Xanthine oxidase inhibition attenuates skeletal muscle signaling following acute exercise but does not impair mitochondrial adaptations to endurance training

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Wadley GD, Nicolas MA, Hiam DS, McConell GK. Xanthine oxidase inhibition attenuates skeletal muscle signaling following acute exercise but does not impair mitochondrial adaptations to endurance training. Am J Physiol Endocrinol Metab 304: E853–E862, 2013. First published March 5, 2013; doi:10.1152/ajpendo.00568.2012.—The aim of this research was to examine the impact of the xanthine oxidase (XO) inhibitor allopurinol on the skeletal muscle activation of cell signaling pathways and adaptations to mitochondrial proteins and antioxidant enzymes following acute exercise and endurance training. Male Sprague-Dawley rats performed either acute exercise (60 min of treadmill running, 27 m/min, 5% incline) or 6 wk of endurance training (5 days/wk) while receiving allopurinol or vehicle. Allopurinol treatment reduced XO activity to 5% of the basal levels (P < 0.05), with skeletal muscle uric acid levels being almost undetectable. Following acute exercise, skeletal muscle oxidized glutathione (GSSG) significantly increased following allopurinol and vehicle-treated groups despite XO activity and uric acid levels being unaltered by acute exercise (P < 0.05). This suggests that the source of ROS was not from XO. Surprisingly, muscle GSSG levels were significantly increased following allopurinol treatment. Following acute exercise, allopurinol treatment prevented the increase in p38 MAPK and ERK phosphorylation and attenuated the increase in mitochondrial transcription factor A (mtTFA) mRNA (P < 0.05) but had no effect on the increase in peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), nuclear respiratory factor-2, GLUT4, or superoxide dismutase mRNA. Allopurinol also had no impact on the endurance training-induced increases in PGC-1α, mtTFA, and mitochondrial proteins including cytochrome c, citrate synthase, and β-hydroxacyl-CoA dehydrogenase. In conclusion, although allopurinol inhibits cell signaling pathways in response to acute exercise, the inhibitory effects of allopurinol appear unrelated to exercise-induced ROS production by XO. Allopurinol also has little effect on increases in mitochondrial proteins following endurance training.

BENEFICIAL ADAPTATIONS to regular endurance exercise include increases in skeletal muscle mitochondrial biogenesis (synthesis), antioxidant defenses (5), and increased glucose transporter 4 (GLUT4) mRNA and protein (14). Our understanding of how these pathways are regulated following endurance exercise is still not completely understood but is important, given the crucial role they play in skeletal muscle metabolism. The potential role of reactive oxygen species (ROS) in the regulation of skeletal muscle mitochondrial biogenesis with exercise/contraction is controversial, with some studies supporting such a role (5, 11, 25, 29), while others do not (8, 32, 35, 40). In particular, data by Kang et al. (11) suggested that ROS generated by xanthine oxidase (XO), which is located in the endothelium of skeletal muscle (7), may be regulating some of the increased skeletal muscle mitochondrial biogenesis in response to an acute bout of very exhaustive exercise in rodents (11). Indeed, two studies have now established that the XO inhibitor allopurinol attenuates (11) or abolishes (4) the increase in skeletal muscle phosphorylation of the p38 mitogen-activated protein kinase (p38 MAPK) during an acute bout of exercise. These aforementioned findings have implications for the regulation of exercise-induced mitochondrial biogenesis, since the exercise-induced increase in phosphorylation of p38 MAPK is implicated in the activation of skeletal muscle mitochondrial biogenesis (1). Further supporting the role of XO-produced ROS in mitochondrial biogenesis is that allopurinol also attenuated or abolished the exercise-induced increase in mitochondrial biogenesis markers such as peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a master regulator of mitochondrial biogenesis, as well as mitochondrial transcription factor A (mtTFA) and nuclear respiratory factor 1 (NRF-1) (11) and also the increase in antioxidant enzymes during acute exercise (4).

Examination of these aforementioned kinase signaling and gene expression responses following an acute bout of exercise provides mechanistic insights into the regulation of exercise-induced mitochondrial biogenesis. However, it is the increased content or enzyme activity of mitochondrial proteins that will influence, at least in part, the function of the organelle, with this process taking at least a few weeks (10, 20). This process is largely due to the summation of transient increases in the transcription of genes encoding mitochondrial proteins following each acute bout of exercise (i.e., endurance training) (10, 20). Despite evidence showing that allopurinol attenuated skeletal muscle mitochondrial biogenesis markers following acute exercise (11), no study to date has examined the effects of the inhibition of ROS production derived from XO on skeletal muscle mitochondrial biogenesis following endurance training. Consequently, it is reasonable to hypothesize that ROS inhibition with allopurinol would inhibit p38 MAPK phosphorylation and therefore attenuate increases in skeletal muscle mitochondrial biogenesis markers in response to endurance training.

