Thiol-based antioxidant supplementation alters human skeletal muscle signaling and attenuates its inflammatory response and recovery after intense eccentric exercise[1–3]

Yannis Michailidis, Leonidas G Karagounis, Gerasimos Terzis, Athanasios Z Jamurtas, Kontantinos Spengos, Dimitrios Tsoukas, Athanasios Chatzinikolaou, Dimitrios Mandalidis, Renae J Stefanetti, Ioannis Papassotiriou, Spyros Athanasopoulos, John A Hawley, Aaron P Russell, and Ioannis G Fatouros

ABSTRACT

Background: The major thiol-disulfide couple of reduced glutathione (GSH) and oxidized glutathione is a key regulator of major transcriptional pathways regulating aseptic inflammation and recovery of skeletal muscle after aseptic injury. Antioxidant supplementation may hamper exercise-induced cellular adaptations.

Objective: The objective was to examine how thiol-based antioxidant supplementation affects skeletal muscle's performance and redox-sensitive signaling during the inflammatory and repair phases associated with exercise-induced microtrauma.

Design: In a double-blind, crossover design, 10 men received placebo or *N*-acetylcysteine (NAC; 20 mg · kg^{-1} · d^{-1}) after muscle-damaging exercise (300 eccentric contractions). In each trial, muscle performance was measured at baseline, after exercise, 2 h after exercise, and daily for 8 consecutive days. Muscle biopsy samples from vastus lateralis and blood samples were collected before exercise and 2 h, 2 d, and 8 d after exercise.

Results: NAC attenuated the elevation of inflammatory markers of muscle damage (creatine kinase activity, C-reactive protein, proinflammatory cytokines), nuclear factor κB phosphorylation, and the decrease in strength during the first 2 d of recovery. NAC also blunted the increase in phosphorylation of protein kinase B, mammalian target of rapamycin, p70 ribosomal S6 kinase, ribosomal protein S6, and mitogen activated protein kinase p38 at 2 and 8 d after exercise. NAC also abolished the increase in myogenic determination factor and reduced tumor necrosis factor-α 8 d after exercise. Performance was completely recovered only in the placebo group.

Conclusion: Although thiol-based antioxidant supplementation enhances GSH availability in skeletal muscle, it disrupts the skeletal muscle inflammatory response and repair capability, potentially because of a blunted activation of redox-sensitive signaling pathways. This trial was registered at clinicaltrials.gov as NCT01778309. *Am J Clin Nutr* 2013;98:233–45.

INTRODUCTION

Skeletal muscle injury is evident in numerous catabolic states, characterized by marked proteolysis and muscle wasting (eg, cancer cachexia, muscular dystrophy, and sepsis), which results in physical debilitation and an impaired quality of life (1). Injury causes tissue disruption and subcellular damage followed by cytokine release and a rapid and sequential infiltration of leukocyte subpopulations into the muscle (2). This inflammatory phase is followed by a muscle repair/regeneration phase characterized by increased protein synthesis (3). These 2 phases are mechanistically interrelated: proceed in succession and inhibition of the former may hamper the later (4). Strenuous eccentric exercise causes muscle microtrauma characterized by ultrastructural damage, leukocyte infiltration, marked inflammatory response, and oxidative stress but not sepsis (5). These striking similarities between exercise-induced damage and trauma make eccentric exercise a valuable model to investigate the potential intracellular molecular and signaling pathways that contribute to trauma-induced muscle damage and repair in humans.

After injury, infiltrating leukocytes exert antiseptic protection of muscle by releasing reactive oxygen species (ROS)[4] through activation of NADPH oxidase (respiratory burst) (2), which leads to marked perturbations of redox status in myofibers (6). Moreover, cytokines released by neutrophils and injured myofibers activate ROS-generating enzymes (eg, xanthine oxidase and cyclooxygenase-2) (7). Although elevated antioxidants maintain inflammatory response under control, increased ROS may cause secondary damage to injured and noninjured fibers

[1] From the Democritus University of Thrace, Department of Physical Education and Sport Sciences, Komotini, Greece (YM, AC, and IGF); the Nestlé Research Center, Nestec Ltd, Lausanne, Switzerland (LGK); the Athletics Laboratory, School of Physical Education and Sport Science, University of Athens, Athens, Greece (GT, DM, and SA); the Department of Physical Education and Sport Sciences, University of Thessaly, Karies, Trikala, Greece, and the Institute of Human Performance and Rehabilitation, Center for Research and Technology–Thessaly, Trikala, Greece (AZJ); the 1st Department of Neurology, University of Athens, School of Medicine, Eginition Hospital, Athens, Greece (KS); the Department of Toxicology, Medical School, University of Athens, Athens, Greece (DT); the Department of Clinical Biochemistry, "Aghia Sophia" Children's Hospital, Athens, Greece (IP); the Centre for Physical Activity and Nutrition Research, School of Exercise and Nutrition Sciences, Deakin University, Burwood, Victoria, Australia (RJS and APR); and the Exercise Metabolism Research Group, School of Medical Sciences, RMIT University, Bundoora, Australia (JAH and LGK).

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[3] Address correspondence to IG Fatouros, Department of Physical Education and Sports Sciences, University Campus, 69100, Komotini, Greece. E-mail: ifatouro@phyed.duth.gr.

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(2). Reduced glutathione (GSH), a potent antioxidant, is consumed to its oxidized form (GSSG), altering the GSH/GSSG ratio or redox potential of muscles (1, 2). After trauma, this thiol/disulfide couple is a key regulator of important transcriptional pathways controlled by redox-sensitive signaling cascades (8). Thus, immune cell recruitment, adhesion molecule mobilization, antioxidant synthesis, and satellite cell recruitment are regulated by nuclear transcription factor κB (NF-κB), mitogen activated protein kinases (MAPK), and protein kinase B (Akt)/mammalian target of rapamycin (mTOR) mediated pathways (9, 10). Calcium release from damaged sarcoplasmic reticulum activates proteolytic pathways probably in a redox-dependent manner, which further exacerbates muscle dysfunction and inflammation (11). Administration of the powerful thiol-based antioxidant *N*-acetylcysteine (NAC) increases GSH/GSSG and attenuates respiratory burst and MAPK- and NF-κB–mediated proinflammatory cytokine release during inflammation (8, 12, 13). However, it is unclear whether redox changes induced by antioxidant supplementation affect Akt/mTOR signaling. Nevertheless, antioxidant supplementation may be harmful because it disrupts the physiologic adaptation induced by exercise (4, 14, 15).

Although therapies for the treatment of muscle trauma target these aforementioned signaling pathways, there is limited information regarding their redox-dependent regulation in humans. In this investigation we used NAC administration to foster GSH availability during an 8-d period after eccentric exercise-induced muscle damage to test our hypotheses that *1*) NAC supplementation ameliorates skeletal muscle performance by reducing inflammatory processes and exercise-induced muscle injury and *2*) NAC supplementation attenuates intracellular redox-dependent signaling pathways.

SUBJECTS AND METHODS

Subjects

Power analysis determined that a sample size of 10 subjects was required to detect a statistically meaningful treatment effect between consecutive measurements after acute eccentric dynamometry with an α level of 0.90. Accordingly, 10 healthy male volunteers participated in this investigation. Participants were recreationally trained, as evidenced by their maximal oxygen consumption ($\dot{V}O_2$max) level (>45 mL · kg^{-1} · min^{-1}); had been engaged in systematic exercise ≥3 times/wk for ≥12 mo, and were nonsmokers. Participants abstained from any vigorous physical activity and the consumption of caffeine, alcohol, or performance-enhancing or antioxidant supplements and medications before (6 mo) and during the exercise trials. Exclusion criteria included a known NAC intolerance or allergy, a recent febrile illness, and history of muscle lesion and lower limb trauma. Participants signed an informed consent form after they were informed of all risks, discomforts, and benefits involved in the study. Procedures were in accordance with the 1975 Declaration of Helsinki, as revised in 2000, and approval was received from the Institutional Review Board of the University of Thessaly.

