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1 **Hepatic AMPK activation in response to dynamic REDOX balance is a biomarker of**
2 **exercise to improve blood glucose control**
3
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27 **Abstract**

28 Antioxidant intervention is considered to inhibit reactive oxygen species (ROS) and
29 alleviates hyperglycemia. Paradoxically, moderate exercise can produce ROS to improve
30 diabetes. The exact redox mechanism of these two different approaches remains largely
31 unclear. Here, by comparing exercise and antioxidants intervention on type 2 diabetic rats,
32 we found moderate exercise upregulated compensatory antioxidant capability and reached a
33 higher level of redox balance in the liver. In contrast, antioxidant intervention achieved a
34 low-level redox balance by inhibiting oxidative stress. Both of these two interventions could
35 promote glycolysis and aerobic oxidation mediated by hepatic AMPK activation,
36 ameliorating diabetes. During exercise, different levels of ROS generated by exercise have
37 differential regulations on the activity and expression of hepatic AMPK. Moderate exercise-
38 derived ROS promoted hepatic AMPK glutathionylation activation. However, excess
39 exercise increased oxidative damage, and inhibited the activity and expression of AMPK.
40 Overall, our results illustrate that both exercise and antioxidant intervention improve blood
41 glucose in diabetes by promoting redox balance, despite the levels of redox balance are
42 different. Moreover, the activation and expression of AMPK could act as a biomarker to
43 reflect the effective treatment range for diabetes. This finding provides theoretical evidence
44 for the precise regulation of diabetes by antioxidants and exercise.

45

46 **Key words**

47 Redox balance; AMPK; exercise; glutathionylation

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49 **Introduction**

50

51 Diabetes mellitus is a chronic metabolic disorder disease, which has emerged as a global
52 public health problem. According to the latest epidemiological data from the International
53 Diabetes Federation, about 8.8% of the world population, or 415 million people, have
54 diabetes as of 2019, about a 2.4% increase from that of 2010 (6.4%) (*1*). With the
55 development of genomics, proteomics and metabolomics, it has been discovered by many
56 studies that type 2 diabetes is associated with irreversible risk factors such as age, genetics,
57 race, and ethnicity and reversible factors such as diet, physical activity and lifestyle (*2, 3*).
58 Given the essential function of aerobic metabolism in glucose oxidation, mitochondrial
59 damage and oxidative stress have been considered to play a critical role in the occurrence
60 and development of diabetes (*4*). Exercise and antioxidant supplements are often suggested
61 as essential therapeutic strategies in the early stages of type 2 diabetes (*5, 6*), with different
62 mechanisms. It has been reported that chronic exercise training can alleviate oxidative stress
63 and diabetic symptoms by improving cellular mitochondrial function and biogenesis in the
64 diabetic state (*7*). Contradictorily, exercise also increases ROS production, while prolonged
65 or high-intensity exercise could result in mitochondrial functional impairment to aggravate
66 complications of diabetes (*8*). Since the 1970s, studies have demonstrated that 1 hour of
67 moderate endurance exercise can increase lipid peroxidation in humans (*9, 10*). In 1998,
68 Ashton directly detected increasing free radical levels in exercising humans using electron
69 paramagnetic resonance spectroscopy (EPR) and spin capture (*11*). These results led to a
70 great deal of interest in the role of ROS in physical exercise (*12-14*). Regarding the
71 contradiction of exercise on ROS scavenging or production, James D Watson also
72 hypothesized that type 2 diabetes is accelerated by insufficient oxidative stress rather than
73 oxidative stress (*15*), based on the effect of exercise on diabetes treatment. Although
74 Watson's opinions supported that exercise could treat diabetes by producing ROS, whether
75 exercise-induced ROS production is beneficial or detrimental to diabetes is still being
76 debated. The specific regulation of ROS produced by exercise on diabetic blood glucose in
77 *vivo* is unclear. In contrast, the general view of the antioxidant treatment for diabetes is that
78 antioxidants reduce cytotoxic ROS and oxidation products, thus alleviating diabetes and
79 achieving glycemic control (*16*). Our previous study also found that hepatic mitochondrial
80 ROS scavengers and antioxidant substances inhibited the oxidative products such as MDA
81 and 4-HNE in diabetic animals and favored glycemic control (*17, 18*). Exercise-induced
82 oxidation and antioxidant administration, as two opposite approaches, could achieve the

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83 regulation of diabetes, respectively. However, the differences in redox mechanisms between
84 these two approaches to diabetes treatment have not been fully understood.
85 It is well established that the increase of skeletal muscle glucose uptake during exercise is
86 crucial in glycemic control (*19-21*). Considering that liver is another vital organ for
87 maintaining blood glucose homeostasis, including storing, utilizing and producing glucose,
88 exercise-induced hepatic redox metabolism is also significant. The activation of hepatic
89 AMP-activated protein kinase (AMPK), which acts as a 'metabolic master switch', alleviates
90 diabetes symptoms by reducing glycogen synthesis, increasing glycolysis, and promoting
91 glucose absorption in surrounding tissues (*22*). Therefore, the activation of AMPK in the
92 liver is significant for regulating glucose and lipid metabolism in the blood. Zmijewski et al.
93 found that AMPK could be activated by hydrogen peroxide stimulation through direct
94 oxidative modification (*23*). In contrast, other studies suggested that oxidative stress could
95 disrupt the activation of the AMPK signaling pathway (*24, 25*). Our previous study explored
96 the mechanism by which redox status contributes to hepatic AMPK dynamic activation.
97 Under a low ROS microenvironment, GRXs mediated S-glutathione modification activates
98 AMPK to improve glucose utilization. Meanwhile, under an excessive ROS
99 microenvironment, sustained high level ROS might cause loss of AMPK protein (*26*). These
100 studies indicate that oxidative modification can directly regulate AMPK activity in liver cells,
101 thus activating downstream signaling pathways to regulate glucose and lipid metabolism.
102 However, it is unclear why both antioxidant intervention and ROS produced by exercise can
103 promote the seemingly contradictory phenomenon of AMPK activation. Moderate exercise
104 has been proved significantly elevate systemic oxidative stress. At the same time,
105 endogenous antioxidant defences also increased to counteract increased levels of ROS
106 induced by exercise, leading to a higher level of redox balance(*27*). Thus, we hypothesized
107 that both antioxidants and exercise could reach either high-level or low-level redox balance
108 in diabetic individuals. Moreover, the activity and expression of AMPK might be a marker
109 of redox balance *in vivo*.
110 Hence, the present study was designed to understand the different mechanisms of exercise
111 and antioxidant intervention in diabetes and verify the activation of hepatic AMPK as a
112 hallmark of dynamic redox balance. Firstly, we utilized the streptozotocin-high fat diet (STZ-
113 HFD) induced type 2 diabetic (T2DM) model in rats to clarify the hepatic redox status in
114 T2DM rats after the exercise or antioxidant intervention. Then, according to the exercise
115 intensity and mode, we divided the exercise groups into three modes and found that AMPK
116 activation could serve as a biomarker of redox balance and moderate exercise in diabetic

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117 treatment. Taken together, in this study, we found that AMPK activation and expression
118 could reflect the threshold of exercise or antioxidant administration for diabetes treatment. It
119 provides a theoretical basis for the precise regulation of diabetes by antioxidants and exercise.

120

121 **Results**

122

123 **1. Exercise promotes antioxidant levels through producing ROS, leading to a high level**
124 **of REDOX balance in the liver.**

125 To investigate the hepatic redox regulation in diabetes after exercise intervention, we
126 established the T2DM rat model by feeding HFD followed by a low dose of STZ injection
127 (35 mg/kg). The exercise intervention was started from Day 0 to Day 28 (Fig. 1A). According
128 to previous studies, the initial speed of exercise was 15 m/min, and the speed was increased
129 by 3 m/min every 5 min. After the speed reached 20 m/min, the speed was maintained for
130 another 60 min with slope of 5%. The exercise intensity was 64%-76% VO_{2max} (*28*). The
131 low-intensity continuous exercise (CE) can be regarded as aerobic exercise.

