Title page

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

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Running Head: Hypoxia preconditioning and Nrf2

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ABSTRACT

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 the cellular redox homoeostasis 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 pre-conditioned to 48 hours 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 KO and WT mice were exposed to normoxia or hypoxia for 48 hours 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 absence of Nrf2 did not affect the exercise capacity, though the mRNA expression of certain muscular genes involved in antioxidant, glycogen and fatty acid catabolism was decreased in Nrf2 KO mice. However, the 48-hour 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 the HP promotes exercise capacity of mice with the participation of Nrf2 signal in skeletal muscle.

NEW & NOTEWORTHY Hypoxia preconditioning (HP) activated 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-hour HP. Our results indicate that Nrf2 plays an essential role in the exercise

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45 capacity-enhancing effect of HP, possibly by modulating muscular antioxidative responses, the
46 mRNA expression of muscular genes involved in glycogen and fatty acid metabolism, as well as
47 mitochondrial biogenesis, and through the crosstalk with AMPK and HIF-1α signaling.

48

49 INTRODUCTION

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

59 Nuclear factor erythroid 2-related factor 2 (Nrf2, also called Nfe2l2) is activated under
60 oxidative stress, binds to the antioxidant response element (ARE) in the 5'-promoter region of
61 cytoprotective genes, and then increases the expression levels of antioxidant and detoxification
62 genes, such as catalase (*Cat*), NAD(P)H: quinone oxidoreductase 1 (*Nqo1*), and heme oxygenase-1
63 (*Hmox1*), which prepare the cells to withstand oxidative stress (17). Besides mediating
64 stress-stimulated induction of antioxidant genes, accumulating evidence has indicated that Nrf2
65 may also influence substance metabolism and mitochondrial biogenesis. Chromatin
66 immunoprecipitation analysis (50) has demonstrated that Nrf2 binds the upstream promoter

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67 regions of 1,4-a-glucan branching enzyme 1 (*Gbe1*) and phosphorylase kinase regulatory subunit
68 a1 (*Phka1*), which encode glycogen branching enzyme (GBE) (35) and phosphorylase kinase
69 alpha M subunit (7), respectively, the key enzymes of glycogen branching and breakdown in
70 skeletal muscle. In the absence of Nrf2, fatty acid oxidation is suppressed and may lead to the
71 lower ATP levels and mitochondrial dysfunction (14). It has been reported that uncoupling protein
72 3 (UCP3; encoded by *Ucp3*) is a regulator of fatty acid export and helps to maintain muscular fat
73 oxidative capacity (40) and the promoter region of *Ucp3* contains a Nrf2 binding site (1). In
74 addition, nuclear respiratory factor 1 (NRF1; encoded by *Nrf1*) is implicated in the control of
75 nuclear genes required for respiration, mitochondrial DNA transcription and replication (38), and
76 the promoter region of *Nrf1* also contains a Nrf2 binding site and Nrf2 activation can induce its
77 transcription (37). Therefore, Nrf2 may have an important role in skeletal muscle contractile and
78 mitochondrial function (8).

79 We have investigated the effects of acute hypoxia exposure (11.2% oxygen concentration)
80 with different durations (0-48 hours) on the activation of Nrf2-ARE pathway in the C57BL/6J
81 mice. The results showed that the 48-hour hypoxia exposure significantly increased the activation
82 of Nrf2, but not the shorter exposures (20). To continue our research, in the present study, we
83 applied the 48-hour hypoxia exposure as a hypoxia preconditioning (HP) to both Nrf2 knockout
84 (KO) and wild-type (WT) mice and aimed to evaluate the effects of the HP and Nrf2 on exercise
85 capacity, and the exercise-induced changes of antioxidant reaction, substance metabolism and
86 mitochondrial function in skeletal muscle. To date, there has been no report regarding the
87 application of HP to pre-treat the exercised Nrf2 KO mice in the literature. We hypothesized that
88 the deficiency of Nrf2 would impair antioxidant and metabolic adaptations of skeletal muscle to

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89 exercise. Furthermore, the exercise with the HP would upregulate antioxidant reaction and
90 metabolism in skeletal muscle, as well as the exercise capacity of WT mice; while the genetic
91 defect of Nrf2 would diminish these effects.

92 **METHODS**

93 *Animal care*

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

97 *Animals*

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

104 *HP*

105 The HP was achieved by placing the mice in a normobaric chamber (210 cm long, 200 cm
106 wide, and 200 cm high). The chamber was infused with hypoxic air through an air compressor and
107 a nitrogen synthesizing machine, which could reduce the oxygen concentration in the chamber to
108 11.2% (at about simulated altitude of 4500 m) based on the previous work (20). The oxygen
109 concentration in the chamber was monitored throughout the experimental period with an oxygen
110 sensor. The HP was performed for 48 hours.

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111 *Endurance exercise capacity*

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

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

125 *Quantitative PCR analysis*

126 Total RNA was isolated from crushed extensor digitorum longus muscle using TRIzol
127 reagent (life technologies, USA) following the manufacturer's instructions. Real-time PCR was
128 performed in an ABI 7500 Real-time PCR System (Thermo Scientific, Inc., Waltham, MA, USA)
129 using the SYBR Green Real-time PCR Master Mix kit (Toyobo Co., Ltd, Osaka, Japan) with the
130 previously synthesized cDNA (FSQ-101; Toyobo Co., Ltd) as template in a 20 μL reaction volume.
131 The following commercial primers from Qiagen (Germany) were used: *Nqo1* (QT00094367),
132 glutathione reductase (*Gr*; QT01758232), superoxide dismutase 1 (*Sod1*; QT00165039),

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133 superoxide dismutase 2 (*Sod2*; QT00161707), *Cat* (QT01058106), glutamate-cysteine ligase
134 catalytic subunit (*Gclc*; QT00130543), glutathione peroxidase1 (*Gpx1*; QT01195936), *Hmox1*
135 (QT00159915), *Gbe1* (QT00252924), *Phka1* (QT00143514), mitochondrial uncoupling protein 3
136 (*Ucp3*; QT00115339), mitochondrial transcription factor A (*Tfam*; QT00154413), *Nrf1*
137 (QT01051820), and 18S ribosomal RNA (*Rn18s*; QT010036875). In addition, the primer
138 sequences of carnitine palmitoyl transferase 1,2 (*Cpt1, Cpt2)*, peroxisome proliferator-activated
139 receptor gamma coactivator 1-alpha (*Ppargc1a*), ATP-citrate lyase (*Acly*), acetyl-CoA carboxylase
140 1 (*Acaca*), fatty acid synthase (*Fasn*) and stearoyl CoA desaturase (*Scd1*) were listed in the
141 Supplementary Table S1 [DOI: https://doi.org/10.6084/m9.figshare.9630701.
142 https://figshare.com/articles/Supplementary_table_1_Mouse_quantitative_PCR_primer_sequences_docx/9630701]
143 and these primers were synthesized by Invitrogen Trading (Shanghai, China) Co., Ltd. The Rn18s
144 gene is a reliable internal control for comparative analyses of transcription under hypoxia (32),
145 which was assessed using software (ABI 7500RT PCR). The difference in expression between
146 control and experimental samples was calculated using the $2^{-\triangle\triangle Ct}$ method, as described previously
147 (24).

