Hepatic AMPK signaling activation in response to dynamic REDOX balance is a biomarker of exercise to improve blood glucose control

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Abstract

Antioxidant intervention is considered to inhibit reactive oxygen species (ROS) and alleviates hyperglycemia. Paradoxically, moderate exercise can produce ROS to improve diabetes. The exact redox mechanism of these two different approaches remains largely unclear. Here, by comparing exercise and antioxidants intervention on type 2 diabetic rats, we found moderate exercise upregulated compensatory antioxidant capability and reached a higher level of redox balance in the liver. In contrast, antioxidant intervention achieved a low-level redox balance by inhibiting oxidative stress. Both of these two interventions could promote glucose catabolism and inhibit gluconeogenesis through activation of hepatic AMPK signaling, therefore ameliorating diabetes. During exercise, different levels of ROS generated by exercise have differential regulations on the activity and expression of hepatic AMPK. Moderate exercise-derived ROS promoted hepatic AMPK glutathionylation activation. However, excessive exercise increased oxidative damage and inhibited the activity and expression of AMPK. Overall, our results illustrate that both exercise and antioxidant intervention improve blood glucose in diabetes by promoting redox balance, despite different levels of redox balance. These results indicate that the AMPK signaling activation, combined with oxidative damage markers, could act as a sensitive biomarker, reflecting the threshold of redox balance defining effective treatment in diabetes. These findings provide theoretical evidence for the precise treatment of diabetes by antioxidants and exercise.

Key words

Redox balance; AMPK signaling; exercise; antioxidant; glutathionylation; diabetes
Introduction

Diabetes mellitus is a chronic metabolic disorder disease, which has emerged as a global public health problem. According to the latest epidemiological data from the International Diabetes Federation, the global diabetes prevalence in 20–79 year olds was estimated to be 10.5% (536.6 million people) in 2021, and is expected to rise to 12.2% (783.2 million) in 2045 (1). With the development of genomics, proteomics and metabolomics, it has been discovered by many studies that type 2 diabetes is associated with irreversible risk factors such as age, genetics, race, and ethnicity and reversible factors such as diet, physical activity and lifestyle (2, 3).

Aerobic metabolism in glucose oxidation, mitochondrial damage and oxidative stress have been considered to play a critical role in the occurrence and development of diabetes (4). Exercise and antioxidant supplements are often suggested as essential therapeutic strategies in the early stages of type 2 diabetes (5, 6), with different mechanisms. It has been reported that chronic exercise training can alleviate oxidative stress and diabetic symptoms by improving cellular mitochondrial function and biogenesis in the diabetic state (7). Contradictorily, exercise also increases ROS production, while prolonged or high-intensity exercise could result in mitochondrial functional impairment to aggravate complications of diabetes (8). Since 1970s, studies have demonstrated that 1 hour of moderate endurance exercise can increase lipid peroxidation in humans (9, 10). In 1998, Ashton directly detected increasing free radical levels in exercising humans using electron paramagnetic resonance spectroscopy (EPR) and spin capture (11). These results led to a great deal of interest in the role of ROS in physical exercise (12-14). Regarding the contradiction of exercise on ROS scavenging or production, James D Watson also hypothesized that type 2 diabetes is accelerated by insufficient oxidative stress rather than oxidative stress (15), based on the effect of exercise on diabetes treatment. Although Watson’s opinions supported that exercise could treat diabetes by producing ROS, whether exercise-induced ROS production is beneficial or detrimental to diabetes is still being debated. The specific regulation of ROS produced by exercise on diabetic blood glucose in vivo is unclear. In contrast, the general view of the antioxidant treatment for diabetes is that antioxidants reduce cytotoxic ROS and oxidation products, thus alleviating diabetes and achieving glycemic control (16). Our previous study also found that hepatic mitochondrial ROS scavengers and antioxidant substances inhibited the oxidative products such as MDA and 4-
HNE in diabetic animals and favored glycemic control (17, 18). Exercise-induced oxidation and antioxidant administration, as two opposite approaches, could achieve the regulation of diabetes, respectively. However, the differences in redox mechanisms between these two approaches to diabetes treatment have not been fully understood.

It is well established that the increase of skeletal muscle glucose uptake during exercise is crucial in glycemic control (19-21). Considering that liver is another vital organ for maintaining blood glucose homeostasis, including storing, utilizing and producing glucose, exercise-induced hepatic redox metabolism is also significant. The activation of hepatic AMP-activated protein kinase (AMPK), which acts as a ‘metabolic master switch’, alleviates diabetes symptoms by reducing glycogen synthesis, increasing glycolysis, and promoting glucose absorption in surrounding tissues (22). Therefore, the activation of AMPK in the liver is significant for regulating glucose and lipid metabolism in the blood. Zmijewski et al. found that AMPK could be activated by hydrogen peroxide stimulation through direct oxidative modification (23). In contrast, other studies suggested that oxidative stress could disrupt the activation of the AMPK signaling pathway (24, 25). Our previous study explored the mechanism by which redox status contributes to hepatic AMPK dynamic activation. Under a low ROS microenvironment, GRXs mediated S-glutathione modification activates AMPK to improve glucose utilization. In contrast, under an excessive ROS microenvironment, sustained high level ROS might cause loss of AMPK protein (26). These studies indicate that oxidative modification can directly regulate AMPK activity in liver cells, thus activating downstream signaling pathways to regulate glucose and lipid metabolism. However, it is unclear why two seemingly contradictory phenomenon of antioxidant intervention and exercise-induce ROS can promote AMPK activation. Moderate exercise has been proved significantly elevate systemic oxidative stress. At the same time, endogenous antioxidant defences also increased to counteract increased levels of ROS induced by exercise (27). Thus, we hypothesized that both antioxidants and exercise could reach either high-level or low-level redox balance in diabetic individuals. Moreover, the activity and expression of AMPK might be a marker of redox balance in vivo (Fig. 1).

