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RESEARCH ARTICLE

Different fuel regulation in two types of myofiber results in different antioxidant strategies in Daurian ground squirrels (*Spermophilus dauricus*) during hibernation

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ABSTRACT

We previously showed that different skeletal muscles in Daurian ground squirrels (*Spermophilus dauricus*) possess different antioxidant strategies during hibernation; however, the reason for these varied strategies remains unclear. To clarify this issue, we studied REDD1, FOXO4, PGC-1α, FOXO1 and atrogin-1 proteins to determine the potential cause of the different antioxidant strategies in Daurian ground squirrels during hibernation, and to clarify whether different strategies affect atrophy-related signals. Results showed that the soleus (SOL) muscle experienced intracellular hypoxia during interbout arousal, but no oxidative stress. This may be due to increased PGC-1α expression enhancing antioxidant capacity in the SOL under hypoxic conditions. Extensor digitorum longus (EDL) muscle showed no change in oxidative stress, hypoxia or antioxidant capacity during hibernation. The FOXO1 and PGC-1α results strongly suggested differentially regulated fuel metabolism in the SOL and EDL muscles during hibernation, i.e. enhanced lipid oxidation and maintained anaerobic glycolysis, respectively. Atrogin-1 expression did not increase during hibernation in either the SOL or EDL, indicating that protein synthesis was not inhibited by atrogin-1. Thus, our results suggest that different fuel regulation may be one mechanism related to antioxidant defense strategy formation in different kinds of skeletal muscle fibers of Daurian ground squirrels during hibernation.

KEY WORDS: Hibernation, Skeletal muscle, Fuel regulation, Antioxidant defense strategy, Ground squirrels

INTRODUCTION

Unlike non-hibernators under muscle disuse conditions, hibernators experience little atrophy in their skeletal muscles despite months of inactivity (Gao et al., 2012; Hershey et al., 2008; Lohuis et al., 2007). For example, after long-term inactivity during hibernation, small mammal hibernators exhibit only 13% to 33% hindlimb skeletal muscle atrophy (Nowell et al., 2011; Rourke et al., 2004; Steffen et al., 1991). In addition, ischemia–reperfusion following long-term disuse can cause oxidative stress in non-hibernators; in contrast, ischemia–reperfusion can also occur in hibernators during interbout arousal, but without induced oxidative stress. As such, hibernators can be regarded as a natural model of resistance to disuse atrophy and oxidative stress (Chazarin et al., 2019; Ma et al., 2005).

Reactive oxygen species (ROS) are produced in different cellular organs where oxygen consumption is high, including in peroxisomes, endoplasmic reticulum and, particularly, mitochondria (Phaniendra et al., 2015). Under hypoxia or other conditions such as disuse and ischemia–reperfusion, mitochondria produce excessive ROS, which cannot be adequately dealt with by the antioxidative defense system. This, in turn, induces oxidative stress, resulting in an imbalance in protein homeostasis and, finally, muscle atrophy. Many researchers propose that oxidative stress-induced imbalance of protein homeostasis is the main reason leading to disuse atrophy (Gao et al., 2018; Powers et al., 2012).

During hibernation, hibernators transfer fuel from carbohydrates to stored fat in order to survive cold winters with limited food supply. This transformation is accomplished through the high expression of pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) in heart, skeletal muscle and white adipose tissue (WAT), which suppresses pyruvate dehydrogenase activity and increases the consumption of fat in those tissues (Buck et al., 2002).

Our research group previously showed that malondialdehyde content, an indicator of lipid oxidative stress, is significantly lower in extensor digitorum longus (EDL) muscles than in soleus (SOL) muscle; furthermore, differential expression in essential antioxidative protein Nrf-2 occurs in different skeletal muscles (Wei et al., 2019). This suggests that energy metabolism may be involved in different antioxidant defense strategies in different types of skeletal muscle in Daurian ground squirrels (*Spermophilus dauricus* Brandt 1843) during hibernation.