132 Firstly, we detect the expression of antioxidant enzymes and oxidase in liver tissue. As redox
133 proteins regulate the redox state *in vivo*, the protein expression of GRX and TRX were found
134 to be up-regulated during exercise intervention (Fig. 1B-E). Notably, the PRX expression
135 also showed a trend of increase (Fig. 1B-C). Parallelly, the expressions of NADPH oxidase
136 4 (NOX4) and cyclooxygenase 2 (COX2) in the liver were also significantly up-regulated in
137 the exercise group (Fig. 1F-H). Protein carbonylation is a type of protein oxidation that can
138 be promoted by ROS. However, we found that the exercise group did not decrease the protein
139 carbonylation level (Fig. 1I). As shown in Fig. 1J-K, the acetylation level of MnSOD also
140 shows an increase in the exercise group, indicating the inactivation of mitochondrial
141 MnSOD. MDA, a biomarker of lipid peroxidation, was also significantly up-regulated in the
142 diabetic group but decreased in exercise group (Fig.1L). Meanwhile, specific markers related
143 to kidney dysfunction, such as the blood urea nitrogen (BUN) level, were also significantly
144 increased in the diabetic rat group. Exercise intervention reduced the BUN level (Fig.1M).
145 These results indicated that the high ROS production in the exercise group could
146 compensatory increase the antioxidant status to avoid oxidative damage. It suggests that
147 exercise can promote redox to reach a high level of balance, therefore ROS produced by
148 exercise does not lead to oxidative damage.

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149 **2. Antioxidant intervention alleviates blood glucose through reducing oxidative stress,**
150 **leading to a low level of REDOX balance in the liver.**

151 Recent studies have suggested that NADPH oxidase is one of the primary sources of ROS
152 (*29*). Apocynin has already been characterized as an NADPH oxidase inhibitor in the early
153 1980s, and it can also act as an antioxidant (*30*). Our previous study showed that apocynin
154 intervention alleviated blood glucose by inhibiting oxidative products. Compared with the
155 exercise intervention, the antioxidant intervention was also started from Day 0 to D28 in this
156 study (Fig. 2A). We found that apocynin intervention decreased the protein carbonylation
157 level and MDA level in the liver (Fig. 2B-C). Also, the TAOC level increased after apocynin
158 treatment (Fig. 2D). The random blood glucose and oral glucose tolerance (2 h after oral
159 glucose, OGTT) decreased in the apocynin intervention group compared with the diabetic rat
160 group (Fig. 2F-G). Consistent with the apocynin intervention group, the exercise group also
161 showed lower random blood glucose levels and 2h OGTT (Fig. 2H-I). These studies indicated
162 that the apocynin treatment inhibited the protein oxidative damage and alleviated blood
163 glucose.

164 **3. Moderate exercise-generated ROS production promotes phosphorylated activation**
165 **of AMPK and reduces blood glucose level, while excessive exercise- generated oxidative**
166 **stress reduces AMPK expression and exacerbates diabetes.**

167 In order to find out the biomarkers that could reflect moderate exercise to improve blood
168 glucose control, diabetic rats were divided into short-term continuous exercise (CE),
169 intermittent exercise (IE), and excessive exercise (EE) according to the exercise intensity and
170 mode (*28*). We found that the random blood glucose and 2h OGTT in CE and IE treated
171 diabetic rats decreased (Fig. 2A-B). In contrast, EE intervention did not improve blood
172 glucose but increased random and 2h OGTT, without statistical significance (Fig. 3A-B).
173 Next, we detected the expression of antioxidant enzyme and oxidase in the liver tissue of
174 exercise-treated T2D rats. Hepatic MDA concentration showed significant up-regulation in
175 the diabetic group but a decrease in continuous and intermittent exercise (Fig.3D). We found
176 that the CE group did not obviously change the protein carbonylation level. However, the EE
177 intervention promoted the protein carbonylation in the liver, indicating the mode of action is
178 not free radical scavenging but ROS production (Fig.3E).

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179 The ROS-generating NADPH oxidases (NOXs) have been recognized as one of the main
180 sources of ROS production in cells (*31*). Cyclooxygenase 2 (COX2) activity could also act
181 as a stimulus for ROS production (*32*). The increase of NOX4 and COX2 in the EE group
182 indicated the highest oxidation level (Fig. 3K-L). As shown in Fig. 3F-G, although the
183 acetylation level of MnSOD was found to increase significantly in the CE and EE group,
184 which presented the inactivation of MnSOD, the antioxidant enzyme GRX and TRX were
185 found to be up-regulated during CE intervention (Fig. 3I-J). Thus, the CE intervention
186 maintains a high-level balance of redox state. Considering the decrease of antioxidant
187 enzymes in the EE group, the REDOX balance in EE group was disrupted. Therefore, MDA
188 in the CE group did not increase, while the increased MDA in the EE group indicated
189 oxidative damage (Fig. 3D). Among these three exercise modes, the IE group showed the
190 lowest level of oxidation (the minor increase in COX and a slight decrease in carbonylation).
191 Although the levels of antioxidant enzymes such as GRX, TRX, and PRX did not increase,
192 the activity of MnSOD also increased significantly (the level of acetylation decreased) (Fig.
193 3F). The reduction of MDA level also indicates IE group did not form oxidative damage (Fig.
194 3D), indicating the IE group could also maintain a relatively high level of redox balance.

195 Notably, the phosphorylation of AMPK showed different patterns in three kinds of exercise,
196 among which both CE and IE intervention could promote the phosphorylation of AMPK
197 compared to the diabetic rats (Fig. 3O-P). EE intervention did not increase the content of
198 AMPK phosphorylation, which might be caused by the reduction of AMPK level.
199 Meanwhile, the ratio of AMP to ATP was detected, and exercise-activated AMPK did not
200 exhibit AMP-dependent characteristics at this time (Fig. 3N). These results suggested that
201 moderate exercise-generated ROS may directly promote AMPK phosphorylation activation
202 (independent of AMP upregulation) and reduce blood and liver glucose levels. However,
203 excessive exercise-generated oxidative stress reduces AMPK expression and exacerbates
204 diabetes.

205 **4. Moderate exercise promoted glycolysis and mitochondrial tricarboxylic acid cycle in**
206 **the liver of diabetic rats.**

207 Next, we further explored the mechanism by which inhibiting blood glucose during CE and
208 IE intervention. Fructose-2,6-diphosphate (F-2,6-P2; also known as F-2,6-BP), which is a
209 product of the bifunctional enzyme 6-phosphofructose 2-kinase/fructose 2,6-diphosphatase
210 2 (PFK/FBPase 2, also known as PFKFB2), is a potent regulator of glycolytic and

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211 gluconeogenic flux. The phospho-PFKFB2 to PFKFB2 ratio represents the glycolytic rate.
212 A high ratio of phospho-PFKFB2:PFKFB2 leads to an increase in the F-2,6-P2 level and the
213 allosteric activation of phosphor-fructose kinase 1 (PFK1), while a low ratio leads to a
214 decrease in F-2,6-P2 and an increase in gluconeogenesis (*33*). The overexpression of
215 bifunctional enzymes in mouse liver can reduce blood glucose levels by inhibiting hepatic
216 glucose production (*34*). Therefore, bifunctional enzymes are also a potential target for
217 reducing hepatic glucose production. In our study, the p-PFK2:PFK2 ratio decreased in the
218 diabetic rats but was enhanced by CE and IE intervention (Fig. 4A-C), suggesting that CE
219 and IE could reverse gluconeogenesis to glycolysis by enhancing PFK/FBPase. Meanwhile,
220 the substrates of the glycolytic pathway (such as DHAP, Fig. 4D) and the tricarboxylic acid
221 cycle (such as citrate, succinate and malate, Fig. 4D) showed an upward trend. These results
222 illustrated that moderate exercise promoted glucose catabolism in the liver of diabetic rats
223 (Fig. 4E).

224 **5. Moderate exercise inhibited hepatic mitophagy, while excessive exercise promoted**
225 **hepatic mitophagy and inhibited the mitochondrial biogenesis.**

226 The electron transport associated with the mitochondrial function is considered the major
227 process leading to ROS production during exercise (*35*). To further explore the downstream
228 signal of AMPK activation in moderate and excessive exercise, we detected the protein
229 expression of mitochondrial dynamic and mitochondrial biogenesis. According to the result
230 in Figure 5A-E, we found that the mitochondrial fusion protein MFN significantly decreased
231 in the liver of the excessive exercise group, and the mitochondrial fission protein (Fis) and
232 autophagy-related protein ATG5 and LC3B did not change, compared with the diabetic
233 group. Notably, the ATG5 and LC3B levels decreased in the CE and IE group, compared
234 with the diabetic group (Fig. 5A-E). PGC1α is a transcriptional coactivator, a central inducer
235 of mitochondrial biogenesis in cells (*36*). The expression of PGC1α increased in the CE and
236 IE group, but not in the EE group (Fig. 5F). These results indicated that moderate exercise
237 ameliorated mitochondrial biogenesis and autophagy in the liver. However, excessive
238 exercise aggravated mitochondrial fission and did not exhibit autophagy alleviation.
239 Parallelly, the mitochondria structure of the live tissue in the EE group is fragmented and
240 showed greatly diminished cristae and swelling matrix under transmission electron
241 microscopy, which is functionally reflected as a defect in oxidative phosphorylation.
242 However, the CE and IE group showed increased numbers of cristae and a clear structure of

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243 mitochondrial cristae (Fig. 5H). These results all show that the *in vivo* mitochondrial ROS
244 burst caused by excessive exercise inhibits the expression of AMPK and promotes
245 mitophagy. The damage to the mitochondrial dynamics and structure in liver tissue leads to
246 abnormal aerobic oxidation, thereby aggravating diabetes.