Hence, the present study was designed to understand the different mechanisms of exercise and antioxidant intervention in diabetes and verify the activation of hepatic AMPK as a hallmark of dynamic redox balance. Firstly, we utilized the streptozotocin-high fat diet
(STZ-HFD) induced type 2 diabetic (T2DM) model in rats to clarify the hepatic redox status in T2DM rats after the exercise or antioxidant intervention. Then, according to the exercise intensity and mode, we divided the exercise groups into three modes and found that AMPK activation could serve as a biomarker of redox balance and moderate exercise in diabetic treatment. In this study, we found that AMPK activation and its downstream pathways could reflect the threshold of exercise or antioxidant administration for diabetes treatment. This study provides clues for the personalized treatment of diabetes by antioxidants and exercise.

Results

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

To investigate the hepatic redox regulation in diabetes after exercise intervention, we established the T2DM rat model by feeding HFD followed by a low dose of STZ injection (35 mg/kg). The exercise intervention was started from Day 0 to Day 28 (Fig. 2A). According to previous studies, the initial speed of exercise was 15 m/min, and the speed was increased by 3 m/min every 5 min. After the speed reached 20 m/min, the speed was maintained for another 60 min with slope of 5%. The exercise intensity was 64%-76% VO$_2$max (28). The low-intensity continuous exercise (CE) can be regarded as aerobic exercise.

The ROS-generating NADPH oxidases (NOXs) have been recognized as one of the main sources of ROS production in cells (29). Cyclooxygenase 2 (COX2) activity could also act as a stimulus for ROS production (30). The expressions of NOX4 and COX2 in the liver were increased in the diabetic group. After exercise intervention, NOX4 and COX2 level were further up-regulated compared with the diabetic group (Fig. 2B-D). These results indicate the exercise intervention up-regulated ROS production.

Next, we detected the expression of antioxidant enzymes in liver tissue. Nrf2 is the central regulator of the threshold mechanisms of oxidative stress and ROS generation (31). With the increase of ROS level in the development of diabetes, Nrf2 was activated to induce the transcription of several antioxidant enzymes (32, 33). We found an increase in Nrf2
expression in diabetic rats (Fig. 2E-F). After CE intervention, the level of Nrf2 levels further increased, indicating that exercise intervention could activate antioxidant system (Fig. 2E-F). Under stress conditions, Nrf2 translocates to the nucleus and binds to antioxidant response elements (AREs), which results in the expression of diverse antioxidant and metabolic genes, such as thioredoxin (Trx), to relieve oxidative damage (34). Thioredoxin-1 (Trx-1), a type of cytosolic isoform of Trx, has been widely accepted to regulate glutathione metabolism with GRX and PRX. After CE intervention, we found the protein expression of GRX1 and TRX1 were up-regulated (Fig. 2H, 2J-K). Notably, the PRX expression also showed a trend of increase (Fig. 2H-I). Sestrin2 is a cysteine sulfanyl reductase that plays crucial roles in regulation of antioxidant actions (35). As an endogenous antioxidant, the hepatic sestrin2 level also showed a significant increase in the CE group (Fig. 2E,2G).

Since excess ROS can cause the increase of oxidative damage (36), we further detected the protein damage and lipid peroxidation to determine the redox status. 3-Nitrotyrosine (3-NT) and protein carbonylation are biomarkers of reactive nitrogen species (RNS) and ROS modified proteins (37, 38). We found that the CE intervention reduced the 3-Nitrotyrosine level and did not further decrease the protein carbonylation level (Fig. 2L-N). MDA, a biomarker of lipid peroxidation, was also significantly up-regulated in the diabetic group but decreased in exercise group (Fig. 2O). These results indicate that the high ROS production in the CE group could, instead, increase the antioxidant status to avoid oxidative damage. It suggests that CE can promote redox to reach a high level of balance. Therefore, even if exercise increases the ROS-generating enzymes NOX4 and COX2, the increase in ROS production does not lead to oxidative damage.

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

Recent studies have suggested that NADPH oxidase is one of the primary sources of ROS (29, 39). Apocynin has already been characterized as an NADPH oxidase inhibitor in the early 1980s, and it can also act as an antioxidant (40). Our previous study showed that apocynin intervention alleviated blood glucose by inhibiting oxidative products. In this study, the antioxidant supplement was also started from Day 0 to Day 28 in this study (Fig.
We found that apocynin supplement decreased the protein carbonylation level and MDA level in the liver (Fig. 3B-C). Also, the TAOC level was increased after apocynin treatment, indicating the decrease of oxidation level (Fig. 3D). Moreover, as endogenous antioxidant, the Sestrin2 and Nrf2 expression decreased after apocynin intervention (Fig. 3E-G). These results indicate that the antioxidant intervention reduced the ROS in diabetic hepatocytes, thereby decreasing the ROS-induced compensatory upregulation of Sestrin2 and Nrf2.

Consistently, Glut2, a glucose sensor in the liver, was increased in diabetic liver and decreased after the apocynin supplement (Fig. 3E and 3H). The postprandial blood glucose, fasting blood glucose and oral glucose tolerance (2 h after oral glucose, OGTT) were decreased in the apocynin intervention group compared with the diabetic rat group (Fig. 3I-K). Consistent with the apocynin intervention group, the exercise group also showed lower postprandial blood glucose and fasting blood glucose levels and OGTT (Fig. 3I-K). These studies indicate that the apocynin treatment improved the diabetes through inhibiting ROS level and protein oxidative damage to achieve a low-level redox balance.