Study design

Participants received either NAC or placebo immediately after an acute bout of eccentric exercise on an isokinetic dynamometer (not before or during) and daily for 8 d after exercise in a double-blind, crossover, repeated-measures design (**Figure 1**). A 6-wk washout period was adapted between trials (performed in a random order). Before each trial and during their visit, participants were tested for select anthropometric and performance measures ($\dot{V}O_2$max and muscle performance) as described previously (*see* online supplementary material under "Supplemental data" in the online issue). Briefly, $\dot{V}O_2$max was measured during continuous incremental running to volitional fatigue on a treadmill with a pulmonary gas exchange system (Oxycon Mobile; SensorMedics Corporation). $\dot{V}O_2$peak and strength measurements, as indexes of physical conditioning status, ensured that participants had a similar training level, because physical training upregulates antioxidant status, glutathione concentration, and redox status in skeletal muscle (16, 17). Muscle and blood samples were collected before exercise and 2 h, 48 h, and 8 d after exercise. Muscle performance and soreness were measured before exercise, 2 h after exercise, and daily for 8 d. Testing was performed at the same time of day to prevent circadian variations.

Exercise protocol

Participants performed a protocol consisting of 300 eccentric unilateral repetitions (20 sets, 15 repetitions/set, 30-s rest between sets) with the quadriceps muscle group at a speed of 30°/s on an Isoforce (TUR Gmbh) isokinetic dynamometer as described previously (18). The effectiveness of this protocol to induce myofibrillar disruption was previously documented with electron microscopy and immunohistochemistry (18).

Supplementation

In total, 20 mg NAC/kg per day was administered orally (UniPharma) in 3 daily dosages. NAC was dissolved in a 500-mL drink that contained water (375 mL), a sugar-free cordial (125 mL), and a 2-g low-calorie glucose/dextrose powder to improve palatability. On the basis of pilot trials (with blind tasting of the NAC/placebo solutions), the addition of the cordial and dextrose made the 2 solutions indistinguishable in taste or flavor. The placebo solution was formulated to be identical to the NAC solution, except for the NAC content.

Muscle function

Maximal knee extensor eccentric peak torque at 60°/s on an isokinetic dynamometer (Isoforce) was used as a measure of muscle force-generating capacity, as described previously (18), after a familiarization period (CV for repeated measures was 4.1%). Delayed onset of muscle soreness (DOMS) was determined by palpation of the muscle belly and the distal region of the vastus

[4] Abbreviations used: Akt, protein kinase B; CK, creatine kinase; CRP, C-reactive protein; DOMS, delayed onset of muscle soreness; GSH, reduced glutathione; GSSG, oxidized glutathione; MAPK, mitogen activated protein kinases; mTOR, mammalian target of rapamycin; MyoD, myogenic determination factor; NAC, *N*-acetylcysteine; NF-κB, nuclear factor κB; $p38^{MAPK/Thr180–Tyr182}$, p38 mitogen activated protein kinase, serine/threonine mitogen activated protein kinase; p70S6k, p70 ribosomal S6 kinase; PC, protein carbonyls; ROS, reactive oxygen species; rpS6, ribosomal protein S6; TBARS, thiobarbituric acid–reactive substances; $\dot{V}O_2$max, maximal oxygen consumption.

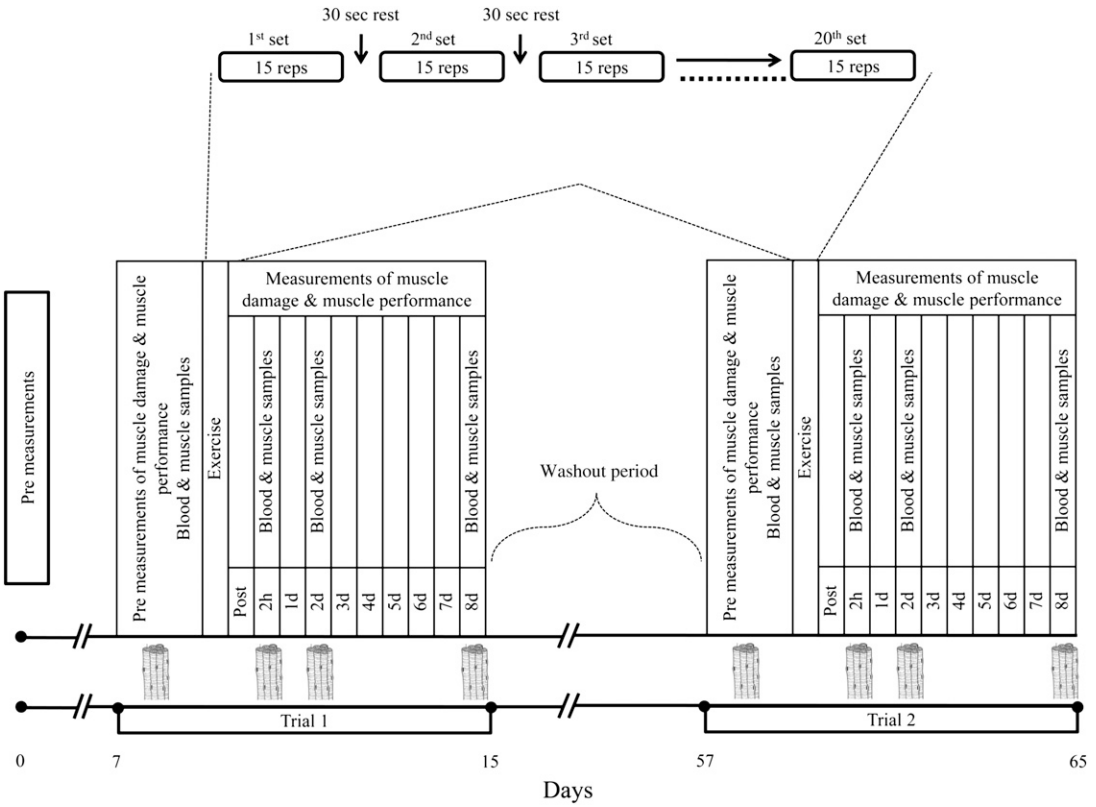

FIGURE 1. The experimental protocol. Post, after exercise; Pre, at baseline.

medialis, vastus lateralis, and rectus femoris after a squat position with the muscles relaxed as described previously (19).

Diet records

In an attempt to standardize diet and antioxidant status, 5-d diet recalls (1 recall/d) were completed before each trial. Dietary intake was monitored with diet recalls daily during the first trial, and participants repeated the same dietary plan during the second trial. Each subject was shown how to complete the diet-recall questionnaires and determine food servings and sizes by a trained qualified dietitian. Diet records were analyzed by using the Science Fit Diet 200A (Science Technologies).

Blood sampling and assays

Subjects reported that they abstained from alcohol and caffeine consumption as well as intense physical activity during the entire experimental period. Blood samples (15 mL) were drawn from the antecubital fossa with a 20-gauge disposable needle equipped with a Vacutainer tube holder (Becton Dickinson) while in a seated position at 0700 after an overnight fast. Serum was separated by centrifugation (1500 × *g*, 4°C, 15 min), placed into separate microcentrifuge Eppendorf tubes in multiple aliquots, and frozen at −75°C for later analyses of cytokines, C-reactive protein (CRP), and creatine kinase (CK) activity. A blood portion was collected into tubes containing EDTA and centrifuged (1370 × *g*, 10 min, 4°C), and the plasma was collected. Another blood portion (2 mL) was immediately mixed with EDTA to prevent clotting for hematologic analysis. A complete blood count was determined by using an automated hematology analyzer (Sysmex K-1000 autoanalyzer; TOA Electronics). CK activity was determined with a Cobas Integra Plus-400 chemistry analyzer (Roche Diagnostics) as described previously (19). CRP was analyzed with a Cobas Integra 800 Clinical Chemistry System (Roche Diagnostics). Multiple analyte profiling (Luminex-100 IS Integrated System; Luminex Corporation) technology was used to measure serum concentrations of the cytokines IL-1β, IL-6, IL-8, and IL-10 (MILLIPLEX Human Cytokine Panel; Millipore Corp). Interassay variabilities for individual cytokines ranged from 1.0% to 9.8%, and intraassay variabilities ranged from 3.6% to 12.6%. Blood samples were stored in multiple aliquots at –80°C and thawed only once before analysis. The inter- and intraassay CVs in all assays performed were 3.5–8.5% and 4.6–12%, respectively.

