Exercise-induced immunosuppression: roles of reactive oxygen species and 5′-AMP-activated protein kinase dephosphorylation within immune cells

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Moir H, Hughes MG, Potter S, Sims C, Butcher LR, Davies NA, Verheggen K, Jones KP, Thomas AW, Webb R. Exercise-induced immunosuppression: roles of reactive oxygen species and 5′-AMP-activated protein kinase dephosphorylation within immune cells. J Appl Physiol 108: 1284–1292, 2010. First published February 18, 2010; doi:10.1152/japplphysiol.00737.2009.—We previously proposed 5′-AMP-activated protein kinase (AMPK) dephosphorylation within immune cells as an intracellular mechanism linking exercise and immunosuppression. In this study, AMPK phosphorylation underwent transient (<1 h) decreases (53.8 ± 7.2% basal) immediately after exercise (45 min of cycling at 70% VO2max) in a cohort of 16 adult male participants. Similar effects were seen with running. However, because exercise-induced inactivation of AMPK was previously shown to occur in an AMP-independent manner, the means by which AMPK is inactivated in this context is not yet clear. To investigate the hypothesis that exercise-induced inactivation of AMPK is mediated via signaling mechanisms distinct from changes in cellular AMP-to-ATP ratios, reactive oxygen species (ROS) and intracellular Ca2+ signaling were investigated in mononuclear cells before and after exercise and in cultured monocytic MM6 cells. In vitro studies, treatment with an antioxidant (ascorbic acid, 4 h, 50 μM) decreased MM6 cell intracellular ROS levels (88.0 ± 5.2% basal) and induced dephosphorylation of AMPK (44.7 ± 17.6% basal). By analogy, the fact that exercise decreased mononuclear cell ROS content (32.8 ± 16.6% basal), possibly due to downregulation (43.4 ± 8.0% basal) of mRNA for NOX2, the catalytic subunit of the cytoplasmic ROS-generating enzyme NADPH oxidase, may provide an explanation for the AMPK-dephosphorylating effect of exercise. In contrast, exercise-induced Ca2+ signaling events did not seem to be coupled to changes in AMPK activity. Thus we propose that the exercise-induced decreases in both intracellular ROS and AMPK phosphorylation seen in this study constitute evidence supporting a role for ROS in controlling AMPK, and hence immune function, in the context of exercise-induced immunosuppression.

5′-adenosine monophosphate-activated protein kinase; mononuclear cells

THE LOSS OF GLOBAL IMMUNE FUNCTION induced by prolonged, intense exercise has been linked to decreases in the ability of isolated immune cells to respond to a challenge following strenuous exercise (3). However, there has as yet been little research into the intracellular biochemical mechanisms underpinning this effect. We previously demonstrated that suppression of global immune function immediately after a single bout of intense exercise [45 min of cycling at an intensity equivalent to 70% of the maximal rate of oxygen consumption (70% VO2max)] coincides with dephosphorylation of the 5′-AMP-activated protein kinase (AMPK) within immune cells (23), and we therefore suggested that immune cell energy depletion may be responsible for exercise-induced immunosuppression.

AMPK, the “metabolic master switch,” is a ubiquitous signaling agent activated in cells by increases in the ratio of cellular AMP to ATP (14). Classically, AMPK is allosterically activated by AMP to become a substrate for constitutively active upstream kinases such as LKB-1 (38), but it also can be activated in an AMP-independent manner, by oxidative stress (35), or by signaling agents such as calmodulin-dependent protein kinase kinase β (CaMKKβ) (16), which may themselves be activated by extracellular stimuli (33). Once activated, AMPK phosphorylates substrate proteins, stimulating biochemical reactions that generate ATP within the cell, while switching off ATP-consuming processes that are not essential for short-term cell survival. In our previous study (23), we confirmed that AMPK is expressed in immune cells: specifically, we demonstrated that the AMPKα1 catalytic isoform is expressed in both cultured monocytic MM6 cells (40) and in mononuclear cells, the agranulocyte leukocytic fraction that comprises monocytes and lymphocytes.