3. Moderate exercise-generated ROS promotes activation of AMPK by phosphorylation and reduces blood glucose level, while excessive exercise-generated oxidative stress reduces AMPK expression and exacerbates diabetes.

In order to find out the biomarkers that could reflect moderate exercise to improve blood glucose control, diabetic rats were divided into short-term continuous exercise (CE), intermittent exercise (IE), and excessive exercise (EE) according to the exercise intensity and mode (28). We found that the random blood glucose and 2h OGTT in CE and IE treated diabetic rats were decreased (Fig. 4A-B). In contrast, EE intervention did not improve blood glucose but slightly increased random and 2h OGTT (Fig. 4A-B).

We detected the increase of ROS production-related enzymes, such as NOX4 and COX2 in the EE group, indicating the highest oxidation level (Fig. 4C-E). Next, we detected the expression of antioxidant enzymes and oxidative damage in the liver tissue of exercise-treated T2D rats. As shown in Fig. 4F-G, IE intervention increased the activity of MnSOD as shown by decreased level of acetylation compared with the diabetic rats. The expression
of GRX and TRX were up-regulated after CE intervention (Fig. 4F, 4H-I). Furthermore, we detected the oxidative damage in these three modes. The results showed that the CE and IE group did not obviously change the protein carbonylation level. However, the EE intervention promoted the protein carbonylation in the liver, indicating this mode of action is not due to free radical scavenging but oxidative damage (Fig. 4J). In addition to the protein damage, hepatic MDA concentration showed significant up-regulation in the diabetic group but decreased in CE and IE group (Fig. 4K), while increased MDA in the EE group indicates oxidative damage. Among these three exercise modes, the IE group showed the lowest level of oxidation (the minor increase in NOX4 and a slight decrease in carbonylation). Although the levels of antioxidant enzymes such as GRX and TRX did not increase, the activity of MnSOD also increased significantly (Fig. 4F-4G). The reduction of MDA level also indicates IE group did not form oxidative damage (Fig. 4K), indicating the IE group could also maintain a relatively high level of redox balance. Nevertheless, the decrease of antioxidant enzymes and increase of oxidative damage in the EE group indicates that the REDOX balance in the EE group was disrupted.

Notably, the phosphorylation of AMPK showed different patterns in three kinds of exercise, among which both CE and IE intervention could promote the phosphorylation of AMPK compared to the diabetic rats (Fig. 4M-N). EE intervention did not increase the content of AMPK phosphorylation, which might be caused by the reduction of AMPK level. Meanwhile, the ratio of AMP to ATP was detected, and exercise-activated AMPK did not exhibit AMP-dependent characteristics at this time (Fig. 4L). These results suggest that moderate exercise-generated ROS may directly promote AMPK activation by phosphorylation without AMP upregulation and reduce blood and liver glucose levels. However, excessive exercise-generated oxidative stress reduces AMPK expression and exacerbates diabetes.

### 4. Moderate exercise promoted glycolysis and mitochondrial tricarboxylic acid cycle and inhibited the gluconeogenesis in the liver of diabetic rats.

Next, we further explored the mechanism by which inhibiting blood glucose during CE and IE intervention. Fructose-2,6-diphosphate (F-2,6-P2; also known as F-2,6-BP), which is a product of the bifunctional enzyme 6-phosphofructose 2-kinase/fructose 2,6-diphosphatase 2 (PFK/FBPase 2, also known as PFKFB2), is a potent regulator of glycolytic and gluconeogenic flux. The phospho-PFKFB2 to PFKFB2 ratio represents the glycolytic rate.
A high ratio of phospho-PFKFB2:PFKFB2 leads to an increase in the F-2,6-P2 level and the allosteric activation of phosphor-fructose kinase 1 (PFK1), while a low ratio leads to a decrease in F-2,6-P2 and an increase in gluconeogenesis (41). The overexpression of bifunctional enzymes in mouse liver can reduce blood glucose levels by inhibiting hepatic glucose production (42). Therefore, bifunctional enzymes are also a potential target for reducing hepatic glucose production. In our study, the p-PFK2:PFK2 ratio decreased in the diabetic rats but was enhanced by CE and IE intervention (Fig. 5A-C), suggesting that CE and IE could reverse gluconeogenesis to glycolysis by enhancing PFK/FBPase. Meanwhile, the substrates of the glycolytic pathway (such as DHAP, Fig. 5D) and the tricarboxylic acid cycle (such as citrate, succinate and malate, Fig. 5D) showed an upward trend.

Besides glycolysis, gluconeogenesis are critical in maintaining liver and blood glucose homeostasis. FoxO1 has been tightly linked with hepatic gluconeogenesis through inhibiting the transcription of gluconeogenesis-related PEPCK and G6Pase expression (43, 44). Herein, we found the expression of FoxO1 was increased in the diabetic group but reduced in the CE and IE groups (Fig. 5E-F). Meanwhile, the mRNA level of Pepck and G6PC also decreased in the CE and IE groups (Fig. 5H-I). These results indicate that moderate exercise (CE and IE) inhibited gluconeogenesis through down-regulating FoxO1.

For the glucose uptake, we detected the protein expression of GLUT2 in the liver tissue, which helps in the uptake of glucose by the hepatocytes for glycolysis and glycogenesis. We found the level of GLUT2 was increased in diabetic mice, but down-regulated by the CE and IE intervention (Fig. 5E and 5G). Taken together, these results illustrated that moderate exercise promoted glucose transport, glucose catabolism and inhibited the gluconeogenesis in the liver of diabetic rats (Fig. 5J).

