaling Technology), Akt substrate of 160 kDa [total AS160; Millipore, JBC1361857, phospho-(SerThr) Akt substrate antibody, no. 9611; Cell Signaling Technology], extracellular signal-regulated kinases (p-Erk1/2 Thr\(^{202}\)/Tyr\(^{204}\), no. 4370; Cell Signaling Technology), mechanistic target of rapamycin [total mTOR (no. 5536), Cell Signaling Technology; p-mTOR Ser\(^{2448}\) (no. 600–401–422), Rockland, p38 mitogen-activated protein kinase [total p38 MAPK (no. 9212) and p-p38 MAPK Thr\(^{180}/182\) (no. 9215), Cell Signaling Technology], p53 [total (no. 2524) p-p53 Ser\(^{15}\) (no. 9284) Cell Signaling Technology], p70S6 kinase (p-p70S6K Thr\(^{389}\), no.9234; Cell Signaling Technology), ribosomal protein S6 (p-S6Ser\(^{235/236}\), no. 2211; Cell Signaling Technology), signal transducer and activator of transcription 3 (p-STAT3 Tyr\(^{705}\), no. 9131; Cell Signaling Technology), and eukaryotic translation initiation factor 4E-binding protein 1 [total 4E-BP1 (no. 9452) and p-4E-BP1 Thr\(^{37/46}\) (no. 2855) Cell Signaling Technology].

**Protein Carbonylation Assay**

Protein carbonylation analysis was performed on snap-frozen muscle samples using the OxyBlot Protein Oxidation Detection kit (Millipore, Billerica, MA) as per the manufacturer’s instructions, except that lysis was performed without the addition of β-mercaptoethanol (21). Protein carbonylation was then determined via electrophoresis and immunoblotting as per Western blot analysis.

**Glycogen Content Assay**

Glycogen content assay was performed on freeze-dried muscle samples using a commercially available kit, following the manufacturer’s instructions (ab65620; Abcam, Cambridge, UK). Glycogen content was normalized to dry muscle sample weight.

**Glutathione Content Assay**

Total (tGSH) and oxidized (GSSG) muscle glutathione content was determined spectrophotometrically using a commercially available kit (Bioxytech GSH/GSSG-412; Oxis Health Products, Portland, OR), as described previously (39, 51). Freeze-dried muscle was dissected free of connective tissue, divided into two aliquots, and then powdered and...
was centrifuged at 23,000 g for 15 min at 4°C. The resulting supernatant was diluted 1:25 (tGSH) and 1:20 (GSSG) in assay buffer. Samples, standards, and blanks (50 µl) were added to a 96-well plate in triplicate, followed by 50 µl of chromagen, 50 µl of glutathione reductase, and, just prior to measurement, 50 µl of NADPH. Change in absorbance (reduction of dithiobis-2-nitrobenzoic acid) was measured at 412 nm at 30-s intervals for 4.5 min in a spectrophotometer (xMark; Bio-Rad Laboratories, Hercules, CA). Pellets remaining from the centrifuged homogenate were dissolved in 1 N NaOH, heated at 60°C with agitation, and then assayed for protein content (Bio-Rad Laboratories). Glutathione values were then reported normalized to protein content.

Plasma Interleukin-6 Assay

Plasma interleukin-6 (IL-6) was determined using a commercially available ELISA kit according to the manufacturer’s instructions (no. EZHIL6; Millipore, Bedford, MA). The intra- and interassay coefficients of variation were 3.07 and 2.63, respectively.

Statistical Analysis

Data were analyzed by two-way (treatment × time) analysis of variance with repeated measures on both factors (IBM SPSS Statistics version 20). Where significant main effects were detected, post hoc analyses were conducted with Student’s t-tests for pairwise comparisons and adjusted for multiple comparisons. Single comparisons (IS) were analyzed by using a two-tailed paired Student’s t-test. Statistical significance was accepted at P < 0.05. Raw data are presented as means ± SD for n = 7, and change scores are reported as fold change of the mean or percent difference where appropriate.

RESULTS

Physiological Responses to Exercise

Exercise intensity for CON and NAC trials, was 68 ± 8% and 69 ± 6% V02peak, respectively, for 55 min and 83 ± 9 and 84 ± 5% V02peak, respectively, for the final 5 min. Heart rate for CON and NAC trials was 161 ± 9 and 164 ± 6 beats/min, respectively, for 55 min and then 176 ± 3 and 178 ± 2 beats/min for the final 5 min. Muscle glycogen was similar at BASE and reduced by a similar degree after exercise (EX) in both CON and NAC (fig. 2A).