Muscle biopsy sampling

The initial (baseline) muscle biopsy sample was taken from the middle portion of the vastus lateralis ~25–30 cm from the midpatella at a depth between 4 and 5 cm after administration of the local anesthetic xylocaine (1%) while the subjects were in a supine position as previously described (20). Subsequent biopsy samples (second, third, and fourth) were obtained 3 cm proximal to each previous biopsy site. Two samples at opposite directions (weight: 120–180 mg) were obtained from the same incision site by using the Bergstrom needle technique with the application of manual suction (20). Adipose tissue was removed, and samples were immediately frozen in liquid nitrogen and stored at −80°C for further analysis of protein phosphorylation

and GSH, GSSG, protein carbonyl (PC), and thiobarbituric acid–reactive substance (TBARS) concentrations. For the histochemistry analysis, samples were removed from the needle, aligned, placed in embedding compound, and immediately frozen in isopentane precooled to its freezing point (21). Samples were kept at −80°C until analyzed.

Muscle thiols and oxidative stress markers

Muscle samples were homogenized in phosphate-buffered saline and protein inhibitors as described previously (22). After being homogenized, the samples were vigorously mixed, sonicated, placed on ice, and centrifuged (12,000 × *g*, 4°C, 30 min) and then the supernatant fluid was collected. Muscle GSH, GSSG, PC, and TBARS concentrations were measured spectophotometrically (Hitachi 2001 ultraviolet/VIS; Hitachi Instruments Inc) as described elsewhere (*see* online supplementary material under "Supplemental data" in the online issue) (21, 22). Reagents were purchased from Sigma-Aldrich. Absorbancies were read in duplicate. The inter- and intraassay CVs in all assays performed were 4.2–8.81% and 4.2–7.1%, respectively.

Immunohistochemistry

Transverse cryostat sections (6 μm thick) were labeled by using immunohistochemistry for binding of mouse monoclonal antibody CD68 (1:50, KP1-70761; Santa Cruz Biotechnology). Samples were air-dried and incubated with the primary antibody for 1 h and sequentially with biotinylated anti-mouse Ig for 30 min and with avidin-peroxidase for 30 min (Mouse ExtrAvidin Peroxidase Staining Kit; Sigma-Aldrich), according to the manufacturer's instructions. Peroxidase was localized by using 3-amino-9-ethylcarbazole substrate (Sigma-Aldrich) and prepared according to the manufacturer's instructions. Sections were incubated with the substrate solution for 5 to 10 min or until the red label was sufficiently intense. Sections were then mounted with glycerol gelatin. Tonsil tissue was used in every slide as a positive control. Negative controls were performed by omission of the primary antibody. Identification of the number of macrophages in each sample was performed by 2 investigators who were blind to the sample identity as described previously (23). The mean value of the 2 counts was used in further analyses. Macrophages were expressed as the total number of positive cells divided by the total number of muscle fibers in each sample.

Antibodies

Monoclonal anti-phospho-AKT^{Ser473}, polyclonal anti-phospho-mTOR^{Ser2448}, p70 ribosomal S6 kinase (p70S6k^{Thr389}), p38 mitogen activated protein kinase (p38$^{MAPK/Thr180\text{-}Tyr182}$), NF-$\kappa$B^{Ser536}, and TNF-$\alpha$ were purchased from Cell Signaling Technology Inc. Monoclonal α-tubulin was purchased from Sigma-Aldrich Corp. Myogenic determination factor (MyoD) antibody was purchased from Santa Cruz Biotechnology. Anti-rabbit secondary antibody and enhanced chemiluminescence reagents were from Amersham Biosciences and Pierce Biotechnology.

Measurement of intracellular signaling related proteins

Samples were homogenized in ice-cold buffer (50 mmol/L Tris-HCL, pH 7.5, 1 mmol EDTA/L, 1 mmol EGTA/L, 10% glycerol, 1% triton-X, 50 mmol NaF/L, 5 mmol $Na_4P_2O_7$/L, 1 mmol dithiothreitol/L, 10 μg trypsin inhibitor/mL, 2 μg aprotinin/mL, 1 mmol benzamidine/L, and 1 mmol phenylmethylsulfonyl fluoride/L). The lysate was centrifuged (12,000 × *g*, 20 min, 4°C). Proteins were separated by using SDS-PAGE electrophoresis and subsequently transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with 5% nonfat milk/tris-buffered saline for 90 min. All primary antibodies were incubated overnight at 4°C. Membranes were washed with TBS-T and incubated with secondary antibody for 60 min. Membranes were washed before being exposed, visualized by chemiluminescence, and quantified by densitometry (Bio-Rad Chemidoc). All densitometry values were then expressed relative to a corresponding α-tubulin control from the equivalent sample lysate. Protein concentrations were measured by using a BCA protein measurement kit (Pierce).

Statistical analysis

Changes in dependent variables were examined with a 2-factor, repeated-measures ANOVA [trial (placebo and NAC) × time (before exercise, 2 h after exercise, 48 h after exercise, and 8 d after exercise)]. When a significant interaction was detected, post hoc analysis was performed through the Bonferonni test. Baseline differences in anthropometric characteristics and physical conditioning status ($\dot{V}O_2$max) before each trial were determined with a 1-factor, repeated-measures (on time) ANOVA. Data are expressed as means ± SDs. Significance was set at α = 0.05. The analyses were performed with SPSS software (15.0; SPSS Inc).

RESULTS

Participants' characteristics (**Table 1**), dietary profiles (**Table 2**), and baseline values of all dependent variables were similar between trials (*see* Figures 3–8). Muscle measures during exercise were comparable between trials (**Figure 2**). These results confirm that the exercise protocols were performed under the same conditions in both trials and that the 6-wk period between trials effectively washed out any systemic inflammatory responses developed by the first trial. Participants reported no adverse side effects attributed to NAC consumption.

TABLE 1
Participants' physical characteristics before each trial[1]

	Placebo trial	NAC trial
Age (y)	23.5 ± 2.5	23.5 ± 2.5
Body mass (kg)	76.3 ± 9.0	76.0 ± 9.7
Height (m)	1.77 ± 0.1	1.77 ± 0.1
BMI (kg/m^2)	24.4 ± 2.2	24.3 ± 2.2
Body fat (%)	14.3 ± 3.5	14.2 ± 3.2
Lean mass (kg)	65.2 ± 7.0	65.1 ± 7.3
$\dot{V}O_2$max (mL · kg^{-1} · min^{-1})	47.7 ± 4.6	47.5 ± 4.4

[1] All values are means ± SDs. The participants had comparable anthropometric characteristics and physical-conditioning values at baseline (n = 10 per trial). One-factor (time) repeated-measures ANOVA was used. NAC, *N*-acetylcysteine; $\dot{V}O_2$max, maximal oxygen consumption.

TABLE 2
Dietary antioxidant profiles and caloric intakes during the course of the 2 trials[1]

	Placebo trial	NAC trial
Energy (kcal)	2693.4 ± 121.8	2587.1 ± 145.2
Carbohydrate (% of total energy)	55.8 ± 3.5	55.5 ± 4.1
Fat (% of total energy)	27.6 ± 3.1	28.2 ± 3.2
Protein (% of total energy)	16.6 ± 1.9	16.3 ± 1.3
Selenium (μg/d)	44.6 ± 4.2	41.9 ± 3.7
Zinc (mg/d)	12.1 ± 1.3	12.6 ± 0.9
Vitamin C (mg/d)	124.2 ± 9.5	129.6 ± 11.8
Vitamin E (mg/d)[2]	8.6 ± 0.8	8.9 ± 0.7

[1] All values are means ± SDs. The participants had comparable dietary intakes before and during the 2 trials (n = 10 per trial). One-factor (time) repeated-measures ANOVA was used. NAC, *N*-acetylcysteine.
[2] In α-tocopherol equivalents.

Redox status and oxidative stress changes in muscle

PC and TBARS (**Figure 3**, A and B, respectively) increased with placebo at 2 h, peaked at 48 h, and subsided at 8 d without reaching baseline values (TBARS: P = 0.001–0.004; placebo: P = 0.001), but increased increased only at 2 h (P = 0.001) and 48 h ($P < 0.001$) with NAC. PC and TBARS were lower (PC: P = 0.001–0.008; TBARS: P = 0.001–0.002) with NAC than with placebo at 2 h, 48 h, and 8 d. GSH (Figure 3C) decreased (P = 0.001) with placebo at 2 h, reached its lowest value at 48 h, and remained reduced at 8 d, whereas with NAC it declined (P = 0.001) at 2 h, reached its lowest value at 48 h, and recovered thereafter. NAC attenuated (P = 0.001–0.009) the decline in GSH throughout recovery. GSSG increased (P = 0.001–0.035) at 2 h and peaked at 48 h in both trials and remained elevated at 8 d only with placebo. NAC administration resulted in a lower (P = 0.001–0.017) GSSG concentration (Figure 3D) than did placebo at 2 and 48 h. GSH/GSSG (Figure 3E) declined (P = 0.001) at 2 h and reached its lowest value (P = 0.001) at 48 h in both trials, but remained elevated at 8 d only with placebo (P = 0.001). NAC maintained a higher (P = 0.001–0.014) GSH/GSSG ratio than did placebo throughout recovery.