5. Moderate exercise inhibited hepatic mitophagy, while excessive exercise exhibited opposite effect and inhibited the mitochondrial biogenesis.

The electron transport associated with the mitochondrial function is considered the major process leading to ROS production during exercise (45). To further explore the downstream signal of AMPK activation in moderate and excessive exercise, we detected the protein expression of mitochondrial dynamic and mitochondrial biogenesis. According to the result in Fig. 6A-E, we found that the mitochondrial fusion protein MFN was significantly decreased in the liver of the excessive exercise group, and the mitochondrial fission protein...
Fis and autophagy-related protein ATG5 and LC3B did not change, compared with the diabetic group. Notably, the ATG5 and LC3B levels decreased in the CE and IE group, compared with the diabetic group (Fig. 6A-E). Since PGC-1α is a transcriptional coactivator, a central inducer of mitochondrial biogenesis in cells (46), we measured the expression of PGC-1α and found that its expression was increased in CE and IE group, but not in the EE group (Fig. 6F).

These results indicates that moderate exercise promoted mitochondrial biogenesis and ameliorated autophagy in the liver. However, excessive exercise aggravated mitochondrial fission and did not alleviate autophagy. Similarly, the mitochondria structure of the live tissue in the EE group was fragmented and showed greatly diminished cristae and swelling matrix under transmission electron microscopy, reflecting a defect in oxidative phosphorylation. However, the CE and IE group showed increased numbers of cristae and a clear structure of mitochondrial cristae (Fig. 6H). These results all showed that the in vivo mitochondrial ROS burst caused by excessive exercise inhibited the expression of AMPK and promoted mitophagy. The damage to the mitochondrial dynamics and structure in liver tissue led to abnormal aerobic oxidation, thereby aggravating diabetes.

6. Moderate ROS activates AMPK through GRX-mediated glutathionylation

To further illustrate the effect of moderate ROS on the activation of AMPK, primary hepatocytes were intervened with H2O2 to mimic ROS production in vivo. H2DCFDA and dihydroethidium dyes were used to assess intracellular ROS levels in the H2O2-treated primary hepatocytes. As shown in Fig. 7A-7B, the levels of ROS (DCF), such as H2O2, and O2•− accumulation (dihydroethidium) were dose-dependently increased in the primary hepatocytes treated with 50-200 μM H2O2. Consistent with our previous study (26), we found moderate ROS would activate AMPK through GRX-mediated S-glutathionylation. As shown in Fig. 7C, exposure to 50 and 100 μmol/l H2O2 led to an increase of GSS-protein adduct, concomitant with the AMPK phosphorylation (Fig. 7C-F), suggesting that the ROS level within redox balance threshold could induce glutathionylation and phosphorylation of AMPK to activate AMPK. However, when the concentration of ROS was higher (200 μmol/l H2O2), both of AMPK glutathionylation and phosphorylation were decreased (Fig. 7E-7H). These results indicates that activation of AMPK by moderate ROS might be mediated through GRX-mediated S-glutathionylation.
Meanwhile, we also detected the substrates of glycolysis and aerobic oxidation at different concentrations of H$_2$O$_2$. We found the exposure to 20-100 μmol/l H$_2$O$_2$, which made cells within the redox balance threshold, showed a trend of increase on glycolysis and aerobic oxidation substrates, indicating the increase of hepatic glucose catabolism (data not shown).

**Discussion**

It has been shown that both antioxidants and exercise can be beneficial in substantially ameliorating hyperglycaemia through ROS-mediated mechanisms in diabetes patients. However, antioxidant intervention reduces oxidative stress, while exercise produces ROS. It is imperative to explore the mechanisms underlying these seemingly paradoxical approaches for effective diabetes treatment.

The remission of diabetes by antioxidant intervention has been well documented. Some compounds in food that have substantial antioxidant activities or inhibit NADPH oxidase, such as polyphenols and flavonoids (47), have been shown to improve blood glucose and relieve type 2 diabetes in animal experiments. Several clinical trials also demonstrated the relief of diabetic hyperglycaemia by antioxidants (48, 49). Our previous study found that hepatic mitochondrial ROS scavenger and antioxidant substances inhibited the oxidative products such as MDA and 4-HNE in diabetic mice and rats and improved blood glucose control (18). These results indicate that reducing the oxidative level of diabetic animals could treat diabetes. Hepatic AMPK regulates cellular and whole-body energy homeostasis, signals to stimulate glucose uptake in skeletal muscles, fatty acid oxidation in adipose and other tissues, and reduces hepatic glucose production (50, 51). Numerous pharmacological agents, including the first-line oral drug metformin, natural compounds, and hormones are known to activate AMPK (52-55). Moreover, our previous study found that antioxidant intervention in diabetic rats could promote the phosphorylation and activation of AMPK protein, thereby regulating hepatic glucose metabolism (56). Taken together, we found that the activation of AMPK by antioxidant intervention was accompanied by a decrease in oxidative stress level in diabetic rats, resulting in a low level of redox balance to benefit diabetic hyperglycaemia.

In the meantime, regular exercise have been recommended to mitigate symptoms of many diseases, including psychiatric, neurological, metabolic, cardiovascular, pulmonary,
musculoskeletal, and even cancer (57). John Holloszy’s studies found that exercise improved insulin sensitivity in patients with type 2 diabetes and provided a better understanding of how muscle adapts to endurance exercise (19-21, 58-60). Although the benefits of exercise are irrefutable, excessive exercise is harmful (8), suggesting the importance of the amount and intensity of exercise. Recently, Chrysovalantou et al. found that NADPH oxidase 4 (NOX4) is a crucial exercise-related protein to regulate adaptive responses and prevent insulin resistance (61). We found that exercise could indeed increase NOX4 expression, but NOX4 was also upregulated in excessive exercise. Although Chrysovalantou's study highlights the role of the redox environment in exercise, the biomarkers of effective intervention of moderate exercise on diabetes remain unclear.