Thus, in the present study, we selected SOL (representative of oxidative myofiber) and EDL (representative of glycolytic myofiber) muscles to study the relationship between energy metabolism and antioxidant defense strategies in different types of myofiber. This was achieved by analyzing oxygen supply, oxidative stress response, antioxidant defense capacity and energy metabolism regulation based on the expression levels of the REDD1, FOXO4, PGC1 and FOXO1 proteins. The REDD1 (also known as RTP801) promoter contains a hypoxia response element, which reacts quickly to and is an indicator of intracellular hypoxia (Adams et al., 2009; Canal et al., 2014; Ono et al., 2017; Shoshani et al., 2002). The FOXO4 transcription factor can function as an indicator of oxidative stress in myofibers under different oxygen conditions (Essers et al., 2004). The expression of PGC-1α, which mainly exists in high-energy demanding tissues with abundant mitochondria, can reflect the antioxidant defense capacity of tissues (Austin and St-Pierre, 2012; Xu et al., 2013). Furthermore, PGC-1α and FOXO1, which are upstream transcription factors of PDK4, can

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enhance lipid oxidation in the cell (Buck et al., 2002; Connaughton et al., 2010). We also detected the expression of atrogin-1 to study whether energy metabolism in different myofibers affects protein synthesis during hibernation.

MATERIALS AND METHODS

Animal treatment

All animal experiments were approved by the Laboratory Animal Care Committee of the China Ministry of Health, and this study was reviewed and approved by the Northwest University Ethics Committee.

Daurian ground squirrels were captured in mid-August from the Wei Nan region, Shaanxi Province, China. The squirrels were kept in cages maintained at 18–25°C and provided with food and water *ad libitum* until they entered hibernation and finished the pre-hibernation phase (mid-September). After this, they were transferred to a hibernaculum (5–8°C) to facilitate entry into torpor (Gao et al., 2012; Yang et al., 2014). Body temperature (T_b) was measured based on thermal imaging twice daily using a visual thermometer (Fluke VT04 Visual IR Thermometer, Fluke, Everett, WA, USA) throughout hibernation. The different experimental groups were as follows (Table 1): (i) pre-hibernation group (PRE): animals maintained a stable T_b of 36–38°C in mid-September; (ii) hibernation group (HIB): animals established regular torpor bouts lasting longer than 2 months and entered into a new torpor bout with T_b maintained at 5–8°C for 5 days; (iii) interbout arousal group (IBA): animals hibernated for 2 months and fully aroused from torpor with a T_b of 36–38°C for ∼12 h; and (iv) post-hibernation group (POST): animals awoke from hibernation and maintained a T_b of 36–38°C for more than 3 days in March (spring) of the following year. The squirrels were weight-matched and randomly allocated into one of four groups (n=8 in each group). All animals were anesthetized with urethane and killed by cervical dislocation. Hindlimb skeletal muscle samples, including slow-twitch SOL and fast-twitch EDL, were immediately collected, dissected and weighed to determine wet muscle mass. The hindlimb skeletal muscle samples were frozen in liquid nitrogen and stored at −80°C until later use.

Total protein extraction and western blot analysis

Total soluble protein extracts from frozen skeletal muscle samples from three to six animals in each stage of hibernation (PRE, HIB, IBA and POST) were prepared as described previously (Yang et al., 2014). In brief, frozen tissue samples (∼0.1 g) were homogenized with RIPA lysis buffer and 10 µl ml^{-1} protease inhibitor cocktail (both from Heart, China). Samples were centrifuged at 4°C for 15 min at 15,172 ***g*** to obtain the supernatant. Soluble protein concentration was determined using a Pierce™ BCA protein quantification kit (Thermo Fisher Scientific, USA). The samples were then adjusted to a final protein concentration of 2.5 µg µl^{-1} with 1× SDS loading buffer (Boster, China) and homogenizing buffer. The final protein samples were stored at −80°C until further use.

