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RESEARCH ARTICLE

Hypoxia preconditioning promotes endurance exercise capacity of mice by activating skeletal muscle Nrf2

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[1]*School of Sport Science, Beijing Sport University, Beijing, China;* [2]*State Key Laboratory of Natural and Biomimetic Drugs, Department of Molecular and Cellular Pharmacology, Peking University School of Pharmaceutical Sciences, Beijing, China;* [3]*Faculty of Health, Engineering, and Sciences, University of Southern Queensland, Toowoomba, Queensland, Australia; and* [4]*Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey*

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Wang L, Yang S, Yan L, Wei H, Wang J, Yu S, Kong AT, Zhang Y. Hypoxia preconditioning promotes endurance exercise capacity of mice by activating skeletal muscle Nrf2. *J Appl Physiol* 127: 1267–1277, 2019. First published September 5, 2019; doi: 10.1152/japplphysiol.00347.2019.—Elite endurance athletes are used to train under hypoxic/high-altitude conditions, which can elicit certain stress responses in skeletal muscle and helps to improve their physical performance. Nuclear factor erythroid 2-related factor 2 (Nrf2) regulates cellular redox homeostasis and metabolism in skeletal muscle, playing important roles in adaptation to various stresses. In this study, Nrf2 knockout (KO) and wild-type (WT) mice were preconditioned to 48 h of hypoxia exposure (11.2% oxygen), and the effects of hypoxia preconditioning (HP) on exercise capacity and exercise-induced changes of antioxidant status, energetic metabolism, and mitochondrial adaptation in skeletal muscle were evaluated. Nrf2 knockout (KO) and wild-type (WT) mice were exposed to normoxia or hypoxia for 48 h before taking incremental treadmill exercise to exhaustion under hypoxia. The skeletal muscles were collected immediately after the incremental treadmill exercise to evaluate the impacts of HP and Nrf2 on the exercise-induced changes. The results indicate the absence of Nrf2 did not affect exercise capacity, although the mRNA expression of certain muscular genes involved in antioxidant, glycogen and fatty acid catabolism was decreased in Nrf2 KO mice. However, 48-h HP enhanced exercise capacity in WT mice but not in Nrf2 KO mice, and the exercise capacity of WT mice was significantly higher than that of Nrf2 KO mice. These findings suggest HP promotes exercise capacity of mice with the participation of the Nrf2 signal in skeletal muscle.

NEW & NOTEWORTHY Hypoxia preconditioning (HP) activated the nuclear factor erythroid 2-related factor 2 (Nrf2) signal, which was involved in HP-elicited adaptation responses to hypoxia, oxidative, and metabolic stresses in skeletal muscle. On the other hand, Nrf2 deficiency abolished the enhanced exercise capacity after the 48-h HP. Our results indicate that Nrf2 plays an essential role in the exercise capacity-enhancing effect of HP, possibly by modulating muscular antioxidative responses, the mRNA expression of muscular genes involved in glycogen and fatty acid metabolism, as well as mitochondrial biogenesis, and through the cross talk with AMPK and hypoxia-inducible factor-1α signaling.

exercise; hypoxia preconditioning; Nrf2; skeletal muscle

INTRODUCTION

High-altitude/hypoxia training can build up endurance capacity in skeletal muscles of elite athletes (9, 39). It has been demonstrated that endurance exercise training under stimulated hypoxia conditions significantly promoted glutathione (GSH) system, antioxidant enzyme activities (3, 10), mitochondrial biogenesis, glucose uptake, and metabolism (43, 44) in skeletal muscle of humans or rats, compared with the outcomes from exercise training in normoxia conditions. This evidence implies that the enhanced endurance capability may, at least in part, be attributed to the hypoxia-induced increases in antioxidant capacity and aerobic metabolism in skeletal muscles. However, the molecular mechanisms by which hypoxia stimulates antioxidant reaction, metabolic, and energy-balance regulation in skeletal muscle are to be further investigated.

Nuclear factor erythroid 2-related factor 2 (Nrf2, also called Nfe2l2) is activated under oxidative stress, binds to the antioxidant response element (ARE) in the 5′-promoter region of cytoprotective genes, and then increases the expression levels of antioxidant and detoxification genes, such as catalase (*Cat*), NAD(P)H:quinone oxidoreductase 1 (*Nqo1*), and heme oxygenase-1 (*Hmox1*), which prepare the cells to withstand oxidative stress (17). Besides mediating stress-stimulated induction of antioxidant genes, accumulating evidence has indicated that Nrf2 may also influence substance metabolism and mitochondrial biogenesis. Chromatin immunoprecipitation analysis (50) has demonstrated that Nrf2 binds the upstream promoter regions of 1,4-a-glucan branching enzyme 1 (*Gbe1*) and phosphorylase kinase-regulatory subunit a1 (*Phka1*), which encode glycogen branching enzyme (GBE) (35) and phosphorylase kinase αM subunit (7), respectively, the key enzymes of glycogen branching and breakdown in skeletal muscle. In the absence of Nrf2, fatty acid oxidation is suppressed and may lead to lower ATP levels and mitochondrial dysfunction (14). It has been reported that uncoupling protein 3 (UCP3; encoded by *Ucp3*) is a regulator of fatty acid export and helps to maintain muscular fat oxidative capacity (40) and the promoter region of *Ucp3* contains a Nrf2 binding site (1). In addition, nuclear respiratory factor 1 (NRF1; encoded by *Nrf1*) is implicated in the control of nuclear genes required for respiration, mitochondrial DNA transcription, and replication (38), and the promoter region of *Nrf1* also contains a Nrf2 binding site, and Nrf2 activation can induce its transcription (37). Therefore,

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Nrf2 may have an important role in skeletal muscle contractile and mitochondrial function (8).

We have investigated the effects of acute hypoxia exposure (11.2% oxygen concentration) with different durations (0–48 h) on the activation of the Nrf2-ARE pathway in C57BL/6J mice. The results showed that the 48-h hypoxia exposure significantly increased the activation of Nrf2, but not the shorter exposures (20). To continue our research, in the present study we applied the 48-h hypoxia exposure as hypoxia preconditioning (HP) to both Nrf2 knockout (KO) and wild-type (WT) mice and aimed to evaluate the effects of HP and Nrf2 on exercise capacity, and the exercise-induced changes in antioxidant reaction, substance metabolism, and mitochondrial function in skeletal muscle. To date, there has been no report in the literature regarding the application of HP to pretreat the exercised Nrf2 KO mice. We hypothesized that the deficiency of Nrf2 would impair antioxidant and metabolic adaptations of skeletal muscle to exercise. Furthermore, the exercise with HP would upregulate antioxidant reaction and metabolism in skeletal muscle, as well as the exercise capacity of WT mice, while the genetic defect of Nrf2 would diminish these effects.

METHODS

Animal care. The experimental procedure was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Beijing Sport University (Beijing, China). The protocol was approved by the Animal Care and Use Committee of Beijing Sport University.