These findings were in line with those of Marsin et al. (21), who have proposed an important role for AMPK within monocytes. Because the hypoxia seen in sites of inflammation curtails the use of aerobic respiration, Marsin et al. suggested that, after extravasation and recruitment to an inflammatory site, immune cells respond by activating anaerobic respiration via AMPK-mediated phosphorylation of the glycolytic enzyme inducible 6-phosphofructo-2-kinase (21). According to this view, AMPK seems to be vital, particularly during inflammatory episodes, for the ability of mononuclear cells to carry out important functions such as phagocytosis, antigen presentation and cytokine secretion (monocytes), and antigen recognition and production of immunoglobulin (lymphocytes), all of which require correct maintenance of cellular energy status.

In our previous report (23), we demonstrated that exercise-induced AMPKα1 dephosphorylation occurred in an AMPK-independent manner in mononuclear cells (and also in purified monocyte samples). Therefore, we stated that “elucidation of the source of the AMPK inactivation observed in the present study is a current aim of our research group” (23). In the present study, we present data tentatively identifying reactive oxygen species (ROS)-mediated inactivation of AMPKα1 within immune cells such as monocytes and lymphocytes as an intracellular mechanism that may potentially underpin exercise-induced immunosuppression.

The immune response to exercise is known to be elevated when the activity induces muscle damage (27), which activates...
immune cells to release inflammatory signaling molecules and thus initiate immune responses. Whereas our previous work on the exercise-induced changes in mononuclear AMPKn1 phosphorylation status (23) only used cycling, where minimal muscle damage would be anticipated (4), the present study also compared the influence of exercise mode (running vs. cycling) to investigate whether the extent to which the AMPKα1 response occurs is consistent with the amount of tissue damage associated with the mode of exercise being performed.

MATERIALS AND METHODS

Materials. Reagents were obtained from Sigma-Aldrich (Poole, UK) unless stated otherwise.

Participant recruitment and exercise procedures. A cohort of 16 healthy active male individuals (n = 16: age, 23.1 ± 0.5 yr; height, 175.4 ± 1.6 cm; body mass, 75.4 ± 2.7 kg) were recruited to participate in an exercise study. The entire cohort fasted overnight before undertaking cycling exercise; a subset of eight individuals from the same cohort (age, 21.1 ± 1.2 yr; height, 182.6 ± 6.5 cm; body mass, 85.6 ± 10.0 kg) also undertook running. In all cases, standardization of food intake and physical activity before undertaking exercise was achieved via use of health/activity questionnaires. Ethical approval was granted by the School of Health Sciences Ethical Committee at the University of Wales Institute, Cardiff, UK, with all participant understanding and signing an approved informed consent form, health/activity questionnaire, and blood collection form; all information was confidential. Exercise procedures were performed on a Monark 824E cycle ergometer (Monark Exercise, Varberg, Sweden) or a Woodway Desmo treadmill (Woodway, Waukesha, WI). Participants first performed an incremental cycling test to exhaustion. Each stage of the test lasted 3 min, and the required power output increased by 30 W at every stage until volitional exhaustion. Once VO2max had been established (56.3 ± 1.1 ml·kg−1·min−1),2 the power output corresponding to 70% VO2max was calculated (190.4 ± 10.4 W); this intensity was used in subsequent sustained (45 min) exercise bouts. Because of the higher VO2max that would be expected during running compared with cycling (20), we attempted to match the demands of the different exercise modes by making minor adjustments to the treadmill speed so that the heart rate responses were matched to the results obtained during the cycling trials. These adjustments were made based on the mean heart rate results from consecutive 30-s intervals. In all cases, heart rate was monitored throughout with the use of a Polar S410 HR monitor (Polar Electro, Oy, Finland). VO2 was monitored using a Jaeger Oxycon Delta system (Erich Jaeger, Höchberg, Germany) every 10 min, and capillary blood samples were taken every 15 min using finger capillary samples for determination of blood lactate concentration with an Analox GM7 Microstat analyzer (Analox Instruments, London, UK). The physiological stresses involved, indicated by mean heart rate, blood lactate concentration, and VO2 resulting from the bouts of exercise, were not significantly different, indicating that exercise was performed at a comparable intensity in both cases.