Also, the aforementioned inhibitory effects of allopurinol following acute exercise have so far been established only with models that utilize exercise to exhaustion (3, 10). However, very exhaustive exercise is not indicative of most people’s common exercise experience, since normal endurance training...
mostly involves acute bouts of nonexhaustive exercise. Therefore, if allopurinol were to inhibit signaling pathways known to regulate mitochondrial biogenesis during acute nonexhaustive exercise, it may have long-term implications for endurance training, as it may inhibit beneficial training adaptations to skeletal muscle mitochondria.

Therefore, given the potential for allopurinol to interfere with beneficial endurance training adaptations to skeletal muscle mitochondria, the overall aim of this research was to examine the impact of allopurinol treatment on skeletal muscle mitochondrial adaptations following acute endurance exercise (*study 1*) and endurance exercise training (*study 2*). The aim of *study 1* was to examine whether allopurinol treatment during a single bout of nonexhaustive endurance exercise attenuated increases in skeletal muscle metabolic signaling and increases in expression of genes involved with regulation of mitochondrial biogenesis, GLUT4, and antioxidant enzymes after exercise. The aim of *study 2* was to examine whether prolonged allopurinol treatment during accumulative bouts of acute exercise (i.e., endurance training) attenuated the increases in markers of skeletal muscle mitochondrial biogenesis such as PGC-1α, mtTFA, NRF-1 and -2, and mitochondrial proteins involved with the electron transport chain such as cytochrome c, the TCA cycle (i.e., citrate synthase), and proteins involved in fatty acid oxidation such as β-hydroxyacyl-CoA dehydrogenase (β-HAD) in skeletal muscle. Since allopurinol prevents the increase in the mRNA of the antioxidant enzyme manganese superoxide dismutase (MnSOD) following a single bout of exercise (4), we also aimed to determine whether the increase in rat skeletal muscle MnSOD protein following endurance training is attenuated by allopurinol treatment. Importantly, for both studies we included a nonexercised (rested) allopurinol-treated control group to account for any inhibitory effects of allopurinol on basal XO activity that might be independent of its attenuation of XO during exercise.

We hypothesized in *study 1* that inhibiting skeletal muscle XO activity with allopurinol during acute exercise would attenuate oxidative stress and the activation of key redox-sensitive kinases such as p38 MAPK and ERK and prevent increases in the expression of genes involved with regulation of mitochondrial biogenesis, GLUT4, and antioxidant enzymes after exercise. We hypothesized in *study 2* that chronic treatment with allopurinol would attenuate skeletal muscle mitochondrial and antioxidant proteins following endurance training.

**MATERIALS AND METHODS**

**Animal Care and Dietary Treatment**

In both studies, Male Sprague-Dawley rats aged ∼6 wk old and weighing 234 ± 4 g were obtained from The University of Melbourne Pharmacology and Physiology Animal House. Animals were housed in an environmentally controlled laboratory (temperature 22°C) with a 12:12-h light-dark cycle. One week prior to experimentation, animals were familiarized to treadmill running on two separate days for 15 min each day at speeds progressing up to 27 m/min at a 5% incline. The University of Melbourne Animal Experimentation Ethics Committee approved all experimental procedures.

*Study 1: Acute Exercise Study*

On the morning of the experiment, food was withdrawn, and rats received either an intraperitoneal (ip) injection of allopurinol (32 mg/kg body wt; Sigma, St. Louis, MO) or vehicle, with this dose having previously been shown to inhibit skeletal muscle XO activity in rodents (4). The allopurinol was dissolved in 350 μL of DMSO and diluted with 350 μL of olive oil immediately prior to injection (4). The vehicle consisted 350 μL of DMSO and 350 μL of olive oil. All animals then rested in their cage for 30 min before either running on a treadmill for 60 min or remaining in their cages for 60 min.

This procedure produced three groups of vehicle- and three groups of allopurinol-treated rats that either rested for 60 min and were then killed (Rest; *n = 6*), ran on a motor-driven treadmill at 27 m/min on a 5% incline for 60 min and were then killed immediately (Ex; *n = 6*), or ran on a motor-driven treadmill at 27 m/min on a 5% incline for 60 min and then recovered in their cage for 4 h before being killed (Rest; *n = 6*). Animals in the Ex and Ex+Allo groups had ad libitum access to drinking water.