Animals. Nrf2-deficient mice of C57BL/6J background were kindly provided by Dr. M. Yamamoto at Tohoku University (16). Male $Nrf2^{-/-}$ mice and $Nrf2^{+/+}$ littermates (24 ± 2 g, 8 wk old), herein referred to as Nrf2 KO and WT mice, respectively, were housed in a temperature- and light-controlled environment (20–25°C and 12-h light-dark cycle). Food and water were supplied ad libitum. Male Nrf2 KO and WT mice were randomly allocated to four groups: WT-no HP, WT-HP, KO-no HP, and KO-HP, with 8–10 mice in each group.

HP. HP was achieved by placing the mice in a normobaric chamber (210 cm long, 200 cm wide, and 200 cm high). The chamber was infused with hypoxic air through an air compressor and a nitrogen synthesizing machine, which could reduce the oxygen concentration in the chamber to 11.2% (at about simulated altitude of 4,500 m) based on the previous work (20). The oxygen concentration in the chamber was monitored throughout the experimental period with an oxygen sensor. HP was performed for 48 h.

Endurance exercise capacity. Before HP, all mice were familiarized with treadmill running for 3 days in normoxia. The treadmill was equipped with an electric stimulation grid at the rear. The duration of these familiarization runs was 10 min with a speed of 10 m/min and an incline of +5°. After the 48-h normoxia (WT-no HP and KO-no HP) or hypoxia exposure (WT-HP and KO-HP), an incremental treadmill test to exhaustion (13) in hypoxia (11.2% oxygen) was performed for all mice. Briefly, this test was started with an incline of +5° and a speed of 10 m/min for 5 min. After this initial phase, the speed was progressively increased by 3 m/min every 3 min until the mouse spent longer than 10 s on the shock grid without attempting to continue running (31). Once exhaustion was reached, the power of the shock grid was turned off and running duration and distance were recorded.

After the incremental treadmill hypoxic exercise, the mice were immediately euthanized by cervical dislocation. Skeletal muscles were collected, cleaned, and quick-frozen in liquid nitrogen, and then stored at −80°C. Different muscles were used in the different tests.

Quantitative PCR analysis. Total RNA was isolated from crushed extensor digitorum longus muscle using TRIzol reagent (Life Technologies) following the manufacturer's instructions. Real-time PCR was performed in an ABI 7500 Real-time PCR System (Thermo Scientific, Waltham, MA) using a SYBR Green Real-time PCR Master Mix kit (Toyobo, Osaka, Japan) with the previously synthesized cDNA (FSQ-101; Toyobo) as template in a 20-μL reaction volume. The following commercial primers from Qiagen (Germany) were used: *Nqo1* (QT00094367), glutathione reductase (*Gr*; QT01758232), superoxide dismutase 1 (*Sod1*; QT00165039), superoxide dismutase 2 (*Sod2*; QT00161707), *Cat* (QT01058106), glutamate-cysteine ligase catalytic subunit (*Gclc*; QT00130543), glutathione peroxidase1 (*Gpx1*; QT01195936), *Hmox1* (QT00159915), *Gbe1* (QT00252924), *Phka1* (QT00143514), mitochondrial uncoupling protein 3 (*Ucp3*; QT00115339), mitochondrial transcription factor A (*Tfam*; QT00154413), *Nrf1* (QT01051820), and 18S ribosomal RNA (*Rn18s*; QT02448075). In addition, the primer sequences of carnitine palmitoyl transferase 1,2 (*Cpt1, Cpt2*), peroxisome proliferator-activated receptor gamma coactivator 1-α (*Ppargc1α*), ATP-citrate lyase (*Acly*), acetyl-CoA carboxylase 1 (*Acaca*), fatty acid synthase (*Fasn*), and stearoyl CoA desaturase (*Scd1*) are listed in Supplementary Table S1 (https://doi.org/10.6084/m9.figshare.9630701), and these primers were synthesized by Invitrogen Trading (Shanghai, China). The Rn18s gene is a reliable internal control for comparative analyses of transcription under hypoxia (32), which was assessed using software (ABI 7500RT PCR). The difference in expression between control and experimental samples was calculated using the $2^{-\Delta\Delta Ct}$ method, as described previously (24).

Western blotting. Total proteins were isolated from the extensor digitorum longus muscles using RIPA protein extraction reagents (P0013B; Beyotime, Beijing, China). Protein concentration was measured using the BCA protein assay kit (Pierce 23225; Thermo Fisher Scientific). Twenty micrograms of proteins were separated on Bolt 4–12% Bis-Tris PlusGels (NW04125BOX; Thermo Fisher Scientific) by electrophoresis, and the fractionated proteins were subsequently transferred to a nitrocellulose membrane using iBlot Gel Transfer Stacks Nitrocellulose (IB23001; Thermo Fisher Scientific). The blots were probed using the following antibodies: Nrf2 (sc-722;), hypoxia-inducible factor-1α (HIF-1α; sc-10790), Kelch-like ECH-associated protein 1 (Keap1; sc-33569), NQO1 (sc-16464), GR (sc-133245), SOD1 (sc-11407), SOD2 (sc-30080), CAT (sc-50508), GCLc (sc-22755), AMP-activated protein kinase α (AMPKα; sc-74461), and β-actin (sc-47778). The above-mentioned antibodies were all from Santa Cruz Biotechnology. Also used were Thr172-phosphorylated (p)-AMPKα (2535; Cell Signaling Technology), acetyl-CoA carboxylase (ACC; 3662, Cell Signaling Technology), Ser79-p-ACC (3661; Cell Signaling Technology), total oxidative phosphorylation (OXPHOS) complexes, and rodent WB antibody cocktail (ab110413; Abcam Trading Shanghai Company). The density of protein bands was analyzed using Bio-Rad imaging software (Bio-Rad Laboratories, Hercules, CA). The individual values were originally expressed as a ratio of a standard (β-actin content) and then expressed as a fold-change of the control group value.

Reactive oxygen species generation. According to the manufacturer's instructions for the kit (GMS10016.3; GENMED, Shanghai, China), 50 mg soleus and quadriceps femoris muscles were homogenized with reagent C in the kit, respectively. The supernatants were used to yield the reactive oxygen species (ROS) samples (2 μg protein/μL). These steps were performed at 4°C.

Then, ROS samples were incubated with the chloromethyl derivative of (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; CM-H_2DCFDA) at 37°C for 20 min in 96-well plates, and the ROS levels were detected by a fluorescence plate reader at λexc: 490 nm and λem: 520 nm (Bio Tek Synergy H1, Bio Tek Instruments).

Mitochondrial DNA copy number. Genomic DNA in quadriceps femoris muscles was extracted using a TIANamp Genomic DNA kit (Tiangen, Beijing, China) according to the kit protocol. Using the DNA, the relative copy numbers of mitochondrial DNA (mtDNA) and

nuclear DNA (nDNA) were determined by quantitative PCR as described (52).

The following primers (Invitrogen, Shanghai, China) were used in this assessment: mtDNA (F, 5′-CCCTAAAACCCGCCACATCT-3′; R, 5′-GAGCGATGGTGAGAGCTAAGGT-3′) or nDNA (F, 5′-CGAGTCGTCTTT CTCCTGATGAT-3′; R, 5′-TTCTGGATTC-CAA TGCTTCGA-3′) (33).

Citrate synthase activity. Quadriceps femoris muscles were prepared as described above for Western blot analysis. Citrate synthase activity was determined using a commercially available kit from Solarbio (Beijing, China). For its determination, the formation of 5-thio-2-nitro-benzoic acid was measured spectrophoretically at 412 nm.