Equal amounts of protein from each sample (7 µl) were loaded onto 10% (molecular weight more than 25 kDa and less than 200 kDa) and 15% (molecular weight less than 25 kDa) polyacrylamide gels containing 0.5% trichloroethanol (TCE), with electrophoresis run at 120 V for 60–100 min (Ladner et al., 2004). Fluorescence images of gels were captured using a G:BOX XR5 Bioimaging system (Syngene, Cambridge, UK) to normalize protein loading irregularities and analyze total protein expression. Proteins in the gel were then transferred to polyvinylidene fluoride membranes (0.20 µm pore size, Roche, Germany) using a Bio-Rad tank transfer apparatus with electroblotting at 40 V for 30 min in heated transfer buffer (>70°C) containing 25 mmol l^{-1} Tris and 192 mmol l^{-1} glycine (Kurien and Scofield, 2015). Membranes were then blocked with 4% Mowiol® PVA-203 (Aladdin, China) in Tris-buffered saline (TBS) for 10 min (Gholap et al., 2005; Rodda and Yamazaki, 1994), and incubated with REDD1 (Proteintech, 10638-1-AP), atrogin-1 (Proteintech, 12866-1-AP), PGC-1α (Novus, NBP1-04676SS), FOXO1 (C29H4) rabbit mAb (CST#2880), phospho-FOXO1 (Ser256) antibody (CST#9461), FOXO4 (CST#9472) and FOXO4 (phospho-Thr451) (Signalway antibody, 12053) in 0.1% TBST (TBS with 0.1% Tween 20) containing 2% polyvinylpyrrolidone (PVP-40; Amresco, USA, 0507-500G) at 4°C overnight. Membranes were washed and subsequently incubated with horseradish peroxidase (HRP)-linked anti-rabbit IgG secondary antibody (1:3000 to 1:10,000 v:v dilution, depending on targets, Thermo Fisher Scientific, A27014) in TBST for 2 h at room temperature. After again washing with TBST (4×5 min), the membranes were incubated with chemiluminescence solutions (Thermo Fisher Scientific, cat. no. 34080) and bands were detected using the G:BOX XR5 Bioimaging system.

The fold-change of every target protein relative to pre-hibernation levels was calculated. Immunoblot band density in each lane was standardized against the summed intensity of total protein. The enhanced chemiluminescence (ECL) band intensities were analyzed using GeneTools (v4.3.7.0). The sum of a group of stably expressed proteins in the TCE-containing gel was analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). The ECL bands were standardized against a group of stably expressed proteins within the same lane in the TCE-containing gels to control against irregularities in protein loading (Gilda and Gomes, 2013; Gurtler et al., 2013; Ladner et al., 2004; Vigelsø et al., 2015). Total protein normalization is more effective than using a single housekeeping gene in natural model systems (Wijenayake et al., 2017). For time-course western blot quantification, not all protein homogenates could be run on the same gel owing to lane limitations, so one of the samples (e.g. PRE1) was run on each gel, and the band densities of the other samples were standardized to the same sample (e.g. PRE1 on all blots).

Table 1. Details of the different experimental groups used in the present study

State	Sample time	T_b of animals
PRE	Mid-September	36–38°C
HIB	After 2 months of hibernation, animals entered a new hibernation bout	Stable 5–8°C for 5 days
IBA	Same as HIB	36–38°C for ∼12 h
POST	March of following year	36–38°C for more than 3 days

T_b, body temperature; PRE, pre-hibernation group; HIB, hibernation group; IBA, interbout arousal group; POST, post-hibernation group.

Data and statistical analysis

Data are presented as means±s.e.m. normalized to the PRE group mean. Statistical analysis was performed using one-way ANOVA followed by a Tukey's *post hoc* test (P<0.05) using SPSS Statistics v19 (IBM, Armonk, NY, USA). Outlier testing was also conducted using SPSS Statistics v19. All figures were constructed using GraphPad Prism software (San Diego, CA, USA).

RESULTS

REDD1 expression

REDD1 is located downstream of HIF-1 and responds quickly to hypoxia (Shoshani et al., 2002). As shown in Fig. 1, REDD1