The mechanisms of exercise for the treatment of diabetes have previously been studied mainly around the skeletal muscle, as the activation of AMPK in skeletal muscle during exercise is considered mainly caused by the increase of intracellular AMP:ATP ratio and phosphorylation of Thr172 on the “activation loop” of the α-subunit (62). The activation of AMPK leads to the inhibition of mTORC1 activity and activation of PGC-1α, which enhances mitochondrial biogenesis and further increases muscle uptake of glucose from the blood (63). However, liver energy state also plays an essential role in the activation of AMPK (64) and liver is known to be critical in whole-body glucose tolerance (65). In addition, AMPK is also considered as a redox-sensitive protein, and its cysteine 299 and 304 sites are likely to be regulated by the oxidation of hydrogen peroxide (23, 66, 67). Thus, AMPK might be activated not only by the increase of AMP, but also by phosphorylation through ROS regulation during exercise.

Currently there is no appropriate biomarkers to differentiate moderate and excessive exercises. According to the exercise intensity and mode, we divided the exercise groups into three modes: CE, IE for moderate exercises and EE for excessive exercise. Our study, for the first time, found that hepatic AMPK activation could act as a biomarker of dynamic redox balance during exercise to improve glycaemic control in diabetic rats. ROS generated by different exercise intensities could profoundly alter the cellular redox microenvironment and directly regulate the activity and expression of hepatic AMPK through a redox-related mechanism. Moderate exercise produced optimal ROS directly promoted AMPK activation via glutathionylation in hepatocytes. The activated AMPK signaling pathway can phosphorylate and activate PFK-2 to promote glycolysis and aerobic oxidation (Fig. 8A).
Furthermore, AMPK activation can phosphorylate and inhibit the CRCT2 and class IIa HDACs pathways in the liver, thus affecting the binding of class IIa HDACs to the FOXO-family of transcription factors (68, 69). Since the gluconeogenesis related mRNA expression of PEPCK and G6Pase was mainly transcribed by FOXO1, the reduction of FOXO transcriptional activity induced by AMPK indicates the inhibition of gluconeogenesis. Therefore, under moderate exercise, the metabolic balance between glycolysis and gluconeogenesis is regulated through the activation of AMPK phosphorylation, which promotes glucose catabolism and inhibits gluconeogenesis. However, excessive ROS inhibits the activity and expression of AMPK in hepatocytes cells, which might be related with the oxidative stress induced protein degradation (Fig. 8A). In addition, the number of mitochondria and the function of aerobic oxidation in the EE group was significantly lower than those in the moderate exercise group. In the EE group, the autophagy and fission of liver mitochondria were also up-regulated as compared with the moderate exercise, accompanied by the increase of MDA, an indicator of lipid peroxidation damage. These results indicate that AMPK signaling and oxidative damage-related index could be investigated as sensitive biomarkers for redox status changes during exercise intervention in diabetic rats. This finding can be applied to analyze the thresholds of redox balance that discriminates moderate and excessive exercise.

Exercise is considered to improve blood glucose by promoting ROS levels, which seems to be contradictory to antioxidant interventions of inhibiting ROS. Zsolt Radak et al proposed a bell-shaped dose-response curve between normal physiological function and level of ROS in healthy individuals, and suggested that moderate exercise can extend or stretch the levels of ROS while increases the physiological function (70). Our results validated this hypothesis and further proposed that moderate exercise could produce ROS meanwhile increase antioxidant enzyme activity to maintain high level redox balance according to the bell-shaped curve, whereas excessive exercise would generate a higher level of ROS, leading to reduced physiological function. In this study, we found the state of diabetic individuals is more applicable to the description of a S-shaped curve, due to the high level of oxidative stress and decreased reduction level in diabetic individuals (Fig.8B). With the increase of ROS, the physiological function of diabetic individuals gradually decreases. Moderate exercise shifts the S-shaped curve into a bell-shaped dose-response curve, thus reducing the sensitivity to oxidative stress in diabetic individuals and restoring redox homeostasis. However, with excessive exercise, ROS production increases beyond the
threshold range of redox balance, resulting in decreased physiological function (Fig.8B, see the decreasing portion of the bell curve to the right of the apex).

Nevertheless, the antioxidant intervention increased physiological activity by reducing ROS levels in diabetic individuals, restoring a bell-shaped dose-response curve at low level of ROS (Fig.8B). Therefore, redox balance could be achieved either at low level of ROS mediated by antioxidant intervention or at high level of ROS mediated by moderate exercise, both of which were regulated by AMPK activation. Therefore, both high and low levels of redox balance can lead to high physiological function as long as they are in the redox balance threshold range. Then, the activation of AMPK is an important sign of exercise or antioxidant intervention to obtain redox dynamic balance which helps restore physiological function. Accordingly, we speculate that the antioxidant intervention based on moderate exercise might offset the effect of exercise, but antioxidants could be beneficial during excessive exercise. The human study also supports that supplementation with antioxidants may preclude the health-promoting effects of exercise (71). Therefore, personalized intervention with respect to redox balance will be crucial for the effective treatment of diabetes patients.

