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RESEARCH ARTICLE

Assessment of fatigue-related biochemical alterations in a rat swimming model under hypoxia

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ABSTRACT

It is well known that exercise-induced fatigue is exacerbated following hypoxia exposure and may arise from central and/or peripheral mechanisms. To assess the relative contribution of peripheral and central factors to exercise-induced fatigue under hypoxia, a rat model of fatigue by a bout of exhaustive swimming was established and fatigue-related biochemical changes in normoxic and severe hypoxic conditions were compared. Rats were randomly divided into four groups: normoxia resting (NR), exhaustive swimming (NE), hypoxia resting (HR) and exhaustive swimming (HE). The swimming time to exhaustion with a weight equal to 2.5% of their body weight reduced under hypoxia. There were lower blood lactate levels, lower gastrocnemius pAMPK/AMPK ratios and higher gastrocnemius glycogen contents in the HE than in the NE groups, which all suggested a lower degree of peripheral fatigue in the HE group than in the NE group. Meanwhile, there was a significant increase in striatal 3,4-dihydroxyphenylacetic acid (DOPAC) caused by exhaustive swimming under normoxia, whereas this increase was almost blunted under severe hypoxia, indicating that hypoxia might exacerbate exercise-induced central fatigue. These biochemical changes suggest that from normoxia to severe hypoxia, the relative contribution of peripheral and central factors to exercise-induced fatigue alters, and central fatigue may play a predominant role in the decline in exercise performance under hypoxia.

KEY WORDS: High altitude, Peripheral fatigue, Central fatigue, Exercise

INTRODUCTION

Exposure to high altitude is well known to be detrimental for lowlanders' performance of aerobic exercise (Goodall et al., 2014; Millet et al., 2012; Romer et al., 2007). Understanding such an effect would be beneficial to lowlanders who are working and living in a high-altitude environment, thus potentially improving their physical performance and quality of life. While researchers agree that the lack of oxygen is the main cause of their low physical performance, the underlining mechanisms are not fully understood. For decades, there existed abundant evidence showing decreased muscle contractility and increased muscle metabolite accumulation during an exhaustive exercise under acute hypoxia, which are important indicators for peripheral fatigue (Amann et al., 2007; Howlett and Hogan, 2007; Morales-Artacho et al., 2015). In addition, some other evidence showed there might also be a linkage between central fatigue in hypoxia and poor exercise performance (Garner et al., 1990; Kayser et al., 1994). Further, Amann et al. (2006) found that a lack of oxygen for central nervous system (CNS) could result in a reduced central motor drive, hence limiting exercise performance. In addition, Goodall et al. (2014) detected decreased supraspinal maximal voluntary activation in severe hypoxia when subjects were exhausted. Ruggiero et al. (2017) reported that motor neuron excitability declined to 40% of the baseline value during the fatiguing task in acute hypoxia.

The most up-to-date viewpoint is that both peripheral and central fatigue are associated with decreased exercise performance under hypoxia (Rupp et al., 2015; Smirmaul et al., 2017; Twomey et al., 2017). When seeking optimal strategies of ameliorating physical fatigue in humans in hypoxic environments, e.g. high-altitude environments, it is important to explicitly ascertain the determinants of fatigue. Yet, the contributions of peripheral and central factors to exercise-induced fatigue still remain largely unknown. One reason for this is the difficulty of collecting adequate experimental data in humans and the lack of animal models of exercise-induced fatigue in such a harsh environment. Another reason is the deficiency of reliable indicators for quantitative and qualitative evaluations of both peripheral and central fatigue.