Effects of NAC Infusion on Muscle Oxidative Stress Markers

Plasma NAC concentration reached 288.2 ± 53.5 µmol/l at the end of exercise/infusion, which was cleared to 12.4 ± 5.1 µmol/l 1 h after cessation of the infusion. NAC was not detected in plasma during the CON trial or before NAC infusion (data not shown).

Protein carbonylation tended to increase by 38% from BASE to EX in CON (P = 0.06) and 31% in NAC (P = 0.057), with a tendency for NAC to attenuate protein carbonylation relative to CON immediately post-EX by −13.5%, (P = 0.08; Fig. 2B).

![Image](http://ajpendo.physiology.org/)

**Fig. 2.** Effect of NAC or CON infusion. Exercise and insulin stimulation on human skeletal muscle glycogen content (A) and muscle total protein carbonylation with representative blot from 1 participant (B), along with reduced (C) and oxidized (D) muscle glutathione content expressed as a ratio (E). Data are means ± SD for n = 7. *Significantly different, NAC vs. CON (P < 0.05), P = 0.07 and P = 0.08 denote trend for NAC vs. CON; †significantly different from BASE (P < 0.05).
Muscle content of both reduced (GSH; Fig. 2C) and oxidized glutathione (GSSG; Fig. 2D) remained stable throughout the experiment in the CON condition. NAC infusion tended to increase GSH concentration after EX (10.94 ± 2.21 vs. 7.67 ± 2.26 μmol/g protein, \( P = 0.07 \)), decrease GSSG at REC (\( P < 0.05 \)), and increase the GSH/GSSG ratio 2.2-fold post-EX (\( P = 0.08 \)) and 1.6-fold with 3-h REC (\( P < 0.05 \)), returning to baseline at PI (Fig. 2E).

Effects of NAC on Insulin Sensitivity

In the final 30 min of the hyperinsulinemic euglycemic clamp, mean glucose infusion rate was 7.29 ± 1.58 and 7.13 ± 1.25 mg·kg\(^{-1}\)·min\(^{-1}\) for CON vs. NAC, respectively (\( P = 0.501 \)). There was a trend for plasma insulin to be elevated with NAC vs. CON (\( P = 0.12 \)), neither exercise nor NAC treatment had a significant influence on the degree of phosphorylation of either Akt site. Akt substrate of 160-kDa phosphorylation, with the PAS (Ser/Thr) antibody, was not significantly increased immediately after exercise but rose at 3-h REC and was further increased PI relative to BASE (\( P < 0.05 \)) in both conditions. Despite this and the decrease in whole body IS with NAC, there was no effect of NAC observed on the degree of AS160 phosphorylation (Fig. 5C).

Protein translation regulation. There was a trend for a time \times treatment interaction with mTOR phosphorylation (\( P = 0.087 \); Fig. 6A). Phosphorylation of p70S6K was increased in response to insulin stimulation, but this was blunted 48.2% with NAC vs. CON (\( P < 0.05 \); Fig. 6B). Phosphorylation of ribosomal protein S6 was increased with exercise and insulin above BASE (\( P < 0.05 \)) without an effect of NAC treatment (Fig. 6C). Exercise dephosphorylated 4E-BP1 Thr\(^{374/46} \) compared with BASE (\( P < 0.05 \); Fig. 6D), which returned to baseline levels at 3-h REC and PI, with no effect from NAC infusion. STAT3 phosphorylation was increased ~30-fold after 3-h REC relative to BASE (\( P < 0.05 \)), which occurred following an ~5.5-fold increase in plasma IL-6 after EX, although neither of these changes was influenced by NAC (Fig. 6E).

DISCUSSION

Antioxidants have been shown to impair adaptations to chronic exercise training in some (10, 37, 46) but not all (14, 51, 56) studies. Since chronic training responses are the result of repeated bouts of acute exercise, it is important to elucidate the effects of ROS after a single bout of exercise. Here, we provide novel in vivo evidence that ROS play a role in the enhancement of postexercise whole body IS, which occurs independently of the hypothesized distal insulin-signaling pathway from Akt to AS160 in human skeletal muscle. In addition, NAC reduced phosphorylation of p70S6K in response to the insulin infusion, suggesting an influence of ROS in aspects of the regulation of protein synthesis.

The present insulin clamp findings are, to the best of our knowledge, the first to examine the effect of NAC on postexercise IS after a single bout of aerobic exercise in humans. The ~6% impairment in the postexercise enhancement of insulin sensitivity is a small yet statistically significant effect determined via the hyperinsulinemic euglycemic clamp, a technique commonly used as a gold-standard measurement of IS. Although we did not measure resting vs. postexercise IS, it has been shown previously that whole body IS was elevated by 32% 18 h after exercise in sheep (24) and to a similar degree 3 h postexercise in an older overweight population (19). Since exercise is an effective means of improving IS for not only healthy individuals but those with insulin resistance (36), it can be suggested that any impairment to this beneficial effect is clinically relevant. Although a one-off, ~6% impairment in
postexercise IS may represent a small effect, there may be further clinical implications if there are cumulative or chronic effects of impaired IS due to antioxidants with repeated bouts of exercise (i.e., training), as demonstrated by Ristow et al. (37).

