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Expression of *Ldh-c* (Sperm-Specific Lactate Dehydrogenase Gene) in Skeletal Muscle of Plateau Pika, *Ochotona curzoniae*, and its Effect on Anaerobic Glycolysis

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YW, ZC and JW conceived and designed the study. YW, ZC, MT, HZ and XY contributed in experimental work, acquisition of data analyzed and drafting of the manuscript. ZC, MT and SM statistically evaluated the data. JW, YW and MAA wrote the article.

Key words
Sperm-specific lactate dehydrogenase, Anaerobic glycolysis, Skeletal muscle, *Ochotona curzoniae* Plateau pika, *Ldh-c*, *N*-isopropyl oxamate, LDH activity.

ABSTRACT

The plateau pika (*Ochotona curzoniae*) has a strong adaptability to hypoxic plateau environment. We found that the sperm-specific lactate dehydrogenase (LDH-C_4) gene *Ldh-c* is expressed in plateau pika skeletal muscles. In order to shed light on the effect of LDH-C_4 on the anaerobic glycolysis in plateau pika skeletal muscle, 20 pikas were randomly divided into two groups the inhibitor group (experimental) and the control group, reach of 10 pikas. The pikas of experimental group were injected with 1 mL of 1 mol/L *N*-isopropyl oxamate, a specific LDH-C_4 inhibitor, in biceps femoris muscle of hind legs, each leg with 500 μL. The pikas of control group were injected with the same volume of normal saline. The mRNA and protein expression levels of *Ldh-c* in plateau pika skeletal muscles were determined by real-time PCR and Western blot. The LDH activities, lactate contents and ATP levels in skeletal muscle were compared between the experimental group and the control group. The results showed that 1) the expression levels of *Ldh-c* mRNA and protein were 0.804±0.059 and 0.979±0.176, respectively; 2) 30 min after administration of 1 mL of 1 mol/L *N*-isopropyl oxamate injected in biceps femoris muscle, the concentration of *N*-isopropyl oxamate in blood was 0.08 mmol/L; 3) in skeletal muscles of the inhibitor group and the control group, the LDH activities were 6.30±0.50 U/mg and 10.01±0.59 U/mg, the contents of LD were 0.28±0.039 mmol/g and 0.84±0.16 mmol/g, and the ATP level were 8.15±1.03 nmol/mg and 12.06±1.23 nmol/mg (P<0.01); 5) the inhibition rates of *N*-isopropyl oxamate to LDH, LD and ATP were 37.12%, 66.27%, and 32.42%, respectively. The results suggest that *Ldh-c* expresses in skeletal muscles of plateau pika, and the pika skeletal muscle may get at least 32.42% ATP for its activities by LDH-C_4 catalyzed anaerobic glycolysis, which reduces the dependence on oxygen and enhances the adaptation to the hypoxic environments.

INTRODUCTION

The Qinghai-Tibet Plateau is the highest plateau on earth at an average of over 4,000 m above sea level, which possesses a unique harsh environment of climate and geography, such as hypoxia, cold, and strong ultraviolet radiation. Hypoxia is the most obvious climate characters on the plateau, which expectedly have profound effects on animal survival. Over long years evolution, many plateau-native animals have developed their own unique mechanisms to the adaptation in the plateau environment.

Plateau pika (*Ochotona curzoniae*), a dominant

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small mammal in the alpine meadow ecosystems across the Qinghai-Tibetan plateau, China (Smith and Foggin, 1999; Lai and Smith, 2003). Pikas play an important role as a keystone species in maintaining ecosystem functions for providing food for predators and underground nests for small birds, and in promoting nutrient recycling within alpine ecosystems (Yang *et al*., 2007). Within perpetual evolution, the pika underwent a series of adaptative changes to the harsh environment. First, the pika obtained oxygen effectively by larger pulmonary alveoli superficial and higher capillary density (Wang *et al*., 2008a), thin walled pulmonary arterioles and blunted hypoxic pulmonary vasoconstriction (Ge *et al*., 1998), an increase in erythrocyte count (Wang *et al*., 2008b), reduction in the mean corpuscular volume (Ye *et al*., 1994), changes in hemoglobin (Hb) (He *et al*., 1994) and 2,3-diphosphoglycerate concentrations (Ge *et al*., 1998), and an increase in the oxygen affinity to Hb (He *et al*., 1994). Moreover, pika has a strong cardiac pumping function by having a larger heart and smaller right-to-left ventricular plus septum weights (Qi *et al*., 2008); Thirdly, pika has a high ratio of oxygen utilization by increasing capillary and mitochondrial densities (Wei *et al*., 2006), and concentration of myoglobin in tissues (Wang *et al*., 2008; Qi *et al*., 2008). In addition to these physiological mechanisms, pika reduces dependence on oxygen by increasing anaerobic glycolysis in skeletal muscle (Zhu *et al*., 2009) and gluconeogenesis in liver (Sun *et al*., 2013). The molecular basis of these adaptations in the pika have occurred because of a series of genetic evolutionary changes, including HIF-1α (Li *et al*., 2009; Zhao *et al*., 2004), hemoglobin (Yang *et al*., 2007), vascular endothelial growth factor (VEGF) (Li *et al*., 2013; Zheng *et al*., 2011) testis-specific lactate dehydrogenase (LDH-C_4) (Wang *et al*., 2013), pyruvate carboxylase (Sun *et al*., 2013), myoglobin (Qi *et al*., 2008), cytochromec oxidase (Luo *et al*., 2008), neuron nitric oxide synthase (nNOS) (Pichon *et al*., 2009), and leptin (Yang *et al*., 2006, 2008).