Isolation of human peripheral mononuclear cells and serum samples from whole blood. Blood samples from the antecubital vein were obtained via phlebotomy from each subject at baseline (“pre”), following exercise (immediately postexercise; “post”), and 1 h after exercise (“1 h”). For isolation of peripheral blood mononuclear cells, 10 ml of heparinized blood was diluted 1:1 in RPMI, layered over 10 ml of Histopaque-1077 Ficoll-Hypaque, and centrifuged at 400 G for 20 min. The mononuclear cell suspension was carefully removed from the Ficoll-Hypaque interface and washed four times (500 G, 10 min) in 0.4 ml of Active Motif phosphate inhibitor solution and 7.6 ml of phosphate-buffered saline (PBS). Samples were disqualified if cell viability was <95% as measured by trypan blue staining. An additional 5 ml of blood was also collected into EDTA-treated tubes at each time point and centrifuged at 1,500 G for 5 min. Serum samples were aspirated off as supernatants above the resulting pellet, aliquoted, and stored at −80°C for subsequent analysis.

Salivary IgA measurements. Saliva was collected from each subject at each of the above time points by placing a cotton salivette under the tongue for 2 min. Extraction of liquid saliva was carried out by centrifugation at 3,000 rpm for 5 min and storage at −80°C. The samples were analyzed via enzyme-linked immunosorbant assay (ELISA) using anti-IgA capture antibody and horseradish peroxidase (HRP)-conjugated secondary antibody (Bethyl Labs, Montgomery, TX). As recommended (1), salivary IgA (sIgA) levels (ng/ml) were normalized for protein concentration on the assumption that total protein concentration in saliva does not change with exercise.

Maintenance of cells in culture. Cells from the monocytic MM6 cell line (40), obtained from ATCC (Teddington, UK), were allowed to grow and propagate under controlled conditions (37°C in a humidified 5% CO2 atmosphere) in Roswell Park Memorial Institute cell culture medium 1640 (RPMI) supplemented with 1% (vol/vol) penicillin-streptomycin, 10% (vol/vol) fetal calf serum, 1% (vol/vol) nonessential amino acids, 1% glutamine, and 1% (vol/vol) sodium pyruvate. Cells were seeded at ~0.3 × 106 cells/ml and passed at ~1 × 106 cells/ml after a growth period of 3–4 days [MM6 doubling time in suspension is 50 h (40)]. Experiments were routinely performed with cells at passage ≤25 and at viability >95% as measured by trypan blue exclusion. MM6 samples (5 × 106 cells) were incubated with the macrolide antibiotic oligomycin (a mitochondrial ATP synthase inhibitor; 1 μM), the pharmacological AMPK activator 5-aminooimidazole-4-carboxamid riboside (AICAR; a precursor of the AMP analog ZMP; 1.5 mM), or the antioxidant ascorbic acid (50 μM) at 37°C for the indicated times.

Western blot analysis. AMPK Western blot experiments were carried out as described previously (23). Briefly, total protein extracts from mononuclear or MM6 cells were prepared by treatment with 100 μl of protein extraction/lysis buffer, containing 1 mM Protease inhibitor cocktail and 1 mg/ml calyculin A phosphatase inhibitor (Active Motif, Rixensart, Belgium). Protein content was then estimated using a Bio-Rad protein assay (Bio-Rad Laboratories, Basingstoke, UK). Samples (50 μg of protein) were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-AMPKn1 or anti-phosphoAMPK primary antibodies (16 h, 1:1,000 dilution; Cell Signaling Technology, Danvers, MA), followed by HRP-labeled anti-rabbit IgG antibody (2 h, 1:2,000 dilution; Cell Signaling Technology). Activation of AMPK was expressed as the ratio of phosphorylated to total AMPKn1, detected as 63-kDa immunogenic bands on Western blots using antibodies directed against phospho-AMPK (threonine residue 172) and total AMPKn1, respectively. For detection of protein phosphatase 2C (PP2C) or CaMKKβ, samples were probed with anti-PP2C or anti-CaMKKβ primary antibodies (16 h, 1:1,000 dilution; BD Biosciences, San Jose, CA), followed by HRP-labeled anti-rabbit IgG antibody (2 h, 1:2,000 dilution; Cell Signaling Technology). PP2C and CaMKKβ were detected as phosphorylated [48 kDa (pPP2C) or 64 kDa (pCaMKKβ)] and nonphosphorylated [42 kDa (PP2C) or 56 kDa (CaMKKβ)] immunogenic bands on Western blots.