Enhanced postexercise IS does not appear to be due to altered proximal insulin signaling, since insulin receptor substrate-1 tyrosine phosphorylation and phosphoinositide-3-kinase (PI3K) activity are not increased by exercise (54, 55). Rather, exercise amplifies downstream insulin signaling (9), leading to greater postexercise GLUT4 translocation to the plasma membrane (22). This may be due to the convergence of contraction-mediated and insulin-stimulated signal transduction at two serine-threonine kinase substrates [AS160 (TBC1D4) and TBC1D1], an important step in the signaling cascade leading to GLUT4 translocation. Our findings of increased AS160 phosphorylation 3 h after exercise, along with an additive effect of insulin stimulation (fig. 5C), are consistent with previous findings (9, 15, 43, 48). With regard to ROS mediation of IS, when glutathione peroxidase knockout (Gpx1−/−) mice were treated with NAC, postexercise IS was reverted to that of the WT mice (20). Although the Loh et al. (20) study shows that ROS may mediate postexercise IS via the degree of Akt Ser473 phosphorylation and its kinase activity, in the present study the small reduction in postexercise whole body IS with NAC infusion was not associated with decreases in either of these (Fig. 5, B and C). Intriguingly, there appears to be a tendency for Akt Thr308 to be elevated with NAC vs. CON after the insulin clamp (P = 0.12; Fig. 5A). This increased phosphorylation may potentially occur due to via PI3K, sensing the tendency for elevated plasma insulin concentration with NAC vs. CON, yet no downstream effects are seen with NAC since dual phosphorylation of both the serine and threonine sites are required for kinase activity (1). It may also be speculated that the detection methods (i.e., Western blotting) lacked the sensitivity to measure the potentially very minor accompanying changes in Akt Ser473 and AS160 phosphorylation associated with the small yet physiologically relevant decrease in whole body IS with NAC. Alternatively, the possibility that the distal insulin-signaling pathway is less affected by ROS in humans than in rodents cannot be excluded. Regardless, further research is required to fully understand these interesting in vivo human findings.

The Akt/mTOR pathway integrates input from upstream signals due to nutrient availability and growth factors (including insulin) and has also been shown to be redox sensitive (41). Full activation of Akt is achieved by the dual phosphorylation at positions Thr308 and Ser473 by phosphoinositide-dependent
protein kinase-1 (PDK1) and mTOR, respectively, and this upregulation usually occurs with exercise and insulin stimuli (23, 53). Exercise-induced Akt phosphorylation is not reflected in our data (Fig. 5), although this may be explained by the timing of our biopsy samples immediately post-exercise and at 3 h of recovery, since AktSer327 phosphorylation has been shown to peak at −1 h postexercise and then return to baseline by 3 h (23). Similarly, although increased mTOR phosphorylation has previously been reported after 1 h of endurance exercise at −75% VO2 peak (2, 23, 53), we did not observe mTOR phosphorylation immediately or 3 h after exercise (Fig. 6A). Therefore, it is a limitation of the present study that we were unable to include an additional 1 h postexercise biopsy to compare phosphorylation events with previous findings. The lack of exercise-induced mTOR phosphorylation in the present study may also be due to the greater-intensity final 5 min (85% VO2 peak) of our exercise protocol causing a more potent AMPK-induced inhibition of mTOR phosphorylation (4, 7), and it also precludes the possibility of identifying any redox-sensitive mTOR response to exercise.

Typically, the phosphorylation of mTOR Ser2448 activates downstream p70S6K, which in turn phosphorylates the ribosomal protein S6 and 4E-BP1, leading to the initiation of protein synthesis (3). Intriguingly, in the present study, phosphorylation of p70S6K induced by the insulin clamp was blunted by NAC treatment by −50% (Fig. 6B). This occurred despite the lack of any difference in the degree of phosphorylation of upstream (mTOR) and downstream phosphorylation targets (S6 and 4E-BP1) due to NAC. This interesting finding suggests that the degree of insulin-stimulated p70S6K phosphorylation may occur via discrete signaling pathways such as PDK1/PKC, in response to insulin (35), and p38 MAPK, which has been shown to be sensitive to ROS (11), or that specific phosphorylation sites within the p70S6K protein are altered by ROS-mediated posttranslational modifications. Nonetheless, further investigation is required to fully elucidate the underlying mechanism(s) of this finding.