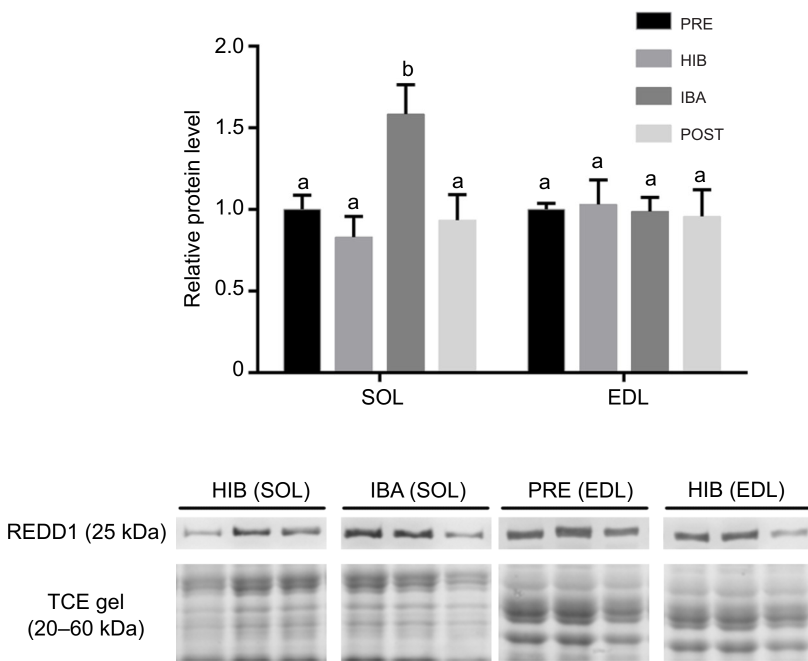

Fig. 1. Expression of REDD1 in SOL and EDL muscles of Daurian ground squirrels during hibernation. Protein expression levels were visualized at four sampling points: pre-hibernation (PRE), hibernation (HIB), interbout arousal (IBA) and post-hibernation (POST). See Materials and Methods for more extensive definitions. Representative immunoblots and Stain-Free total protein loading controls are shown below for selected pairs of sampling points and muscles that are labeled on the top of the blot. Data were analyzed using an ANOVA with a *post hoc* Tukey's test ($P<0.05$); values that are not statistically different from each other share the same letter notation.

expression in the SOL muscle increased significantly by 1.58-fold in the IBA group compared with the PRE group ($P<0.05$). In contrast, REDD1 expression in the EDL muscle showed no significant change.

FOXO expression and phosphorylated level

As shown in Fig. 2, in the EDL muscle, total protein expression and phosphorylation of FOXO4 remained stable during hibernation; in the SOL muscle, however, the phosphorylated level of FOXO4 was reduced by 63.2% ($P<0.05$) in the IBA group compared with that in the PRE group. In the EDL muscle, total protein expression of FOXO1 remained stable during hibernation (Fig. 3), whereas its phosphorylated level decreased significantly by 77.4% ($P<0.01$) and 72.4% ($P<0.05$) in the POST group compared with that in the HIB and IBA groups, respectively. In the SOL muscle, total expression and phosphorylation of FOXO1 increased 1.72-fold ($P<0.01$) and 1.91-fold ($P<0.05$), respectively, in the HIB group compared with that in the PRE group.

PGC-1α expression

As seen in Fig. 4, PGC-1α protein expression in the SOL muscle showed a significant 1.57-fold increase ($P<0.01$) in the HIB group compared with that in the PRE group. In the EDL muscle, however, no significant changes were observed in any group.

Atrogin-1 expression

We detected atrogin-1 expression in the EDL and SOL muscles. As shown in Fig. 5, there was no change in atrogin-1 protein expression

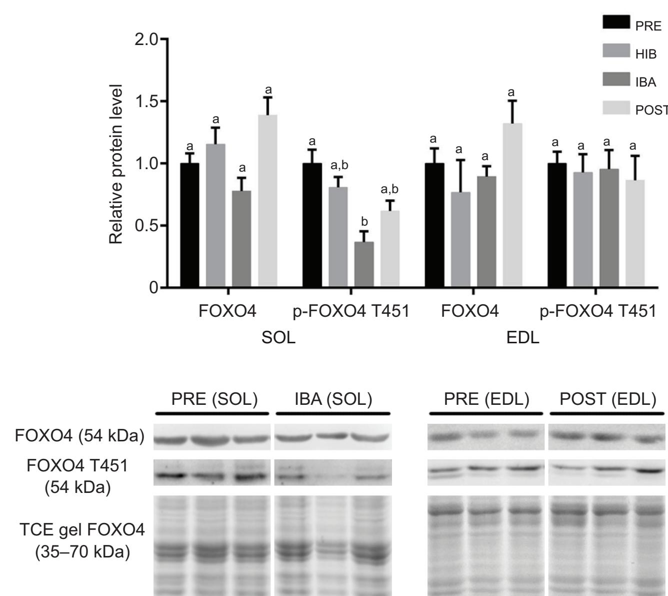

Fig. 2. FOXO4 expression and phosphorylation in skeletal muscles of Daurian ground squirrels during hibernation. Other information as in Fig. 1.