148 *Western blotting*

149 Total proteins were isolated from the extensor digitorum longus muscles using RIPA protein
150 extraction reagents (P0013B; Beyotime, Inc. Beijing). Protein concentration was measured using
151 the BCA protein assay kit (Pierce 23225; Thermo Fisher Scientific, Inc.). 20 μg proteins were
152 separated on Bolt 4–12% Bis-Tris PlusGels (NW04125BOX; Thermo Fisher Scientific, Inc.) by
153 electrophoresis, and the fractionated proteins were subsequently transferred to a nitrocellulose
154 membrane using iBlot Gel Transfer Stacks Nitrocellulose (IB23001; Thermo Fisher Scientific,

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155 Inc.). The blots were probed using the following antibodies: Nrf2 (sc-722;), hypoxia-inducible
156 factor-1 alpha (HIF-1α; sc-10790), Kelch-like ECH-associated protein 1 (Keap1; sc-33569),
157 NQO1 (sc-16464), GR (sc-133245), SOD1 (sc-11407), SOD2 (sc-30080), CAT (sc-50508), GCLc
158 (sc-22755), AMP-activated protein kinase α (AMPKα; sc-74461) and β-actin (sc-477778),
159 above-mentioned antibodies were all from Santa Cruz Biotechnology, USA.
160 Thr172-phosphorylated (p)-AMPKα (#2535; Cell Signaling Technology, Inc., USA), acetyl-CoA
161 carboxylase (ACC; #3662, Cell Signaling Technology, Inc., USA), Ser79-p-ACC (#3661; Cell
162 Signaling Technology, Inc., USA), Total oxidative phosphorylation (OXPHOS) complexes rodent
163 WB antibody cocktail (ab110413; Abcam Trading Shanghai Company Ltd.). The density of
164 protein bands was analyzed using Bio-Rad imaging software (Bio-Rad Laboratories, Hercules, CA,
165 USA). The individual values were originally expressed as a ratio of a standard (β-actin content)
166 and then expressed as a fold change of the control group value.

167 *Reactive oxygen species (ROS) generation*

168 According to the manufacturer's instructions of the kit (GMS10016.3; GENMED, Shanghai,
169 China), 50 mg soleus and quadriceps femoris muscles were homogenized with Reagent C in the
170 kit, respectively. The supernatants were used to yield the ROS samples (2 μg protein/μl). These
171 steps were performed at 4°C.

172 Then ROS samples were incubated with the chloromethyl derivative of
173 (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; H_2DCFDA) (CM-H_2DCFDA)
174 at 37°C for 20 minutes in the 96-well plates and the ROS levels were detected by a fluorescence
175 plate reader at λexc: 490 nm and λem: 520 nm (Bio Tek Synergy H1, Bio Tek Instruments, Inc.,
176 USA).

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177 *Mitochondrial DNA copy number*

178 Genomic DNA in quadriceps femoris muscles was extracted using a TIANamp Genomic
179 DNA kit (Tiangen, Beijing, China) according to the kit protocol. Using the DNA, the relative copy
180 numbers of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) were determined by
181 quantitative PCR as described (52)

182 The following primers (Invitrogen, Shanghai, China) were used in this assessment: mtDNA
183 (F, 5'-CCCTAAAACCCGCCACATCT-3'; R, 5'-GAGCGATGGTGAGAGCTAAGGT-3') or
184 nDNA (F, 5'-CGAGTCGTCTTT CTCCTGATGAT-3'; R, 5'-TTCTGGATTCCAA
185 TGCTTCGA-3') (33).

186 *Citrate synthase activity*

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

191 *Muscle glycogen and triglyceride contents*

192 Quadriceps femoris muscles were used for the measurements of glycogen and triglyceride
193 contents. The muscle glycogen and triglyceride contents were determined using the commercial
194 Assay Kits BC0345 and BC0625 (Solarbio, Beijing, China).

195 *Statistics*

196 All values are reported as the mean ± standard error (SE). Statistical calculations were
197 performed using SPSS STATISTICS v. 19 software (IBM Corp., USA). Data were analyzed using

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198 a two-way ANOVA (strain x HP). When a significant interaction effect was obtained, simple main

199 effect analysis with the post hoc LSD test was performed to identify significant mean differences

200 between groups. Statistical significance was set at $p < 0.05$.

201

202 **RESULTS**

203 *Exercise performance, HIF-1α, Nrf2, and Keap1 protein levels*

204 There was no significant difference in running distance and expression of HIF-1α protein

205 between the WT-no HP and KO-no HP groups. However, significant increases in running distance,

206 and expression of HIF-1α protein were observed in the WT-HP group, when compared to those of

207 the WT-no HP group. Moreover, the shorter running distance and the lower expression of HIF-1α

208 protein were shown in the KO-HP group, compared to those of the WT-HP group (Fig.1.A and B).

209 These results demonstrated that the HP significantly improved the exercise capacity and muscular

210 HIF-1α protein level after the hypoxic exercise in normal WT mice, but had no effects in Nrf2 KO

211 mice. The HP significantly increased the protein expression of Nrf2 in the post-hypoxic exercise

212 skeletal muscle in WT-HP group, compared with that of WT-no HP group (Fig.1.C). In addition,

213 there was no significant change in Keap1 protein between the WT-no HP and WT-HP groups.

214 There was also no significant difference in Keap1 protein between the WT-no HP and KO-no HP

215 groups or between the WT-HP and KO-HP groups (Fig.1.D).

216

217 *The mRNA expression of muscular genes involved in antioxidation*

218 The mRNA expressions of almost all measured antioxidative genes were significantly lower

219 in Nrf2 KO mice of both HP and no-HP groups, compared to those of the WT mice. Moreover, the

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220 exercise with the HP produced a significant increase in most of the mRNA expressions of
221 Nrf2-mediated antioxidative genes (*Nqo1*, *Gr, Gclc*, *Gpx1* and *Hmoxo1*) in skeletal muscle of WT
222 mice, compared with those of the WT-no HP group; while there were no such significant changes
223 in Nrf2 KO mice (Fig.2.).

224 *The protein expression of muscular genes involved in antioxidation and ROS level*

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

234 *The mRNA expressions of genes involved in glycogen metabolism and glycogen content*

235 To investigate effects of Nrf2 deficiency and the HP on muscular glycogen metabolism, the
236 mRNA expression of two important glycogen metabolism-related genes, *Gbel and Phka1,* as well
237 as muscle glycogen content were measured. Nrf2 deficiency strongly reduced muscular mRNA
238 levels of *Gbe1* and *Phka1*, compared with those of WT mice, in both HP and no HP groups.
239 Moreover, the WT-HP group had higher expression of muscular glycogen metabolism-related
240 genes than those of WT-no HP group, while there was no such change between KO-HP and

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241 KO-no HP groups. In addition, there were not any significant differences in muscle glycogen
242 content among the four groups (Fig.4.)