*Study 2: Pilot Study*

To avoid the need for daily injections and minimize any unnecessary stress to the rats throughout the 6-wk training study, a pilot investigation involving ingestion of allopurinol was performed. This was to determine the most effective dose of allopurinol to dissolve in drinking water that would inhibit XO activity and attenuate p38 MAPK phosphorylation during acute exercise to a similar extent as previous studies (4) and *study 1*. To determine a suitable dose, we chose to investigate 0.25 mg/ml and 0.4 mg/ml allopurinol, as previous studies had shown these oral doses to inhibit XO activity (13, 33, 38) and were low enough to avoid the potential nonspecific effects of allopurinol such as the scavenging of hydroxyl radicals (13, 18).

Rats were randomly divided into six groups: 1) rest and treated with 0.4 mg/ml allopurinol (Rest + 0.4 mg/ml; *n = 4*), 2) acute exercise and treated with 0.4 mg/ml allopurinol (Ex + 0.4 mg/ml; *n = 5*), 3) rest and treated with 0.25 mg/ml allopurinol (Rest + 0.25 mg/ml; *n = 4*), and 4) acute exercise and treated with 0.25 mg/ml allopurinol (Ex + 0.25 mg/ml; *n = 4*). Non-allopurinol-treated rats were also used: 5) rest (Rest; *n = 4*) and 6) acute exercise (Ex; *n = 4*).

On the experimental day, rats from the three exercise groups (Ex, Ex + 0.25 mg/ml, and Ex + 0.4 mg/ml) completed an acute bout of exercise identical to *study 1* and were killed immediately. Rats from the three rested groups were then placed on an identical motor-driven treadmill and remained stationary for 1 h and then killed using the identical procedure described below.

*Endurance Training Study*

In the pilot study, we found that 0.25 mg/ml allopurinol in the drinking water inhibited p38 MAPK phosphorylation with acute exercise to a similar extent as ip injection of allopurinol in *study 1* (see RESULTS and Fig. 5). Therefore, *study 2* involved allopurinol ingestion instead of daily ip allopurinol injections to reduce distress on the rats.

Rats were randomly assigned to one of the four groups depending on whether they were 1) given ad libitum access to untreated drinking water and remained sedentary (SedWater; *n = 6*), 2) administered allopurinol-treated drinking water and remained sedentary (SedAllo; *n = 6*), 3) endurance trained with ad libitum access to untreated drinking water (ExWater; *n = 6*), or 4) administered allopurinol-treated drinking water and endurance trained (ExAllo; *n = 6*). The exercised rats underwent endurance training over a period of 6 wk, whereas sedentary rats remained on a stationary treadmill for an identical period of time as the endurance-trained groups throughout the study. All animals in *study 2* had ad libitum access to food throughout the study except for when they were on the treadmill.

Twenty-four hours after the second familiarization session, and 3 days prior to the beginning of training, rats were treated with 0.25 mg/ml allopurinol (dose determined from the pilot study; see RESULTS) placed in the drinking water. Fresh solutions of allopurinol were provided every 48 h as suggested previously by others (33).
The training protocol was similar to a previous study (5) and involved 5 days of treadmill running per week on a 5% incline for 6 wk. Exercised rats commenced endurance training on day 1 at a treadmill speed of 24 m/min for 20 min. Duration and intensity of training were progressively increased until rats ran at a treadmill speed of 30 m/min for 90 min. Sedentary rats remained on a stationary treadmill for an identical period of time as endurance-trained rats. Minor encouragement was provided for all endurance-trained rats in the form of blowing medium-pressure compressed air. Fluid ingestion and food consumption were recorded daily throughout the training study in all four experimental groups. All rats were handled and weighed daily. After the last training session, all rats rested for 48 h to eliminate the influence of the exercise-induced effects of the final exercise bout (5) before being killed. During the 48-h rest period, alloprilol-treated rats continued their alloprilol treatment until they were killed.

For all studies, the rats were killed by an overdose of pentobarbital sodium via ip injection (Sigma, St. Louis, MO; 170 mg/kg) followed by cervical dislocation. Following this, gastrocnemius muscles were rapidly excised, frozen, and stored in liquid N2. Just prior to analysis, whole gastrocnemius muscles were crushed into a powder under liquid N2 with a mortar and pestle to obtain a homogenous sample. Blood samples were obtained via cardiac puncture and placed in tubes containing lithium-heparin. Blood was spun and the plasma immediately stored at −80°C until analysis.

Gene Expression

RNA was isolated from frozen rat gastrocnemius using the Micro-to-Midi Total RNA Purification System kit with TRizol and DNase on-column digestion (Invitrogen, Melbourne, Australia). RNA integrity was verified and the concentration determined on the Experion Automated Electrophoresis System (Bio-Rad Laboratories, NSW, Australia). First-strand cDNA was generated from 0.5 μg of RNA using AMV Reverse Transcriptase (Promega, Madison, WI) as previously described (37). Following reverse transcription, the remaining RNA was degraded by treatment with RNase H (Invitrogen) for 20 min at 37°C. The amount of single-stranded DNA was then determined in each sample compared with an oligonucleotide standard in an assay using OliGreen reagent (Invitrogen), which was incubated in 10 min at 37°C. The amount of single-stranded DNA was then determined in each sample compared with an oligonucleotide standard in an assay using OligoGreen reagent (Invitrogen), which was incubated in the dark at 80°C for 5 min prior to the measurement of fluorescence (24, 35).