Measurements of serum levels of reduced glutathione and E-selectin. In a subset of the original cycling cohort (n = 8), E-selectin and reduced glutathione (GSH) levels were measured in serum samples (collected at each of the above time points) via commercially available ELISA methodologies using anti-E-selectin capture antibodies and HRP-conjugated secondary antibodies (R&D Systems, Oxon, UK) and anti-GSH capture antibodies and HRP-conjugated secondary antibodies [Calbiochem Biosciences (Merck, Nottingham, UK), respectively.

Flow cytometric measurements of MM6 or mononuclear cell [ROS]cyt and [Ca2+],cyt. MM6 cells, or samples from a subset of the original cycling cohort (n = 8), were subjected to flow cytometric analysis. Leukocytes [extracted from whole blood via incubation for 10 min with OptiLyse C lysis solution (Beckman Coulter, High.
Wycombe, UK) to lyse red blood cells] or MM6 cells were centrifuged (300 G, 5 min), washed, and resuspended in PBS at a concentration of 10^5 cells/ml.

For quantitation of intracellular Ca^2+ concentration ([Ca^2+]_{cyto}), 100-μl mononuclear cell aliquots were incubated in fluo-3 (Molecular Probes, Eugene, OR; 3 μM, 15 min, 4°C) for 10 min on ice. After dye uptake, cells were washed in PBS, centrifuged (300 G, 3 min) and resuspended in 500 μl of PBS for analysis using a Beckman FACS FC500 flow cytometer (Becton Dickinson, Mountain View, CA).

Control samples for elevated [Ca^2+]_{cyto} (F_{max}) and minimal Ca^2+ (F_{min}) were prepared by preincubation with thapsigargin (50 mM) and EGTA (10 mM) for 10 min, respectively. By using the Gryniewicz equation and K_d values for fluo-3’s affinity for Ca^2+ (11, 22), the fluorescence intensities of samples [excitation wavelength (λ_ex) = 495 nm; emission wavelength (λ_em) = 524 nm] were used to estimate absolute [Ca^2+]_{cyto} values.

Quantitation of intracellular ROS concentration ([ROS]_{cyto}) was achieved by following the method of Hirpara et al. (15) with minor modifications. Cell aliquots (100 μl) of mononuclear cells taken pre- and postexercise or MM6 cells treated with ascorbic acid (50 μM, 4 h) were loaded with dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes; 2.5 μM, 10 min, 4°C), and the resulting fluorescence (λ_ex = 495 nm; λ_em = 524 nm) was analyzed using a Beckman FACS FC500 flow cytometer (Becton Dickinson). [ROS]_{cyto} values were estimated for samples taken pre- and postexercise and for positive control samples for elevated levels of [ROS]_{cyto} [obtained by incubation with phorbol 12-myristate 13-acetate (PMA; 25 ng/ml, 10 min)].

In both cases, separate electronic gates were set on the dot plot of the forward and side scatter to differentiate between different subpopulations of leukocytes, and thus to specifically quantify [ROS]_{cyto} and [Ca^2+]_{cyto} within mononuclear cells (monocytes and lymphocytes). To ensure consistency, the photomultiplier value of the detector in the different channels was set at 450 V throughout all experiments.

Isolation of RNA and RT-PCR assays. In a subset of the original cycling cohort (n=8), samples were subjected to RT-PCR analysis. The extraction of total RNA was carried out using a RiboPure-Blood kit (Ambion, Huntingdon, UK) according to the manufacturer’s instructions. Isolation of RNA and RT-PCR assays (assay IDs: Hs00166163_m1 and Hs00156308_m1, respectively) was semi-quantitatively using commercially available gene expression assays (whose expression is under the control of AMPK) [whose expression is under the control of AMPK (12)].