Together, our study revealed the mechanisms underlying seemingly contradictory effects of level of ROS by either antioxidant intervention or moderate exercise for the treatment of diabetes. Moderate exercise promoted hepatic ROS production and up-regulated antioxidant capability, achieving a high-level balance of redox state. In contrast, antioxidant intervention scavenged the hepatic free radical to form a delicate low-level balance of redox state. Moreover, excessive exercise led to redox imbalance due to excess ROS levels. Hepatic AMPK signaling activation could act as a sign and hallmark of moderate exercise and dynamic redox balance to guide appropriate exercise or antioxidant intervention. These results illustrate that it is necessary to develop a moderate exercise program according to the REDOX microenvironment of diabetes patients and provide theoretical evidence for the precise treatment of diabetes by antioxidants and exercise.
Materials and Methods

1. Materials

T-AOC kit was supplied by Changzhou Redox Biological Technology Corporation (Jiangsu, CN). Antibodies against Actin, Acetylated-Lysine, P-PFK2, Ace-SOD2, ATG5, LC3A/B, GAPDH, MFN1 and IgG-HRP were purchased from Cell Signaling Technology (USA). Antibodies against CAT, PRX1, AMPKa1, GRX1, GRX2, SOD2, HSP90, COX1, COX2 and PFK2 were purchased from ProteinTech (Wuhan, CN). Antibodies against 3-NT, 4HNE, NOX4 and PGC-1α were purchased from Abcam. Antibodies against p-AMPKα1/α2 were purchased from SAB (Signalway Antibody, USA). The detailed antibody information is shown in Supplementary file 1a. High-fat diet (HFD) were purchased from Shanghai SLRC laboratory animal Company Ltd (Shanghai, China) and the nutritional composition is shown in Supplementary Table S2.

2. Animal

Male SD rats (150–160 g body weight, 6-8 weeks) were purchased from Fudan University Animal Center (Shanghai, China). Normal chow and high-fat diet (HFD) were purchased from Shanghai SLRC laboratory animal Company Ltd (Shanghai, China) and the nutritional composition was shown in Supplementary file 1b. All animal care and experimental procedures were approved by the Fudan University Institutional Laboratory Animal Ethics Committee (NO. 20170223-123). Animals were housed in a pathogen free environment with 12 h dark/light cycles.

3. Establishment of diabetic rat model

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

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

Continuous exercise:
The initial speed was 15 m/min, and the speed was increased by 3 m/min every 5 min. After the speed reached 20 m/min, the speed was maintained for another 60 min with slope of 5%. The exercise intensity was 64%-76% VO2max (28, 72, 73).

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

Excessive exercise:
The initial speed was 15 m/min, and the speed was increased by 3 m/min every 5 min. After the speed reached 50 m/min, the speed was maintained for another 60 min with slope of 5%. The exercise intensity was higher than 80% VO2max.

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

4. Cell culture

Normal Human Hepatic Cell Line L02 cells (Cell Bank of Chinese Academy of Sciences) were grown in DMEM supplemented with 10% FBS (GIBCO, USA) in a humidified incubator (Forma Scientific) at 37 °C and 5% CO2 as described previously. The medium were supplemented with 10% FBS (GIBCO, USA), 2 mmol/l glutamine, 1 mmol/l sodium pyruvate, 10 mmol/l HEPES, 50 μmol/l β-mercaptoethanol, 105 U/l penicillin and streptomycin. Glutamine and sodium pyruvate were purchased from Sinopharm Chemical
Reagent Co., Ltd, HEPES were purchased from Beyotime Biotechnology (Shanghai, CN). All cell lines used in the study were tested for mycoplasma and were STR profiled.

5. Flow cytometry

For measurement of intracellular Superoxide, primary hepatocytes were stained with 5 μM hydroethidine (superoxide indicator) (Thermo Fisher Scientific, USA). Stained cells were analyzed with NovoCyte Quanteon flow cytometer (Agilent Technologies, Inc.), and acquired data were analyzed with NovoExpress software (Agilent Technologies, Inc.) and FlowJo software (TreeStar, Ashland, OR).

6. ATP and AMP content analysis

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

7. SOD activity assay

SOD activity assay was carried out using a chemiluminometric detector (Lumat LB9507, Berthold). Superoxide anions were generated by adding xanthine oxidase (XO) into the reaction system consisting of xanthine and Lucigenin. The drop of luminescence within 2 min was recorded as the relative SOD activity.

8. MDA content assay

MDA from the oxidative polyunsaturated fatty acids (PUFA) degradation is determined by the reaction of thiobarbituric acid (TBA) with MDA to generate the stable end product of MDA-TBA adduct. Liver tissue lysis was reacted with TBA to form a red product which can be detected using fluorometric (Ex/Em: 532/553 nm) plate reader.

9. S-Glutathionylation of AMPK and detection of GSS-AMPK adduct formation
GSS-AMPK adduct were measured as described previously (23, 26). Primary hepatocytes (2×10^6 cell/well) were incubated with ethyl ester GSH-biotin (6 mM) for 1 h. Cells were then washed twice with culture buffer to remove the excess of GSH and treated with H_2O_2 for 30 min. Cell lysates were prepared in the presence of N-ethylmaleimide (5 mM) and then passed through Bio-Gel P10 to remove free GSH-biotin and N-ethylmaleimide. The level of GSS-protein conjugates was determined using non-reducing Western blot analysis with streptavidin-HRP, whereas GSS-AMPK subunit levels were measured after pull-down with streptavidin-agarose (60 min at 4 °C), followed by reducing SDS-PAGE and Western blot analysis with antibodies against AMPK α subunit.