243 *The mRNA expression of muscular genes involved in fatty acid metabolism and triglyceride*
244 *content*

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

258 *Mitochondrial adaptation*

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

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262 reported in the literature. Therefore, in addition to the muscular antioxidation, glycogen and fatty
263 acid metabolism, we also investigated effects of Nrf2 deficiency and the HP on mitochondrial
264 volume (mtDNA/nDNA ratio and citrate synthase activity), the mRNA expression of muscular
265 genes involved in mitochondrial biogenesis including *Ppargc1a, Nrf1* and *Tfam,* and
266 mitochondrial OXPHOS protein levels. The mtDNA/nDNA ratio and citrate synthase activity are
267 markers of mitochondrial volume (30). Notably, PGC-1α (encoded by *Ppargc1a*) is a crucial
268 factor for the activation of the full program of mitochondrial biogenesis (18). As a transcriptional
269 coactivator, PGC-1α interacts with transcription factor NRF1 (46). Furthermore, NRF1 increases
270 the transcription of many genes required for mitochondrial biogenesis, such as *Tfam* (47). TFAM
271 (encoded by *Tfam*) as a transcription factor for mitochondrial DNA, is critical for the regulation of
272 mitochondrial DNA and replication (38). Meanwhile, OXPHOS is a major source of ATP
273 production in eukaryotic cells, and it takes place in the inner mitochondrial membrane via five
274 OXPHOS complexes including NADH dehydrogenase (Complexes I; CI), succinate
275 dehydrogenase (Complexes II; CII), ubiquinone cytochrome c oxidoreductase (Complexes III;
276 CIII), cytochrome c oxidase (Complexes IV, CIV) and ATP synthase (Complexes V, CV) (23). In
277 the present study, the Nrf2 deficiency did not affect mitochondrial DNA copy number, citrate
278 synthase activity, the basal mRNA expression of *Ppargc1a, Nrf1* and *Tfam*, and protein levels of
279 CI, CIII and CIV, but the expression levels of CII and CV protein were lower than those in
280 skeletal muscle of WT-no HP mice. Moreover, the citrate synthase activity, the expression levels
281 of these measured genes (*Ppargc1a, Nrf1* and *Tfam*) and protein (CI, CII, CIII and CV) were
282 decreased in Nrf2 KO-HP group, compared with those of WT-HP mice (Fig.6.). Thus, the
283 influence of Nrf2 on mitochondrial adaptation may be one potential mechanism responsible for

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284 the altered muscular respiration and ATP production under HP.

285 *The expression levels of p-AMPKα (Thr172) and p-ACC (Ser79)*

286 Immunoblotting revealed the p-AMPKα/AMPKα and p-ACC/ACC ratios were strongly

287 induced under the hypoxic exercise with HP, but these inductions were diminished by the

288 concomitant knockout of Nrf2. These results thus demonstrate that AMPK signaling was impaired

289 with consequent inhibitory phosphorylation of ACC, a target for AMPK, to decrease fatty acid

290 oxidation or catabolism in response to acutely increase the energy level of the skeletal muscle for

291 the stress.

292

293 **DISCUSSION**

294 The main findings of this study revealed that the 48-hour HP significantly increased the

295 endurance exercise performance in WT mice, but not in Nrf2 KO mice. These results support the

296 hypotheses of the present study. Moreover, new evidence has been found that the increased

297 exercise capacity following HP may be achieved, at least in part, through the Nrf2-mediated

298 improvements in antioxidation, the mRNA expression of muscular genes involved in glycogen and

299 fatty acid catabolism, as well as mitochondrial biogenesis, and the protein levels of AMPKα

300 phosphorylation and HIF-1α. To the best of our knowledge, this is the first study which

301 demonstrated the role of Nrf2 in the HP-induced improvement of exercise performance.

302 Elite endurance athletes usually seek to boost their physical performance by exercise under

303 hypoxic/high altitude conditions (42) and hypoxia elicits specific molecular responses in skeletal

304 muscles (15). Previous studies have shown that HP leads to skeletal muscle adaptation, which

305 counteracts the hypoxic effects by augmentation of aerobic respiration and mitochondrial

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306 biogenesis, protects skeletal muscles from exercise-induced oxidative damage, and enhances
307 endurance performance (43, 44). The present study showed that 48 hours of hypoxia exposure as a
308 HP could improve WT mice's endurance performance, but not in Nrf2 KO mice. This evidence
309 implies that Nrf2 signaling may play an important role under the HP condition. In addition, we
310 noticed that the Nrf2 KO-no HP mice displayed similar running distance in the treadmill exercise
311 test to those of their WT counterparts, suggesting the absence of Nrf2 did not hinder the running
312 performance. The present outcome did not match the research of Oh et al (33), which reported that
313 the mice deficient for Nrf2 had a marked reduction in running distance. We speculated that this
314 inconsistency arose from the differences of endurance exercise test protocol and its determined
315 environment. In Oh et al. study, the test was determined under normoxia, and an initial running
316 speed of 5 m/min; so that the running distance of Nrf2 KO mice was about 1358 m. However, in
317 our study the determined test was under hypoxia, and the running distance of Nrf2 KO mice was
318 just only about 600 m with the initial running speed of 10 m/min. Thus, the effect of Nrf2
319 deficiency on exercise capacity needs to be studied in the future. Moreover, the HP significantly
320 increased the protein expression of Nrf2 in the post-hypoxic exercise skeletal muscle in WT-HP
321 group, compared with that of WT-no HP group, but there was not any significant difference in the
322 protein expression of Keap1 between the two groups. It was speculated that no changes in the
323 expression of Keap1 protein may be associated with the regulatory flexibility in the Nrf2-mediated
324 stress response by conformational cycling of the Keap1-Nrf2 protein complex (4).

325 Hypoxia or HP enhancing physical performance is related to muscular antioxidative capacity
326 (10, 44). To further investigate the role of Nrf2 in the muscular anti-oxidative actions to the HP-
327 promoted endurance exercise performance, the expressions of Nrf2 and its downstream

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328 antioxidant target genes, and ROS level in skeletal muscle of WT and Nrf2 KO mice with or
329 without HP were measured. The results showed that after the same exercise test, both Nrf2
330 KO-HP and KO-no HP mice exhibited a significant decrease in most of the mRNA and protein
331 expressions of Nrf2-mediated antioxidative genes and a remarkable increase in ROS level in
332 skeletal muscle, compared to their WT mice. Furthermore, the WT-HP group significantly
333 increased the muscular expression of Nrf2 and its target genes, and reduced ROS level, compared
334 with those of the WT-no HP group; while the KO-HP group did not present the relevant changes
335 in skeletal muscle. These data suggest that with the HP, the Nrf2 deficiency-mediated
336 diminishment of muscular antioxidant gene expression may be an important factor to hinder the
337 exercise capacity of the Nrf2 KO mice.

338 Enhancing physical performance through HP is also closely associated with increased aerobic
339 respiration and mitochondria biogenesis in skeletal muscles (43). Skeletal muscles store glucose as
340 glycogen, which is used to generate glucose metabolites when energy is required; consequently,
341 efficient skeletal muscle glycogen utilization is an important factor of exercise ability and glucose
342 homeostasis (11). The regulation of *Gbe1* and *Phka1* genes is the critical molecular target for
343 improving glycogen utilization in skeletal muscle (50). Moreover, fatty acids are also an important
344 fuel for contracting muscle (19). *Acly, Acaca, Fasn and Scd1* encode four critical enzymes of fatty
345 acid synthesis, they are ATP-citrate lyase (ACL), acetyl-CoA carboxylase 1 (ACC1), fatty acid
346 synthase (FAS) and stearoyl CoA desaturase 1 (SCD1), respectively (22, 55, 57). ACL catalyzes
347 citrate to generate acetyl-CoA, which is conversed to malonyl-CoA via ACC1, and malonyl-CoA
348 is channeled towards the synthesis of saturated fatty acids, through the activity of FAS. Then,
349 saturated fatty acids are conversed to unsaturated fatty acids by SCD1. Malonyl-CoA is a potent

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350 allosteric inhibitor of carnitine palmitoyl transferase (CPT, encoded by *Cpt1 and Cpt2*), which
351 mediates the transport of long-chain fatty acids into the mitochondria by binding them to carnitine,
352 and is thus considered as the rate-limiting step in the mitochondrial oxidation of long-chain fatty
353 acids (12). In addition, UCP3 has consistently been shown to facilitate skeletal muscle fatty acid
354 oxidation *in vitro,* as well as in animal models (5, 27).