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Upon return of the samples to the laboratory, RNA was reverse transcribed to cDNA using the Quantitect reverse transcriptase kit (Qiagen) according to the manufacturer’s instructions. The expression of target genes was quantified by quantitative real-time PCR using the LightCycler (Roche) or a 7500 real-time PCR system. Gene expression of peroxisome proliferator-activated receptor-γ (PPARγ) was analyzed using the LightCycler (Roche) or a 7500 real-time PCR system. Gene expression of peroxisome proliferator-activated receptor-γ (PPARγ) was analyzed using the LightCycler (Roche) or a 7500 real-time PCR system.

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monocyte \([\text{Ca}^{2+}]_{\text{cyto}}\) using flow cytometry. As shown in Fig. 3A, exercise led to increased \([\text{Ca}^{2+}]_{\text{cyto}}\) (pre, 60 ± 27 nM; post, 278 ± 10 nM; \(P < 0.05\)). However, although Western blot analysis showed that CaMKKβ is expressed in mononuclear cells (as 2 distinct bands at 64 and 56 kDa, representing pCaMKKβ and nonphosphorylated CaMKKβ, respectively), no difference in the phosphorylation status of this enzyme could be detected as a result of exercise [i.e., similar pCaMKKβ/CaMKKβ ratios were observed in both pre- (0.74 ± 0.09) and postexercise (0.75 ± 0.06) samples; \(P > 0.05\); Fig. 3B and C].

To investigate the report by Toyoda et al. (35) that AMPK activation is regulated by ROS, we treated cultured MM6 monocytic cells with the antioxidant ascorbic acid (50 μM, 4 h). This brought about a borderline significant decrease in \([\text{ROS}]_{\text{cyto}}\) (88.0 ± 5.2% basal; ANOVA (\(P < 0.10\); Fig. 4A), as detected by flow cytometric analysis of \(2\)-[7-(diethylamino)phenyl]-1,3,8-naphthalocyanine (DPA)-loaded MM6 cells. In contrast, the AMP:ATP-dependent AMPK activator oligomycin and AICAR had no significant effect on \([\text{ROS}]_{\text{cyto}}\) (data not shown). The effect of antioxidant treatment on pAMPKα1 levels was then measured using Western blotting; a significant transient decrease in phosphorylation was seen that was maximal (44.7 ± 17.6% basal) after 4 h [ANOVA with Bonferroni’s post hoc analysis (\(P < 0.05\) where indicated); Fig. 4B]. Similarly, as shown in Fig. 5A, a marked decrease in basal \([\text{ROS}]_{\text{cyto}}\) was observed in \(2\)-[7-(diethylamino)phenyl]-1,3,8-naphthalocyanine-loaded mononuclear cells postexercise (pre, 49.7 ± 7.9 μM; post, 16.3 ± 8.9 μM, or 32.8 ± 16.6% basal; paired \(t\)-test (\(P < 0.05\)). Neither exercise nor antioxidant treatment brought about changes in phosphorylation or protein expression of the upstream AMPK phosphatase PP2C (19) in mononuclear cells or MM6 cells [data not shown; ANOVA (\(P > 0.05\)).

The expression of genes linked to cytoplasmic ROS production was assessed via RT-PCR. Expression of the catalytic NOX2 (also known as gp91phox) subunit of the cytoplasmic ROS-generating enzyme NADPH oxidase decreased following exercise (43.4 ± 8.0% of preexercise value; \(P < 0.05\); Fig. 5B); this is in accordance with Adams et al. (1), who reported exercise-induced decreases in NOX2 in endothelial cells. The influence of exercise on \([\text{ROS}]_{\text{cyto}}\) was mirrored by the diminished increase in \([\text{ROS}]_{\text{cyto}}\) seen in response to treatment with PMA in cells taken from participants after exercise (pre+PMA, 111.5 ± 19.3 μM; post+PMA, 74.4 ± 6.4 μM; paired \(t\)-test (\(P < 0.05\)), indicating that the cells’ potential capacity to produce ROS, even when maximally stimulated, was diminished as a result of exercise. In contrast, exercise did not induce changes in mononuclear cell mRNA expression for the antioxidant defense gene catalase at any of the time points measured [data not shown; ANOVA (\(P > 0.05\)).