Muscle function

With placebo, mean torque decreased (**Figure 4**A; P = 0.001) at 2 and 24 h, reached its lowest value at 48 h, and then progressively recovered, reaching baseline values at 8 d. With NAC, mean torque declined (Figure 4A, P = 0.001) at 2 and 24 h, reached its lowest value at 48 h, and then progressively increased until 8 d without normalizing to baseline values. NAC ingestion attenuated (P = 0.02–0.016) the loss in muscle function at 24 and 48 h, whereas placebo showed a greater (P = 0.012) performance output compared with NAC at 8 d. DOMS (Figure 4B) increased at 2 and 24 h, peaked at 48 h, remained elevated until 5 d (placebo: P = 0.002–0.039; NAC: P = 0.003–0.013), and subsided thereafter in both trials.

Inflammatory changes

CK (**Figure 5**A) increased (placebo: P = 0.01–0.048; NAC: P = 0.001–0.046) with exercise in both trials at 2 h, peaked at 48 h, and remained above baseline at 8 d. NAC resulted in a lower CK elevation than did placebo at 2 and 48 h (2 h: P = 0.001; 48 h: P = 0.007). CRP (Figure 5B) increased (P = 0.001) at 2 h, peaked at 48 h, and normalized thereafter in both trials. NAC administration resulted in a smaller (P = 0.041) increase in CRP than did placebo at 48 h. Macrophage infiltration in muscle (Figure 5C) increased (placebo: P = 0.004–0.011; NAC: P = 0.001–0.003) progressively in both trials in a similar manner throughout recovery. Although NAC resulted in a 30% lower macrophage infiltration at 48 h, this difference was not statistically significant. Neutrophils (Figure 5D) peaked at 2 h and remained elevated at 48 h (placebo: P = 0.001; NAC: P = 0.001) in both trials, with NAC resulting in lower (P = 0.013) values than placebo at 2 h.

Cytokine IL-1β (**Figure 6**A) increased at 2 h (placebo: P = 0.001; NAC: P = 0.08) and 48 h (placebo: P = 0.017; NAC: P = 0.046) but had returned to baseline levels by 8 d of recovery in both trials. However, IL-1β was lower (2 h: P = 0.001; 48 h: P = 0.009) with NAC than with placebo both at 2 and 48 h. IL-6 (Figure 6B) increased (P = 0.001) at 2 h, peaked at 48 h, and remained elevated at 8 d in both trials. This IL-6 elevation was lower (P = 0.001–0.005) with NAC than with placebo throughout recovery. The cytokine IL-8 (Figure 6C) decreased at 2 h (placebo: P = 0.014; NAC: P = 0.029) and 48 h (placebo: P =

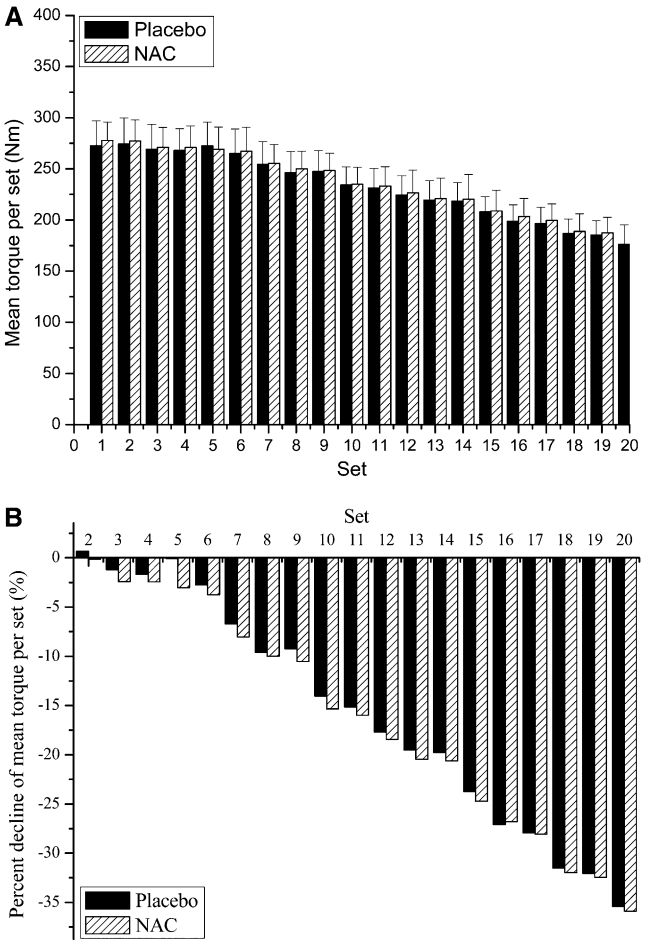

FIGURE 2. Mean (±SD) muscle performance (A) and its average decline (B) during the 2 exercise trials (20 sets; n = 10 per trial). A 2-factor (supplement × time) ANOVA with repeated measures on time and post hoc pairwise comparisons through the Bonferonni test showed no significant differences. NAC, *N*-acetylcysteine.