355 Nrf2 regulates the expression of many metabolism-related genes independently of its role in
356 regulating the oxidative response (51). It has been found that Nrf2 directly regulates the
357 expression of two important glycogen metabolism-related genes, *Gbe1* and *Phka1*, and their
358 increased expression can reduce muscle glycogen content, resulting in improved glucose tolerance
359 (50). Under oxidative stress, the increased ROS production is counteracted by the Nrf2-dependent
360 transcriptional upregulation of *Ucp3* (1). On the contrary, a lower expression of *Cpt* was found in
361 Nrf2 silenced 293 cell (34) and Nrf2-KO mice (29). Moreover, it has been reported that Nrf2
362 negatively regulates the gene expressions of *Acly, Acaca, Fasn* and *Scd1* (12). Furthermore, Nrf2
363 is required for the stresses-induced increases in mRNA level of mitochondrial biogenesis marker
364 genes, such as *Ppargc1a, Nrf1* and *mtTFA* (30, 49). In the current study, Nrf2 KO mice exhibited
365 attenuated mRNA expression of *Gbe1*, *Phka1 Cpt1* and *Cpt2*, and protein expression of CII and
366 CVI, while increased mRNA expression of *Acly, Acaca, Fasn* and *Scd*1, and triglyceride content
367 in skeletal muscle, referred to the WT mice. These results were in accordance with those obtained
368 in skeletal muscle-specific Keap1 (Nrf2 negative regulator) knockdown mice (50) and Nrf2 KO
369 mouse liver (29). Although these data suggest that Nrf2 KO-no HP mice might have reduced
370 utilization and catabolism of glycogen and fatty acid in exercised skeletal muscle, they did not
371 exhibit a decrease in running distance compared with WT-no HP (Fig.1.A). Endurance exercise

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372 capacity is the results of multiple factors, only the changes of above-mentioned indexes were not
373 strong enough to evaluate the overall change in endurance exercise capacity. On the other side,
374 compared with the WT-HP group, the Nrf2 KO-HP mice displayed significant downregulation of
375 *Gbe1*, *Phka1, Cpt1, Cpt2, Ucp3, Ppargc1a, Nrf1,* and *mtTFA* mRNA levels, and upregulation of
376 *Acly, Acaca, Fasn* and *Scd1* expression. These findings indicate that Nrf2 could upregulate energy
377 consumption-related gene expression in skeletal muscle by Nrf2-dependent transcriptional
378 network under the condition of HP.

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

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394 observations suggest that with the HP, the AMPK signaling effect is significantly weaker when
395 Nrf2 is deleted; whereas it is stronger when Nrf2 activity is constitutively high.

396 Hypoxia inducible factor-1 (HIF-1) is a master regulator of several genes that are primarily
397 responsible for systemic and muscular adaptation to hypoxia by enhancing physiological attributes
398 like erythropoiesis, angiogenesis, glucose uptake and metabolism (53). In skeletal muscle, these
399 physiological adjustments can lead to the increases in oxygen delivery and metabolite utilization,
400 and then enhance endurance performance (58). There is mounting evidence that Nrf2 signaling
401 plays a role in activating and sustaining the HIF-1 response. Several studies have shown that
402 knockdown of Nrf2 is enough to decrease HIF-1α at the post-translational level (21, 25); however,
403 these results were mainly from the experiments on cancer cells. To further assess the role of Nrf2
404 in normal muscular HIF-1 changes at the post-translational level with or without HP, we identified
405 the muscular protein expression of HIF-1α in WT and Nrf2 KO mice. The western blotting
406 revealed that, after the hypoxic exercise with the HP, the muscular protein expression of HIF-1α
407 of WT mice was significantly increased, compared with that of the WT-no HP group; but Nrf2
408 KO mice did not make such a change. The data indicated that the hypoxic exercise with the
409 48-hour HP stimulates the protein expression of HIF-1α in mouse skeletal muscle, which is similar
410 to our previous report (20); but under the same HP, the deficiency of Nrf2 displayed a remarkable
411 inhibition of HIF-1α protein expression.

412 In conclusion, HP significantly enhanced the endurance exercise performance of WT mice
413 under hypoxia condition, potentially by up-regulating Nrf2, HIF-1α, AMPK signaling and the
414 expression of genes involved in antioxidant, glycogen and fatty acid metabolism and mitochondria
415 biogenesis. Interestingly, almost all these effects were blunted or even abolished in Nrf2-KO mice,

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416 accompanied by increased oxidative stress upon HP. Taken together, our results suggest that Nrf2

417 signaling plays an essential role in enhanced endurance exercise performance by HP.

418

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423

424 **DISCLOSURES**

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

426

427 **AUTHER CONTRIBUTIONS**

428 L.W., S.Y., and Y.Z. designed the research; L.W., S.Y., L.Y., and H.W. performed the

429 experiments. L.W. analyzed the data and visualization. Y.Z. contributed reagents/materials, and

430 Y.Z., J.W., S.Y., and A.T.K. wrote and revised the paper.

431

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595

596 **Figure legends**

597 Fig.1. Effects of the HP on the running distance (A), expression of HIF-1α protein (C), and Keap1
598 (D) in skeletal muscles of the hypoxic exercised WT and Nrf2 KO mice. Nrf2 protein (B) showed
599 the change from WT mice only. Values are displayed as the mean ± SE (n=8-10 animals/group).
600 *p < 0.05, HP vs no-HP; #p < 0.05, ##p < 0.01, Nrf2 KO vs WT mice.

601 Fig.2. Effects of HP on the mRNA expression of muscular genes involved in antioxidation (A-H)
602 in skeletal muscle of the exercised WT and Nrf2 KO mice. Values are displayed as the mean ± SE
603 (n =8-10 animals/group). *p < 0.05, HP vs no HP; #p < 0.05, ##p < 0.01, Nrf2 KO vs WT mice.

604 Fig.3. Effects of HP on the protein expression of muscular genes involved in antioxidation (A-F)
605 and ROS level (G-H) in skeletal muscle of the exercised WT and Nrf2 KO mice. Values are
606 displayed as the mean ± SE (n =8-10 animals/group). *p < 0.05, HP vs no HP; #p < 0.05, ##p <
607 0.01, Nrf2 KO vs WT mice.

608 Fig.4. Effects of the HP on the mRNA expressions of genes involved in glycogen (A-B) and
609 glycogen content (C) in skeletal muscle of the exercised WT and Nrf2 KO mice. Values are
610 displayed as the mean ± SE (n =8-10 animals/group). *p < 0.05, HP vs no HP; #p < 0.05, ##p <
611 0.01, Nrf2 KO vs WT mice.

612 Fig.5. Effects of the HP on the mRNA expressions of genes involved in fatty acid metabolism

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613 (A-G) and triglyceride content (H) in skeletal muscle of the hypoxic exercised WT and Nrf2 KO
614 mice. Values are displayed as the mean ± SE (n =8-10 animals/group). *p < 0.05, HP vs no HP; #p
615 < 0.05, ##p < 0.01, Nrf2 KO vs WT mice.

616 Fig.6. Effects of HP on mitochondrial DNA copy number (A), citrate synthase activity (B), mRNA
617 of muscular genes involved in mitochondrial biogenesis (C-E), and mitochondrial OXPHOS
618 protein levels (F) in WT and Nrf2 KO mice. Values are displayed as the mean ± SE (n =8-10
619 animals/group). *p < 0.05, HP vs no HP; #p < 0.05, Nrf2 KO vs WT mice.