Here, we established a rat model of fatigue in a hypobaric chamber using a weight-loaded swimming test to assess the biochemical changes in peripheral and central fatigue. Blood lactate, glycogen content and phosphorylation of AMP-activated protein kinase (pAMPK) in gastrocnemius were used to indicate the magnitude of peripheral fatigue. The levels of serotonin (5-HT), dopamine (DA) and their metabolites, 5-hydroxyindole acetic acid (5-HIAA) and 3,4-dihydroxyphenylacetic acid (DOPAC), in the striatum were used to measure the degree of central fatigue. Our study aims to explore the relative contribution of peripheral and central factors to swimming-induced fatigue in rats when exposed to acute severe hypoxia.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (age 8–10 weeks, mass 190±10 g) were used. Rats were housed in a light- (lights on at 07:00 h and off at 19:00 h), humidity- (50±5%) and temperature- (22±1°C) controlled environment for 1 week before conducting the tests. Food (standard rodent chow) and water were available *ad libitum*. The experimental protocol, according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International, was approved by the Ethics Committee of the Army Medical University.

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Received 3 February 2019; Accepted 24 June 2019

Journal of Experimental Biology (2019) 222, jeb199711. doi:10.1242/jeb.199711

Experimental design and procedures

Thirty-two rats were randomly divided into four groups: normoxia resting (NR) and exhaustive swimming (NE) groups, hypoxia resting (HR) and exhaustive swimming (HE). A week before the exhaustive-swimming tests, rats in the NE and HE groups began a general adaptation period by performing an adaptive swimming training every other day (10 min day^{-1}) in an aquatic environment – a water tank with a diameter of 40 cm and a depth of 70 cm. After this period, rats in the HR and HE groups were exposed to hypoxia in a hypobaric chamber (simulated altitude of 5000 m) for 24 h. After entering the hypobaric chamber through a transition chamber, the exhaustive-swimming tests were performed and specimens were drawn. Meanwhile, rats in the NR and NE groups were housed in the same room as they were housed before (altitude of 300 m). A rat model of exercise-induced fatigue using a weight-loaded swimming test was established as described previously with some minor modifications (Tanaka et al., 2003).

In the simulated 5000 m hypobaric chamber, obvious air bubbles attached to the rats' fur. Those newly developed bubbles seemed to increase buoyancy and allowed the rats to float in the water with little movement, even after adding a weight equal to 2.5% of their body weight at the base of their tails. The air bubbles, however, hindered us from obtaining an objective judgement of rats' motor ability. Thus, prior to the tests, we boiled the swimming water to eliminate the bubbles, let it naturally cool down to 34±1°C then maintained it at that temperature.

NE and HE rats both swam individually with a load of lead ring attached to the base of their tails, which weighed 2.5% of their body weight. The swimming-exhaustion time, that is, the period from the beginning of swimming with weight to the point at which the rats could not return to the surface of the water for a consecutive 10 s, was recorded. When rats in the NE and HE groups swam to exhaustion, blood samples were collected from the tails. After dislocation of the neck, the brain and gastrocnemius muscle from the left hindlimb of each rat were rapidly excised and stored in liquid nitrogen. The same procedure was conducted for rats in the resting groups.

Determination of muscle glycogen

The white portions of gastrocnemius muscles that were surgically removed from the left hindlimb were treated in 30% KOH at 100°C for 20 min. Double distilled water was added to the vials to make 5% homogenate. Then, 0.1 ml of homogenate, 0.9 ml 0.2% anthrone (0.2 g of anthrone in 100 ml of freshly prepared 98% sulfuric acid) and 2 ml distilled water were mixed, and the vials were placed in boiling water for 5 min. The optical density of the reaction mixture was determined by photometry at 620 nm.

Determination of blood lactate

Blood samples were collected from the end of the tails of rats; the 25 µl samples were quickly transferred to an electrochemical analyzer (YSI 1500 Sport, Yellow Springs Co., USA) and whole blood lactate concentrations were determined.

Western blot analysis of AMPK and pAMPK

White gastrocnemius muscles surgically removed from the left hindlimb were homogenized in a total protein extraction lysis buffer containing phosphatase inhibitor. The concentration of proteins was determined using the bicinchoninic acid (BCA) assay. The proteins were probed with primary antibodies as follows: polyclonal rabbit anti-AMPK (1:1000; Cell Signaling Technology, USA), and polyclonal rabbit anti-pAMPK (1:1000; Cell Signaling Technology). The immunobands were visualized using the ECL kit (Pierce, USA). The expression bands of target proteins were analyzed and the ratios between pAMPK and AMPK were calculated.