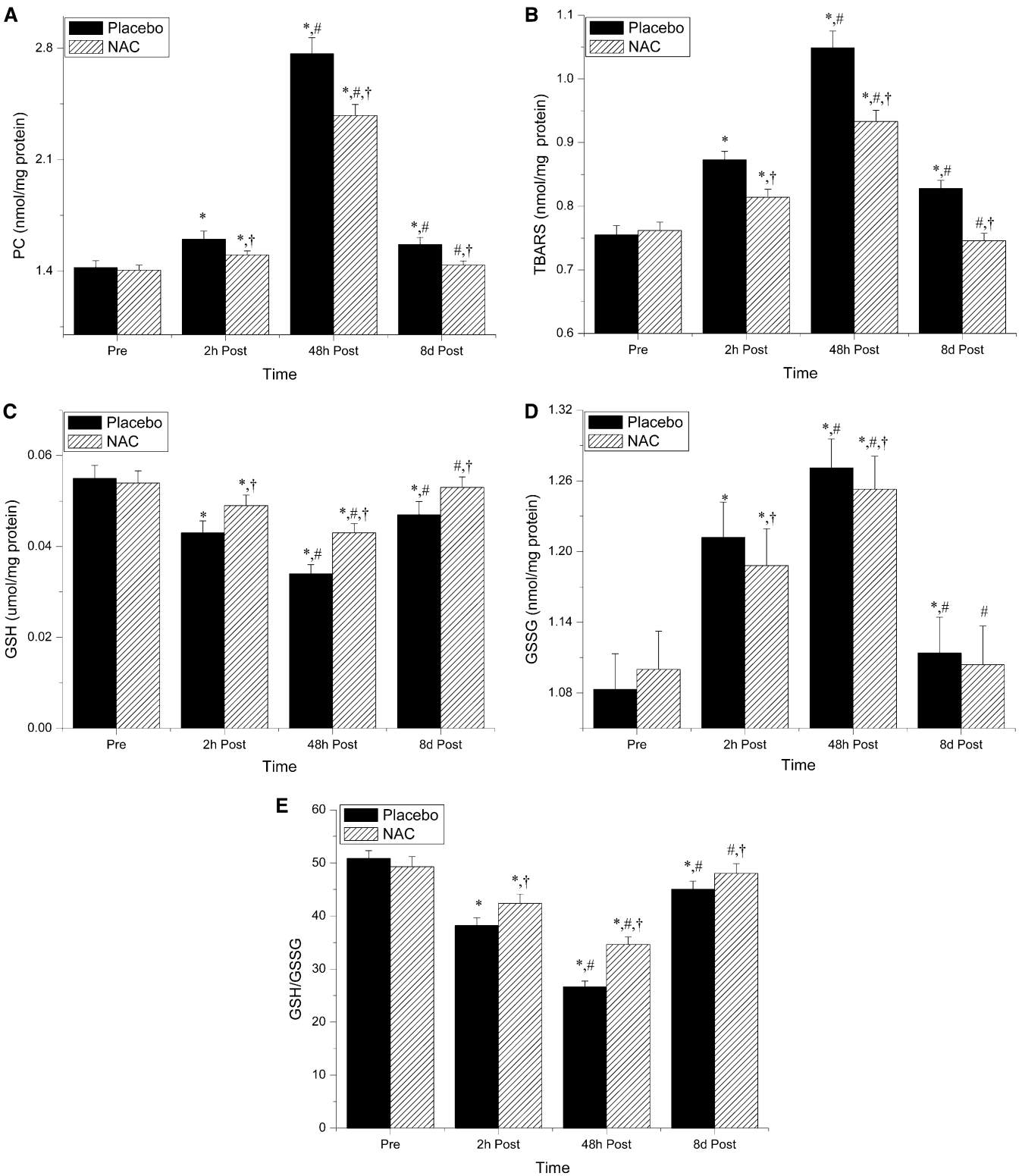

FIGURE 3. Mean (±SD) changes in muscle oxidative stress markers (A and B) and redox status (C, D, and E) at baseline and during postexercise recovery in the 2 trials (n = 10 per trial). Two-factor (supplement × time) ANOVA with repeated measures on time and post hoc pairwise comparisons through the Bonferonni test were used. P-interaction was significant. *Significantly different from baseline, $P < 0.05$. #Significantly different from the previous time point, $P < 0.05$. †Significant difference between trials, $P < 0.05$. GSH, reduced glutathione; GSSG, oxidized glutathione; NAC, N-acetylcysteine; PC, protein carbonyls; Post, after exercise; Pre, at baseline; TBARS, thiobarbituric acid–reactive substances.

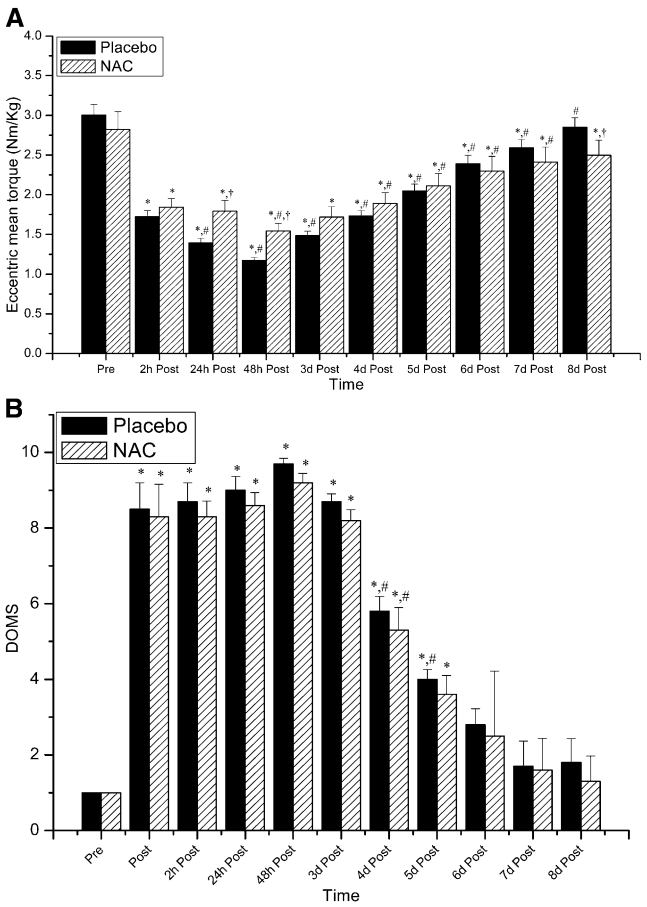

FIGURE 4. Mean (±SD) changes in muscle function (A) and muscle soreness (B) during the 2 trials (n = 10 per trial). Two-factor (supplement × time) ANOVA with repeated measures on time and post hoc pairwise comparisons through the Bonferonni test were used. P-interaction was significant. *Significantly different from baseline, $P < 0.05$. #Significantly different from the previous time point, $P < 0.05$. †Significant difference between trials, $P < 0.05$. DOMS, delayed onset of muscle soreness; NAC, *N*-acetylcysteine; Post, after exercise; Pre, at baseline.

0.039; NAC: $P = 0.004$) of recovery in both trials and returned to baseline at 8 d. NAC elicited a greater reduction in IL-8 than did placebo at 48 h ($P = 0.015$) and on 8 d ($P = 0.04$). Cytokine IL-10 (Figure 6D) increased at 2 and 48 h and peaked at 8 d in both trials (placebo: $P = 0.001$–0.002; NAC: $P = 0.001$–0.007), with NAC inducing a greater ($P = 0.003$) increase in IL-10 at 8 d.

Intracellular signaling proteins

Akt phosphorylation (**Figure 7**A) increased in both trials at 48 h ($P = 0.035$–0.046) and 8 d ($P = 0.012$–0.022). Akt phosphorylation tended to be greater ($P = 0.083$) with placebo than with NAC at 8 d. mTOR phosphorylation (Figure 7B) increased in placebo at 2 h ($P = 0.01$), 48 h ($P = 0.035$), and 8 d ($P = 0.088$) after exercise, whereas with NAC it remained unaltered during the first 48 h but lower ($P = 0.001$) than baseline at 8 d. p70S6K Phosphorylation (Figure 7C) increased with placebo at 2 h ($P = 0.001$), 48 h ($P = 0.002$), and 8 d ($P = 0.072$), whereas with NAC it increased only at 2 h ($P = 0.002$). Ribosomal protein S6 (rpS6) phosphorylation (Figure 7D) increased with placebo 16-fold at 2 h ($P = 0.001$), 6-fold at 48 h ($P = 0.015$), and 3-fold at 8 d ($P = 0.033$), whereas with NAC it increased only at 2 h (14-fold; $P = 0.001$). Placebo induced greater rpS6 phosphorylation than did NAC at 48 h ($P = 0.018$) and 8 d ($P = 0.048$). MyoD protein expression (Figure 7E) increased with placebo only at 8 d ($P = 0.046$), whereas with NAC it decreased at 8 d ($P = 0.022$). Phosphorylated $p38^{MAPK}$ (**Figure 8**A) increased at 2 h (placebo: $P = 0.001$; NAC: $P = 0.016$) and returned to basal levels at 48 h in both trials. However, at 8 d, $p38^{MAPK}$ increased again with placebo ($P = 0.087$) and decreased with NAC (by 37% when compared with baseline, although not statistically significant: $P = 0.147$), with the former maintaining higher ($P = 0.008$) levels of phosphorylated $p38^{MAPK}$ than the latter. Phosphorylated NF-κB (Figure 8B) increased only with placebo at 48 h ($P = 0.03$). NAC resulted in lower ($P = 0.027$) phosphorylated levels of NF-κB than did placebo at 48 h. Although TNF-α (Figure 8C) remained unaltered in both trials, NAC resulted in lower levels than did placebo at 8 d ($P = 0.006$).

DISCUSSION

Inflammatory responses

In this study, eccentric exercise induced severe muscle damage and inflammatory response (days 1–3) as evidenced by the pronounced elevation of DOMS, CK, CRP, proinflammatory cytokines, oxidative stress markers, and leukocytosis and a substantial decrease in muscle function. As in other conditions of increased ROS production (17, 24), NAC increased muscle GSH throughout recovery. Because NAC ingestion leaves GSH regeneration from GSSG unaffected (24), enhanced GSH availability should be attributed to increased cysteine availability (24) because of its enhanced active transport from extracellular compartments via the γ-glutamyl cycle (16, 25). Moreover, NAC ingestion elevates the NAC content in muscle (24), where it produces free cysteine by deacetylation (25). Because cysteine is a potent ROS scavenger (24), NAC may also contribute to intramuscular ROS scavenging, resulting in GSH sparing (26), preventing GSH depletion and thus altering muscle's redox status after exercise. As a substrate for the glutathione peroxidase reaction that controls H_2O_2 removal (25), GSH may further contribute to the attenuation of the GSH/GSSG decrease and the blunted rise of oxidative stress markers throughout recovery. NAC consumption accelerates the normalization of redox status after exercise (27).