NRF-1 (cat. no. Rn01455954_m1) was assessed using predesigned/prevalidated FAM-labeled Assays-on-Demand from Applied Biosystems (Applied Biosystems, Foster City, CA). The primer sequences were obtained from gene sequences from GenBank: PGC-1α-5′-AAGGACCTAGATCGAAGATCAG-3′ and 5′-GACACATCTTCTTCAATTCCT-3′; for NRF-2 were 5′-CCCGTACTGTCATGTTGAT-3′ and 5′-GTTCCTGAGTTGACTCTC-3′; for mTFA were 5′-GACCATTAGTGGATTGGGATGTT-3′ and 5′-CTGCGGTCATGTGTCCTGAGGAAT-3′; for GLUT4 were 5′-GACCCAAAGTCACGCTTGATA-3′ and 5′-AGCCATGTGGAGGGAAG-3′; for MnSOD were 5′-CAACCTTCCTCTGTCTAGAAC-3′ and 5′-GCTTCTTTGTCTGGATTTTC-3′; for phospho-p38 Thr180Tyr182 MAPK were 5′-GCTTCTTTGTCTGGATTTTC-3′ and 5′-GCTTCTTTGTCTGGATTTTC-3′; for phospho-AMPKα Thr172 were 5′-CAACCTTCCTCTGTCTAGAAC-3′ and 5′-GCTTCTTTGTCTGGATTTTC-3′; for phospho-ERK1/2 were 5′-CAACCTTCCTCTGTCTAGAAC-3′ and 5′-GCTTCTTTGTCTGGATTTTC-3′; for phospho-ATF2 Thr71 were 5′-CAACCTTCCTCTGTCTAGAAC-3′ and 5′-GCTTCTTTGTCTGGATTTTC-3′; for phospho-AMPKα Thr172 were 5′-CAACCTTCCTCTGTCTAGAAC-3′ and 5′-GCTTCTTTGTCTGGATTTTC-3′; for phospho-ATF2 Thr71 were 5′-CAACCTTCCTCTGTCTAGAAC-3′ and 5′-GCTTCTTTGTCTGGATTTTC-3′; for phospho-AMPKα Thr172 were 5′-CAACCTTCCTCTGTCTAGAAC-3′ and 5′-GCTTCTTTGTCTGGATTTTC-3′.

Preparation of Skeletal Muscle for Immunoblotting

Frozen muscle (10 μl buffer/ml muscle) was homogenized in freshly prepared ice-cold buffer [50 mM Tris at pH 7.5 containing 1 mM EDTA, 10% vol/vol glycerol, 1% vol/vol Triton X-100, 50 mM NaF, 5 mM Na2PO4, 1 mM DTT, 1 mM PMSF, and 5 μM Protease Inhibitor Cocktail (P8340, Sigma)]. Tissue lysates were incubated on ice for 20 min and then spun at 16,000 g for 20 min at 4°C. Protein concentration was determined using a bichinonic (BCA) protein assay (Pierce, Rockford, IL) with BSA as the standard.

Immunoblotting

Whole cell lysates for determination of p38 MAPK, ERK, AMP-activated protein kinase-α (AMPKα), and activating transcription factor-2 (ATF2) signaling and PGC-1α, mTFA, cytochrome c, and MnSOD protein abundance were solubilized in Laemmli sample buffer. Equal amounts of proteins were separated by SDS-PAGE, and electrotransfer of proteins from the gel to PVDF membranes (25 mmol/l Tris, pH 8.3, 192 mmol/l glycine, and 20% vol/vol methanol) was performed for 90 min at 95 V (constant). Blots were probed with the following antibodies: phospho-p38 Thr180Tyr182 MAPK (pThr180Tyr182 p38 MAPK), AMPKα, phospho-Thr202Tyr204 ERK1/2 (pThr202Tyr204 ERK1/2), and phospho-ATF2 Thr71 (pThr71 ATF2), which were all rabbit polyclonal from Cell Signaling Technology (Hartfordshire, England), and PGC-1α mouse monoclonal (Calbiochem, Darmstadt, Germany), anti-mtTFA rabbit polyclonal (GenWay Biotech, San Diego, CA), anti-NRF-2 rabbit polyclonal (Santa Cruz, CA), anti-cytochrome c mouse monoclonal (BD Bioscience Pharmigen, San Diego, CA), and anti-MnSOD rabbit polyclonal (Abcam, Cambridge, MA). Binding was detected with IRDye 800-conjugated anti-rabbit IgG (Rockland, Gilbertsville, PA) or IRDye 680-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) fluorescent secondary antibodies. As a loading control, blots were reprobed with anti-α-tubulin mouse monoclonal antibody (Sigma). Data were expressed as the ratio of integrated intensity following infrared detection (Odyssey Imaging system; LI-COR Biosciences, Lincoln, NE). For p38 MAPK, ERK, and AMPKα signaling, membranes were then stripped (2% SDS, wt/vol, in 25 mM glycine, pH 2.0), and successful stripping was verified by incubation with anti-α-tubulin IgG (Upstate Biotechnology, New York, NY), and phosphorylation was expressed relative to p38 MAPK, ERK, or AMPKα protein abundance, respectively.