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

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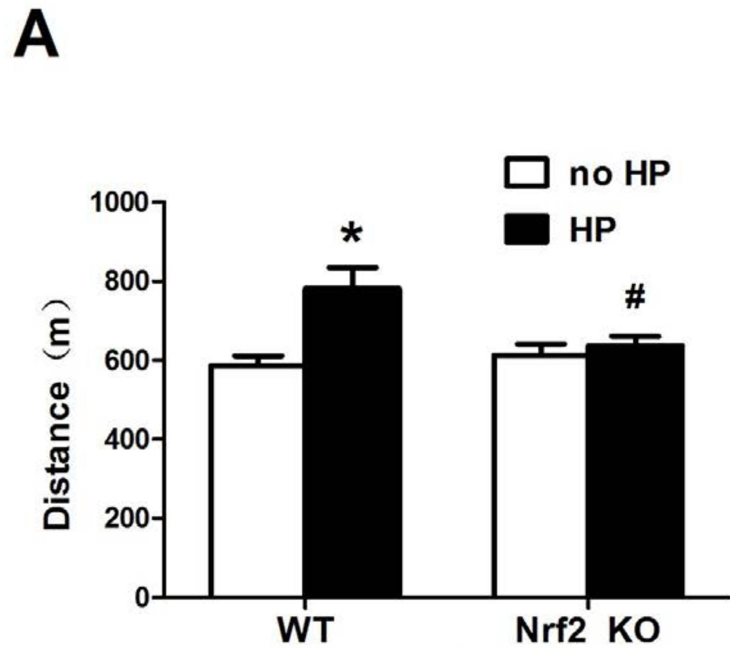