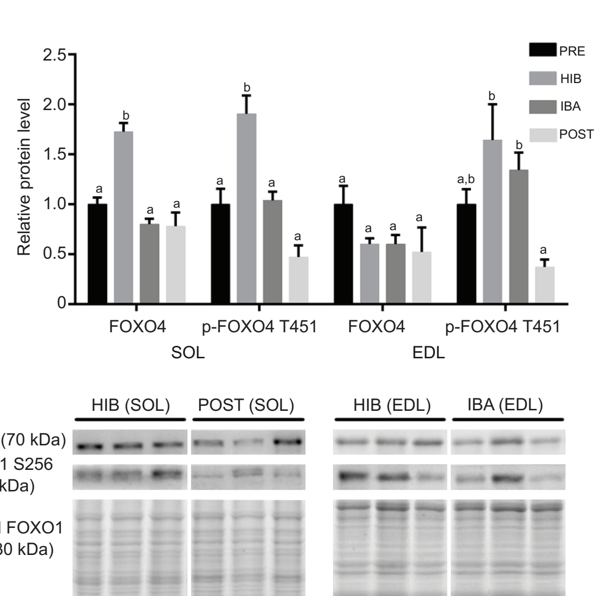

Fig. 3. FOXO1 expression and phosphorylation in skeletal muscles of Daurian ground squirrels during hibernation. Other information as in Fig. 1.

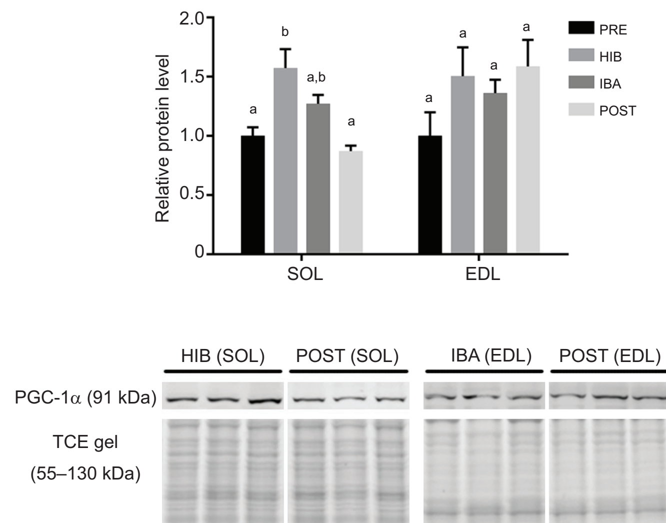

Fig. 4. PGC-1α expression in different muscles of Daurian ground squirrels during hibernation. Other information as in Fig. 1.

in the SOL muscle; in the EDL muscle, however, expression showed a significant 42.3% decrease ($P<0.01$) in the HIB group compared with that in the PRE group.

DISCUSSION

Skeletal muscles of hibernators face challenges from disuse, fasting, hypoxia and ischemia–reperfusion during the hibernation period. These challenges can increase the risk of oxidative stress to skeletal muscle. For example, in non-hibernators under disuse conditions, oxidative stress can lead to muscle atrophy (Moylan and Reid, 2007; Powers et al., 2012). Hibernators survive cold winter conditions by switching fuel to fat reserves stored during pre-hibernation. A high-fat diet and long-chain fatty acid oxidation can induce oxidative stress in non-hibernator skeletal muscles (Seifert et al., 2010; Yuzefovych et al., 2013). Thus, when studying the mechanism of resistance to muscle atrophy in hibernators, researchers should consider not only the multiple stresses of hibernation, but also the potential effects of different energy metabolism on different myofibers. Here, we selected SOL and EDL muscles as representatives of oxidative and glycolytic myofibers, respectively, in order to analyze the relationship between oxidative stress, antioxidant strategies and metabolic regulation in different myofibers. Our results indicated differentiated metabolic regulation in different myofibers of hibernating Daurian ground squirrels, which may be the reason for the different antioxidant strategies in different skeletal muscles.