Exercise-induced muscle damage induces early (2–24 h) neutrophil and late (24–48 h) machrophage mobilization and infiltration in traumatized muscle (1, 2, 7) in response to the action of proinflammatory cytokines (ie, IL-1β, TNF-α, IL-6, and IL-8) (1, 2, 10, 28). NAC mitigated this response, as evidenced by the dampened neutrophil count and $CD68^+$ macrophage infiltration in muscle, which suggests a redox-dependent regulation of their recruitment (12, 13). NAC-mediated redox status perturbations were also accompanied by the simultaneous attenuation of an increase in IL-1β, IL-6, and IL-8, which coincides with reports of an NAC-induced inhibition of IL-1β, IL-6, and IL-8 at the protein and mRNA level (10). This blunted cytokine response may be related to the attenuation of NF-κB/MAPK signaling observed here. NF-κB phosphorylation is essential for the production and release of proinflammatory cytokines, either directly by muscle and/or leukocytes (1, 2) or

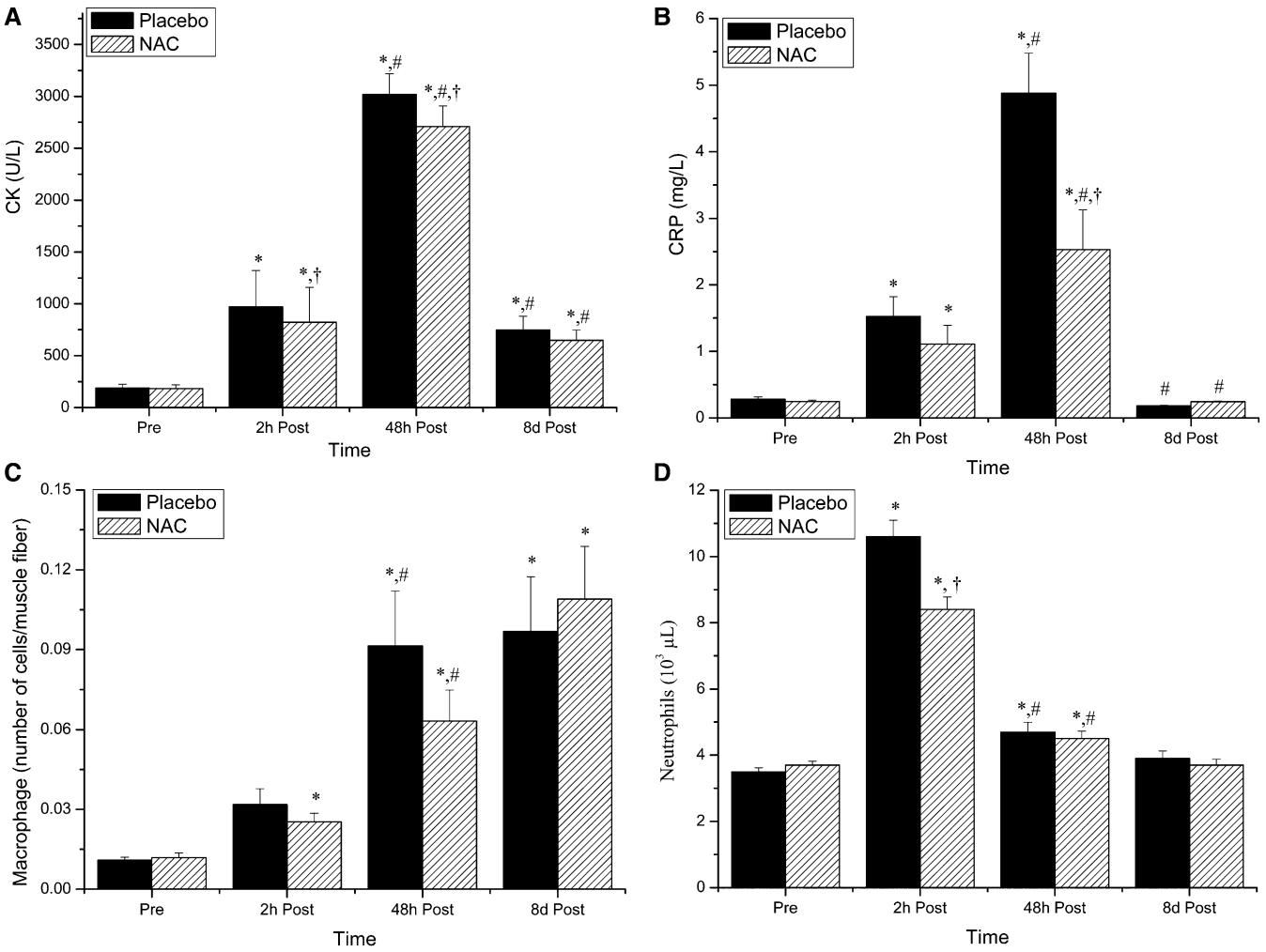

FIGURE 5. Mean (±SD) changes in inflammatory markers (A: CK; B: CRP; C: muscle's macrophage infiltration; D: neutrophils) during the 2 trials (n = 10 per trial). Two-factor (supplement × time) ANOVA with repeated measures on time and post hoc pairwise comparisons through the Bonferonni test were used. P-interaction was significant. *Significantly different from baseline, $P < 0.05$. #Significantly different from the previous time point, $P < 0.05$. †Significant difference between trials, $P < 0.05$. CK, creatine kinase; CRP, C-reactive protein; NAC, *N*-acetylcysteine; Post, after exercise; Pre, at baseline.

indirectly through IL-10 upregulation (28). IL-1β and IL-8 production is NF-κB–dependent, whereas IL-6 induction requires $p38^{MAPK}$ activation in a redox-dependent manner in vitro (28, 29). The attenuated $p38^{MAPK}$ and NF-κB phosphorylation at 48 h suggests that this concept may also be valid for humans after aseptic injury. A redox-sensitive mitigation in the $p38^{MAPK}$-mediated rise in IL-6 and NF-κB–dependent elevation in IL-1β has been reported in animals after muscle activation (8, 28). Disruption of exercise-induced NF-κB signaling has also been induced by other antioxidants (14).

Neutrophil depletion before eccentric contractions reduces muscle damage, which indicates their implication in muscle's secondary damage (30). In this study, NAC induced an attenuated leukocyte infiltration, and phagocytotic action in injured muscle, the result of a blunted cytokine response, might have led to reduced secondary damage to muscle because of lower ROS release by NADPH oxidase, as evidenced by the lower CK and CRP values (2). This may explain the attenuated loss of muscle function in NAC 1–3 d during recovery, as shown previously in humans and animals after damaging exercise (25, 31, 32). In contrast, GSH deficiency impairs muscle performance (33). Alternatively, NAC might have prevented the decline in the Na^+/K^+ pump activity in muscle (34). This can occur as a result of a calcium-induced increase in proteolytic enzyme activity because of sarcoplasmic reticulum damage (2). This occurs in a redox-dependent manner (35), because administration of NAC or other antioxidants can protect muscle by downregulating ubiquitin-proteosome and calpain activity (11, 36).

Regeneration phase responses

After trauma, regeneration of injured myofibers depends heavily on an attenuation of proinflammatory signaling; the ability of satellite cells to proliferate, differentiate, and fuse with the injured fibers; and muscle's potential to synthesize protein via activation of the Akt/mTOR pathway by various anabolic agents (3, 37–40). Akt promotes MyoD-mediated satellite cell differentiation and survival after injury by stimulating myokine production (41). Satellite cell proliferation via the Akt/MyoD pathway may be redox-dependent because ROS produced by NADPH oxidase upregulate this pathway (39). This concept may be also true for humans because the NAC-induced