Levels of Xanthine, XO Activity, and Uric Acid

Skeletal muscle was homogenized in ice-cold 100mM Tris at pH 7.5 containing 5μl buffer/ml Protease Inhibitor Cocktail (P8340, Sigma). Tissue lysates were then spun at 10,000 × g for 15 min at 4°C. Protein concentration was determined using a BCA protein assay with BSA as the standard. Levels of xanthine, XO activity, and uric acid in the supernatant and levels of xanthine in the plasma were determined fluorometrically using commercially available kits according to the manufacturer’s instructions (Invitrogen).

Skeletal Muscle Oxidized Glutathione Levels

Oxidized glutathione (GSSG) and reduced glutathione (GSH) was determined in freeze-dried skeletal muscle spectrophotometrically using a commercially available kit (Biooxygen GSH/GSSG-412; Oxis Health Products, Portland, OR) as previously described (28, 35).

Enzyme Activities

All enzyme activities were examined at room temperature using a spectrophotometer using muscle homogenates and expressed as mi-
Cromoles per minute per gram of total protein (34, 36). β-HAD (β-oxidation of fatty acids) activity was measured at a wavelength of 340 nm by examining the disappearance of nicotinamide adenine dinucleotide (NADH). Citrate synthase activity (TCA cycle) activity was measured by examining the increase of 5,5-dithiobis-2-nitrobenzoate (DTNB) at a wavelength of 412 nm (30).

**Statistical Analyses**

Results were analyzed using two-factor analysis of variance (exercise and allopurinol) with Bonferroni post hoc analysis if the ANOVA revealed significant differences. All data are presented as means ± SE. The level of significance was set at \( P < 0.05 \).

**RESULTS**

**Study 1: Acute Exercise Study**

**Effect of allopurinol and acute exercise on levels of xanthine, XO activity, and uric acid.** In skeletal muscle, allopurinol treatment reduced XO activity to \( \sim 5\% \) of the non-allopurinol-treated levels (Fig. 1A), while uric acid levels were almost undetectable following allopurinol treatment (Fig. 1B, \( P < 0.05 \)). Allopurinol treatment also significantly increased xanthine levels in skeletal muscle and plasma by \( \sim 900\% \) above untreated levels (Fig. 1, C and D, \( P < 0.05 \)). Plasma xanthine levels were significantly higher following a single bout of acute nonexhaustive exercise (Fig. 1D, \( P < 0.05 \)). Importantly, an acute bout of nonexhaustive exercise with or without allopurinol did not significantly increase skeletal muscle XO activity, the levels of uric acid, or xanthine (Fig. 1, \( P > 0.05 \)), although there was a significant increase in plasma xanthine levels following acute exercise with allopurinol treatment.

**Effect of allopurinol and acute exercise on skeletal muscle GSSG levels.** Immediately following a single bout of acute exercise, GSSG levels were significantly higher in vehicle- and allopurinol-treated groups (\( P < 0.05 \) main effect for exercise; Fig. 2A). Surprisingly, GSSG levels were significantly higher overall following allopurinol treatment (\( P < 0.05 \) main effect for allopurinol; Fig. 2A). Although there appeared to be a main effect for an increase in the GSSG/GSH ratio following acute exercise, this was not significant (\( P = 0.099 \); Fig. 2B). There were also no effects of allopurinol or exercise on GSH levels (\( P > 0.05 \); Fig. 2C).