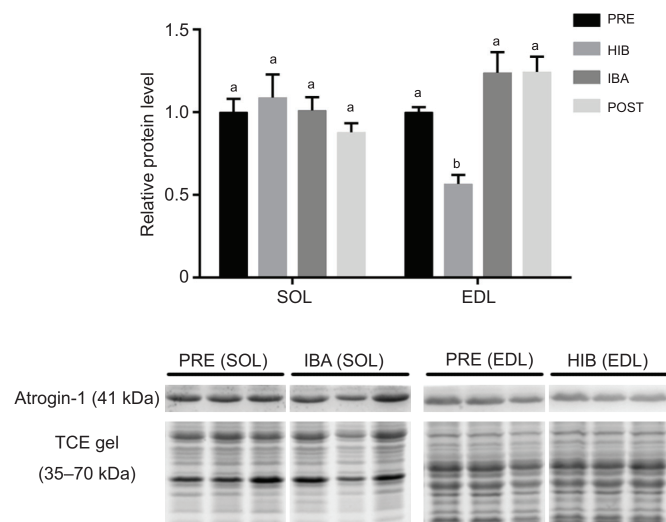

Fig. 5. Atrogin-1 expression in different muscles of Daurian ground squirrels during hibernation. Other information as in Fig. 1.

REDD1 (also known as RTP801) is a downstream transcription factor of hypoxia marker HIF-1 and is upregulated under a variety of stress conditions (Shoshani et al., 2002). The REDD1 promoter contains a hypoxia response element, which responds to and is an indicator of intracellular hypoxia (Adams et al., 2009; Canal et al., 2014; Shoshani et al., 2002). In addition, the REDD1 promoter can be used as a sensor in hypoxia-responsive transgene expression systems (Ono et al., 2017). Our results showed that REDD1 expression in the SOL was increased in the IBA group compared with the PRE group; in contrast, no change occurred in the EDL muscle. This indicates that SOL myofibers experience intracellular hypoxia during IBA, but that oxygen supply has no effect on the EDL. This difference may be due to the different oxidative capacity of the two skeletal muscles. Our previous study showed that myoglobin (Mb) content is increased in the SOL (by 1.31-fold) during hibernation, but shows no change in the EDL, indicating that the oxidative capacity of the SOL increases during hibernation (Jiang et al., 2015). This increase in oxidative capacity may lead to increased oxygen consumption in the SOL during IBA, as well as low S_{pO_2} (peripheral oxygen saturation) (Zhang et al., 2019). These two factors may thus induce intracellular hypoxia, resulting in the upregulation of REDD1.

To clarify whether the different respiration-type myofibers experienced oxidative stress under different oxygen conditions, we analyzed FOXO4, a transcription factor that responds to oxidative stress (Essers et al., 2004). FOXO4 is a member of the FOXO subfamily, which plays a role in anti-oxidative stress, proteostasis, cell cycle, autophagy and cellular metabolism (Canal et al., 2014; Ferdous et al., 2010; McLaughlin and Broihier, 2018). Phosphorylated FOXO4 (Thr451), which is located in the nucleus, functions in oxidative stress response and transcription of antioxidants such as MnSOD and catalase to reduce intracellular ROS (Eijkelenboom and Burgering, 2013; Essers et al., 2004). Thus, the phosphorylation of FOXO4 in the Thr-451 site can reflect intracellular oxidative stress levels (Eijkelenboom and Burgering, 2013; Essers et al., 2004). Our results indicated that total expression of FOXO4 showed no change in the SOL, but p-FOXO4 (Thr451) decreased by 63.2% ($P<0.05$) in the IBA group compared with that in the PRE group, suggesting that oxidative stress in the SOL decreased significantly under IBA conditions, opposite to the increase observed in REDD1. This conflict implies that the SOL experienced hypoxia during IBA, but that oxidative stress levels were lower than that in the PRE group. In the EDL muscle, no changes in REDD1 and FOXO4 were observed, indicating that oxygen supply and oxidative stress did not cause significant fluctuations during hibernation. Based on this paradox between hypoxia and low oxidative stress, we detected the expression of PGC-1α, a critical factor in antioxidant defense (Austin and St-Pierre, 2012).