Muscle glycogen and triglyceride contents. Quadriceps femoris muscles were used for the measurements of glycogen and triglyceride contents. The muscle glycogen and triglyceride contents were determined using the commercial assay kits BC0345 and BC0625 (Solarbio, Beijing, China).

Statistics. All values are reported as means ± SE. Statistical calculations were performed using SPSS STATISTICS v. Nineteen software (IBM). Data were analyzed using a two-way ANOVA (strain × HP). When a significant interaction effect was obtained, a simple main effect analysis with the post hoc least significant difference test was performed to identify significant mean differences between groups. Statistical significance was set at $P < 0.05$.

RESULTS

Exercise performance, HIF-1α, Nrf2, and Keap1 protein levels. There was no significant difference in running distance and expression of HIF-1α protein between the WT-no HP and KO-no HP groups. However, significant increases in running distance and expression of HIF-1α protein were observed in the WT-HP group compared with those of the WT-no HP group. Moreover, the shorter running distance and the lower expression of HIF-1α protein were shown in the KO-HP group compared with those of the WT-HP group (Fig. 1, *A* and *B*). These results demonstrated that HP significantly improved the exercise capacity and muscular HIF-1α protein level after the hypoxic exercise in normal WT mice, but had no effects in Nrf2 KO mice. HP significantly increased the protein expression of Nrf2 in the post-hypoxic exercise skeletal muscle in the WT-HP group compared with that of the WT-no HP group (Fig. 1*C*). In addition, there was no significant change in Keap1 protein between the WT-no HP and WT-HP groups. There was also no significant difference in Keap1 protein between the WT-no HP and KO-no HP groups or between the WT-HP and KO-HP groups (Fig. 1*D*).

mRNA expression of muscular genes involved in antioxidation. The mRNA expressions of almost all measured antioxidative genes were significantly lower in the Nrf2 KO mice of both the HP and no-HP groups compared with those of the WT mice. Moreover, the exercise with HP produced a significant increase in most of the mRNA expressions of Nrf2-mediated antioxidative genes (*Nqo1*, *Gr*, *Gclc*, *Gpx1*, and *Hmoxo1*) in skeletal muscle of WT mice compared with those of the WT-no HP group, while there were no such significant changes in the Nrf2 KO mice (Fig. 2).

Protein expression of muscular genes involved in antioxidation and ROS level. The expressions of some muscular antioxidative proteins measured were lower and ROS levels of

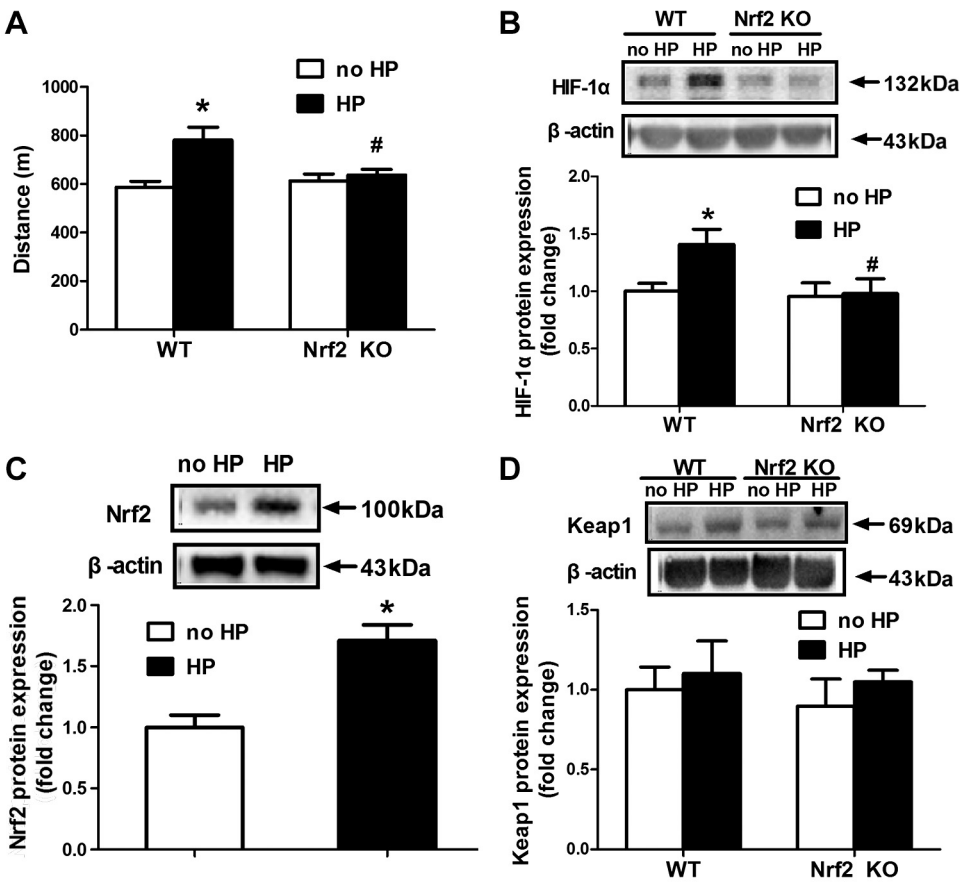

Fig. 1. Effects of hypoxia preconditioning (HP) on running distance (*A*), expression of hypoxia-inducible factor (HIF)-1α protein (*C*) and Keap1 (*D*) in skeletal muscles of the hypoxic exercised wild-type (WT) and nuclear factor erythroid 2-related factor 2 knockout (Nrf2 KO) mice. Nrf2 protein (*B*) showed the change from WT mice only. Values are means ± SE ($n = 8$–10 animals/group). *$P < 0.05$, HP vs. no-HP. #$P < 0.05$, Nrf2 KO vs. WT mice.