**Effect of allopurinol on acute exercise-induced mitochondrial biogenesis signaling.** Phosphorylation of p38 MAPK Thr\(^{180}\)Tyr\(^{182}\) and ERK1 Thr\(^{202}\)Tyr\(^{204}\), and ATF2 Thr\(^{71}\) were significantly increased \( \sim 2.4\-, 1.9\-, \) and 4.4-fold, respectively, immediately following 60 min of treadmill running, (\( P < 0.05 \); Fig. 3). Allopurinol treatment prevented the increase in phosphorylation of p38 MAPK Thr\(^{180}\)Tyr\(^{182}\) and ERK1 Thr\(^{202}\)Tyr\(^{204}\) but not ATF2 Thr\(^{71}\) immediately after acute exercise (\( P < 0.05 \); Fig. 3, A–C, respectively). Interestingly, allopurinol treatment increased basal phosphorylation of ERK1 Thr\(^{202}\)Tyr\(^{204}\). Acute exercise also tended to increase phosphorylation...
of AMPKα Thr\textsuperscript{172} ($P = 0.08$, main effect for exercise; Fig. 3D), although there was no significant interaction between allopurinol and acute exercise on the phosphorylation of AMPKα Thr\textsuperscript{172} ($P = 0.15$; Fig. 3D). Acute exercise did not alter phosphorylation of ERK2 Thr\textsuperscript{185}/Tyr\textsuperscript{187}; however, allopurinol treatment significantly increased its basal phosphorylation ($0.42 \pm 0.07$ vs. $0.38 \pm 0.06$ vs. $1.58 \pm 0.13$ vs. $0.38 \pm 0.07$ arbitrary units for Rest vs. Ex vs. Rest+Allo vs. Ex+Allo, respectively, $P < 0.05$). Protein abundance of p38 MAPK, ERK1 and -2, AMPKα, and α-tubulin were not significantly different between groups ($P > 0.05$; data not shown).

Effect of allopurinol on mitochondrial biogenesis mRNA markers and GLUT4 mRNA 4 h following acute exercise. Four hours following 60 min of treadmill running the mRNA of mtTFA, NRF-2, PGC-1α, and GLUT4 were significantly increased by $\sim 38$, $32$, $900$, and $51\%$ ($P < 0.05$; Fig. 4, A–D, respectively). Allopurinol treatment prevented the acute exercise-stimulated increase in mtTFA mRNA ($P < 0.05$; Fig. 4A); however, it did not alter the acute exercise-induced increases in PGC-1α, NRF-2, and GLUT4 mRNA ($P < 0.05$; Fig. 4, B–D). The mRNA of NRF-1 was not affected by acute exercise or allopurinol (data not shown, $P > 0.05$).

Skeletal muscle MnSOD mRNA was increased 4 h after treadmill running, and this increase was not altered by prior treatment with allopurinol ($P < 0.05$; Fig. 4E). We also chose to examine ecSOD, since skeletal muscle levels of ecSOD mRNA had previously been reported to be increased 6 h following acute exercise in mice (9). There was a significant exercise effect on ecSOD mRNA 4 h after acute exercise, and allopurinol had no effect (Fig. 4F).

Effect of allopurinol on PGC-1α protein following acute exercise. Protein abundance of PGC-1α was not increased immediately after or 4 h after acute exercise and was unaltered by allopurinol treatment ($0.039 \pm 0.004$ vs. $0.035 \pm 0.003$ vs. $0.041 \pm 0.005$ vs. $0.042 \pm 0.007$ vs. $0.045 \pm 0.004$ vs. $0.044 \pm 0.004$ arbitrary units for Rest vs. Ex vs. Rest+Allo vs. Ex+Allo, respectively, $P > 0.05$). Protein abundance of α-tubulin was not significantly different between groups ($P > 0.05$, data not shown).

Study 2: Pilot Study

Acute exercise increased p38 MAPK phosphorylation compared with rested control rats ($P < 0.05$; Fig. 5). Compared
with acute exercised rats, allopurinol in the drinking water at
doses of 0.25 and 0.4 mg/ml significantly attenuated increases
in p38 MAPK phosphorylation to a similar extent ($P < 0.05$; Fig. 5).
Importantly, the effect of allopurinol in the drinking water was similar to what we found with ip allopurinol (Fig. 3). Since both concentrations of allopurinol in drinking water prevented the increase in p38 MAPK phosphorylation during acute exercise, the lower dose of 0.25 mg/ml was employed for study 2. Also, during the pilot study, rats weighed 250 g and ingested 35 ml of fluid per day. Therefore, a dose of 0.25 mg/ml equates to a daily allopurinol dose of 35 mg/kg, which is very similar to the 32 mg/kg ip-injected dose that was used in the acute exercise study.

**Endurance Training Study**

**Food/fluid intake and body weight.** At the conclusion of the 6-wk training, rats weighed 494 ± 6 g. Food consumption and the increase in body weight were not different between groups throughout the experimental period ($P > 0.05$, data not shown). There was a small but significant effect of endurance training and allopurinol on fluid uptake between groups (37.5 ± 0.8 vs. 40.9 ± 0.6 vs. 36.1 ± 0.7 vs. 39.0 ± 1.0 ml fluid/day for SedWater vs. ExWater vs. SedAllo vs. ExAllo, respectively; $P < 0.05$ main effect for exercise training, $P < 0.05$ main effect for allopurinol), with the exercise groups drinking more.