247 **6. ROS differentially regulated AMPK activation through GRX-mediated**
248 **glutathionylation within redox balance threshold**

249 To further elucidate the relationship between redox balance and AMPK activation in cellular
250 environment, L02 cells were intervened with different concentrations of H_2O_2 (50-200
251 μmol/l) to mimic the different ROS levels *in vivo* (Fig. 6A). We found the expression of 3-
252 NT and 4-HNE increased after H_2O_2 intervention (100-200 μmol/l, Fig. 6B). The acetylation
253 level of Mn-SOD also increased after H_2O_2 intervention (Fig. 6C). Significantly, oxidative
254 stress marker 3-nitrotyrosine (3-NT) level highly increased at the concentration of 200
255 μmol/l, indicating the H_2O_2 intervention at 200 μmol/l exceeded the threshold of redox
256 balance, thus causing oxidative damage. Our previous study shows that optimal ROS would
257 activate AMPK through GRX-mediated S-glutathionylation (*26*). As shown in Fig. 6D-F,
258 exposure to 50 and 100 μmol/l H_2O_2 led to an increase of GSS-protein adduct, concomitant
259 with the AMPK phosphorylation and glutathionylation (Fig. 6E-F), suggesting that the ROS
260 level within redox balance threshold could induce glutathionylation and phosphorylation of
261 AMPK. However, when the concentration of ROS was too high to exceed the redox balance
262 threshold, the AMPK protein would be partially degraded, thereby inhibiting its activity.
263 Exposure to 200 μmol/l H_2O_2 led to a decrease in AMPK glutathionylation, phosphorylation
264 as well as protein content (Fig. 6D-F). These results indicated that the increased ROS within
265 the redox balance threshold could promote the AMPK activation through GRX-mediated S-
266 glutathionylation.

267 At the same time, we also detected the substrates of glycolysis and aerobic oxidation at
268 different concentrations of H_2O_2. We found the exposure to 20-100 μmol/l H_2O_2, which made
269 cells within the redox balance threshold, showed a trend of increase on glycolysis and aerobic
270 oxidation substrates, indicating the increase of hepatic glucose catabolism (data not shown).

271

272 **Discussion**

273

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274 It is known that both antioxidants and exercise can substantially benefit and ameliorate
275 hyperglycaemia through ROS-mediated mechanisms in diabetes patients. However,
276 antioxidant intervention reduces oxidative stress, while exercise produces ROS. The
277 differences in redox mechanism between these two opposite approaches to diabetes treatment
278 have not been fully explored. Therefore, it is still unclear how to use exercise and antioxidants
279 scientifically and rationally to treat diabetes effectively.

280 The remission of diabetes by antioxidant intervention has been well documented. Some
281 compounds in food that have a vigorous antioxidant activity or inhibit NADPH oxidase, such
282 as polyphenols and flavonoids (*37*), have been confirmed to improve blood glucose and
283 relieve type 2 diabetes in animal experiments. Several clinical trials also demonstrated the
284 relief of antioxidants in diabetic hyperglycaemia (*38, 39*). Our previous study found hepatic
285 mitochondrial ROS scavenger and antioxidant substances inhibited the oxidative products
286 such as MDA and 4-HNE in diabetic mice and rats and benefitted blood glucose control (*18*).
287 These evidence indicates that reducing the oxidative level of diabetic animals could treat
288 diabetes. Hepatic AMPK regulates cellular and whole-body energy homeostasis, signals to
289 stimulate glucose uptake in skeletal muscles, fatty acid oxidation in adipose (and other)
290 tissues, and reduces hepatic glucose production (*40, 41*). Numerous pharmacological agents
291 (including the first-line oral drug metformin), natural compounds, and hormones are known
292 to activate AMPK (*42-45*). Moreover, our previous found that antioxidant (apocynin)
293 intervention in diabetic rats could promote the phosphorylation and activation of AMPK
294 protein, thereby regulating hepatic glucose metabolism (*46*). Taken together, we found that
295 the activation of AMPK by antioxidant intervention was accompanied by a decrease in
296 oxidative stress level in diabetic rats, resulting in a low level of redox balance to benefit
297 diabetic hyperglycaemia.

298 Many medical research and public health recommendations support regular exercise to
299 mitigate symptoms of many diseases, including psychiatric, neurological, metabolic,
300 cardiovascular, pulmonary, musculoskeletal, and even cancer (*47*). John Holloszy's studies
301 found that exercise improved insulin sensitivity in patients with type 2 diabetes and provided
302 a better understanding of how muscle adapts to endurance exercise (*19-21, 48-50*). Although
303 the benefits of exercise are irrefutable, excessive exercise is harmful (*8*). It is unclear how to
304 control the amount and intensity of exercise. Recently, Chrysovalantou et al. found that
305 NADPH oxidase 4 (NOX4) is a crucial protein of exercise to regulate adaptive responses and

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306 prevent insulin resistance (*51*). We found that exercise can indeed increase NOX expression,
307 but NOX is also upregulated in excessive exercise. Although Chrysovalantou's research puts
308 more emphasis on the role of the redox environment in exercise, the biomarkers of moderate
309 exercise for diabetes remain uncertain. In this study, according to the exercise intensity and
310 mode, we divided the exercise groups into three modes: CE, IE and EE. We found that
311 moderate exercise (including CE and IE) promoted hepatic ROS production and up-regulated
312 a compensatory increase in antioxidant capability, forming a higher level balance of redox
313 state. However, excessive exercise increases mitochondrial oxidative stress level and cause
314 oxidative damage, which is not beneficial for glycaemic control in diabetes.

315 During exercise, the activation of AMPK in skeletal muscle was considered mainly caused
316 by the increase of intracellular AMP:ATP ratio and phosphorylation of Thr172 on the
317 "activation loop"7 of the α-subunit (*52*). The activation of AMPK leads to the inhibition of
318 mTORC1 activity and activation of PGC-1α, thereby enhancing mitochondrial biogenesis
319 (*53*). Early studies clarified that the activation of AMPK is related to the liver energy state
320 during exercise (*54*). There are some papers against the role of AMPK in regulating glucose
321 uptake during exercise (*55-57*), but these studies mainly focused on skeletal muscle glucose
322 uptake but not the liver. Although the glucose was uptake by primarily skeletal muscle after
323 exercise, the liver is prominent in whole-body glucose tolerance (*58*). Increasing evidence
324 showed that AMPK is a redox-sensitive protein, and its cysteine 299 and 304 sites are likely
325 to be regulated by the oxidation of hydrogen peroxide (*23, 59, 60*). Thus, the phosphorylation
326 of AMPK might be directly activated through ROS regulation during exercise, not only
327 depending on the increase of AMP. Our previous study found that both oxidation and
328 reduction can promote AMPK activation (*26*), actually, this is because the redox balance
329 state induced by oxidation or reduction intervention activates AMPK. In this study, we
330 demonstrates that both antioxidant intervention and moderate exercise can activate AMPK
331 oxidative phosphorylation while achieving redox balance, of which antioxidant intervention
332 achieves a low-level redox balance and moderate exercise achieves a high-level redox
333 balance.

334 Our study, for the first time, found hepatic AMPK activation could act as a biomarker of
335 dynamic redox balance during exercise to benefit glycaemic control in diabetic rats. Different
336 intensities of exercise-induced ROS production could profoundly alter the cellular redox
337 microenvironment and directly regulate the activity and expression of hepatic AMPK

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338 through a redox-related mechanism. Moderate exercise (including CE and IE) appropriately
339 promotes oxidation in the liver, thus, compensatory promotes the level of reduction, forming
340 a high level of redox balance. Excessive exercise producing ROS exceeding the redox
341 threshold caused oxidative damage with a significant increase in MDA and urea nitrogen
342 levels. Specifically, optimal ROS directly promoted AMPK activation via glutathionylation
343 and upregulated glycolysis and aerobic oxidation in L02 cells, which was consistent with the
344 animal experiments. However, excessive ROS inhibits the activity and expression of AMPK
345 in L02 cells, which might be related with the oxidative stress induced protein degradation
346 (Fig. 7). Accordingly, we speculate that the antioxidant intervention based on moderate
347 exercise might offset the effect of exercise, but the antioxidant intervention after excessive
348 exercise could restore redox balance. In addition, the number of mitochondria and the
349 function of aerobic oxidation in the IE group was significantly higher than those in the CE
350 group. In the EE group, the autophagy and fission of liver mitochondria are also up-regulated.
351 These results indicate that the phosphorylation and expression of AMPK can act as a sensitive
352 biomarker during exercise in diabetic rats, reflecting the threshold of redox balance and the
353 range of exercise appropriateness.