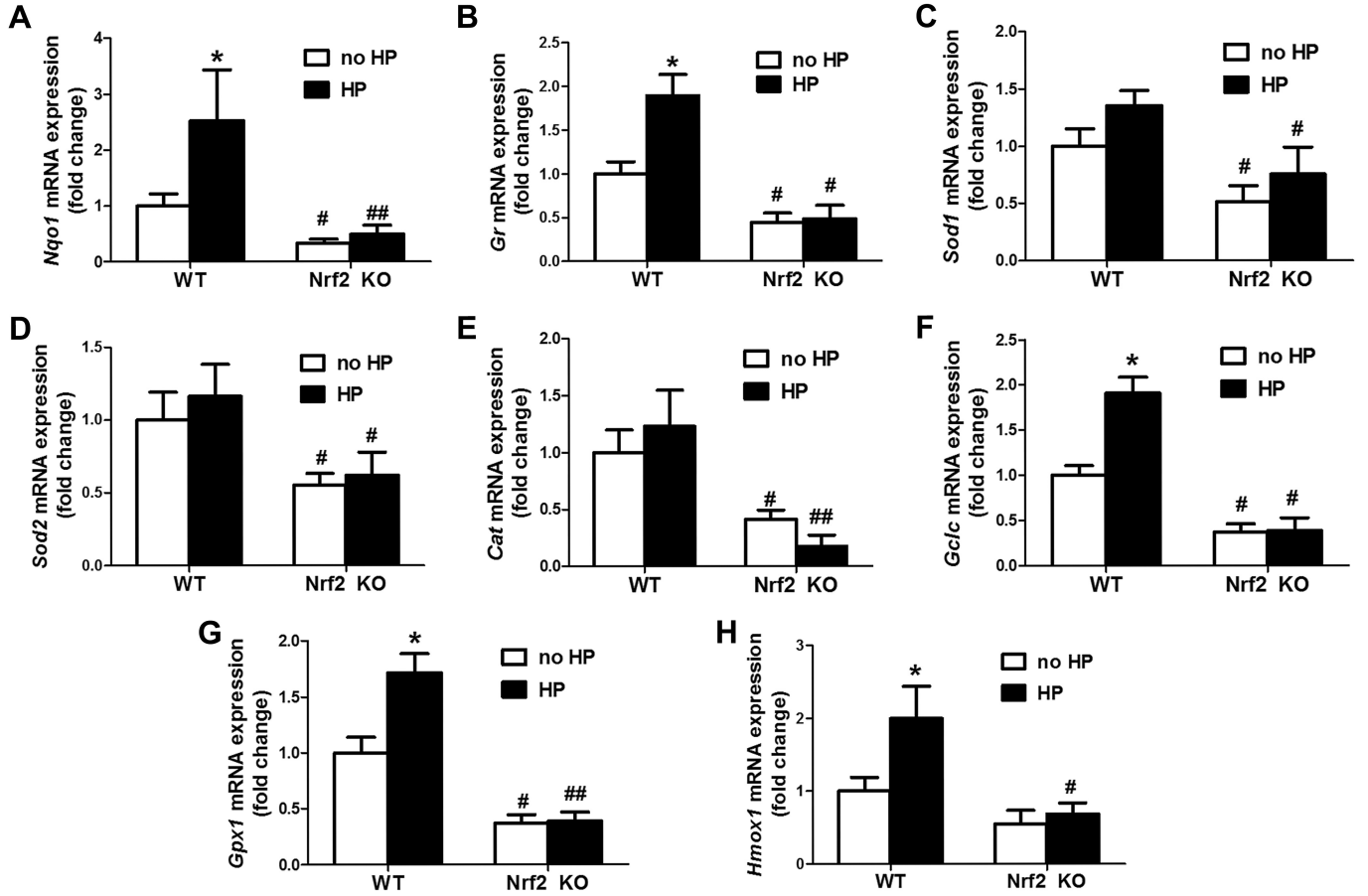

Fig. 2. Effects of HP on the mRNA expression of muscular genes involved in antioxidation (*A–H*) in skeletal muscle of the exercised WT and Nrf2 KO mice. Values are means ± SE (n = 8–10 animals/group). *$P < 0.05$, HP vs. no HP. #$P < 0.05$, ##$P < 0.01$, Nrf2 KO vs. WT mice.

soleus and quadriceps femoris muscles were higher in the Nrf2 KO mice of both the HP and no-HP groups compared with those of the WT mice. In contrast, compared with mRNA expression, the protein expression levels (such as SOD1 and CAT) in these groups were less significant. The exercise with HP had a significant increase in the protein expressions (NQO1, GR, and GCLc) of Nrf2-mediated antioxidative genes, and a reduction in the ROS level in soleus muscle of WT mice (Fig. 3, *A*, *B*, *F*, and *G*), compared with those of the WT-no HP group, while there were no such changes in the Nrf2 KO mice. Taken together, these findings suggest that the deficiency of Nrf2 affected muscular antioxidative responses to the hypoxic exercise stress with or without HP.

mRNA expressions of genes involved in glycogen metabolism and glycogen content. To investigate effects of Nrf2 deficiency and HP on muscular glycogen metabolism, the mRNA expression of two important glycogen metabolism-related genes, *Gbel* and *Phka1*, as well as muscle glycogen content were measured. Nrf2 deficiency strongly reduced muscular mRNA levels of *Gbe1* and *Phka1* compared with those of WT mice in both the HP and no HP groups. Moreover, the WT-HP group had higher expression of muscular glycogen metabolism-related genes than those in the WT-no HP group, while there was no such change between the KO-HP and KO-no HP groups. In addition, there were not any significant differences in muscle glycogen content among the four groups (Fig. 4).

mRNA expression of muscular genes involved in fatty acid metabolism and triglyceride content. To obtain more molecular evidence of the effects of Nrf2 deficiency and HP on the regulatory factors associated with muscular fatty acid metabolism, the mRNA expressions of key enzyme genes involved in fatty acid oxidation (*Cpt1*, *Cpt2*, and *Ucp3*), fatty acid synthetase (*Acly*, *Acaca*, *Fasn*, and *Scd1*), and triglyceride content in skeletal muscle were measured. Nrf2 deficiency downregulated muscular mRNA levels of *Cpt1* and *Cpt2* (Fig. 5, *A* and *B*) and upregulated expression of *Acly*, *Acaca*, *Fasn*, and *Scd1* (Fig. 5, *D–G*) compared with those of WT mice in both the HP and no HP groups. Also, muscle triglyceride content was increased significantly in the KO-no HP group compared with that in the WT-no HP group (Fig. 5*H*). Furthermore, the WT-HP group had higher expression of muscular *Ucp3* than that of the WT-no HP group, and the mRNA expression of *Ucp3* in the Nrf2 KO-HP group was also significantly increased compared with that of the Nrf2 KO-no HP group (Fig. 5*C*); however, it was still remarkably lower than that of the WT-HP group. Therefore, the genetic ablation of Nrf2 affected the muscular fatty acid utilization under the hypoxic exercise stress with HP or without HP.

Mitochondrial adaptation. Nrf2 has been implicated in the regulation of exercise-induced mitochondrial biogenesis and function in skeletal muscle (26, 30, 33), whereas effects of HP on mitochondrial biogenesis markers in skeletal muscle of hypoxic exercised WT and Nrf2 KO mice have not been