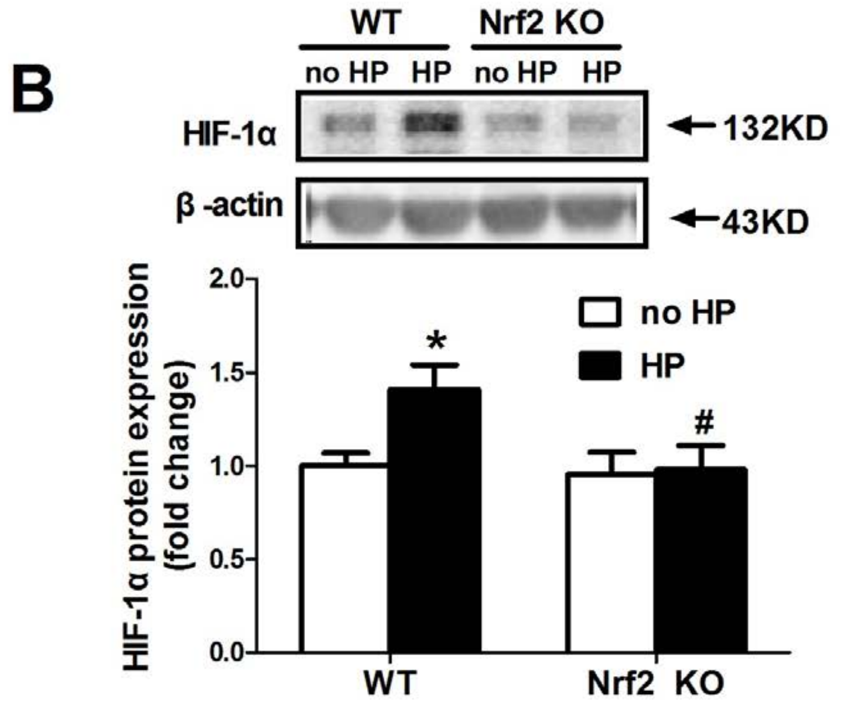

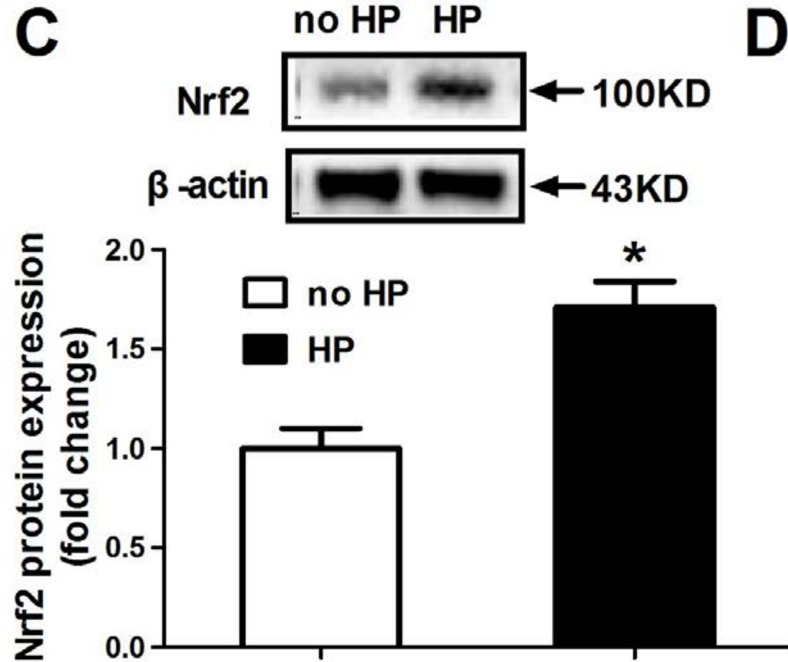

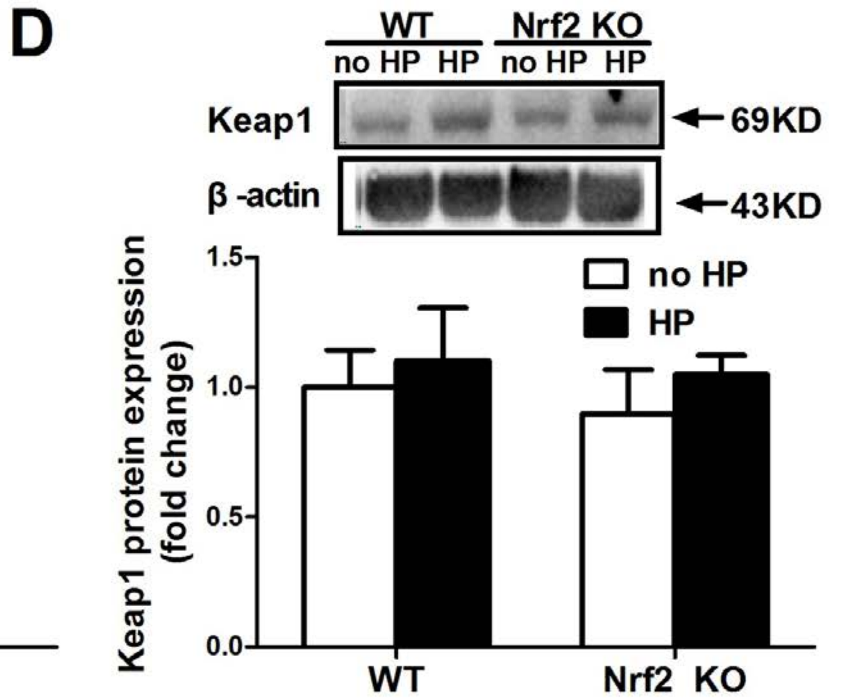

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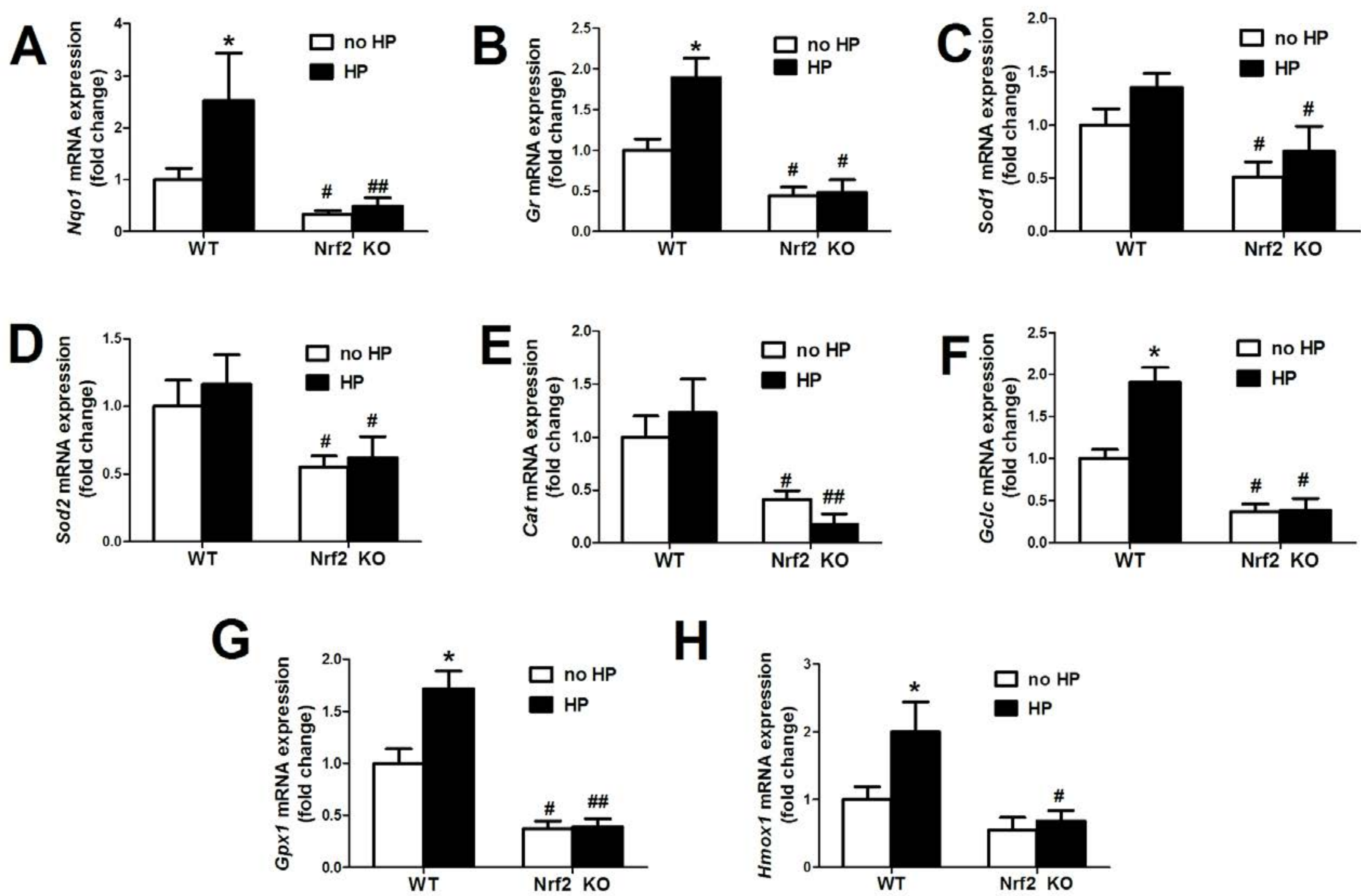

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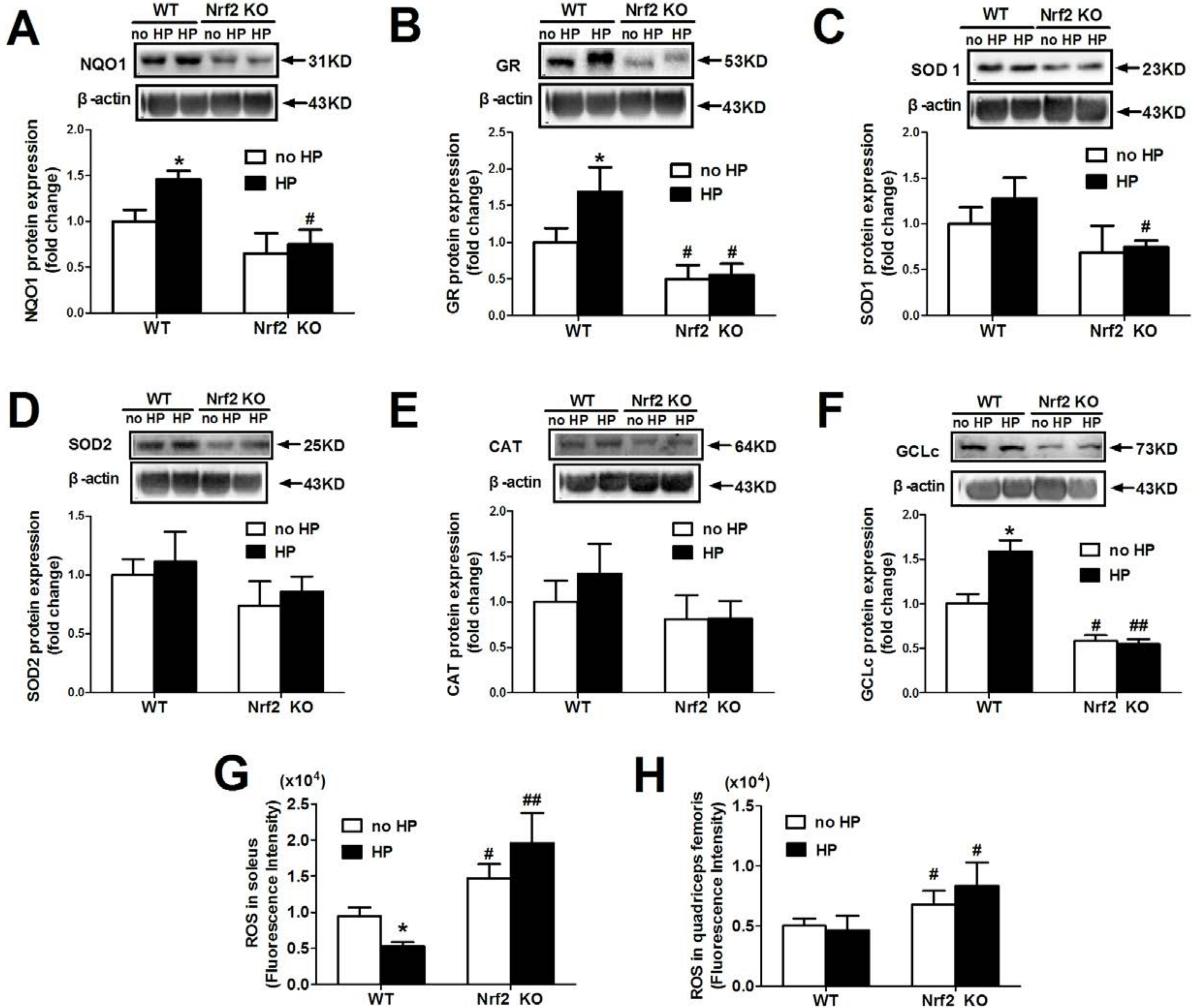