354 Together, our results illustrate the different regulatory mechanisms of exercise and
355 antioxidant intervention on redox balance and blood glucose level in diabetes. Moderate
356 exercise promoted hepatic ROS production and up-regulated a compensatory increase
357 antioxidant capability, forming a high-level balance of redox state. In contrast, antioxidant
358 intervention scavenged the hepatic free radical to form a delicate low-level balance of redox
359 state. Moreover, excessive exercise led to redox imbalance due to excess ROS levels. Hepatic
360 AMPK activation could act as a sign and hallmark of moderate exercise and dynamic redox
361 balance to guide appropriate exercise or antioxidant intervention. These results illustrate that
362 it is necessary to develop a moderate exercise program according to the REDOX
363 microenvironment of diabetes patients and provide theoretical evidence for the precise
364 regulation of diabetes by antioxidants and exercise.

365

366 **Materials and Methods**

367

368 **1. Materials**

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369 T-AOC kit was supplied by Changzhou Redox Biological Technology Corporation (Jiangsu
370 , CN). Antibodies against Actin, Acetylated-Lysine, P-PFK2, Ace-SOD2, ATG5, LC3A/B,
371 GAPDH, MFN1 and IgG-HRP were purchased from Cell Signaling Technology (USA).
372 Antibodies against CAT, PRX1, AMPKa1, GRX1, GRX2, SOD2, HSP90, COX1, COX2
373 and PFK2 were purchased from ProteinTech (Wuhan, CN). Antibodies against 3-NT, 4HNE,
374 NOX4 and PGC1-α were purchased from Abcam. Antibodies against p-AMPKα1/α2 were
375 purchased from SAB (Signalway Antibody, USA). The detailed antibody information is
376 shown in Supplementary Table S1. High-fat diet (HFD) were purchased from Shanghai
377 SLRC laboratory animal Company Ltd (Shanghai, China) and the nutritional composition is
378 shown in Supplementary Table S2.

379

380 **2. Animal**

381 Male SD rats (150–160 g body weight, 6-8 weeks) were purchased from Fudan University
382 Animal Center (Shanghai, China). All animal care and experimental procedures were
383 approved by the Fudan University Institutional Laboratory Animal Ethics Committee (NO.
384 20170223-123). Animals were housed in a pathogen free environment with 12 h dark/light
385 cycles.

386

387 **3. Establishment of diabetic rat model**

388 Rats were divided into six groups in a non-blinded, randomized manner: Control (Ctl),
389 STZ+HFD diabetic rat (T2D), Continuous exercise+STZ+HFD diabetic rat (T2D+CE),
390 intermittent exercise+STZ+HFD diabetic rat (T2D+IE), excessive exercise+STZ+HFD
391 diabetic rat (T2D+EE) and Apocynin+STZ+HFD diabetic rat (T2D+APO) (n=8 per group).
392 The sample size was calculated according to the Power Curve. The diabetic rats model was
393 established by 12hr-fasting followed by intraperitoneal injection of 0.1 M streptozotocin
394 (STZ) citrate solution (pH 4.5) at a dose of 35 mg/kg for Day1, and 35 mg/kg for Day 2 at
395 the 5th week. The HFD was started from the 1st week to the 8th week. After 8 weeks of
396 intervention, the mice were sacrificed. The tissues and plasma were collected and preserved
397 at −80 °C for further analysis.

398 Rat were acclimated to treadmill running for 3 days before the initiation of the experiments
399 and the exercise training intervention was continued for 4 weeks (5 times per week). All
400 animals were randomized before the initiation of exercise tests.

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401 *Continuous exercise*:

402 The initial speed was 15 m/min, and the speed was increased by 3 m/min every 5 min. After
403 the speed reached 20 m/min, the speed was maintained for another 60 min with slope of 5%.
404 The exercise intensity was 64%-76% VO_{2max} (*28, 61, 62*).

405 *Intermittent exercise*:

406 The initial speed was 15 m/min, and the speed was increased by 3 m/min every 5 min. After
407 the speed reached 20 m/min, the speed was maintained for 20 min and then 5 min rest at 5
408 m/min. The training was continued for 3 times, and the total running time is 60 min with two
409 5 min rest with slope of 5%.

410 *Excessive exercise*:

411 The initial speed was 15 m/min, and the speed was increased by 3 m/min every 5 min. After
412 the speed reached 50 m/min, the speed was maintained for another 60 min with slope of 5%.
413 The exercise intensity was higher than 80% VO_{2max}.

414 OGTT was performed in the fasting mice with intraperitoneal injection of glucose at 1 g/kg
415 of body weight, and glucose was measured at 15min, 30 min, 60 min and 120 min,
416 respectively. Blood glucose was determined by glucometer (Roche, Switzerland).

417

418 **4. Cell culture**

419 Normal Human Hepatic Cell Line L02 cells (Cell Bank of Chinese Academy of Sciences)
420 were grown in DMEM supplemented with 10% FBS (GIBCO, USA) in a humidified
421 incubator (Forma Scientific) at 37 °C and 5% CO_2 as described previously. The medium were
422 supplemented with 10% FBS (GIBCO, USA), 2 mmol/l glutamine, 1 mmol/l sodium
423 pyruvate, 10 mmol/l HEPES, 50 μmol/l β-mercaptoethanol, 105 U/l penicillin and
424 streptomycin. Glutamine and sodium pyruvate were purchased from Sinopharm Chemical
425 Reagent Co., Ltd, HEPES were purchased from Beyotime Biotechnology (Shanghai, CN).
426 All cell lines used in the study were tested for mycoplasma and were STR profiled.

427

428 **5. Flow cytometry**

429 For measurement of intracellular Superoxide and H_2O_2, hepatocytes were stained with 5 μM
430 hydroethidine (superoxide indicator) and 10 μM H_2DCFDA (Thermo Fisher Scientific,

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431 USA). Stained cells were analyzed with NovoCyte Quanteon flow cytometer (Agilent
432 Technologies, Inc.), and acquired data were analyzed with NovoExpress software (Agilent
433 Technologies, Inc.) and FlowJo software (TreeStar, Ashland, OR).

434

435 **6. ATP and AMP content analysis**

436 Liver tissue (20–30 mg) were homogenized on ice by perchloric acid. Homogenized samples
437 were centrifuged for 12,000 rpm at 4 °C (30 min). Supernatant was then neutralized with 4
438 M K2CO3, followed by further centrifugation for 12,000 rpm at 4 °C for 20 min. Supernatant
439 was obtained for the determination of ATP and AMP content by high performance liquid
440 chromatography (HPLC). The detection wavelength was 254 nm.

441

442 **7. Metabolite profiling detection**

443 Cellular metabolites were extracted and analysed by LC-MS/MS. Ferulic acid was added as
444 an internal standard to metabolite extracts, and metabolite abundance was expressed relative
445 to the internal standard and normalized to cell number. Mass isotopomer distribution was
446 determined by LC-MS/MS (AB SCIEX Triple-TOF 4600) with selective reaction monitoring
447 (SRM) in positive/negative mode

448

449 **8. Western blot analysis**

450 Cells and tissues were lysed in a buffer containing 1% Nonidet P-40, 0.25% sodium
451 deoxycholate, 150 mmol/l NaCl, 10 mmol/l Tris, 1 mmol/l EGTA, 1% proteinase and
452 phosphatase inhibitor cocktails (Sigma-Aldrich) at 4 °C for 30 min. Cell lysates were
453 resolved by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis,
454 transferred to polyvinylidene fluoride (PVDF) membranes, and immunoblotted with primary
455 antibodies. Membranes were incubated with HRP-conjugated secondary antibodies and
456 visualized using chemiluminescent substrate (ECL; Tanon, CN) and Tanon-5200
457 Chemiluminesent Imaging System (Tanon, CN).

458

459 **9. Transmission electron microscope (TEM)**

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460 Rat liver tissue (1 mm*1 mm) were fixed by paraformaldehyde. The samples were examined
461 with a Jeol Jem-100SV electron microscope (Japan) which was operated at 80 Kv after fixed
462 by 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) at Institute of Electron microscopy,
463 Shanghai Medical College of Fudan University.

464

465 **10. Statistics**

466 The experimental data were expressed as mean ± SEM. One-way ANOVA was used to
467 compare among groups. Data analysis was conducted by Graphpad prism 9 statistical
468 analysis software. $p < 0.05$ was considered statistically significant. Data are expressed as
469 means ± SEM; $n = 3$ for cells experiment ($n = 3$ represents three times of individual
470 experiment); $n = 8$ for animal experiment.