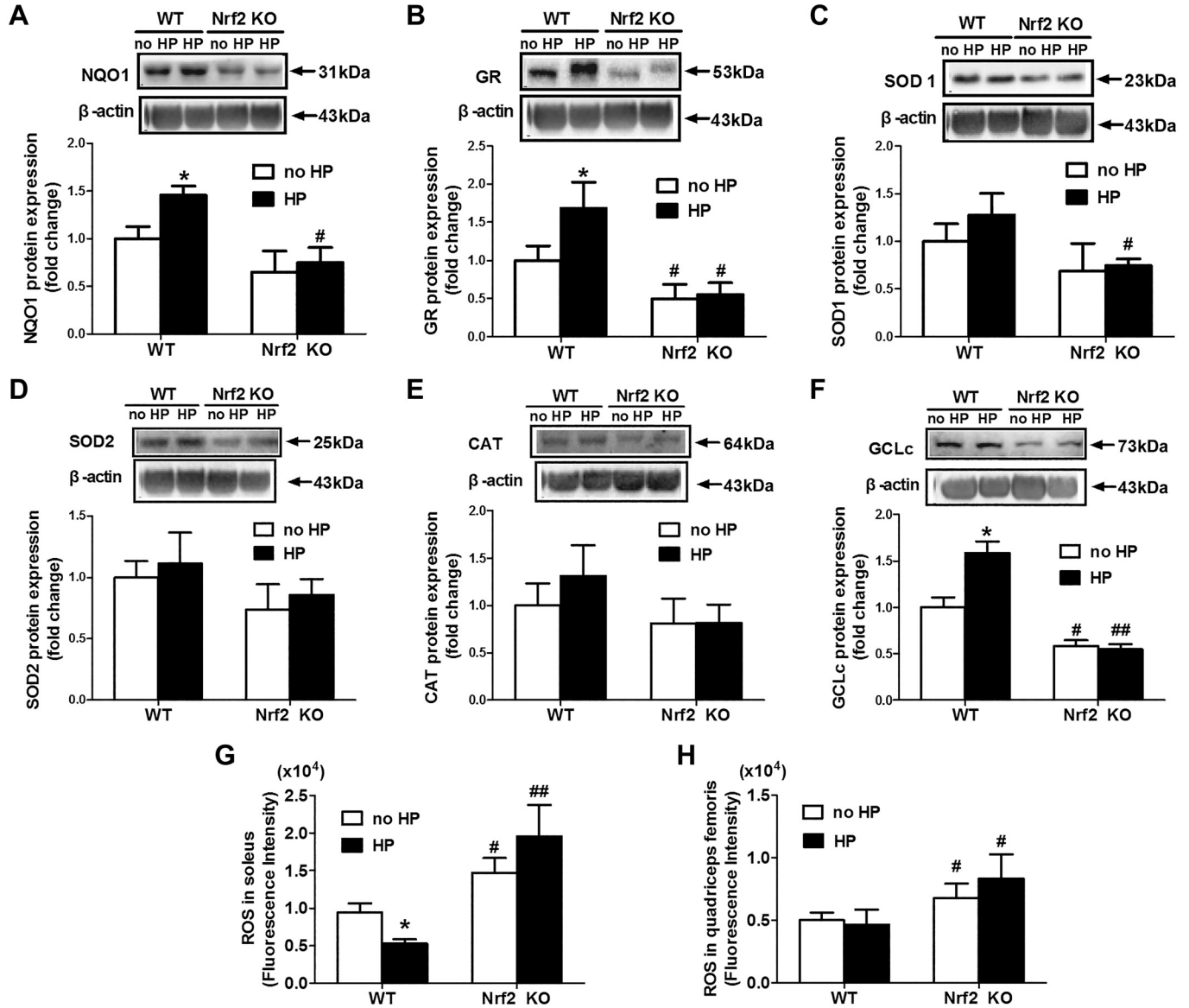

Fig. 3. Effects of HP on the protein expression of muscular genes involved in antioxidation (*A–F*) and reactive oxygen species (ROS) level (*G* and *H*) in skeletal muscle of the exercised WT and Nrf2 KO mice. Values are means ± SE (n = 8–10 animals/group). *$P < 0.05$, HP vs. no HP. #$P < 0.05$, ##$P < 0.01$, Nrf2 KO vs, WT mice.

reported in the literature. Therefore, in addition to muscular antioxidation, glycogen and fatty acid metabolism, we also investigated effects of Nrf2 deficiency and HP on mitochondrial volume (mtDNA/nDNA ratio and citrate synthase activity), the mRNA expression of muscular genes involved in mitochondrial biogenesis, including *Ppargc1a*, *Nrf1*, and *Tfam*, and mitochondrial OXPHOS protein levels. The mtDNA/nDNA ratio and citrate synthase activity are markers of mitochondrial volume (30). Notably, PGC-1α (encoded by *Ppargc1a*) is a crucial factor for the activation of the full program of mitochondrial biogenesis (18). As a transcriptional coactivator, PGC-1α interacts with the transcription factor NRF1 (46). Furthermore, NRF1 increases the transcription of many genes required for mitochondrial biogenesis, such as *Tfam* (47). TFAM (encoded by *Tfam*), as a transcription factor for mitochondrial DNA, is critical for the regulation of mitochondrial DNA and replication (38). Meanwhile, OXPHOS is a major source of ATP production in eukaryotic cells, and it takes place in the inner mitochondrial membrane via five OXPHOS complexes including NADH dehydrogenase (Complexes I; CI), succinate dehydrogenase (Complexes II; CII), ubiquinone cytochrome *c* oxidoreductase (Complexes III; CIII), cytochrome *c* oxidase (Complexes IV; CIV), and ATP synthase (Complexes V; CV) (23). In the present study, the Nrf2 deficiency did not affect mitochondrial DNA copy number, citrate synthase activity, the basal mRNA expression of *Ppargc1a*, *Nrf1*, and *Tfam*, and protein levels of CI, CIII, and CIV, but the expression levels of CII and CV protein were lower than those in skeletal muscle of WT-no HP mice. Moreover, the citrate synthase activity, the expression levels of these measured genes (*Ppargc1a*, *Nrf1*, and *Tfam*) and protein (CI, CII, CIII, and CV) were decreased in the Nrf2 KO-HP group compared

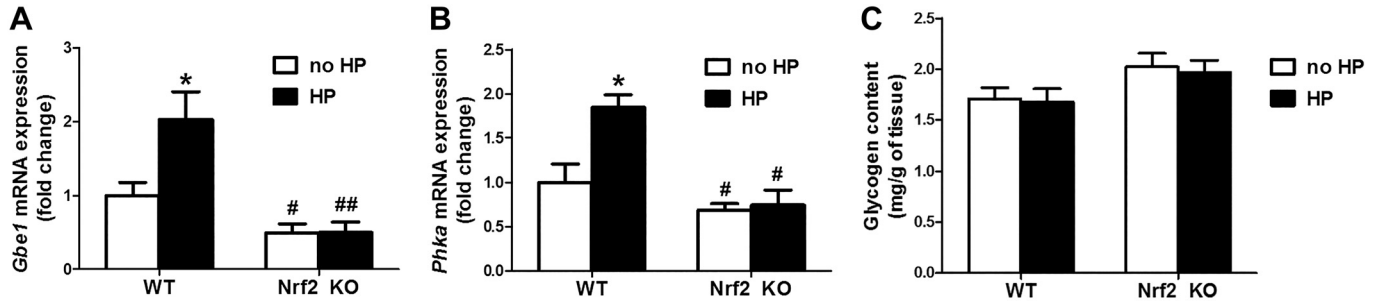

Fig. 4. Effects of HP on the mRNA expressions of genes involved in glycogen (*A* and *B*) and glycogen content (*C*) in skeletal muscle of the exercised WT and Nrf2 KO mice. Values are means ± SE (n = 8–10 animals/group). *$P < 0.05$, HP vs. no HP. #$P < 0.05$, ##$P < 0.01$, Nrf2 KO vs. WT mice.

with those of WT-HP mice (Fig. 6). Thus the influence of Nrf2 on mitochondrial adaptation may be one potential mechanism responsible for the altered muscular respiration and ATP production under HP.

Expression levels of p-AMPKα (Thr172) and p-ACC (Ser79). Immunoblotting revealed the p-AMPKα/AMPKα and p-ACC/ACC ratios were strongly induced under hypoxic exercise with HP, but these inductions were diminished by the concomitant knockout of Nrf2. These results thus demonstrate that AMPK signaling was impaired with consequent inhibitory phosphorylation of ACC, a target for AMPK, to decrease fatty acid oxidation or catabolism in response to acutely increasing the energy level of the skeletal muscle for the stress (Fig. 7).