Finally, to distinguish between ROS that have been generated at different sites, we used ELISA to measure serum levels...
of reduced glutathione ([GSH]_{serum}), which reduces serum ROS to harmless forms while it itself is converted to oxidized glutathione. As shown in Fig. 6, exercise induced a decrease in [GSH]_{serum} [pre, 73.5 ± 8.0 μM; post, 52.4 ± 10.0 μM, or 68.1 ± 7.7% basal; paired t-test (P < 0.05)], suggesting that exercise triggers increases in serum levels of ROS, a pattern of ROS generation distinct from that observed within immune cells.

**DISCUSSION**

As noted previously (21, 23), many mononuclear cell functions involve active processes that require an energy source, and therefore exercise-triggered inactivation of AMPK would be expected to have a downstream impact on mononuclear cell function. For example, we observed (Fig. 1C) transient decreases in mRNA expression for the metabolic regulator PGC-1α, a gene whose expression is regulated by AMPK (16) and which is key to the responses of many parts of the body (including the immune system) to exercise (13). PGC-1α expression has been reported to change rapidly with a half-life of ~2 h (28), and changes over a similar timescale (~1 h) were seen in the present study. Moreover, as previously reported (23), the exercise-induced decreases in mononuclear cell AMPKα1 phosphorylation (Fig. 1, A and B) seen in the present study followed a pattern similar to those for sIgA production (Fig. 1D).

Decreases in sIgA have clinical consequences; there is known to be a negative correlation between sIgA levels and incidence of upper respiratory tract infections (8), and individ-

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**Fig. 3.** Effect of exercise on intracellular concentrations of Ca²⁺ ([Ca²⁺]_{cyto}) and in monocytic cells. Peripheral mononuclear cells were obtained from a cohort of 8 participants ages 27.8 ± 6.4 yr before and after cycling exercise (45 min of cycling at 70% VO₂max). A: [Ca²⁺]_{cyto} data were obtained via flow cytometry using the Ca²⁺-sensitive dye fluo-3 and the Grynkiewicz equation as described in text. Data are means ± SD; n = 8. *P < 0.05. B: total proteins were extracted and subjected to Western blotting using antibodies directed against calmodulin-dependent protein kinase kinase β (CaMKKβ). Mw, molecular mass. C: the intensities of bands at 64 and 56 kDa, representing phosphorylated (pCaMKKβ) and nonphosphorylated CaMKKβ, respectively, were measured densitometrically. Data are means ± SD representative of 8 separate experiments.

**Fig. 4.** Effect of ascorbic acid on intracellular concentrations of reactive oxygen species ([ROS]_{cyto}) and phosphorylation of AMPKα1 in MM6 cells. MM6 cells maintained in culture (solid bar; 0 h) were treated with 50 μM ascorbic acid for 4 (open bars) or 24 h (shaded bar). A: whole cells were loaded with the ROS-sensitive dye H₂DCFDA and viewed using flow cytometry. RFU, relative fluorescence units. B: total protein samples were extracted and subjected to Western blotting; ratios of the intensities of bands representing phosphorylated AMPKα1 (pAMPK) or total AMPKα1 were determined densitometrically. Data are means ± SD; n ≥ 3. *P < 0.05.
uals with sIgA deficiencies suffer from a higher than normal incidence of such infections (9). Therefore, these findings support our initial hypothesis that exercise-induced inactivation of AMPKα1 disrupts individual immune cell function and thus may be linked to exercise-induced immunosuppression. However, it is possible that the delayed return to basal observed for running reflects the greater tissue damage that would be experienced during running compared with cycling.

Measurement of the effect of exercise on plasma levels of soluble E-selectin showed no significant change in the levels of this marker (Fig. 2), a different pattern from the transient decreases observed for AMPKα1 phosphorylation and sIgA production. Because E-selectin is an inflammatory marker derived from endothelial cells, rather than leukocytes, this suggests that intense exercise, or more likely, the accompanying tissue damage, activates endothelia to release inflammatory signaling molecules such as E-selectin. Although the role of such signals is to activate inflammatory processes within leukocytes, the sIgA results presented in this study (Fig. 1D) and the decreased cytokine release described previously (23) indicate that this inflammatory activation of leukocytes does not occur in the context of intense exercise, possibly because of leukocytic AMPKα1 inactivation. Thus the intracellular signaling events that lead to exercise-induced immunosuppression may be specific to leukocytes and may not occur in other vascular cell types such as endothelial cells.