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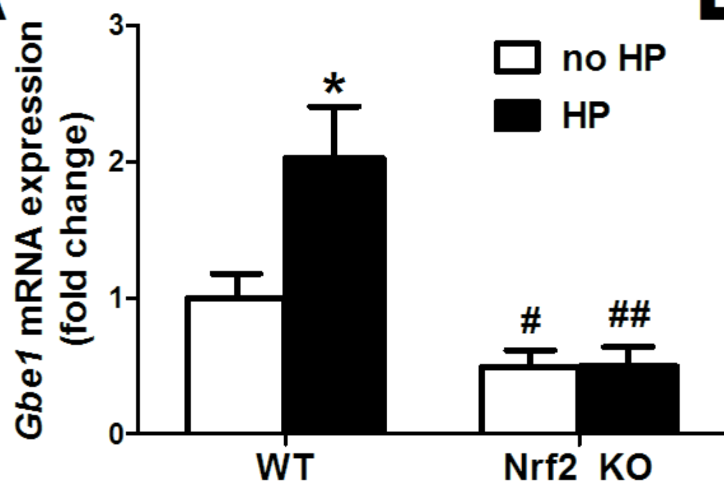

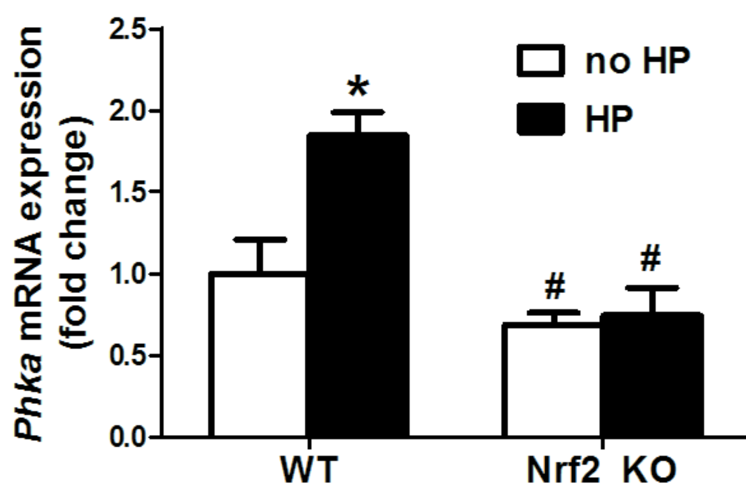

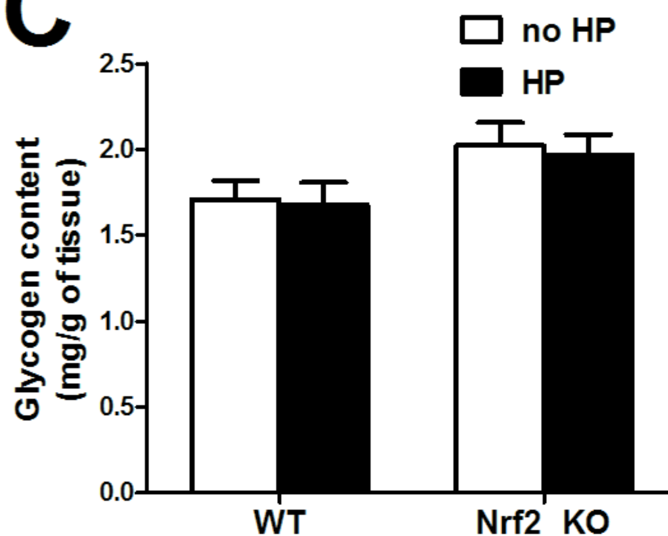

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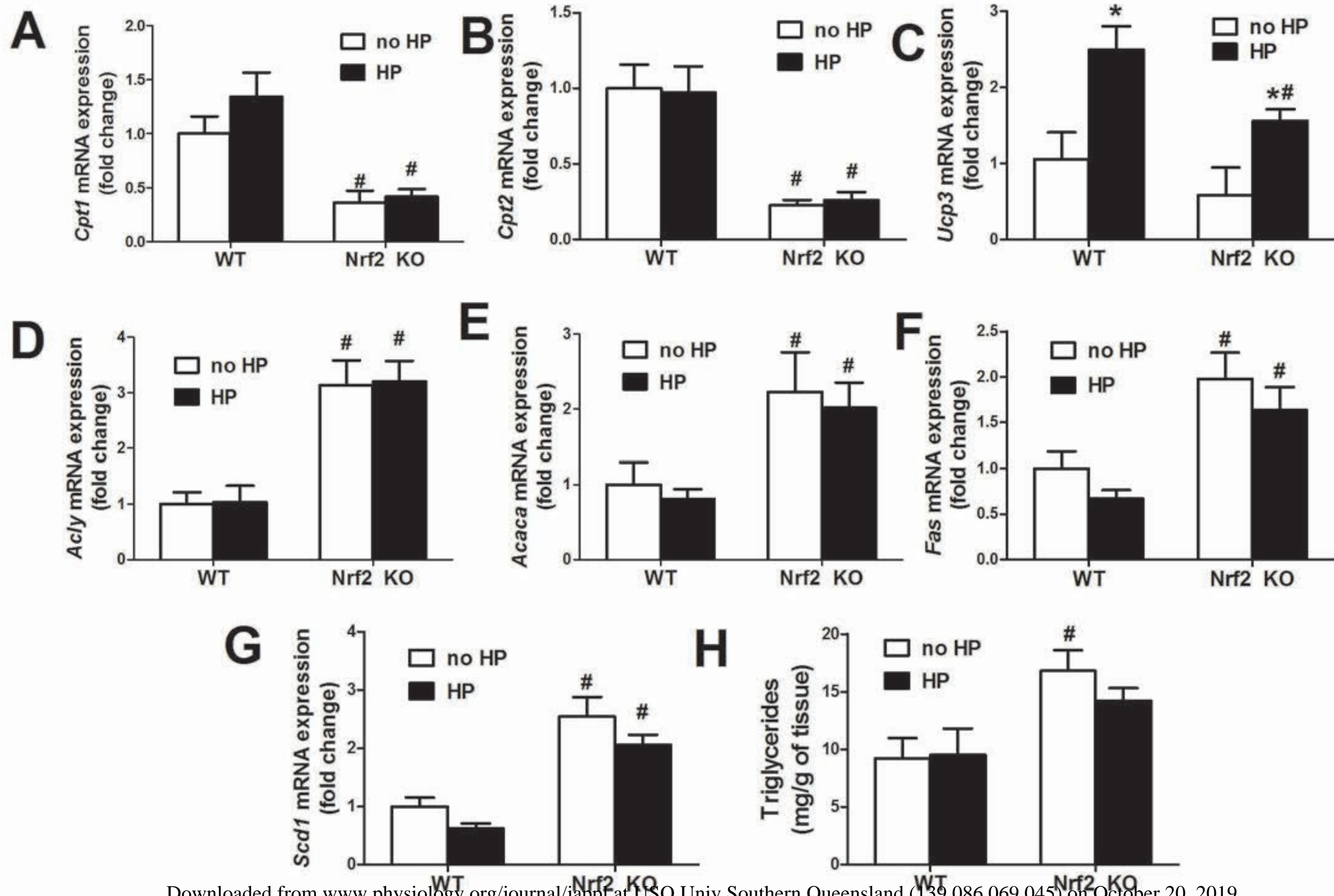