DISCUSSION

The main findings of this study revealed that the 48-h HP significantly increased the endurance exercise performance in WT mice, but not in Nrf2 KO mice. These results support the hypotheses of the present study. Moreover, new evidence has been found that the increased exercise capacity following HP may be achieved, at least in part, through the Nrf2-mediated

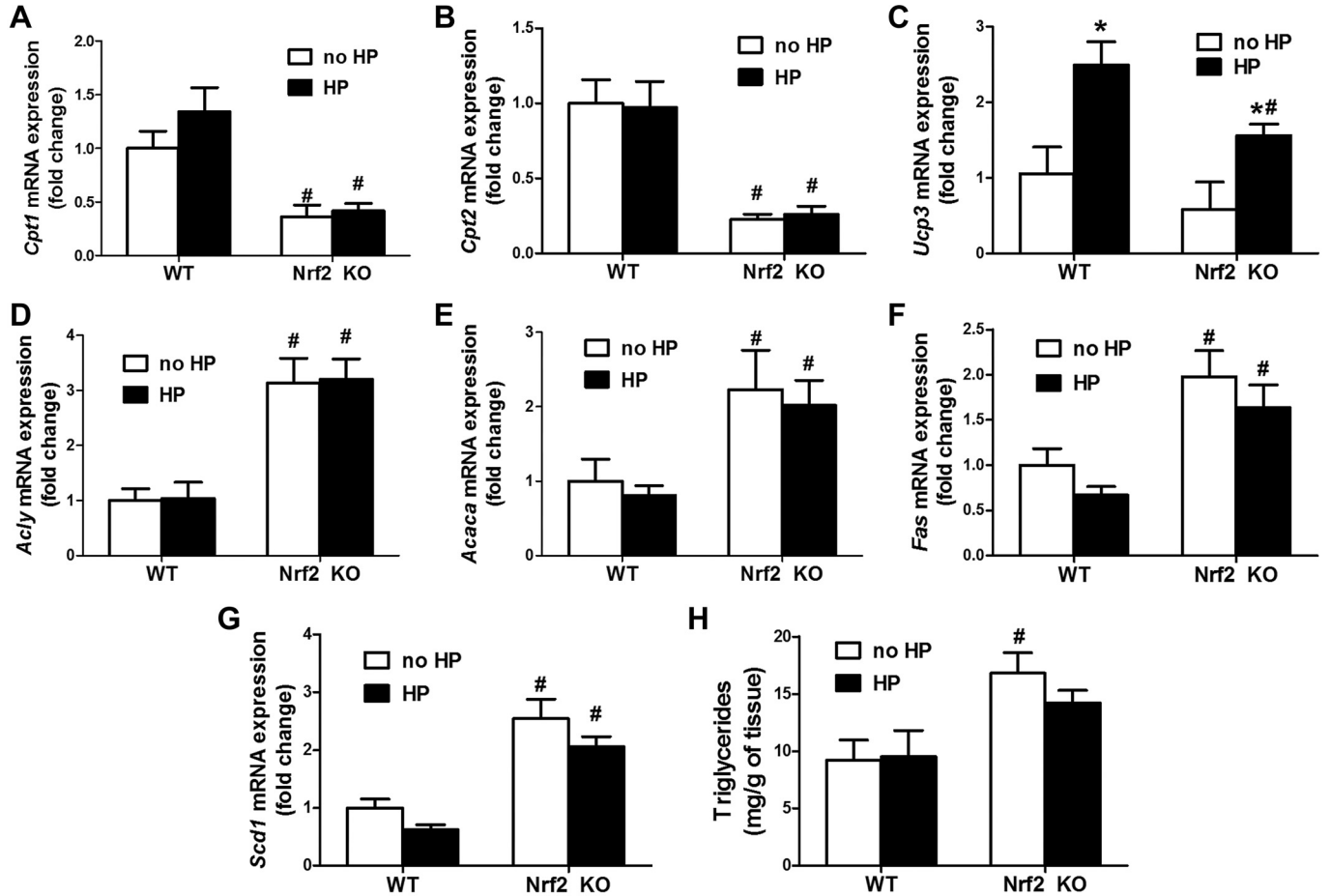

Fig. 5. Effects of HP on mRNA expression of genes involved in fatty acid metabolism (*A*–*G*) and triglyceride content (*H*) in skeletal muscle of the hypoxic exercised WT and Nrf2 KO mice. Values are means ± SE (n = 8–10 animals/group). *$P < 0.05$, HP vs. no HP. #$P < 0.05$, Nrf2 KO vs. WT mice.