As reported previously (23), during intense exercise, AMPKα1 is dephosphorylated in mononuclear cells (and in purified monocyte samples) by a mechanism that does not involve changes in cellular AMP-to-ATP ratios. The current study therefore aimed to identify the AMP:ATP-independent source of the observed AMPKα1 dephosphorylation.

It has been reported that AMPK can be activated in an AMP-independent manner, for example, by oxidative stress (35). ROS are produced as by-products of mitochondrial oxygen consumption (7), and several studies (e.g., Refs. 10, 25) have reported that exercise increases mitochondrial biogenesis and activity within skeletal muscle. Thus exercise-induced increases in serum levels of ROS, indirectly detected as decreases in [GSH]serum in the present study (Fig. 6), may be tentatively attributed to increased ROS generation in skeletal muscle mitochondria during exercise. However, this should be contrasted with the suppression of the intracellular generation of ROS seen in the present study: as shown in Fig. 4A, flow cytometric data using the ROS-sensitive dye H2DCFDA demonstrated that exercise induced a decrease in intracellular ROS levels in monocytes. Thus a specific signaling effect within immune cells seems to be responsible for the different pattern of ROS generation within immune cells compared with that observed extracellularly. Moreover, given that our in vitro data show that antioxidant treatment causes a decrease in monocyte [ROS]cyto levels (Fig. 4A) and that this coincides with an
decrease in AMPKα1 phosphorylation in these cells (Fig. 4B), an exercise-induced decrease in [ROS]cyto is a plausible candidate for decreasing monocyte AMPKα1 activity.

It was not possible in the present study to identify the precise biochemical means by which ROS affect AMPKα1 activity. Although it has been suggested that changes in [ROS]cyto may trigger posttranslational modification and activation of the upstream AMPK phosphatase PP2C (19), in our experiments, no exercise- or antioxidant-induced changes in PP2C expression or phosphorylation could be detected, suggesting that ROS did not regulate AMPKα1 via PP2C in this instance. [However, it should be noted that several alternative candidates also have been proposed, such as the suggestion by Xie et al. (39) that peroxynitrite may be the agent responsible for ROS-mediated activation of AMPK due to its direct effects on PKCζ, which phosphorylates S428 of LKB-1 and so leads to consequent AMPK T172 phosphorylation by LKB-1.]

AMPK also can be activated by Ca2+ signaling, via agents such as CaMKKβ (33). The demonstration (Fig. 3A) that our exercise model caused an increase in [Ca2+], from normal basal levels (~60 nM) to levels similar to those that would be expected in healthy activated leukocytes (~300 nM; Ref. 2) suggests, in line with previous studies (24), that exercise activates Ca2+ signaling in leukocytes. However, this activation did not result in a change in CaMKKβ phosphorylation (Fig. 3, B and C). Moreover, if this activation in Ca2+ signaling were coupled via CaMKKβ to AMPKα1 signaling (33), such an effect would be expected to result in phosphorylation of AMPKα1, rather than the dephosphorylation that was actually observed. Therefore, we conclude that, in this instance, there is no cross talk between the Ca2+ and AMPKα1 signaling pathways and that ROS-mediated modulation of AMPKα1 activity is more likely to be the AMP:ATP-independent mechanism responsible for exercise-induced AMPKα1 inactivation.

It has been reported that >70% of the ROS produced by oxidized LDL-challenged monocytes are derived from a cytoplasmic source, whereas <50% are derived from mitochondria (37). Moreover, Erusalimsky and Moncada (7) have reported that vascular cells are dependent primarily on glycolysis for their energetic demands [e.g., endothelial cells derive only ~15% of their ATP from mitochondria at atmospheric O2 levels (30)] whereas monocyte cells retain ~75% of their ATP content in the presence of oligomycin (23) and so would not be expected to generate large amounts of mitochondrial ROS. Therefore, a distinction should be drawn between ROS derived from these two sources, and it may be inferred that cytoplasmically generated ROS are more relevant to the current study.