471

472 **Acknowledgments**

473 The authors thank Dr. Xiaodong Zhang from Chengdu Brilliant Pharmaceuticals for his proof
474 reading and editing of the manuscript. The authors are also indebted to Dr. Rutan Zhang and
475 Prof. Liang Qiao from Fudan University, for analysis of LC-MS/MS data. The authors thank
476 Mr. Yipei He, Dr. Xiao Zhang, Mrs Lihan Jiang, Mr. Kelei Dong for their assistance in animal
477 experiments. The authors are also indebted to Institute of Electronmicroscopy from Fudan
478 University, for the help on electronmicroscopy analysis.

479 This work was supported by grants from the National Natural Science Foundation of China
480 (Grants No. 31770916).

481

482 **Author contributions:**

483 M.W., A.Z., C.Y., X.L., C.Z. and Q.L. conducted experiments. L.S. and D.S designed the
484 experiments and analyzed data. Y.F. and F.X. provided professional consultation on exercise
485 mode. H.G. provided professional suggestion on electronmicroscopy analysis. L.S. and D.S.
486 supervised the project and wrote the manuscript.

487

488 **Competing interests:**

489 All other authors declare they have no competing interests

490

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491 **Data and materials availability:**

492 All data are available in the main text or the supplementary materials.

493

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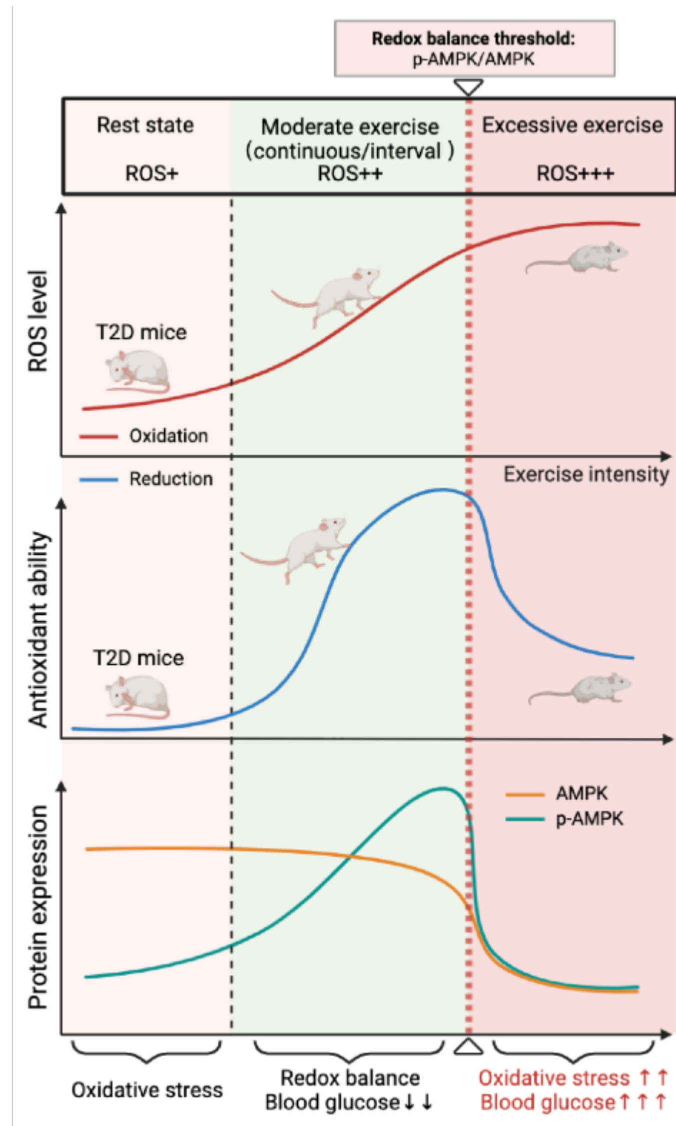
648 **Figure legend:**

649 **Graphic Abstract**



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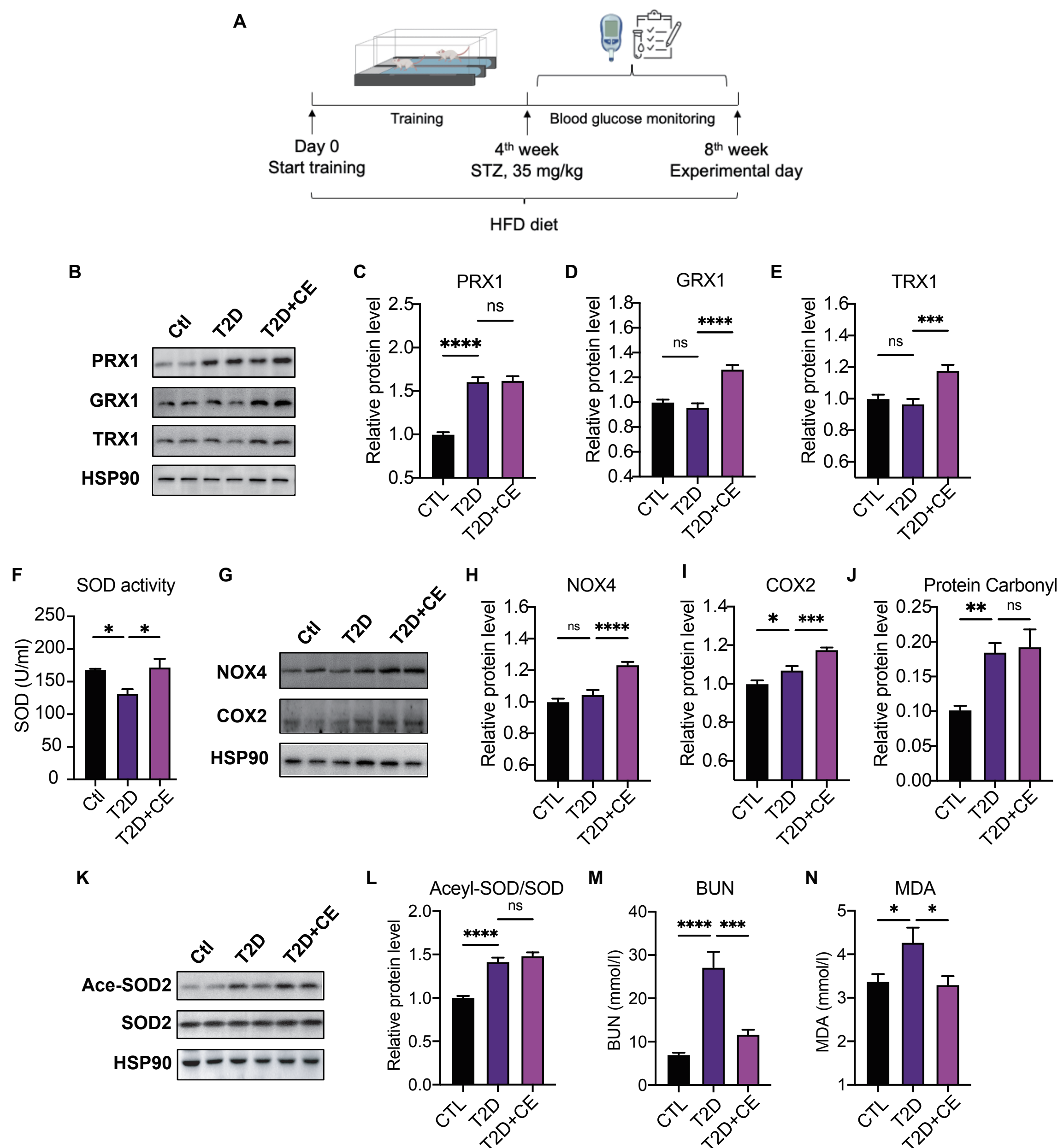

653
654 **Fig. 1 Exercise induced ROS production in exercise group and increased the antioxidant status.**

655 **A.** Experimental design. T2DM rats model was fed by high-fat diet plus a low dose of STZ injection
656 (35 mg/kg). The high-fat diet (HFD, 60% calories from fat) was started from the 1st week to the 8th
657 week. The exercise intervention was started from 1st week to 4th week. **B-E**. Representative protein
658 level and quantitative analysis of PRX1 (27 kDa), Grx1 (17 kDa), Trx1 (12 kDa) and HSP90 (90
659 kDa) in the rats in the Ctl, T2D and T2D + CE groups. The rat livers were homogenized by 1% SDS
660 and analyzed by Western blots with the appropriate antibodies. **F-I**. Representative protein level and
661 quantitative analysis of NOX4 (27 kDa), COX2 (17 kDa) and HSP90 (90 kDa) in the rats in the Ctl,
662 T2D and T2D + CE groups. **J-K**. Representative protein level and quantitative analysis of Ace-SOD2
663 (27 kDa), SOD2 (17 kDa) and HSP90 (90 kDa) in the rat in the Ctl, T2D and T2D + CE groups. **L-**
664 **M**. Liver MDA content (L) and BUN (M) level was detected in the rats of Ctl, T2D and T2D + CE
665 groups. (ns: not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with all
666 groups by one-way ANOVA and Tukey's post hoc test; data are expressed as the mean ± SEM; n =
667 4-8 per group).