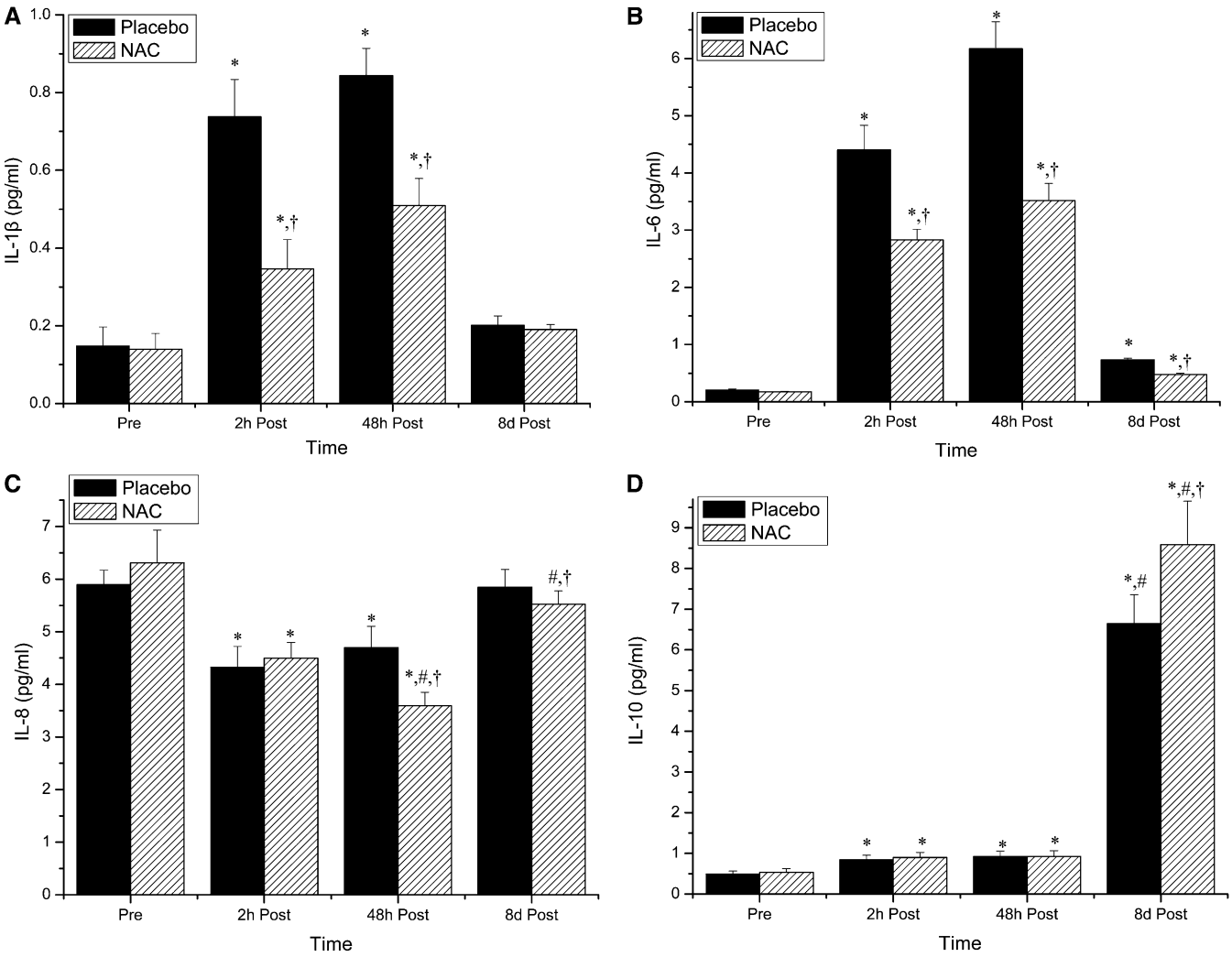

FIGURE 6. Mean (±SD) cytokine responses (A: cytokine 1β; B: cytokine 6; C: cytokine 8; D: cytokine 10) during the 2 trials (n = 10 per trial). Two-factor (supplement × time) ANOVA with repeated measures on time and post hoc pairwise comparisons through the Bonferonni test were used. P-interaction was significant. *Significantly different from baseline, $P < 0.05$. #Significantly different from the previous time point, $P < 0.05$. †Significant difference between trials, $P < 0.05$. NAC, *N*-acetylcysteine; Post, after exercise; Pre, at baseline.

reduction of Akt/mTOR phosphorylation paralleled a block in the increase in MyoD. In the NAC supplementation trial, the attenuation of $p38^{MAPK}$ phosphorylation—a pathway critical for myoblast fusion to myotubes, MyoD-mediated myogenesis, and protein synthesis (1, 42)—after 8 d of recovery provides additional support for a redox-dependent regulation of this pathway; this effect was also seen in animals (40). MyoD reduction may be also related to IL-6 suppression induced by NAC (1, 41). IL-6 may be essential for myoblast proliferation and muscle growth (1, 41), whereas IL-6 mutation impairs myogenesis (43). Although TNF-α promotes myoblast proliferation and satellite cell differentiation, its absence reduces MyoD expression and impairs muscle performance in vitro (1, 44), and its expression in muscle remained unaltered in both trials throughout recovery. However, its reduction with NAC at 8 d compared with placebo may have contributed to the slower rate of muscle strength recovery. Activation of Akt/mTOR signaling leads to the downstream phosphorylation of p70S6k and rpS6, which promotes protein translation and muscle growth (38). NAC decreased or abolished the exercise-induced increase in phosphorylation of all proteins involved in this pathway during late recovery, which provides novel evidence of a redox-dependent regulation of skeletal muscle protein synthesis after injury. ROS were shown to facilitate insulin-like growth factor I synthesis in smooth muscle (45) and growth in cardiac muscle (46)—observations that further explain the attenuation of strength recovery in NAC.

Recent evidence from knockout mice animals and dystrophic models indicates an NF-κB–mediated inhibition of muscle's regeneration (47) after TNF-α stimulation, probably by destabilizing MyoD protein posttranscriptionally (48). However, in line with others, NF-κB phosphorylation increased early rather than late in both trials during recovery (49), whereas TNF-α remained unaffected despite the upregulation of MyoD. Although, the role of NF-κB in regulation of inflammation response in humans is well documented (1, 2, 7), its contribution to muscle's healing is still obscure. NF-κB may be involved early rather than late during skeletal muscle recovery after trauma. Moreover, NF-κB may be operational in interstitium mediating satellite cell activation and/or vascular remodeling rather than in the myocyte itself (49). This may explain the lack