PGC-1α mainly exists in high-energy demanding tissues with abundant mitochondria, such as skeletal muscle, brown adipose tissue, heart, liver and kidney. It functions in ROS detoxification and mitochondrial biogenesis, and its expression level can reflect the antioxidant defense capacity of tissue (Austin and St-Pierre, 2012; Xu et al., 2013). Here, our results showed that expression of

PGC-1α in the SOL increased by 1.57-fold (P<0.01) in the HIB group but was unchanged in the EDL. The increase in PGC-1α expression in the SOL implies an increase in antioxidant capacity, which could promote mitochondrial biogenesis. Coupled with our previous studies (Jiang et al., 2015; Wang et al., 2019), this suggests that the number of mitochondria and oxidative capacity increased in the SOL during hibernation. Wei et al. (2019) showed that Nrf-2 protein expression increases in Daurian ground squirrels during late torpor (same as HIB in the present study). Baldelli et al. (2013) also showed that PGC-1α can activate the Nrf-2/Keap1 signaling pathway and increase the expression of antioxidants. Thus, we speculate that the coexistence of intracellular hypoxia and low oxidative stress may be related to the increased expression of PGC-1α before arousal, which enhanced the antioxidant defense capacity of the SOL during IBA and allowed the muscle to limit oxidative damage under intracellular hypoxia.

Different from that in the SOL, the expression of PGC-1α remained stable in the EDL. This suggests that the EDL, which is primarily composed of glycolytic myofibers (Ma et al., 2019), enhanced glycolytic capacity and activity of lactate dehydrogenase (Jiang et al., 2015) and maintained anaerobic respiration under a fat fuel source, which thus prevented ROS production (Mullarky and Cantley, 2015), as reported under S_{pO_2} conditions during IBA (Zhang et al., 2019). Therefore, the EDL does not need an increased antioxidant defense capacity. Previous work also reported stable Nrf-2 expression in the EDL during hibernation (Wei et al., 2019), corresponding to the stable REDD1 expression and FOXO4 phosphorylation observed in the present study in the EDL during hibernation. This indicates that the EDL does not experience intracellular hypoxia and maintains redox homeostasis.

PGC-1α is an upstream transcription factor of PDK4, which is a crucial regulator of fuel metabolism in skeletal muscle (Connaughton et al., 2010). FOXO1 is also an upstream transcription factor of PDK4, and recruits PGC-1α to the PDK4 promoter to enhance lipid oxidation in the cell (Connaughton et al., 2010; Furuyama et al., 2003; Kwon et al., 2004). To study the regulation of PDK4, we detected the expression of FOXO1 (as well as PGC-1α) to determine the effects of fuel regulation on oxidative stress in different myofibers. PDK4 is a mitochondrial protein that suppresses pyruvate dehydrogenase activity and increases fat consumption in the heart and skeletal muscle (Buck et al., 2002; Connaughton et al., 2010; Crewe et al., 2017). Different from phosphorylated FOXO4, which is located in the nucleus and exhibits transcriptional activity, phosphorylated FOXO1 (Ser-256) is located in the cytoplasm and shows no transcriptional activity, and therefore does not increase PDK4 transcription (Furuyama et al., 2003). In the present study, FOXO1 phosphorylation showed a 1.91-fold increase in the SOL in the HIB group compared with the PRE group. However, total FOXO1 expression increased by 1.72-fold during hibernation, indicating that non-phosphorylated FOXO1 also increased. Thus, these results suggest that the SOL increased PDK4 expression by regulating FOXO1 and PGC1 transcriptional activity to enhance oxidation capacity and lipid consumption. These findings are consistent with the upregulation of PDK4 and enhanced lipid oxidation capacity found in the quadriceps femoris muscles of thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) during hibernation (Buck and Barnes, 2000; Buck et al., 2002).