Neurochemical analysis

Brains stored in liquid nitrogen were taken out and placed on an ice-chilled glass plate. The striatal regions of brains were carefully dissected out, sufficiently homogenized in 0.1 mol l^{-1} cold perchloric acid (100 mg tissue:1 ml perchloric acid) and then centrifuged at 15,000 ***g*** for 20 min. Concentrations of 5-HT, DA and their metabolites, 5-HIAA and DOPAC, were measured using high-performance liquid chromatography (HPLC) as previously described (Leite et al., 2010).

Statistical analysis

All results are expressed as means±s.e.m. Student's *t*-test was used to estimate the significance of exhaustion time between the NE and HE groups. Two-way ANOVA was used to examine the differences in biochemical changes of peripheral and central fatigue between groups. The two experimental factors were 'condition' (normoxia or hypoxia) and 'physical activity' (resting or exhaustive swimming). Differences between groups were tested by Tukey's HSD test. The statistical significance level for all analyses was set at 0.05.

RESULTS

Exhaustion time

After successfully establishing a rat model of fatigue using a bout of exhaustive swimming, the exhaustion time of normoxic and hypoxic rats was recorded. There was a sharp reduction in the exhaustion time of HE rats compared with that of NE rats (HE 37±9 min versus NE 91±16 min, P<0.05; Fig. 1).

Blood lactate

As shown in Fig. 2A, under normoxic conditions, a single bout of exhaustive swimming led to a significant increase in blood lactate (NE 14.1±1.4 mmol l^{-1} versus NR 1.2±0.2 mmol l^{-1}, P<0.05). Under hypoxic conditions, a similar increase of blood lactate was observed in HE rats (HE 10.9±2.1 mmol l^{-1} versus HR 1.5±0.3 mmol l^{-1}, P<0.05). Interestingly, blood lactate levels of HE rats were much lower than those of NE rats (HE 10.9±2.1 mmol l^{-1} versus NE 14.1±1.4 mmol l^{-1}, P<0.05).

Gastrocnemius glycogen content

As shown in Fig. 2B, basal gastrocnemius glycogen contents were significantly increased after hypoxia exposure for 24 h

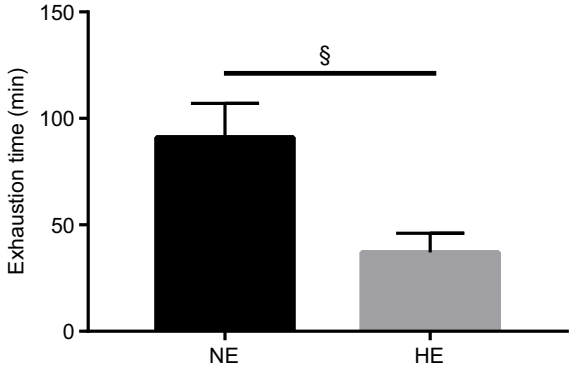

Fig. 1. Severe hypoxia shortens the swimming exhaustion time in rats. NE, normoxia exhaustive exercise; HE, hypoxia exhaustive exercise. Data are means±s.e.m., n=8. $^{§}P$<0.05, compared with the NE group.

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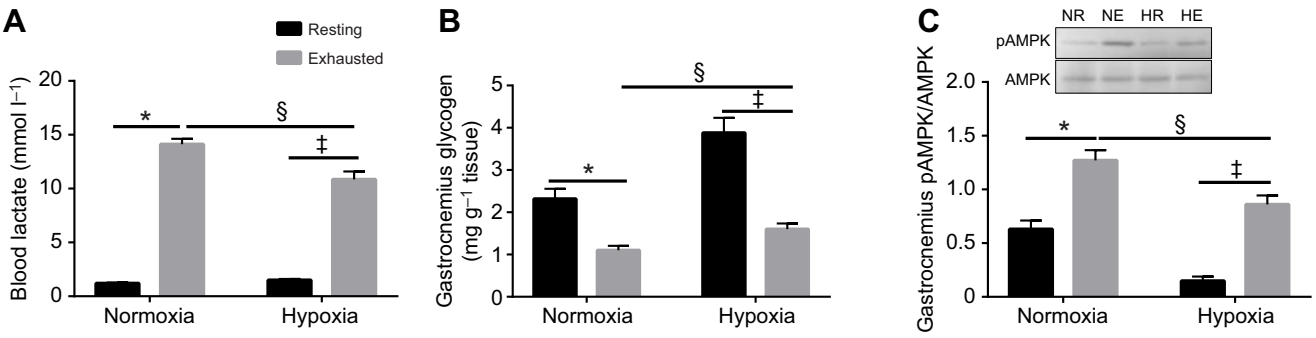