10. Metabolite profiling detection

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

11. Western blot analysis

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

12. Transmission electron microscope (TEM)

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

13. Western blot analysis

Cells were lysed RIPA buffer with phosphatase inhibitor at 4 °C for 30 min. Cell lysates were resolved by 10% SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes, and probed with primary antibodies. Membranes were incubated with peroxidase-conjugated secondary antibodies and visualized using a chemiluminescent substrate (ECL; GE Amersham Pharmacia, Beijing, China) and Tanon-5200 Chemiluminescent Imaging System.

14. Statistics

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

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Competing interests:

All other authors declare they have no competing interests.
Data and materials availability:
All data are available in the main text or the supplementary materials.

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**Figure legend:**

**Fig. 1** A model of the different mechanisms of exercise and antioxidant intervention in diabetes.

A graphical abstract of this study. Moderate exercise upregulated compensatory antioxidant capability and reached a high-level redox balance, whereas antioxidant intervention achieved a low-level redox balance by inhibiting oxidative stress for treating diabetes.

**Fig. 2** Moderate exercise induced ROS production in exercise group and increased the antioxidant status.

A. Experimental design. T2DM rats model was fed by high-fat diet plus a low dose of STZ injection (35 mg/kg). The high-fat diet (HFD, 60% calories from fat) was started from the 1st week to the 8th week. The exercise intervention was started from 1st week to 4th week. B-D. Representative protein level and quantitative analysis of NOX4 (27 kDa), COX2 (17 kDa) and Actin (45 kDa) in the rats in the Ctl, T2D and T2D + CE groups. E-G. Representative protein level and quantitative analysis of Nrf2 (97 kDa), Sestrin2 (56 kDa) and Actin (45 kDa) in the rats in the Ctl, T2D and T2D + CE groups. H-K. Representative protein level and quantitative analysis of PRX1 (27 kDa), Grx1 (17 kDa), Trx1 (12 kDa) and Actin (45 kDa) in the rats in the Ctl, T2D and T2D + CE groups. The rat livers were homogenized by 1% SDS and analyzed by Western blots with the appropriate antibodies. L-M. Representative protein level and quantitative analysis of 3-NT and Actin (45 kDa) in the rat in the Ctl, T2D and T2D + CE groups. N-O. Liver protein carbonylation (O) and MDA content (P) level was detected in the rats of Ctl, T2D, T2D + CE groups. (ns: not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with all groups by one-way ANOVA and Tukey’s post hoc test; data are expressed as the mean ± SEM; n = 4-8 per group).

**Fig. 3** Antioxidant intervention alleviates blood glucose through promoting the upregulation of reducing levels

A. Experimental design. T2DM rats model was fed by high-fat diet plus a low dose of STZ injection (35 mg/kg). The apocynin intervention was started from 1st week to 4th week. B-E. Liver protein carbonylation, MDA content (C) and TAOC (D) level were detected in the rats of Ctl, T2D and T2D + APO groups. E-H. Representative protein level and quantitative analysis of Nrf2 (97 kDa), Sestrin2 (57 kDa), Glut2 (60-70 kDa) and HSP90 (90 kDa) in the rats in the Ctl, T2D and T2D + APO groups. I. Postprandial blood glucose levels of Ctl, T2D, T2D + CE and T2D + APO groups at the end of 8th week. J. Fasting blood glucose levels of Ctl, T2D, T2D + CE and T2D + APO groups at the end of 8th week. K. Blood glucose level after oral glucose administration (0 min, 60min and 120 min) in Ctl, T2D, T2D + CE and T2D + APO groups at the end of 8th week (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with all groups by one-way ANOVA and Tukey’s post hoc test; data are expressed as the mean ± SEM; n = 4-8 per group).
Fig. 4 Moderate exercise-generated ROS promotes activation of AMPK by phosphorylation and reduces blood glucose level, while excessive exercise-generated oxidative stress reduces AMPK expression and exacerbates diabetes.

A. Postprandial blood glucose levels of Ctl, T2D, T2D + CE, T2D + IE and T2D + EE groups at the end of 8th week. B. Blood glucose level after oral glucose administration in Ctl, T2D, T2D + CE, T2D + IE and T2D + EE groups at the end of 8th week. C-E. Representative protein level and quantitative analysis of NOX4 (27 kDa), COX2 (17 kDa) and HSP90 (90 kDa) in the rats in the Ctl, T2D, T2D + CE, T2D + IE and T2D + EE groups. F-J. Representative protein level and quantitative analysis of Ace-SOD2 (27 kDa), SOD2 (17 kDa), Grx1 (17 kDa), Trx1 (12 kDa) and Actin (45 kDa) in the rats in the Ctl, T2D, T2D + CE, T2D + IE and T2D + EE groups. K-L. Liver protein carbonylation content (k), liver MDA content (L) and AMP/ATP ratio (M) were detected in the rats of Ctl, T2D, T2D + CE, T2D + IE and T2D + EE groups. N-O. Representative protein level and quantitative analysis of P-AMPK (67 kDa), AMPK (67 kDa) and Actin (45 kDa) in the rats in the Ctl, T2D, T2D + CE, T2D + IE and T2D + EE groups. (ns: not significant; *P < 0.05, **P < 0.01, ****P < 0.0001 compared with all groups by one-way ANOVA and Tukey’s post hoc test; data are expressed as the mean ± SEM; n = 4-8 per group).

Figure 4_source data_01

Figure 4_source data_02

Fig. 5 Moderate exercise promoted glycolysis and mitochondrial tricarboxylic acid cycle and inhibited the gluconeogenesis in the liver of diabetic rats.