**Effect of endurance training and chronic allopurinol on mitochondrial biogenesis markers, mitochondrial proteins, and mitochondrial enzyme activities.** Six weeks of endurance training significantly increased skeletal muscle protein abundance of mtTFA, PGC-1α, cytochrome c, and MnSOD by 112, 63, 116, and 29%, respectively ($P < 0.05$, main effect of exercise training; Fig. 6). There were no significant effects of allopurinol treatment on mtTFA, PGC-1α, cytochrome c, and MnSOD protein expression for any group.

Endurance training increased both skeletal muscle citrate synthase and β-HAD enzyme activities by 38% ($P < 0.05$, main effect of exercise training; Fig. 7). There were no effects on allopurinol treatment on citrate synthase or β-HAD enzyme activities between any of the groups (Fig. 7).

**DISCUSSION**

We have shown (study 1) for the first time that inhibition of XO with allopurinol attenuated the increase in some redox-sensitive kinases, such as p38 MAPK and ERK1, and the gene expression of mitochondrial biogenesis transcription factor mtTFA following acute nonexhaustive exercise. This attenuation occurred despite the acute bout of exercise not increasing skeletal muscle XO activity. Also, despite these inhibitory effects of allopurinol on mitochondrial biogenesis signaling pathways, the increases in gene expression of PGC-1α, NRF-2,
GLUT4, and antioxidant enzymes following acute exercise were normal. In addition, long-term allopurinol treatment had no impact on the increase in several mitochondrial proteins to endurance training (study 2). Therefore, this suggests that allopurinol does not impair the normal skeletal muscle mitochondrial adaptations observed with endurance training.

The present study found similar ~2.5-fold increases in the phosphorylation of p38 MAPK following acute exercise as those reported by others (4, 11), despite our exercise being nonexhaustive. Also consistent with previous studies was the inhibitory effect of allopurinol on the acute exercise-induced increase in phosphorylation of p38 MAPK that we observed when administered either orally or by ip injection (4, 11). Several lines of evidence suggest that the activation of p38 MAPK is a key step in the regulation of exercise-induced mitochondrial biogenesis in skeletal muscle (1, 22, 39). In particular, p38 MAPK can increase PGC-1α gene expression by directly phosphorylating ATF2, which subsequently increases PGC-1α promoter activity (1, 22). Despite this, study 1 found normal exercise-induced increases in ATF2 phosphorylation and PGC-1α mRNA, despite allopurinol attenuating not only the increase in the phosphorylation in p38 MAPK but also inhibiting the exercise-induced phosphorylation of another MAP kinase, ERK, which can also phosphorylate ATF2 (19). Therefore, our findings imply that the kinase(s) responsible for phosphorylating skeletal muscle ATF2 and subsequently increasing PGC-1α mRNA after exercise in the present study was probably not p38 MAPK or ERK. Thus, the mechanisms accounting for the attenuated activation of the MAPKs despite normal increases in ATF2 phosphorylation and PGC-1α mRNA following XO inhibition is an interesting question that requires further investigation.

The XO-mediated oxidation of hypoxanthine to xanthine and further oxidation of xanthine to uric acid produces superoxide, which is rapidly catalyzed by SOD into oxygen and hydrogen peroxide (6). However, it would appear that the source of skeletal muscle ROS during acute exercise was probably not XO, since there was no significant increase in skeletal muscle XO activity, xanthine, or uric acid levels. Nevertheless, the increased skeletal muscle GSSG levels following acute exercise in study 1 confirms that the exercise protocol was of sufficient intensity to increase skeletal muscle ROS levels. Although elucidating the source of skeletal muscle ROS production during nonexhaustive exercise is beyond the scope of the present study, several potential sources have been suggested (reviewed in Ref. 21), including NADPH oxidase, NOS, and calcium-dependent 14-kDa isoform of phospholipase A2 (PLA2).

Inhibiting XO under basal (nonexercise) conditions also prevents the production of uric acid, and, consistent with this, we found that uric acid levels in skeletal muscle were almost undetectable following allopurinol treatment (Fig. 1). Furthermore, and contrary to our hypothesis, we found evidence that allopurinol increased skeletal muscle oxidative stress, as evidenced by increased levels of skeletal muscle GSSG. Given that uric acid is an antioxidant in humans (2), the most likely mechanism to explain an increased oxidative stress in skeletal muscle following allopurinol treatment appears to be the reduction in skeletal muscle uric acid levels.