Fig. 2. Effect of severe hypoxia on peripheral fatigue indicators after exhaustive swimming in rats. (A) Blood lactate concentrations. (B) Gastrocnemius muscle glycogen content. (C) Representative western blots of pAMPK and AMPK in the gastrocnemius, and densitometric analysis of the pAMPK/AMPK ratio. NR, normoxia resting; NE, normoxia exhaustive exercise; HR, hypoxia resting; HE, hypoxia exhaustive exercise. Data are means±s.e.m., n=8. *P<0.05 compared with the NR group; ‡P<0.05 compared with the HR group; §P<0.05 compared with the NE group.

(HR 3.9±1.0 mg g^{-1} tissue versus NR 2.3±0.7 mg g^{-1} tissue, P<0.05). Following weight-loaded exhaustive swimming, gastrocnemius glycogen contents in both NE and HE rats markedly decreased compared with their corresponding resting controls (NE 1.1±0.3 mg g^{-1} tissue versus NR 2.3±0.7 mg g^{-1} tissue, P<0.05; HE 1.6±0.4 mg g^{-1} tissue versus HR 3.9±1.0 mg g^{-1} tissue, P<0.05). Interestingly, the gastrocnemius glycogen content of HE rats was higher than that of NE rats (HE 1.6±0.4 mg g^{-1} tissue versus NE 1.1±0.3 mg g^{-1} tissue, P<0.05).

AMPK activity in the gastrocnemius

To monitor cellular energy status, AMPK activity was measured using the ratio of pAMPK to AMPK in the gastrocnemius. As shown in Fig. 2C, ratios of gastrocnemius pAMPK/AMPK in both NE and HE rats dramatically increased compared with their corresponding resting controls (NE 1.3±0.3 versus NR 0.6±0.2, P<0.05; HE 0.9±0.2 versus HR 0.2±0.1, P<0.05). It was noted that the pAMPK/AMPK ratio in HE rats was significantly lower than that of NE rats (HE 0.9±0.2 versus NE 1.3±0.3, P<0.05).

5-HT, DA and their metabolites in the striatum

To evaluate the degree of central fatigue, the concentrations of 5-HT, DA and their metabolites (5-HIAA, DOPAC) in the striatum were measured. As shown in Fig. 3A,C, the basal concentrations of 5-HT and DA in the striatum of NR rats were 44.9±8.8 and 508.0±61.4 ng per 100 mg tissue, respectively. In a resting state, hypoxia exposure for 24 h did not result in any change in 5-HT, 5-HIAA, DA or DOPAC contents in the striatum. In contrast, the level of striatal 5-HT increased significantly in both normoxic and hypoxic conditions after exhaustive swimming (NE 67.1±9.9 ng per 100 mg tissue versus NR 44.9±8.8 ng per 100 mg tissue, P<0.05; HE 60.6±11.2 ng per 100 mg tissue versus HR 46.5±10.9 ng per 100 mg tissue, P<0.05; Fig. 3A). No changes in striatal HIAA contents were observed between the groups (Fig. 3B). After a bout of exhaustive swimming, striatal DA contents of NE and HE rats were significantly higher than that of their corresponding resting controls (NE 638.3±66.3 ng per 100 mg tissue versus NR 508.0±61.4 ng per 100 mg tissue, P<0.05; HE 618.4±71.6 ng per 100 mg tissue versus HR 535.9±111.7 ng per 100 mg tissue, P<0.05; Fig. 3C). Although exhaustive swimming resulted in a significant increase in striatal DOPAC under normoxia (NE 173.3±23.7 ng per 100 mg tissue versus NR 133.4±19.5 ng per 100 mg tissue, P<0.05, Fig. 3D), this effect was blunted under severe hypoxia (HE 131.0±17.9 ng per 100 mg tissue versus HR 125.1±32.7 ng per 100 mg tissue, P>0.05, Fig. 3D).