A-B. Representative protein level and quantitative analysis of P-PFK2 (64 kDa), PFK2 (64 kDa) and GAPDH (37 kDa) in the rats in the Ctl, T2D, T2D + CE and T2D + IE groups. C. Liver glucose level after oral glucose administration in Ctl, T2D, T2D + CE and T2D + IE groups at the end of 8th week. D. Relative concentrations of substrates for glycolysis (DHAP and Lactate) and the tricarboxylic acid cycle (citrate, succinate and malate) in the rat of Ctl, T2D, T2D + CE and T2D + IE groups. The concentration of substrates was analyzed by LC-MS/MS. E-G. Representative protein level and quantitative analysis of FoxO1 (82 kDa), GLUT2 (60-70 kDa) and Actin (45 kDa) in the rats in the Ctl, T2D, T2D + CE and T2D + IE groups. H-I. Expression of hepatic Pepck and G6C mRNA in the Ctl, T2D, T2D + CE and T2D + IE groups were evaluated by real-time PCR analysis. Values represent mean ratios of Pepck and G6pase transcripts normalized to GAPDH transcript levels. J. Schematic diagram illustrating the effect of CE and IE on glycolysis, gluconeogenesis and mitochondrial tricarboxylic acid cycle (ns: not significant; *P < 0.05, **P < 0.01, ****P < 0.0001 compared with all groups by one-way ANOVA and Tukey’s post hoc test; data are expressed as the mean ± SEM; n = 6-8 per group).

Figure 5_source data_01

Figure 5_source data_02

Fig. 6 Moderate exercise inhibited hepatic mitophagy, while excessive exercise exhibited opposite effect and inhibited the mitochondrial biogenesis.
A-F. Representative protein level and quantitative analysis of MFN1 (82 kDa), ATG5 (55 kDa), FIS (25 kDa), LC3A/B (14,16 kDa), PGC-1α (130 kDa) and Actin (45 kDa) in the rats in the Ctl, T2D, T2D + CE, T2D + IE and T2D + EE groups. G. TEM analysis of the ultrastructure of hepatocytes in the rats in the T2D, T2D + CE, T2D + IE and T2D + EE groups (The yellow arrows point to mitochondria). (Scale bar = 2 μm; ns: not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with all groups by one-way ANOVA and Tukey’s post hoc test; data are expressed as the mean ± SEM; n = 8 per group).

Fig. 6 Source data_01
Fig. 6 Source data_02

Fig. 7 Moderate ROS activates AMPK through GRX-mediated glutathionylation

A-B. Analysis of superoxide (A) and ROS (B) generation using hydroethidine (A) and H2DCFDA (B) probes in primary hepatocytes under H2O2 stress (50–200 μmol/l, 30 min). The fluorescence intensity was detected by flow cytometry. C-D. Representative protein level and quantitative analysis of GSS-adduct protein and GAPDH in primary hepatocytes under H2O2 stress (50–200 μmol/l). Hepatocytes loaded with EE-GSH-biotin were incubated with/without H2O2 for 30 min, and the amounts of GSS-protein adduct formation were determined using non-reducing SDS-PAGE and Western blot analysis with streptavidin-HRP. E-F. Representative protein level and quantitative analysis of P-AMPK (67 kDa) and AMPK (67 kDa) in primary hepatocytes under H2O2 stress (50–200 μmol/l, 30 min). G-H. AMPK cysteine gel shift immunoblot. Cysteine dependent shifts by incubation of AMPK protein with glutathione reductase and PEG-Mal. PEG2-mal labelled glutathionylation modification shifts AMPK by ~10 kDa above the native molecular weight. Representative protein level and quantitative analysis of GSS-AMPK (72 kDa), AMPK (67 kDa) and GAPDH in primary hepatocytes under H2O2 stress.

Fig. 7 Source data_01
Fig. 7 Source data_02

Fig. 8 Schematic diagram of redox balance threshold.

A. The increased ROS and/or decreased antioxidative capacity (AOD) cause an imbalanced redox state and declined AMPK activity in diabetic individuals. Moderate exercise promotes the activity of antioxidant enzymes by generating benign ROS to reach redox balance, and directly promotes AMPK signaling, thus reducing glucose levels in the blood and liver. Excessive exercise causes excess ROS and exceeds the redox balance threshold, inhibiting AMPK activity and expression, thus leading to exacerbation of diabetes. (AMPK and P-AMPK in grey circles indicate decrease, red circles indicate increase, blue circles indicate no significant changes). B. Dose-response curve of diabetic individuals with exercise and antioxidant intervention. The state of diabetic individuals is applicable to the description of a S-shaped curve, due to the high level of oxidative stress and decreased reduction level in diabetic individuals. With the increase of ROS, the physiological function of diabetic individuals gradually decreases. Moderate exercise shifts the S-shaped curve upward and to the right, forming a bell-shaped dose-response curve, thus reducing the sensitivity to oxidative stress in diabetic individuals and restoring redox homeostasis. However, with excessive exercise, ROS production increases beyond the threshold range of redox balance, resulting in decreased physiological function. The ROS at the peak of the bell-shaped curve for antioxidant interventions (optimal physiological activity) is lower.
than the ROS at the peak for moderate exercise. The intervals on either side of the peak correspond to the range of redox balance thresholds, where antioxidant interventions are at low levels of redox balance and exercise is at high levels of redox balance.

**Supplementary file 1a** Antibody information

**Supplementary file 1b** Chow and HFD diet nutrition composition
A

Severe diabetes

Oxidative stress

Threshold

Increasing cellular ROS level

Excessive Exercise

Improve diabetes

Redox balance

Moderate Exercise

AMPK Signaling

Diabetic state

Redox imbalance

Rest

AMPK

B

Physiological function

Levels of ROS

P-AMPK/AMPK Signaling Pathway

Improve diabetes