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

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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% \(\dot{V}O_2\text{peak}\) plus 5 min at 85% \(\dot{V}O_2\text{peak}\), followed 3 h later by a 2-h hyperinsulinemic euglycemic clamp (40 \(mU\text{min}^{-1}\text{m}^{-2}\)) to determine insulin sensitivity. Four muscle biopsies were taken on each trial day, at baseline before NAC infusion (BASE), after exercise (EX), after 3-h recovery (REC), and post-insulin clamp (PI). Exercise, ROS, and insulin action on protein phosphorylation were evaluated with immunoblotting. NAC tended to decrease postexercise markers of the ROS/protein carbonylation ratio by −13.5% \((P = 0.08)\) and increase the GSH/GSSG ratio twofold vs. CON \((P < 0.05)\). Insulin sensitivity was reduced \((−5.9\%, P < 0.05)\) by NAC compared with CON without decreased phosphorylation of Akt or AS160. Whereas p-mTOR was not significantly decreased by NAC after EX or REC, phosphorylation of the downstream protein synthesis target kinase p70S6K was blunted by 48% at PI with NAC compared with CON \((P < 0.05)\). We conclude that NAC infusion attenuated muscle ROS and postexercise insulin sensitivity independent of Akt signaling. ROS also played a role in normal p70S6K phosphorylation in response to insulin stimulation in human skeletal muscle.

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 elevated 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).

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 postranslational 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 Ser\(^{173}\) 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. 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, VO\textsubscript{2peak} 50.6 ± 4.0 ml·kg\textsuperscript{-1}·min\textsuperscript{-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 VO\textsubscript{2peak}, 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 an overnight-fasted state then during a 1-h exercise bout and received an infusion of NAC in one trial and saline placebo control (CON) immediately postexercise (EX), 3 h postexercise recovery (REC), and insulin 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 VO\textsubscript{2peak} 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. VO\textsubscript{2peak} was determined at the point of volitional fatigue. Throughout the VO\textsubscript{2peak} and during periods in the trials, expired volume was measured using a turbine flowmeter (KL Engineering, Sunnyvale, CA), and mixed expired O\textsubscript{2} and CO\textsubscript{2} contents were analyzed by gas analyzers (S-3A O\textsubscript{2} and CD-3A CO\textsubscript{2}; 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 \( V_{\text{O2peak}} \). Following this, the workload was increased to that which corresponded to 85% of their \( V_{\text{O2peak}} \) 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 anaesthetic into the skin and fascia (1% xylocaine; Astra Zeneca), a small incision was made and a muscle sample taken (~100–200 mg) using a Bergstroem 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 (\( I_g \), mg·kg\(^{-1}\)·min\(^{-1}\)) 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 Na,VO\(_4\)) 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\(^{79}\), 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-AMPKαThr\(^{172}\), no. 2531; Cell Signaling Technology), Akt substrate of 160 kDa [total AS160, Millipore, JBC1361857, phospho-(Ser/Thr) Akt substrate antibody, no. 9611; Cell Signaling Technology], extracellular signal-regulated kinases (p-Erk1/2 Thr\(^{202}/204\), 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. 9234) 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 (Milipore, 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 (gSH) and oxidized (gSSG) muscle glutathione content was determined spectrophotometrically using a commercially available kit (Biosystech 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...
weighed. Muscle was then homogenized with 80 μl/mg dry wt ice-cold 5% metaphosphoric acid with and without 1-methyl-2-vinylpyridinium trifluoromethane sulphonate (10% vol/vol). Homogenate 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 ± 4.94 and 2.63 ± 1.97%, 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% VO₂peak, respectively, for 55 min and 83 ± 9 and 84 ± 5% VO₂peak, 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).

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 in the final 30 min of the clamp, 66.8 ± 20.1 vs. 63.3 ± 17.33 mU/l (P = 0.11), and when expressed as an M-I index (15), IS was significantly reduced by −5.9% with NAC compared with CON: 12.46 ± 4.89 vs. 11.74 ± 4.69 (M-I index units, P = 0.041; Fig. 3).

Effects of NAC on Muscle Signaling Responses

Energy balance sensing. AMPK Thr172 phosphorylation increased approximately fourfold in response to EX relative to BASE (P < 0.05; Fig. 4A), but NAC infusion did not influence the degree of the exercise response. These results were confirmed by phosphorylation of ACC Ser79, AMPK’s downstream target, which showed an approximately ninefold increase in BASE to EX (P < 0.05; Fig. 4B) regardless of NAC treatment.

Cellular stress. Erk1/2 Thr202/Tyr204 phosphorylation increased ~18-fold post-EX and returned to near-baseline values at 3-h REC before rising again ~13-fold at PI, without the influence of NAC treatment (Fig. 4C). There was a lack of effect of NAC, exercise, and insulin on phosphorylation of p53 Ser15 (Fig. 4E); however, there was a main effect for time for p38 MAPK Thr180/182 (P < 0.05; Fig. 4D).

Insulin-signaling cascade. Insulin stimulation induced an approximately ninefold increase in phosphorylation of Akt Thr308 and an approximately threefold increase in phosphorylation of Akt Ser473, PI vs. REC (P < 0.05; Fig. 5, A and B). While there was a trend for Akt Thr308 phosphorylation to be increased PI 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, quantified 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 × 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 Thr37/46 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

Fig. 4. Effect of exercise and insulin stimulation with NAC or CON infusion on phosphorylation of AMP-activated protein kinase (AMPK) Thr172 (A) and acetyl-CoA (ACC) Ser79 (B) proteins and cell stress-signaling proteins Erk1/2 Thr202/Tyr204 (C), p38 MAPK Thr180/Tyr182 (D) and p53 Ser15 (E). Western blots normalized to Ponceau S stain for each lane and representative blots are shown from 1 participant. Data are means ± SD for n = 7. †Significantly different from BASE (P < 0.05). AU, arbitrary units.
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 Akt Ser^473 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% VO_2peak (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% VO_2peak) 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 Ser^2448 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 the 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 Thr^180/Tyr^182 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-
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 Ser245/246 (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).
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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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