DISCUSSION

Exercise-induced fatigue is known to be aggravated under severe hypoxia; however, the underlying mechanisms remain largely unclear. With the aim of investigating the relative contribution of peripheral and central factors to exercise-induced fatigue under hypoxia, we first established a rat model of fatigue by a bout of exhaustive swimming. Then, we compared the fatigue-related biochemical changes in normoxic and severe hypoxic conditions. Our results showed that there was a sharp decline in exercise performance in hypoxic rats, accompanied by a lower degree of peripheral fatigue and a higher degree of central fatigue, compared with normoxic rats. These data suggest that central fatigue may play a more important role than peripheral fatigue in impairing endurance capacity during exercise under severe hypoxic conditions.

Lactate accumulation has been extensively used as an indicator for the development of peripheral fatigue. A higher blood lactate level usually indicates a greater degree of peripheral fatigue (Finsterer, 2012). Because tissue hypoxia causes muscles to generate energy anaerobically during intense exercise, we initially expected that blood lactate would accumulate after exercise, and lactate accumulation would be exacerbated in severe hypoxia. On the contrary, the results showed that blood lactate concentration in HE rats was much lower than in NE rats. A similar phenomenon, called the lactate paradox, also occurred in subjects pedaling a bicycle ergometer to exhaustion at altitude of 6300 m (West, 1986). A possible mechanism of lactate paradox proposed by Hashimoto and Brooks (2008) was that hypoxia exposure enhances the reusage of lactate in active muscles as an alternative energy source, leading to a reduction in the amount of lactate released into the bloodstream. This efficient metabolic pathway allows muscle fibers to create ATP without drawing too much on blood glucose and/or muscle glycogen. Our data supported this view by consistently showing a higher gastrocnemius glycogen content in HE rats than in NE rats. Other findings from Kayser et al. (1994) seemed to suggest that the low lactate accumulation following exhaustive exercise in hypoxia may be the consequence of reduced activation of locomotory muscles driven by motoneurons. Thus, the decreased accumulation of blood lactate during hypoxic exercise may be primarily regulated by the CNS (Noakes, 2012). Therefore, the lactate paradox phenomenon may be the result not only of adaptive responses in muscle to enhance the ability of utilizing lactate during hypoxia, but also of protective regulation by the CNS to prevent failure of whole-body homeostasis.

Glycogen in skeletal muscle serves as an important form of energy storage. The effect of hypoxia on skeletal glycogen content