AMPK is phosphorylated in response to the reduced nutrient availability during exercise, which in turn phosphorylates the downstream fatty acid oxidation regulator ACC, as reflected in our findings (Fig. 4). We did not observe an effect of NAC on the AMPK response to exercise. Sandström et al. (39) reported that NAC blocked 50% of the exercise-induced increase in AMPK phosphorylation. However, their data were derived from an ex vivo experimental condition that may not reflect the in vivo situation. Their findings were inconsistent with those of Merry et al. (29), who showed that NAC affected the increase in glucose uptake during contraction but not the increase in AMPK phosphorylation (28). Therefore, our findings support the notion that AMPK is not redox sensitive in vivo.

Phosphorylation of p38 MAPK Thr180/Tyr182 has been shown to be elevated 2 h after intensive eccentric exercise (30) and 1 h after moderate- to high-intensity aerobic exercise (2), whereas another study reported a only minor change in postexercise p38 phosphorylation and no significant effects of NAC supplementation (33). The latter is in line with the present findings (Fig. 4D), and we speculate that, as per Akt and mTOR phosphorylation, the timing of our muscle biopsies (immediately, and 3 h postexercise) may be too early and too late to observe peak p38 and possibly also p53 phosphorylation. ERK1/2 phosphorylation was greatly increased in response to exercise and insulin as expected (12); however, we did not observe an influence on these responses with NAC, which is in contrast with a previous study (11) that used a selective inhibitor of xanthine oxidase to disturb the in vivo redox balance in rats, which may have alternative effects to that of the NAC infusion of the present study.

Another potential redox sensitive target is STAT3, which is phosphorylated in response to elevated postexercise proinflammatory cytokines such as IL-6 (42, 49), as reflected in the present findings (Fig. 6, E and F). Previously, NAC has been reported to reduce cytokine concentrations after intense eccen-
Nevertheless, with the present exercise protocol, NAC infusion did not influence the level of postexercise plasma IL-6 concentration or the associated STAT3 phosphorylation. A potential limitation of this study is that the NAC infusion was ceased at the end of exercise, which was then rapidly cleared from the plasma. It has been shown that ROS production induces further ROS generation (57), meaning that the effect of NAC on the outcome measures of the present study may be more pronounced if the infusion is maintained throughout the recovery period and possibly also during the insulin clamp. Additionally, despite our focus on skeletal muscle, our whole body IS findings should be interpreted with caution since the liver also plays an important role in glucose metabolism, which can be affected by NAC (32, 44). Finally, although our small sample size may potentially be a limitation, these sample sizes have been used previously in similarly invasive studies, which include several muscle biopsies and hyperinsulinemic euglycemic clamps, across two sessions for each participant (15, 45).

Fig. 6. Effect of exercise and insulin stimulation with NAC or CON infusion on phosphorylation of protein translation regulation proteins mTOR Ser2448 (A), p70S6K Thr389 (B), S6 Ser235/236 (C), eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) Thr37/46 (D), and STAT3 Tyr705 (E). Western blots normalized to Ponceau S stain for each lane, and representative blots from 1 participant are shown. Data are means ± SD for n = 7. *Significantly different, NAC vs. CON (P < 0.05); †significantly different from BASE (P < 0.05).
REFERENCES

- In conclusion, this study revealed novel human in vivo data that attenuation of ROS with antioxidant infusion impaired IS 3 h after exercise, which did not occur not via the Akt-signaling pathway in skeletal muscle. Moreover, phosphorylation of p70S6K, which is involved in protein translation regulation, was shown to be impaired by NAC following insulin stimulation. Further research is required to examine the precise mechanism(s) by which ROS augments postexercise insulin action and protein translation regulation. The elucidation of these mechanisms may reveal potential therapeutic sites to enhance health-promoting responses to exercise, which may have implications for the prevention or improvement of chronic metabolic disorders featuring insulin resistance.

ACKNOWLEDGMENTS

We thank the participants for their generous time and effort; the laboratory staff and students who assisted with conducting exercise trials; Michael McKenna for assistance with study design and data collection; Juleen R. Zierath for providing facilities for biochemical studies; Drs. Larissa Trease, Mitchell Anderson, and Mal Brown for performing medical procedures; and Bernie McInerney from the Australian Proteome Analysis Facility for assisting with the plasma NAC content analysis, which was facilitated using infrastructure provided by the Australian Government through the National Collaborative Research Infrastructure Strategy.

GRANTS

This study was funded by the Victoria University Research Development Grant Scheme, the Swedish Research Council, the Novo Nordisk Foundation, and the Strategic Research Program in Diabetes. I. Levinger is a Foundation Future Leader Fellow (ID No. 100040). N. K. Stepto was supported by the Australian Government Collaborative Research Network Grant Scheme.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