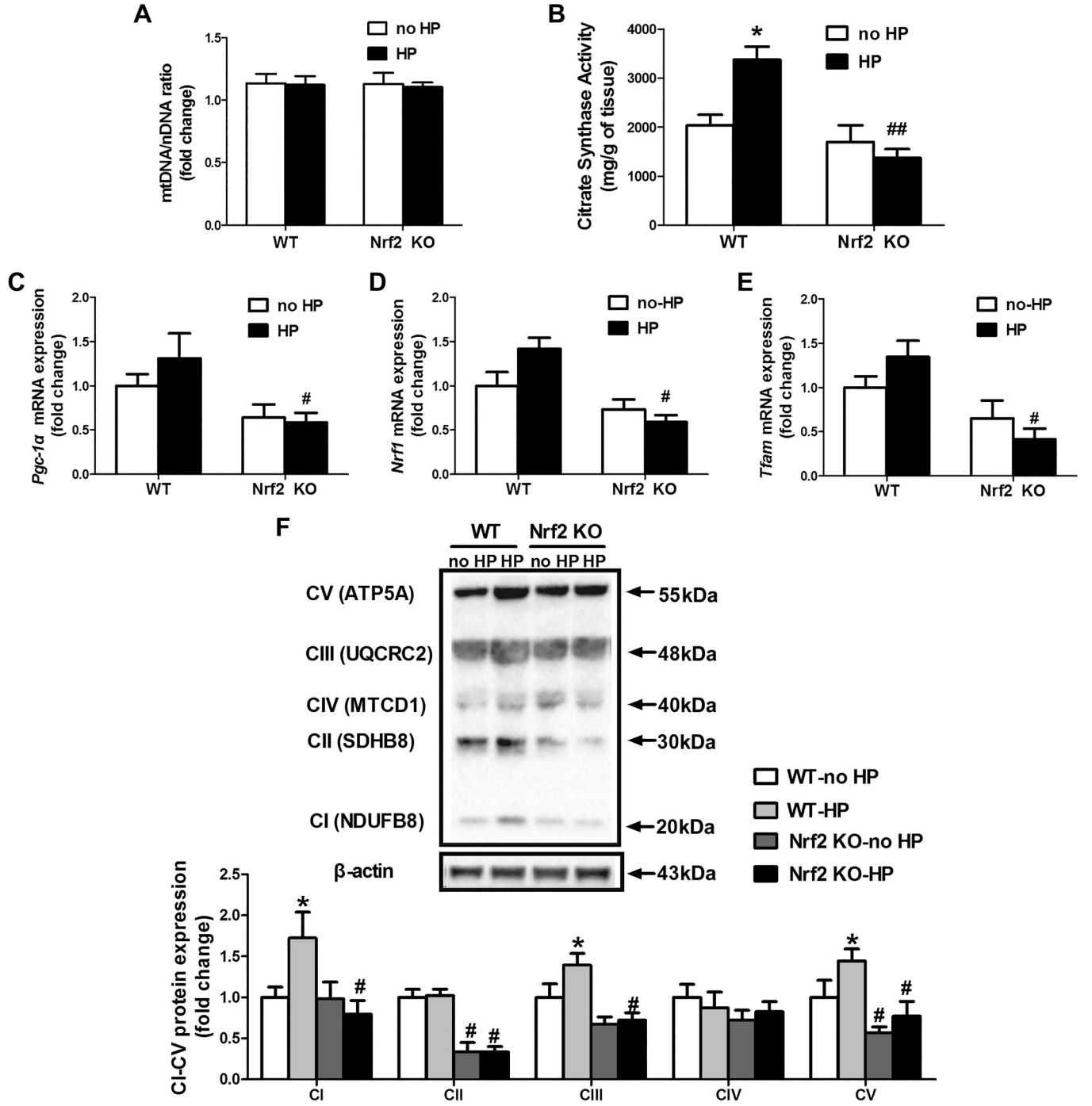

Fig. 6. Effects of HP on mitochondrial DNA copy number (*A*), citrate synthase activity (*B*), mRNA of muscular genes involved in mitochondrial biogenesis (*C–E*), and mitochondrial oxidative phosphorylation (OXPHOS) protein levels (*F*) in WT and Nrf2 KO mice. Values are means ± SE ($n = 8–10$ animals/group). *$P < 0.05$, HP vs. no HP. #$P < 0.05$, Nrf2 KO vs. WT mice.

improvements in antioxidation, the mRNA expression of muscular genes involved in glycogen and fatty acid catabolism, as well as mitochondrial biogenesis, and the protein levels of AMPKα phosphorylation and HIF-1α. To the best of our knowledge, this is the first study which demonstrated the role of Nrf2 in the HP-induced improvement of exercise performance.

Elite endurance athletes usually seek to boost their physical performance by exercising under hypoxic/high-altitude conditions (42), and hypoxia elicits specific molecular responses in skeletal muscles (15). Previous studies have shown that HP leads to skeletal muscle adaptation, which counteracts the hypoxic effects by augmentation of aerobic respiration and mitochondrial biogenesis, protects skeletal muscles from exercise-induced oxidative damage, and enhances endurance performance (43, 44). The present study showed that 48 h of hypoxia exposure as HP could improve WT mice's endurance performance, but not that of Nrf2 KO mice. This evidence

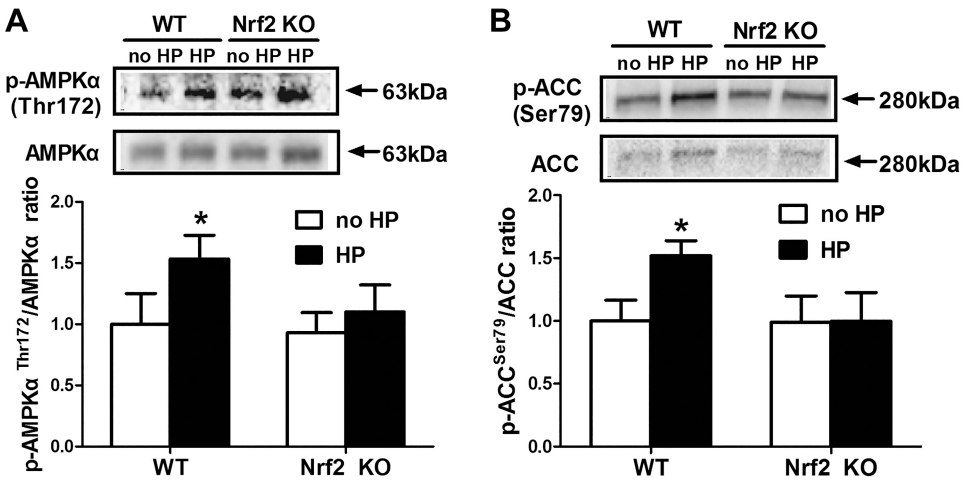

Fig. 7. Effects of HP on the protein levels of p-AMPKα (Thr172; *A*) and p-ACC (Ser79; *B*) in skeletal muscle of the hypoxic exercise WT and Nrf2 KO mice. Values are means ± SE (n = 8–10 animals/group). *$P < 0.05$, HP vs no HP.

implies that Nrf2 signaling may play an important role under the HP condition. In addition, we noticed that the Nrf2 KO-no HP mice displayed similar running distance in the treadmill exercise test to those of their WT counterparts, suggesting the absence of Nrf2 did not hinder running performance. The present outcome did not match the research of Oh et al. (33), which reported that the mice deficient for Nrf2 had a marked reduction in running distance. We speculated that this inconsistency arose from the differences in the endurance exercise test protocol and its determined environment. In the Oh et al. study, the test was determined under normoxia, and an initial running speed of 5 m/min, so that the running distance of Nrf2 KO mice was ~1,358 m. However, in our study the determined test was under hypoxia, and the running distance of Nrf2 KO mice was just only ~600 m, with the initial running speed of 10 m/min. Thus the effect of Nrf2 deficiency on exercise capacity needs to be studied in the future. Moreover, HP significantly increased the protein expression of Nrf2 in the post-hypoxic exercise skeletal muscle in the WT-HP group compared with that of the WT-no HP group, but there was not any significant difference in the protein expression of Keap1 between the two groups. It was speculated that no changes in the expression of Keap1 protein may be associated with the regulatory flexibility in the Nrf2-mediated stress response by conformational cycling of the Keap1-Nrf2 protein complex (4).

Hypoxia or HP enhancing physical performance is related to muscular antioxidative capacity (10, 44). To further investigate the role of Nrf2 in the muscular antioxidative actions of the HP-promoted endurance exercise performance, the expressions of Nrf2 and its downstream antioxidant target genes and ROS levels in skeletal muscle of WT and Nrf2 KO mice with or without HP were measured. The results showed that after the same exercise test, both Nrf2 KO-HP and KO-no HP mice exhibited a significant decrease in most of the mRNA and protein expressions of Nrf2-mediated antioxidative genes and a remarkable increase in the ROS level in skeletal muscle compared with WT mice. Furthermore, the WT-HP group significantly increased the muscular expression of Nrf2 and its target genes, and reduced the ROS level, compared with those of the WT-no HP group, while the KO-HP group did not present the relevant changes in skeletal muscle. These data suggest that with HP, the Nrf2 deficiency-mediated diminishment of muscular antioxidant gene expression may be an important factor to hinder the exercise capacity of the Nrf2 KO mice.