In the EDL muscle, total protein expression of FOXO1 showed no significant change in any group. However, the phosphorylated level of FOXO1 in the HIB and IBA groups was higher than that in the POST group, indicating that the transcriptional activity of FOXO1 was inhibited during hibernation. Combined with the PGC-1α results, these findings indicate that the activities of the two upstream factors of PDK4 were inhibited or remained stable during hibernation; these results correspond with our previous findings, which showed decreased PDK4 expression during hibernation (Chang et al., 2018). Following Jiang et al. (2015), who reported increased lactate dehydrogenase activity in the EDL during hibernation, we speculate that maintained anaerobic glycolysis respiration may prevent oxidative stress in the EDL (Mullarky and Cantley, 2015).

E3 ubiquitin ligase atrogin-1 inhibits protein synthesis by ubiquitination of eif-3f, and enhanced transcriptional activity of FOXO can increase the expression of atrogin-1 (Foletta et al., 2011; Moylan et al., 2008; Mullarky and Cantley, 2015; Pomiès et al., 2016). Research on non-hibernators has shown that atrogin-1 expression and transcription both increase in many atrophy models (Bodine et al., 2001; Gao et al., 2018; Lagirand-Cantaloube et al., 2008). As shown in Fig. 5, atrogin-1 expression in the SOL exhibited no change, indicating that protein synthesis suppression by atrogin-1 did not change during hibernation in the SOL. In the EDL, however, atrogin-1 expression decreased by 42.3% in the HIB group compared with that in the PRE group, indicating that protein synthesis suppression by atrogin-1 decreased under HIB conditions. In SOL, total protein expression and phosphorylation of FOXO1 increased by 1.72-fold and 1.91-fold in the HIB group, respectively. Although the abundance of active FOXO1 increased, the transcriptional level of atrogin-1 remained stable, indicating that other factors inhibited the transcription of atrogin-1. In the EDL, the inhibition of FOXO1 transcriptional activity under HIB and IBA conditions may be the reason for the decrease in atrogin-1 expression in the HIB group. In conclusion, protein synthesis in different skeletal muscles was not suppressed by atrogin-1.

Conclusions

Hibernators experience long-term inactivity combined with hypoxia and ischemia–reperfusion during hibernation; despite this, their skeletal muscle does not exhibit atrophy, unlike that seen in non-hibernators under disuse and stressful environments. This study showed that oxidative skeletal muscle (SOL) experienced intracellular hypoxia during IBA but prevented oxidative stress by pre-enhancement of its antioxidant defense capacity, resulting in lower oxidative stress than that detected in the PRE group and preparation of oxidative stress strategies. Unlike oxidative skeletal muscle, typical glycolytic skeletal muscle (EDL) did not show intracellular hypoxia during hibernation and demonstrated no change in oxidative stress or antioxidant defense capacity. Thus, we reasoned that, under the condition of fuel change from carbohydrates to fat, glycolytic myofibers inhibited FOXO1 transcriptional activity, decreased PDK4 expression and maintained anaerobic glycolysis, which prevented aerobic respiration-induced ROS production and limited oxidative damage. Our results indicate that different myofibers in hibernating Daurian ground squirrels have developed different antioxidant defense strategies adapted to their respiration model. Furthermore, atrogin-1 expression was not increased in the different myofibers, indicating that both antioxidant strategies maintained redox homeostasis, and thus prevented oxidative stress-induced skeletal muscle atrophy.

Competing interests
The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author contributions
Conceptualization: S.X., W.F., H.W., Y.G.; Methodology: S.X.; Software: S.X.; Validation: S.X., W.F.; Formal analysis: S.X.; Investigation: S.X., W.F.; Resources: S.X., W.F.; Data curation: S.X., J.Z.; Writing - original draft: S.X.; Writing - review & editing: W.F., J.Z., H.W., K.D., H.C., Y.G.; Visualization: H.C.; Supervision: J.Z., H.W., K.D., Y.G.; Project administration: Y.G.; Funding acquisition: H.W., K.D., H.C., Y.G.

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