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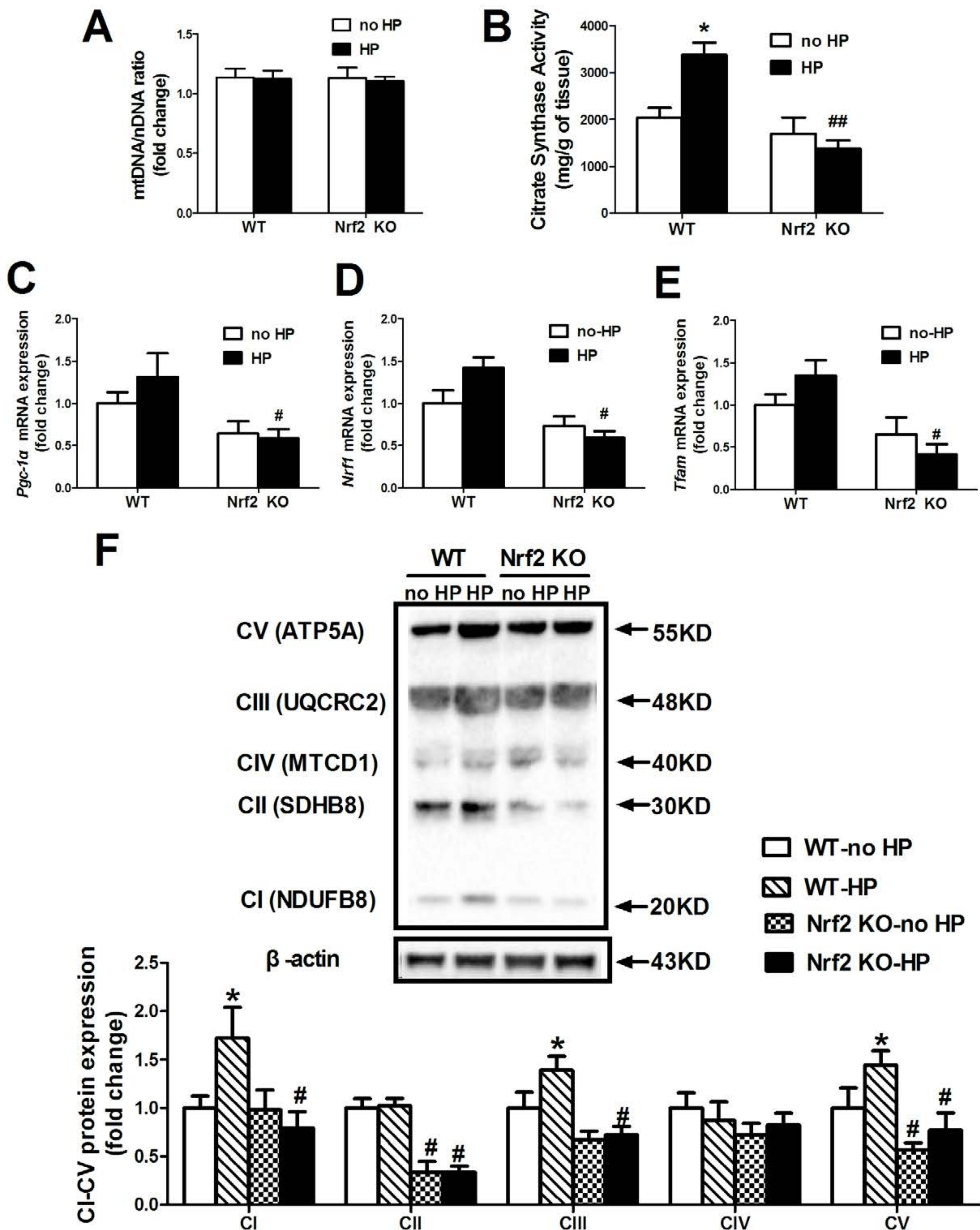

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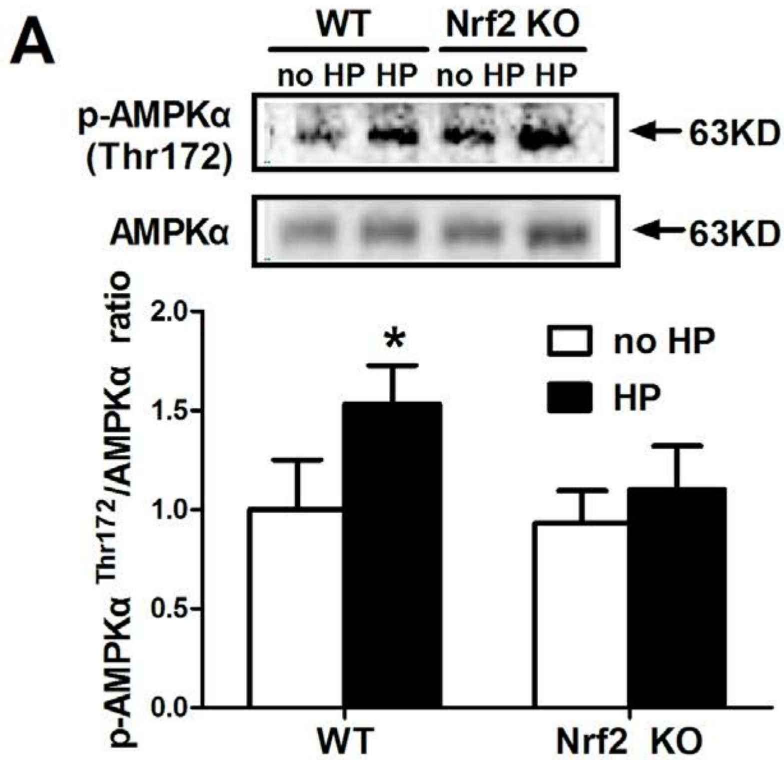

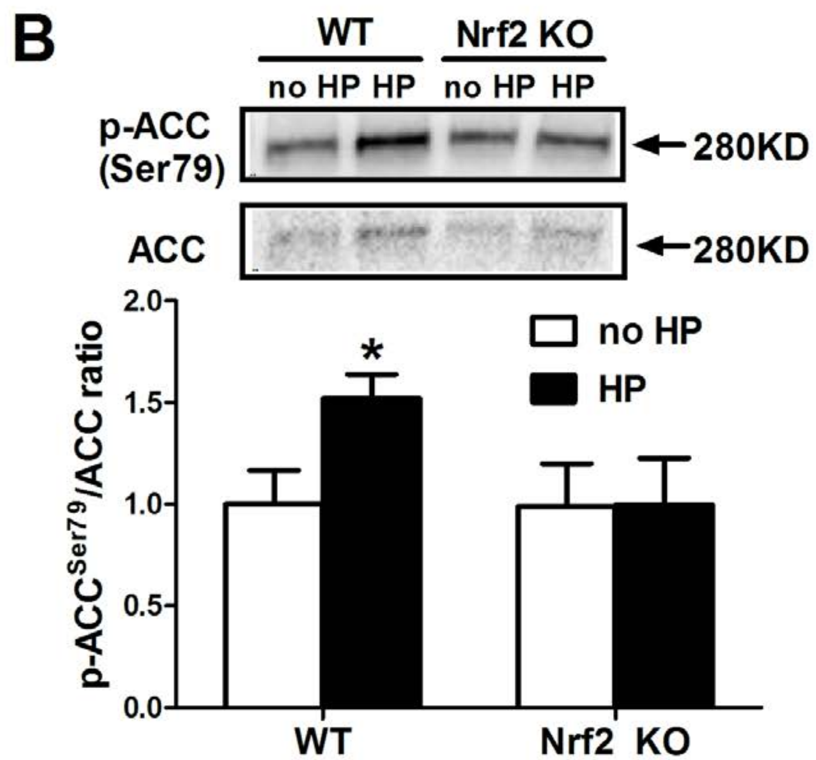

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