Enhancing physical performance through HP is also closely associated with increased aerobic respiration and mitochondria biogenesis in skeletal muscles (43). Skeletal muscles store glucose as glycogen, which is used to generate glucose metabolites when energy is required; consequently, efficient skeletal muscle glycogen utilization is an important factor in exercise ability and glucose homeostasis (11). The regulation of *Gbe1* and *Phka1* genes is the critical molecular target for improving glycogen utilization in skeletal muscle (50). Moreover, fatty acids are also an important fuel for contracting muscle (19). *Acly*, *Acaca*, *Fasn*, and *Scd1* encode four critical enzymes of fatty acid synthesis: they are ATP-citrate lyase (ACL), acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), and stearoyl CoA desaturase 1 (SCD1), respectively (22, 55, 57). ACL catalyzes citrate to generate acetyl-CoA, which is converted to malonyl-CoA via ACC1, and malonyl-CoA is channeled toward the synthesis of saturated fatty acids through the activity of FAS. Then, saturated fatty acids are converted to unsaturated fatty acids by SCD1. Malonyl-CoA is a potent allosteric inhibitor of carnitine palmitoyl transferase (CPT; encoded by *Cpt1* and *Cpt2*), which mediates the transport of long-chain fatty acids into the mitochondria by binding them to carnitine and is thus considered as the rate-limiting step in the mitochondrial oxidation of long-chain fatty acids (12). In addition, UCP3 has consistently been shown to facilitate skeletal muscle fatty acid oxidation in vitro, as well as in animal models (5, 27).

Nrf2 regulates the expression of many metabolism-related genes independently of its role in regulating the oxidative response (51). It has been found that Nrf2 directly regulates the expression of two important glycogen metabolism-related genes, *Gbe1* and *Phka1*, and their increased expression can reduce muscle glycogen content, resulting in improved glucose tolerance (50). Under oxidative stress, the increased ROS production is counteracted by the Nrf2-dependent transcriptional upregulation of *Ucp3* (1). On the contrary, a lower expression of *Cpt* was found in Nrf2-silenced 293 cells (34) and Nrf2-KO mice (29). Moreover, it has been reported that Nrf2 negatively regulates the gene expressions of *Acly*, *Acaca*, *Fasn*, and *Scd1* (12). Furthermore, Nrf2 is required for the

stresses-induced increases in the mRNA level of mitochondrial biogenesis marker genes, such as *Ppargc1a*, *Nrf1*, and *mtTFA* (30, 49). In the current study, Nrf2 KO mice exhibited attenuated mRNA expression of *Gbe1*, *Phka1*, *Cpt1*, and *Cpt2*, and protein expression of CII and CVI, while increased mRNA expression of *Acly*, *Acaca*, *Fasn*, and *Scd*1 and triglyceride content in skeletal muscle referred to the WT mice. These results were in accordance with those obtained in skeletal muscle-specific Keap1 (Nrf2 negative regulator) knockdown mice (50) and Nrf2 KO mouse livers (29). Although these data suggest that Nrf2 KO-no HP mice might have reduced utilization and catabolism of glycogen and fatty acid in exercised skeletal muscle, they did not exhibit a decrease in running distance compared with WT-no HP (Fig. 1*A*). Endurance exercise capacity is the result of multiple factors, only the changes of above-mentioned indexes were not strong enough to evaluate the overall change in endurance exercise capacity. On the other side, compared with the WT-HP group, the Nrf2 KO-HP mice displayed significant downregulation of *Gbe1*, *Phka1*, *Cpt1*, *Cpt2*, *Ucp3*, *Ppargc1a*, *Nrf1*, and *mtTFA* mRNA levels, and upregulation of *Acly*, *Acaca*, *Fasn*, and *Scd1* expression. These findings indicate that Nrf2 could upregulate energy consumption-related gene expression in skeletal muscle by the Nrf2-dependent transcriptional network under the condition of HP.

AMPK is a metabolic master switch in the conditions of hypoxia, exhausting exercise, and caloric restriction (28, 48). When activated, it turns off several anabolic processes while turning on catabolic processes (54). For example, activated AMPK would regulate its downstream target, such as ACC (36) and PGC-1α (45). However, the reduction in AMPK activity would remove the inhibitory phosphorylation (at Ser79) of ACC, which resulted in the high ACC activity and might ultimately increase the levels of the ACC product, i.e., malonyl-CoA, thus decreased fatty acid oxidation. Such outcomes were in agreement with a study of high fat-fed Nrf2 KO mice (29). High Nrf2 activity either by Nrf2 chemical inducers (2, 41) or by Keap1-knockdown (56) can increase AMPK phosphorylation in mouse livers. This statement is supported by the present study; we found impaired muscular AMPK signaling in the Nrf2 KO-HP group compared with that in the WT-HP mice. In addition, it has been shown that the activation of Nrf2 also induces the mRNA expression of *Ppargc1a* (encoding PGC-1α) in human fibroblasts and mouse livers (6, 49). Therefore, this was not surprising that, compared with the WT-HP group, the Nrf2 KO-HP group displayed an attenuated upregulation of *Ppargc1a* mRNA expression and its downstream mitochondrial OXPHOS protein levels (CI, CII, CIII, and CV) after hypoxic exercise. These observations suggest that with HP, the AMPK signaling effect is significantly weaker when Nrf2 is deleted, whereas it is stronger when Nrf2 activity is constitutively high.

HIF-1 is a master regulator of several genes that are primarily responsible for systemic and muscular adaptation to hypoxia by enhancing physiological attributes like erythropoiesis, angiogenesis, glucose uptake, and metabolism (53). In skeletal muscle, these physiological adjustments can lead to the increases in oxygen delivery and metabolite utilization, and then enhance endurance performance (58). There is mounting evidence that Nrf2 signaling plays a role in activating and sustaining the HIF-1 response. Several studies have shown that knockdown of Nrf2 is enough to decrease HIF-1α at the posttranslational level (21, 25); however, these results were mainly from the experiments in cancer cells. To further assess the role of Nrf2 in normal muscular HIF-1 changes at the posttranslational level with or without HP, we identified the muscular protein expression of HIF-1α in WT and Nrf2 KO mice. Western blotting revealed that, after the hypoxic exercise with HP, the muscular protein expression of HIF-1α of WT mice was significantly increased compared with that of the WT-no HP group, but Nrf2 KO mice did not make such a change. The data indicated that hypoxic exercise with 48-h HP stimulates the protein expression of HIF-1α in mouse skeletal muscle, which is similar to our previous report (20), but under the same HP, the deficiency of Nrf2 displayed a remarkable inhibition of HIF-1α protein expression.

In conclusion, HP significantly enhanced the endurance exercise performance of WT mice under the hypoxia condition, potentially by upregulating Nrf2, HIF-1α, AMPK signaling, and the expression of genes involved in antioxidant, glycogen and fatty acid metabolism, and mitochondria biogenesis. Interestingly, almost all these effects were blunted or even abolished in Nrf2-KO mice, accompanied by increased oxidative stress upon HP. Taken together, our results suggest that Nrf2 signaling plays an essential role in enhanced endurance exercise performance by HP.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.W., S. Yang, and Y.Z. conceived and designed research; L.W., S. Yang, L.Y., and H.W. performed experiments; L.W. analyzed data; L.W., J.W. and Y.Z. drafted manuscript; J.W., S. Yu, A.-N.T.K., and Y.Z. edited and revised manuscript; L.W., S. Yang, L.Y., H.W., J.W., S. Yu, A.-N.T.K., and Y.Z. approved final version of manuscript.

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