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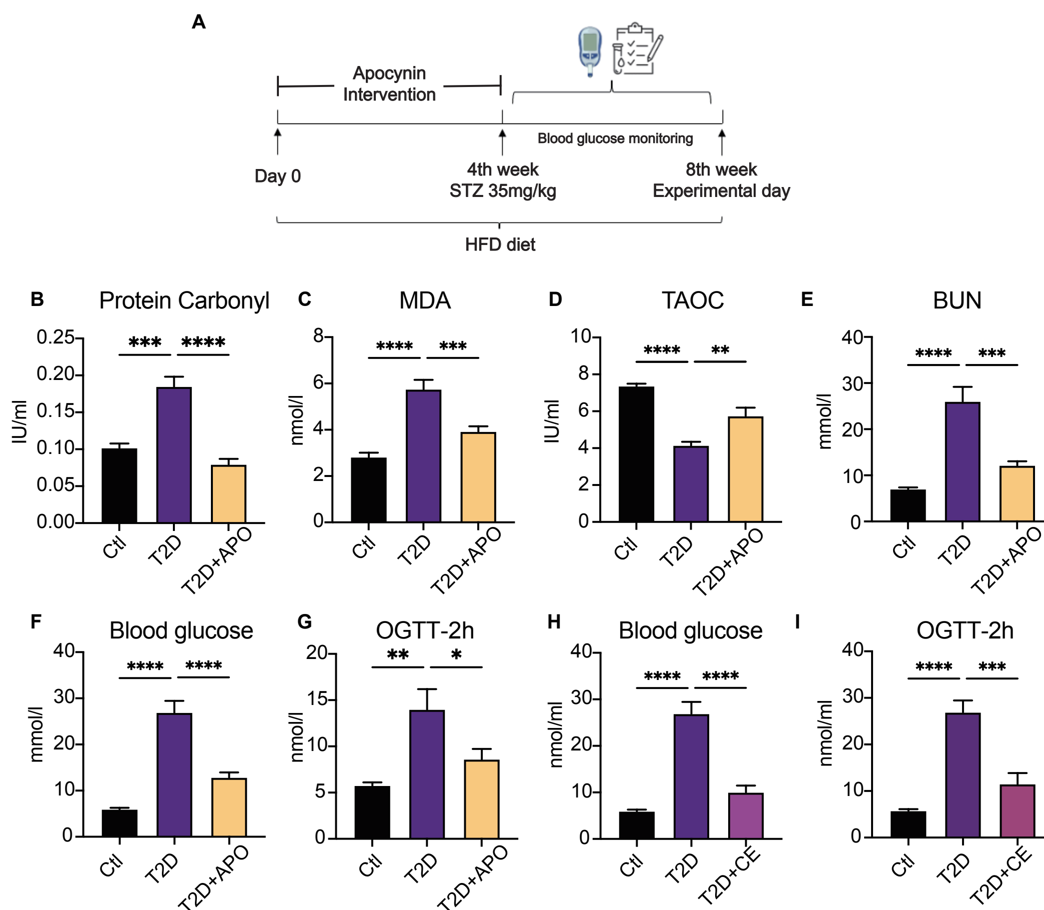

669

670 **Fig.2 Antioxidant intervention alleviates blood glucose through promoting the upregulation of**
671 **reducing levels**

672 **A.** Experimental design. T2DM rats model was fed by high-fat diet plus a low dose of STZ injection
673 (35 mg/kg). The apocynin intervention was started from 1st week to 4th week. **B.** Liver protein
674 carbonylation was detected in the rats of Ctl, T2D and T2D + Apocynin (APO) groups. **C-E.** Liver
675 MDA content (C), TAOC (D) and BUN (E) level was detected in the rats of Ctl, T2D and T2D +
676 APO groups. **F**. Postprandial blood glucose levels of Ctl, T2D and T2D + APO groups at the end of
677 8th week. **G.** Blood glucose level after oral glucose administration in Ctl, T2D and T2D + APO groups
678 at the end of 8th week. **H**. Postprandial blood glucose levels of Ctl, T2D and T2D + CE groups at the
679 end of 8th week. **I.** Blood glucose level after oral glucose administration in Ctl, T2D and T2D + CE
680 groups at the end of 8th week (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ compared with
681 all groups by one-way ANOVA and Tukey's post hoc test; data are expressed as the mean ± SEM; n
682 = 4-8 per group).
683

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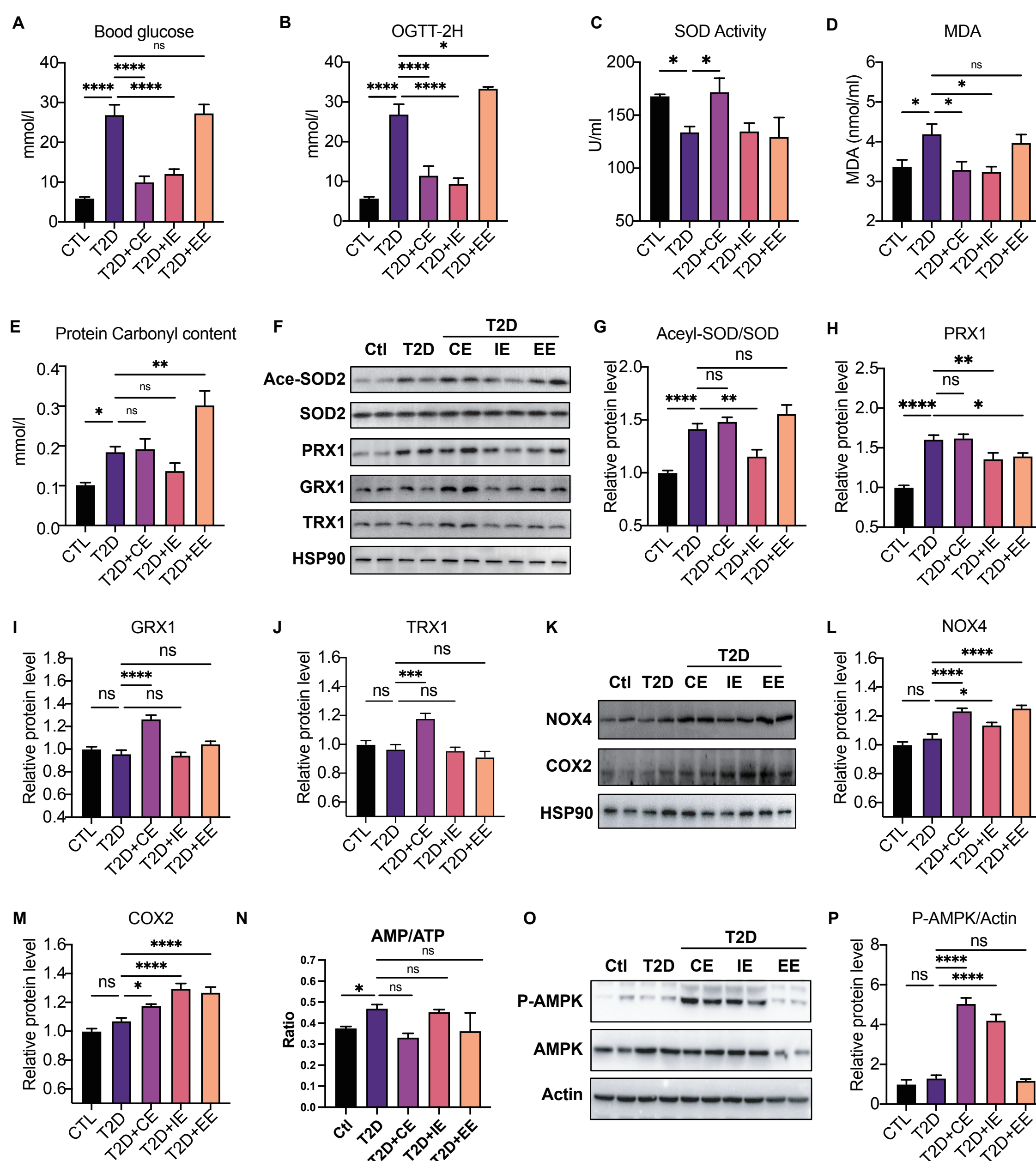

684

685 **Fig.3 Moderate exercise-generated ROS production promotes phosphorylated activation of**
686 **AMPK and reduces blood glucose level, while excessive exercise- generated oxidative stress**
687 **reduces AMPK expression and exacerbates diabetes.**