Exercise not only increases ROS production but also increases antioxidant enzyme expression (4, 5, 25). The exercise-induced increase in the mRNA of MnSOD can be blocked with the administration of allopurinol following acute exhaustive exercise (4, 11). Furthermore, PGC-1α is required for induc-
Therefore, the inhibitory effects we observed in muscle, including increased mitochondrial content (10, 20), of antioxidant enzymes such as SOD (31). Given that the increases in PGC-1α mRNA and protein were not prevented by allopurinol following acute exercise (study 1) and endurance training (study 2), respectively, it is probably not surprising that allopurinol also had no effect on the increased skeletal muscle gene expression of MnSOD or ecSOD following acute exercise.

Accumulative bouts of acute endurance exercise (i.e., endurance training) result in many beneficial adaptations in skeletal muscle, including increased mitochondrial content (10, 20). Therefore, the inhibitory effects we observed in study 1 of allopurinol on key cell signaling pathways and mtTFA gene expression regulating mitochondrial biogenesis in response to acute exercise could potentially be counterproductive, since chronic allopurinol use may also inhibit training-induced skeletal muscle mitochondrial biogenesis. Therefore, we completed an endurance training study to examine whether our findings from study 1 would extend to attenuated mitochondrial proteins following endurance training. Our findings clearly show that allopurinol treatment did not alter the training-induced increase in proteins involved in the regulation of mitochondrial biogenesis, such as PGC-1α and mtTFA. There was also no impact of allopurinol on the increase in the electron transport chain protein cytochrome c, or the activities of mitochondrial enzymes such as citrate synthase and β-HAD. Furthermore, given that citrate synthase activity has recently been shown to be a very good biomarker of skeletal muscle mitochondrial content (15), these findings indicate that chronic allopurinol treatment does not prevent normal increases in mitochondrial content following endurance training.

Given our finding that chronic allopurinol treatment does not prevent endurance training adaptations in rodent muscle, it is tempting to speculate that the inhibitory effects of allopurinol on p38 MAPK during acute exercise suggest that the kinase is not important for the regulation of mitochondrial biogenesis. We would caution against this conclusion, given the considerable redundancy in the mitochondrial biogenesis pathways. Although overexpression of either PGC-1α or PGC-1β in mice is sufficient to increase skeletal muscle oxidative capacity and endurance (3, 16), gene ablation of either PGC-1α or PGC-1β only modestly impairs endurance, whereas the combined knockdown of PGC-1α and -1β is required to dramatically impair endurance and skeletal muscle oxidative capacity (41). Furthermore, what our study demonstrates is that, although endurance training is essentially a series of acute exercise bouts, one cannot assume that altered signaling and gene expression responses during acute exercise will dictate adaptations to endurance training. The findings from this study highlight that exercise studies need to examine both acute and chronic exercise to get a full understanding of the adaptive responses in skeletal muscle.

One of the implications of our findings for human health relates to the interaction between drugs and exercise. Several drugs to treat chronic diseases, such as β-blockers, statins, and nonsteroidal anti-inflammatory drugs, have all been implicated to some extent in reduced mitochondrial adaptations to endurance exercise (26). Given our findings of allopurinol’s inhibitory effects on p38 MAPK signaling and gene expression of mtTFA following acute exercise, this could have been a potential problem for patients with hyperuricemia and chronic gout, as they are often prescribed physical activity as an adjunct therapy to their allopurinol treatment (27). However, our findings suggest that chronic allopurinol treatment is unlikely to interfere with endurance training adaptations in these patients.

A surprise finding from the present study was that allopurinol phosphorylated ERK1 in the rested (nonexercised group) while preventing its phosphorylation following acute exercise. ERK is a redox-sensitive kinase (12); however, it is unlikely that its increased basal phosphorylation was due to the small increase in ROS following allopurinol treatment. Although the product of XO is uric acid, a known antioxidant (2), the basal phosphorylation of p38, which is another redox-sensitive MAPK, was unaffected by allopurinol. Therefore, it would appear that allopurinol is having nonspecific effects on some of the signaling kinases, particularly ERK, via as yet unknown mechanisms, which lie beyond the scope of this study.

In summary, XO inhibition by allopurinol prior to acute nonexhaustive exercise prevented the signaling of p38 MAPK and ERK1 and the increase in mtTFA gene expression that are thought to be involved in the increase in mitochondrial biogenesis following exercise. However, despite these effects on signaling, allopurinol administration did not prevent the increase in most of the mitochondrial biogenesis markers in the hours after an acute exercise bout or the increases in mitochondrial proteins or the antioxidant enzyme MnSOD following acute exercise.
endurance training. The findings from this study also indicate that allopurinol does not prevent endurance training adaptations in rodent skeletal muscle.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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