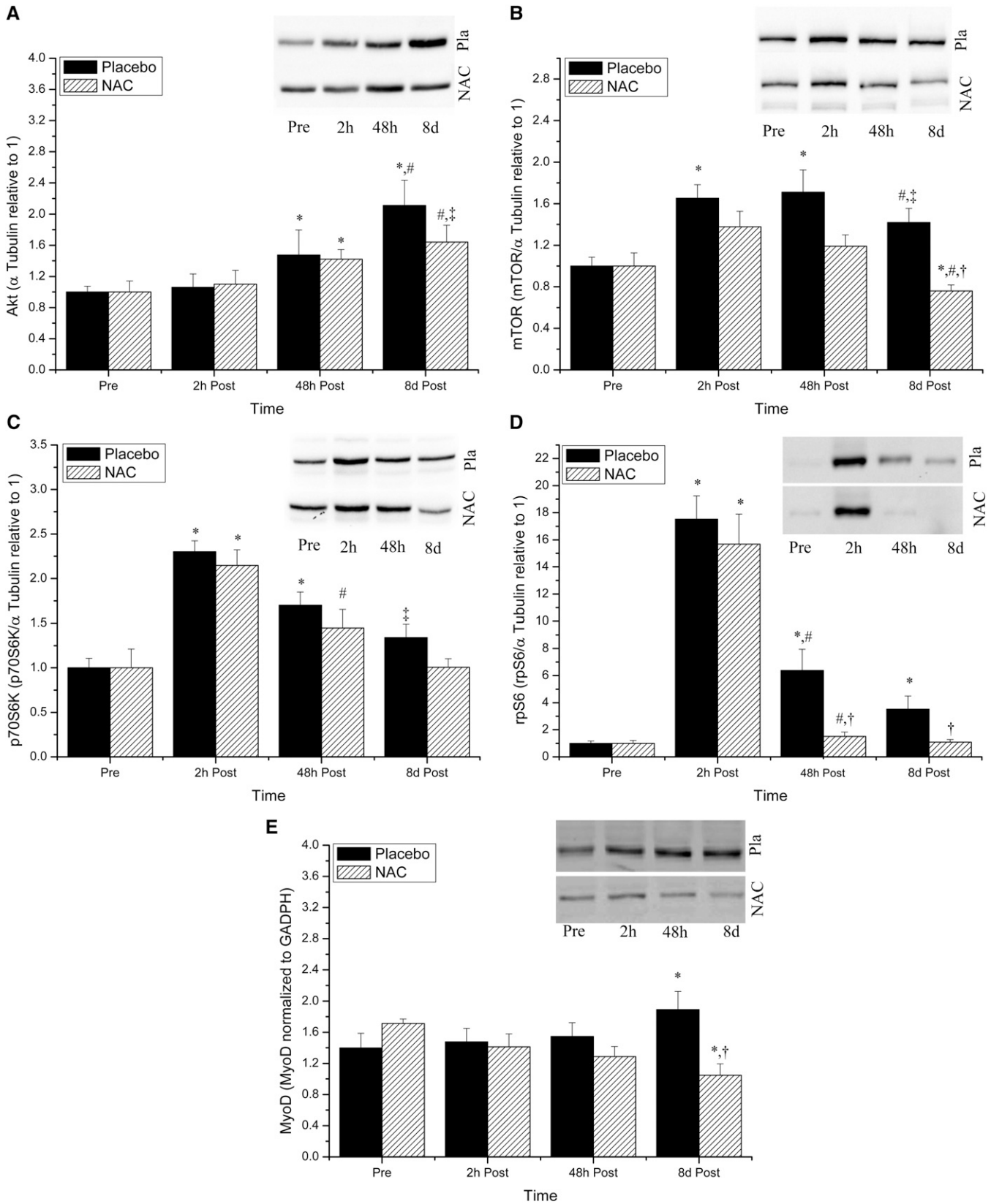

FIGURE 7. Mean (±SD) changes in protein levels of phosphorylated Akt (A), phosphorylated mTOR (B), phosphorylated p70S6K (C), ribosomal protein S6 (D), and MyoD (E) during the 2 trials (n = 10 per trial). Two-factor (supplement × time) ANOVA with repeated measures on time and post hoc pairwise comparisons through the Bonferonni test were used. P-interaction was significant. *Significantly different from baseline, $P < 0.05$. #Significantly different from the previous time point, $P < 0.05$. †,‡Significant difference between trials: †$P < 0.05$, ‡$P < 0.1$. Akt, protein kinase B; mTOR, mammalian target of rapamycin; MyoD, myogenic determination factor; NAC, *N*-acetylcysteine; Pla, placebo; Post, after exercise; Pre, at baseline; p70S6k, p70 ribosomal S6 kinase; rpS6, ribosomal protein 6.

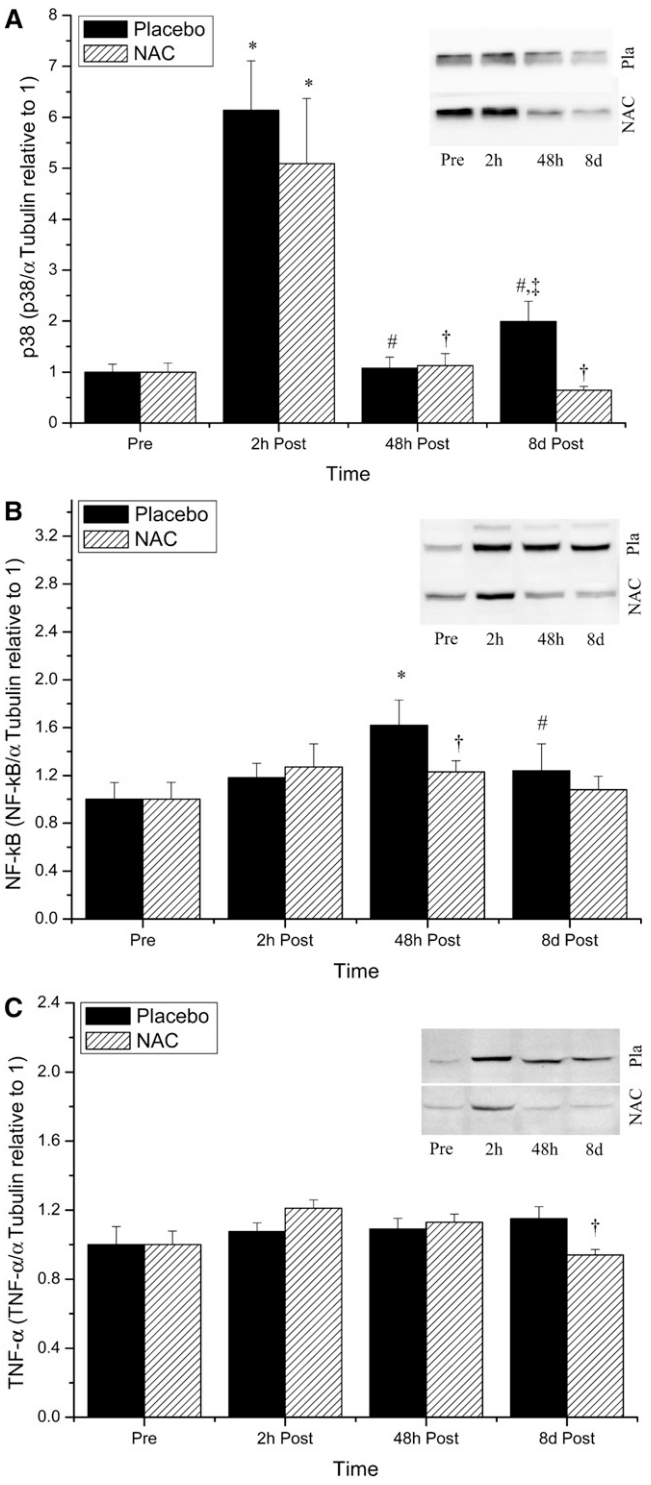

FIGURE 8. Mean (±SD) changes in protein levels of phosphorylated $p38^{MAPK}$ (A), phosphorylated NF-κB (B), and TNF-α (C) during the 2 trials (n = 10 per trial). Two-factor (supplement × time) ANOVA with repeated measures on time and post hoc pairwise comparisons through the Bonferonni test were used. P-interaction was significant. *Significantly different from baseline, $P < 0.05$. #Significantly different from the previous time point, $P < 0.05$. †,‡Significant difference between trials: †$P < 0.05$, ‡$P < 0.1$. NAC, N-acetylcysteine; NF-κB, nuclear factor κB; Pla, placebo; Post, after exercise; Pre, at baseline; $p38^{MAPK}$, serine/threonine mitogen activated protein kinase.

of change in NF-κB in muscle on 8 d. Alternatively, muscle's myogenic potential may override NF-κB activation because interferon-related developmental regulator 1—a MyoD co-activator—deactivates NF-κB by deacetylation, driving its export from nucleus, and increases MyoD expression after muscle damage (48).

Interestingly, NAC supplementation hampered late strength recovery. Use of drugs interrupting the acute inflammatory response after muscle injury attenuates its repair 3–8 d later (3). Similarly, prolonged antioxidant supplementation attenuated performance improvement after endurance training in humans (50) and performance recovery after damaging exercise in animals (15). Tidball and Villalta (1) proposed that transitions of macrophage M1 (proinflammatory phenotype) to M2 (antiinflammatory) are crucial for muscle regeneration via satellite activation after injury. Adequate removal of cellular debris by neutrophils or macrophages may be critical for complete recovery of injured muscle, whereas molecules released by these cells (cytokines, prostaglandins, and chemokines) may regulate muscle repair and growth (1). In fact, NAC treatment resulted in a 30% drop in neutrophil count and macrophage infiltration 48 h after exercise. Whereas not statistically significant, it is of physiologically interest. The NAC-enhanced GSH availability may have induced redox perturbations, downregulating pathways mediating immune cell mobilization. This may result in incomplete clearing of debris, which may be a prerequisite for full recovery of muscle performance. This study has shown that, although redox status alterations attenuate oxidative damage and inflammation and enhance muscle performance shortly after aseptic muscle damage, it may delay its long-term recovery by interfering with intracellular signaling pathways. Therefore, our results corroborate those of previous studies suggesting that heavy use of antioxidants may have an adverse effect on muscle performance and recovery, probably by altering signaling pathways mediating muscle inflammation and recovery and potentially mitochondrial biogenesis and subsequent energy metabolism, as previously suggested (51, 52). Graphic representation of this theoretical model is provided elsewhere (*see* Supplementary Figure 1 under "Supplementary data" in the online issue).

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The authors' responsibilities were as follows—YM, AC, and DM: performed the biochemical determinations, physiological measurements, and training intervention and co-wrote the manuscript; IGF, AZJ, and LGK: conceived of and designed the study and co-wrote the manuscript; DM, SA, and DT: set up the isokinetic measurements, performed the measurements, and co-wrote the manuscript; KS and GT: performed the muscle biopsies and the immunohistochemistry analysis and co-wrote the manuscript; IP: performed the statistical analysis and co-wrote the manuscript; and RJS, JAH, and APR: performed the Western blot analysis and co-wrote the manuscript. The sponsors of the study had no role in the design, implementation, analysis, or interpretation of the data. None of the authors declared a conflict of interest.

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