688 **A**. Postprandial blood glucose levels of Ctl, T2D, T2D + CE, T2D + IE and T2D + EE groups at the
689 end of 8th week. **B.** Blood glucose level after oral glucose administration in Ctl, T2D, T2D + CE,
690 T2D + IE and T2D + EE groups at the end of 8th week. **C-E**. SOD activity (C), liver MDA content
691 (D) and liver protein carbonylation content (E) level was detected in the rats of Ctl, T2D, T2D + CE,
692 T2D + IE and T2D + EE groups. **F-J.** Representative protein level and quantitative analysis of Ace-
693 SOD2 (27 kDa), SOD2 (17 kDa), PRX1 (27 kDa), Grx1 (17 kDa), Trx1 (12 kDa) and HSP90 (90
694 kDa) in the rats in the Ctl, T2D, T2D + CE, T2D + IE and T2D + EE groups. **K-N**. Representative
695 protein level and quantitative analysis of NOX4 (27 kDa), COX2 (17 kDa) and HSP90 (90 kDa) in
696 the rats in the Ctl, T2D, T2D + CE, T2D + IE and T2D + EE groups. **O-P**. Representative protein
697 level and quantitative analysis of P-AMPK (67 kDa), AMPK (67 kDa) and Actin (45 kDa) in the rats
698 in the Ctl, T2D, T2D + CE, T2D + IE and T2D + EE groups. (ns: not significant; *P < 0.05, **P <
699 0.01, ***P < 0.001, ****P < 0.0001 compared with all groups by one-way ANOVA and Tukey's
700 post hoc test; data are expressed as the mean ± SEM; n = 4-8 per group).

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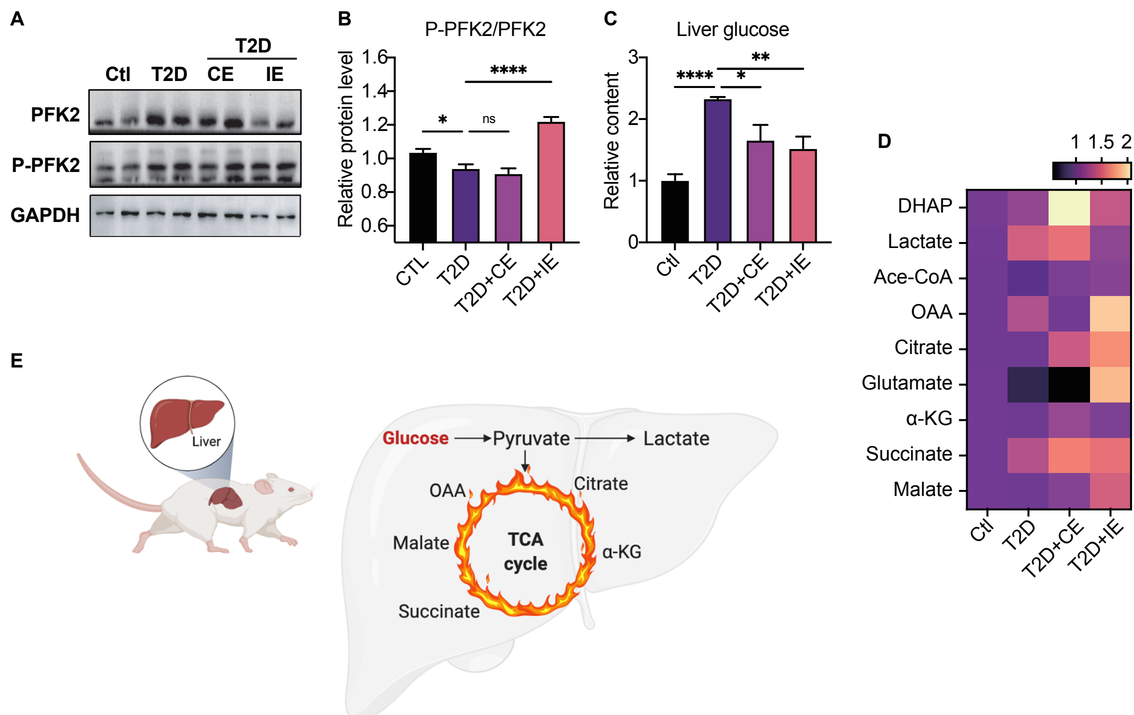

701

702 **Fig.4 Moderate exercise promoted glycolysis and mitochondrial tricarboxylic acid cycle in the**
703 **liver of diabetic rats.**

704 **A-B.** Representative protein level and quantitative analysis of P-PFK2 (64 kDa), PFK2 (64 kDa) and
705 GAPDH (37 kDa) in the rats in the Ctl, T2D, T2D + CE and T2D + IE groups. **C**. Liver glucose level
706 after oral glucose administration in Ctl, T2D, T2D + CE and T2D + IE groups at the end of 8th week**.**
707 **D.** Relative concentrations of substrates for glycolysis (DHAP and Lactate) and the tricarboxylic acid
708 cycle (citrate, succinate and malate) in the rat of Ctl, T2D, T2D + CE and T2D + IE groups. The
709 concentration of substrates was analyzed by LC-MS/MS. **E.** Schematic diagram illustrating the effect
710 of CE and IE on glycolysis and mitochondrial tricarboxylic acid cycle (ns: not significant; *P < 0.05,
711 **P < 0.01, ****P < 0.0001 compared with all groups by one-way ANOVA and Tukey's post hoc
712 test; data are expressed as the mean ± SEM; n = 6-8 per group).
713

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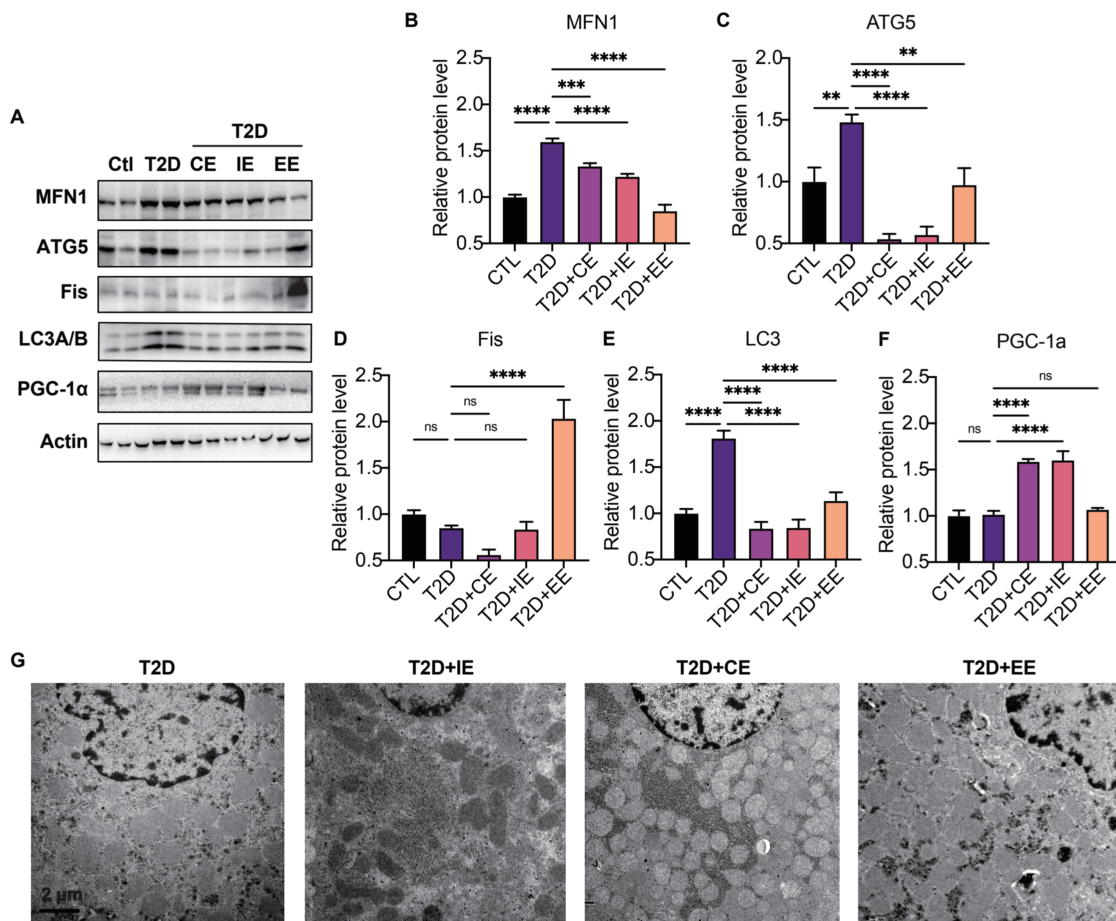

714

715 **Fig.5 Excessive exercise promoted hepatic mitophagy, inhibited the mitochondrial biogenesis**
716 **and destroyed the mitochondrial structure.**

717 **A-F.** Representative protein level and quantitative analysis of MFN1 (82 kDa), ATG5 (55 kDa), FIS
718 (25 kDa), LC3A/B (14,16 kDa), PGC-1α (130 kDa) and Actin (45 kDa) in the rats in the Ctl, T2D,
719 T2D + CE, T2D + IE and T2D + EE groups. **G.** TEM analysis of the ultrastructure of hepatocytes in
720 the rats in the T2D, T2D + CE, T2D + IE and T2D + EE groups. (Scale bar = 2 μm; ns: not significant;
721 *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with all groups by one-way ANOVA
722 and Tukey's post hoc test; data are expressed as the mean ± SEM; n = 8 per group).