Therefore, we investigated the expression of NOX2, which encodes the catalytic subunit of NADPH oxidase, the main nonmitochondrial source of ROS in vascular cells (18). As shown in Fig. 4B, NOX2 was downregulated as a result of participation in exercise, whereas catalase expression did not change. Because Adams et al. (1) have observed linear correlations between NADPH oxidase subunit expression and the generation of ROS in vascular cells, this conforms with our observations that [ROS]cyto levels are decreased postexercise. It has been known since the late 1990s that exercise can suppress leukocyte ROS generation (29) and that monocytes are among the cell types affected: cytoplasmic ROS generation was decreased in the monocytes of participants in a half-marathon (26) and in a cohort recruited to exercise for 40 min at 60% V02max (37). Moreover, rapid changes in expression of genes encoding NADPH oxidase subunits have been reported in response to a variety of stimuli (32), including exercise-mediated decreases in expression of NOX2 and other NADPH oxidase subunits (1). In samples taken postexercise in the present study, increases in ROS production were blunted after maximal stimulation with PMA, which supports the suggestion that NOX2 downregulation may be responsible for the decreased [ROS]cyto levels seen postexercise.

One may therefore speculate that exercise modulates signaling to induce downstream effects including downregulation of NADPH oxidase subunits, and therefore suppression of ROS generation. It is well-known that exercise causes changes in plasma lipid profiles, which can in turn trigger changes in leukocyte cell signaling (6). It has been reported that agents such as PPARγ ligands, which are generated due to participation in exercise (6), downregulate NADPH oxidase subunit expression (17). [Note: these signaling events may also affect NADPH oxidase posttranslationally. For example, NADPH oxidase is activated due to p47phox subunit translocation to the cell membrane only after PKCζ-catalyzed phosphorylation (31, 34), and this process is suppressed in leukocytes after exercise (36).]

Thus our data support the suggestion that exercise at the intensities used in the present study can induce decreased NADPH oxidase expression/activity (possibly via modulation of PPARγ/PKCζ signaling), and thence decreased leukocyte production of ROS. Importantly, our findings can now extend this pathway to include the suggestion that this decrease in ROS may induce AMPKα1 inactivation. It remains to be seen whether any resulting energy depletion would itself immediately reactivate AMPKα1 or whether the AMPK-suppressive effect of decreased levels of ROS would “override” the AMPK-activating effects of energy depletion. As we discussed in our previous paper (23), if the latter is the case, because many mononuclear cell functions involve active processes that require energy source(s), cellular energy depletion following intense exercise would impinge upon the cells’ ability to participate in these processes and, by extension, might lead to suppression of global immune function (3).

In conclusion, Hardie has suggested that “while response to cellular energy depletion may have been AMPK’s original raison d’être, it is becoming clear that during the course of evolution of multicellular organisms, extracellular signaling agents have acquired the ability to regulate the AMPK system” (14). We propose that the current study may represent a novel instance of such regulation, in which exercise-induced generation of extracellular signaling agents triggers decreases in intracellular ROS levels, which act as upstream signaling events bringing about the inactivation of AMPKα1, subsequent energy depletion within individual immune cells, and, ultimately, decreases in these cells’ ability to carry out their functions. It is possible that this AMPKα1 inactivation may have developed as an evolutionary measure to prevent excessive inflammation following the tissue damage that inevitably accompanies intense exercise.

There are several limitations to the current study. For example, nonexercise control groups were not included in any of the experiments. Moreover, the single bout of exercise used as an exercise model in our in vivo experiments is unlikely to induce clinical immunosuppression, which is more usually associated with repeated, intense, monotonous exercise (5). Nevertheless,
we suggest that more prolonged and/or repetitive high-intensity exercise that does lead to clinical immunosuppression may do so via sustained decreases in intracellular ROS levels and prolonged AMPKα1 inactivation within immune cells.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