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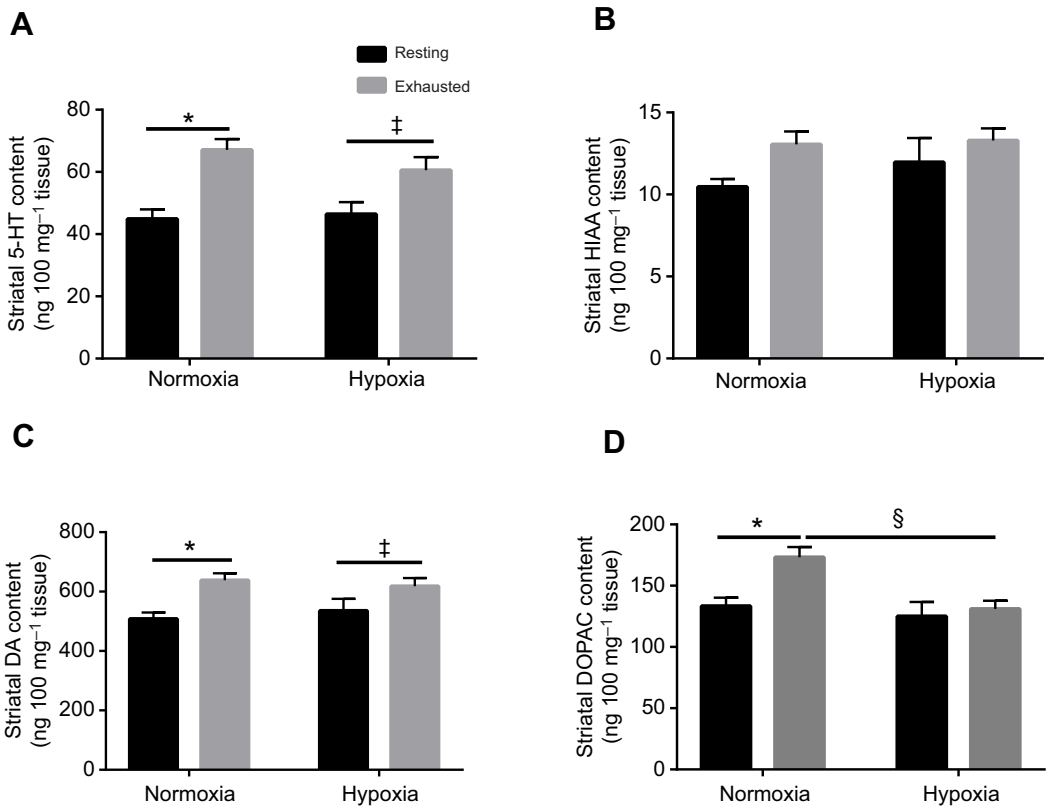

Fig. 3. Effect of severe hypoxia on central fatigue factors after exhaustive swimming in rats. (A) Serotonin (5-HT), (B) 5-hydroxyindole acetic acid (5-HIAA), (C) dopamine (DA) and (D) 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations in the striatum. NR, normoxia resting; NE, normoxia exhaustive exercise; HR, hypoxia resting; HE, hypoxia exhaustive exercise. Data are means±s.e.m., n=8. *P<0.05 compared with the NR group; ‡P<0.05 compared with the HR group; §P<0.05 compared with the NE group.

depends on the duration and extent of hypoxia exposure. Petukhov (1960) found that acute oxygen deficiency (simulated 10,500 m) for 1 h resulted in a marked reduction in glycogen content of skeletal muscle. An *in vitro* study performed by Ren et al. (1992) showed that glycogen concentration of epitrochlearis muscle incubated in the absence of oxygen rapidly dropped by 70% over an 80-min period. However, in a hypoxia-acclimated model where mice were held in hypobaric chambers at the equivalent of 4300 m for 6–8 weeks, no significant effects of hypoxia on muscle glycogen were observed (Lau et al., 2017). In our study, we found that after rats were subjected to simulated 5000 m of high altitude in a hypobaric chamber for 24 h, glycogen content in the gastrocnemius was markedly elevated. Similarly, the phenomenon of hypoxia-induced glycogen accumulation has been observed in several cell lines. Pescador et al. (2010) showed that hypoxia (1% O_2) resulted in accumulation of glycogen in mouse myocytes and hepatocytes. They further demonstrated that enzymes involved in glycogen biosynthesis, such as GYS1, UGP2 and GBE1, induced by hypoxia, mediate glycogen accumulation. Consequently, hypoxia-preconditioned cells with glycogen accumulation were more resistant to cell death when exposed to anoxia than non-preconditioned cells. Therefore, the hypoxia-induced glycogen accumulation may be a protective adaptation of cells for ensuring adequate energy reserve to cope with further hypoxic conditions.