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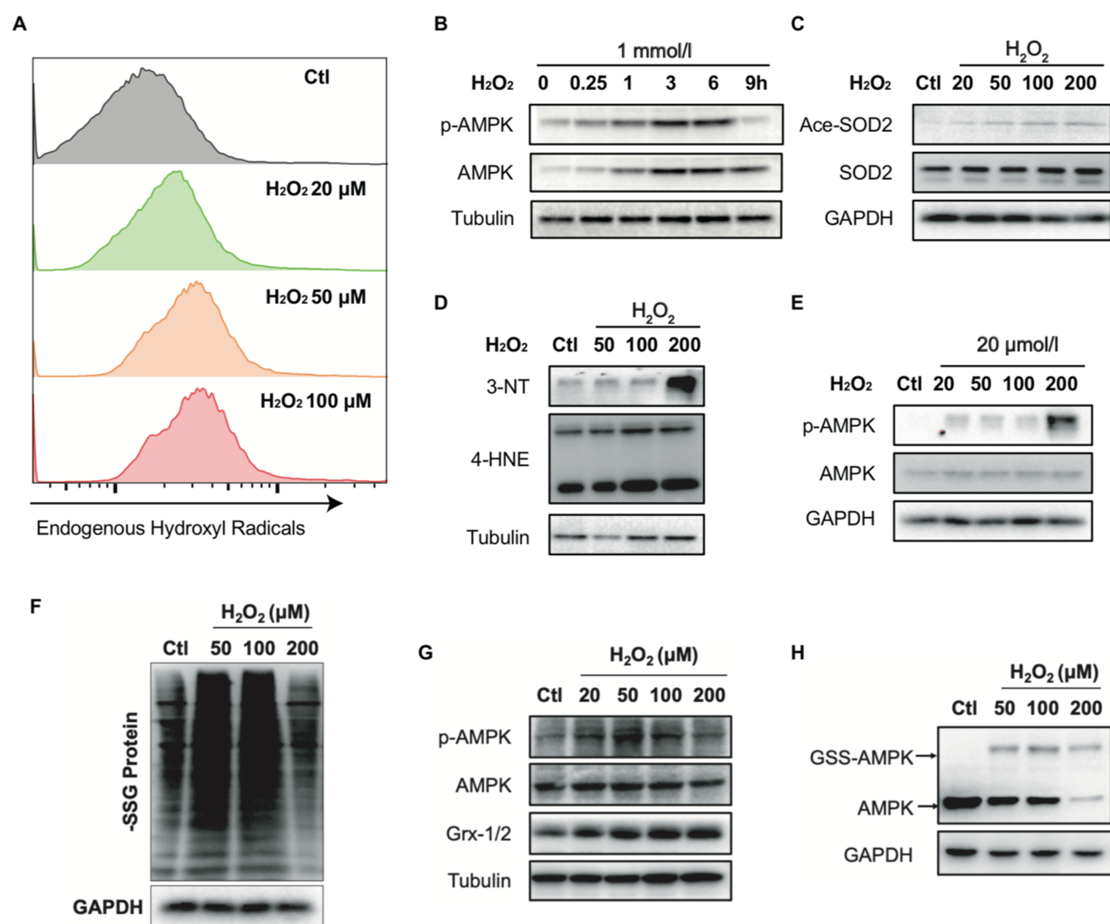

725

726 **Fig. 6 ROS differentially regulated AMPK activation through Grx-mediated glutathionylation**
727 **within redox balance threshold.**

728 **A.** Analysis of superoxide generation using flow cytometry and a hydroethidine probe in L02 cells
729 under H_2O_2 stress (50–200 μmol/l, 30 min). **B.** Representative protein level of 4-HNE, 3-NT and
730 GAPDH (37 kDa) in L02 cells under H_2O_2 stress (50–200 μmol/l, 30 min). **C.** Representative protein
731 level of Aceyl-SOD2, SOD2 and GAPDH (37 kDa) in L02 cells under H_2O_2 stress (50–200 μmol/l,
732 30 min). **D.** GSS-adduct protein level in L02 cells under H_2O_2 stress (50–200 μmol/l). L02 cells
733 loaded with EE-GSH-biotin were incubated with/without H_2O_2 for 30 min, and the amounts of GSS-
734 protein adduct formation were determined using non-reducing SDS-PAGE and Western blot analysis
735 with streptavidin-HRP. **E.** Representative protein level of P-AMPK (67 kDa), AMPK (67 kDa), Grx-
736 1/2 (12 kDa) and Tubulin (55 kDa) in L02 cells under H_2O_2 stress (50–200 μmol/l, 30 min). **F**. AMPK
737 cysteine gel shift immunoblot. Cysteine dependent shifts by incubation of AMPK protein with
738 glutathione reductase and PEG-Mal. PEG2-mal labelled glutathionylation modification shifts AMPK
739 by ~10 kDa above the native molecular weight.

740

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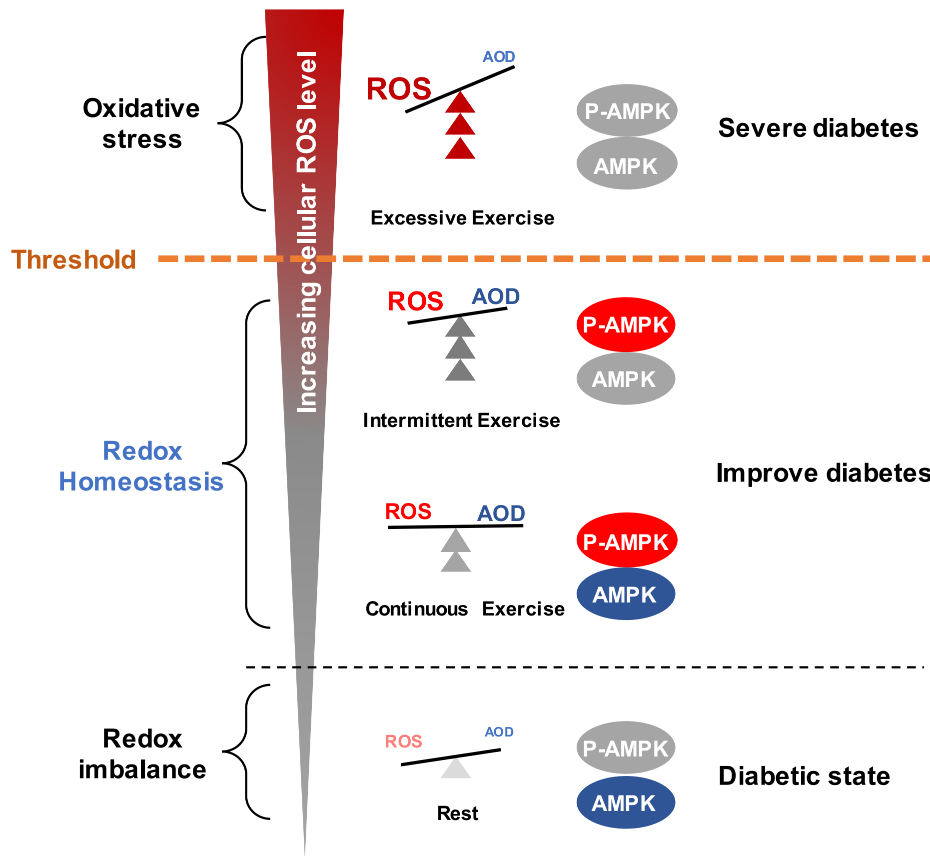

742

743 **Fig.7 Schematic diagram of redox balance threshold.**

744 Moderate exercise promotes the activity of antioxidant enzymes by generating benign ROS, directly
745 promotes AMPK-mediated glycolysis and aerobic oxidation, thus reduces glucose levels in blood and
746 liver. Excessive exercise causes excess ROS and exceed the redox balance threshold, inhibiting
747 AMPK activity and expression, thus leading to exacerbation of diabetes. (AMPK and P-AMPK in
748 grey circles indicate decrease, red circles indicate increase, blue circles indicate no significant
749 changes).