During prolonged aerobic activity, a depletion of muscle glycogen often occurs and is correlated with the inability to maintain muscle contraction force (Britto et al., 2018; Ørtenblad et al., 2013). Glycogen stores in skeletal muscles exhibited a dramatic reduction after exercise in both normoxic and hypoxic conditions (Baldwin et al., 1975). A similar phenomenon was observed in our swimming-induced fatigue model. In addition, after swimming to exhaustion, HE rats showed a higher gastrocnemius glycogen content than NE rats, indicating a better glycogen store in muscles. In order to further assess the energy status in the gastrocnemius, the pAMPK/AMPK ratio was measured. A high pAMPK/AMPK ratio usually represents an energy crisis, whereas a low ratio indicates sufficient energy supply (Hardie et al., 2012; Herzig and Shaw, 2018; Kahn et al., 2005). Our data showed that the pAMPK/AMPK ratio in HE rats was significantly lower than that in NE rats. This provides further evidence for a better energy reserve in skeletal muscles of hypoxic rats than in those of normoxic rats after exercise. These data, together with the lactate paradox phenomenon, suggested that a bout of exhaustive swimming with a 2.5% body weight load resulted in less peripheral fatigue in hypoxic rats than in normoxic rats.

Central fatigue during prolonged exercise is considered to be affected by accumulation or depletion of neurotransmitters, such as 5-HT and DA (Leite et al., 2010; Meeusen and Piacentini, 2003; Meeusen et al., 2006). High 5-HT activity is associated with lethargy and loss of central drive/motivation. It has been shown that high concentrations of 5-HT in the preoptic area and hypothalamus after running exercise were associated with decreased running performance (Lin and Kuo, 2013). Our data showed that, under normoxic conditions, there was an increased striatal 5-HT level in exhaustive rats after swimming exercise, which is consistent with previous studies. A similar elevation of 5-HT concentrations was observed after exhaustive swimming under hypoxic conditions. Furthermore, we assessed the level of HIAA, a metabolite of 5-HT,

and found no changes after exhaustive swimming in both normoxia and hypoxia.

Central DA synthesis and metabolism have been shown to be enhanced in several brain regions during exercise (Foley and Fleshner, 2008). Meeusen et al. (1997) showed that 60 min of exercise significantly increased DA content in the striatum. Increased DA concentrations have also been observed in the hypothalamus and hippocampus during exercise (Chaouloff, 1989). We found a similar increase in DA levels from striatal homogenates of rats in both normoxic and hypoxic conditions after a bout of exhaustive swimming, which suggests an enhanced synthesis of DA. In addition, pharmacological experiments demonstrated that either stimulation of DA release with amphetamine or inhibition of DA reuptake with bupropion could significantly improve exercise performance (Bhagat and Wheeler, 1973), and thus testified to a potent effect of functional DA release on the delay of fatigue. We found that striatal DOPAC, a metabolite of DA, was elevated in rats after exhaustive swimming under normoxic conditions, which indicates an elevated metabolism of DA and its functional release. This result is consistent with the evidence of an increased concentration of striatal DA in treadmill-running, exhausted rats (Heyes et al., 1988). However, the elevation of DOPAC induced by exhaustive exercise was blunted by severe hypoxia exposure, which reflected a poor DA release of exhausted rats in hypoxia. Together, these results imply that exhaustive-swimming-induced central fatigue is more severe in hypoxic than in normoxic rats.

In summary, we established a rat model of fatigue using a weight-loaded swimming test and found a dramatic reduction in the endurance performance of hypoxic rats, accompanied by a lower degree of peripheral fatigue and a greater degree of central fatigue than those of normoxic rats. These findings indicate that hypoxia exposure alters the relative contribution of peripheral and central factors during exercise-induced fatigue, and central fatigue may play a predominant role in impairing endurance capacity during exercise in severe hypoxic conditions. Thus, targeting central factors might be of great potential for improving endurance performance at higher altitude.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Methodology: F.S., T.Y.; Validation: T.Y.; Investigation: F.S., T.Y., J.L.; Data curation: F.S., T.Y., J.L.; Writing - original draft: F.S., Q.-Y.H.; Writing - review & editing: F.S., J.L., Q.-Y.H.; Supervision: Q.-Y.H.; Project administration: Q.-Y.H.; Funding acquisition: Q.-Y.H.

Funding

This work was supported by grants from the National Natural Science Foundation of China (31671238) and Military Creative Project (16CXZ014).

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