The lactate dehydrogenase (LDH) family enzymes catalyze the inter-conversion of pyruvate to lactate with the concomitant oxidation/reduction of NADH to NAD^+ (Everse and Kaplan, 1973). Different forms of LDH are the product of three different genes: *Ldh-a*, *Ldh-b*, and *Ldh-c* which encode A, B and C subunits, respectively (Li, 1989; Li *et al*., 1989). LDH consists of A and B subunits that assemble into homo- or hetero- tetramers that are distributed in the body in various combinations reflecting the metabolic requirements of different tissues and are consistent with the catalytic properties of the isozymes (Cahn *et al*., 1962; Fine *et al*., 1963). However, the homotetramer LDH-C_4 was previously only detected in testis and spermatozoa and not in any other tissues or cells (Goldberg, 1964, 1975, 1984; Coonrod *et al*., 2006). In later studies, it was found that *Ldh-c* is expressed not only in testis and sperm, but also in different types of human cancers (Gupta, 1999, 2012; Koslowski *et al*., 2002). LDH-C_4 is an iso-, allo-, and an auto-antigen. Animals, after immunization its LDH-C_4 show enhanced fertility in homologous species and reduced fertility in heterologous species (Gupta and Syal, 1997; Gupta, 1999). In our previous study, we identified that *Ldh-c* is expressed not only in testis and sperm, but also in somatic tissues of plateau pika (Wang *et al*., 2013). We also found that pika LDH-C_4 has an affinity for pyruvate that is 90-fold higher than that for lactate in our prevoious study, and it was beneficial to catalyze the conversion of pyruvate to lactate even at high concentration of lactate in our previous study (Wang *et al*., 2016).

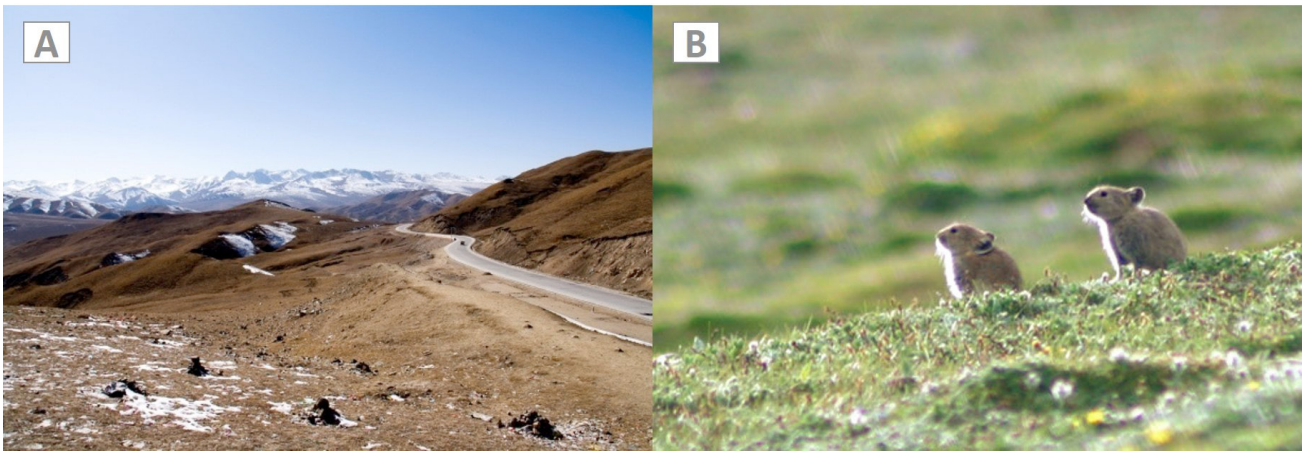

Fig. 1. The plateau pika and its inhabitation environment on Qinghai-Tibetan Plateau. **A,** Qinghai-Tibetan Plateau; **B,** Plateau pika on plateau.

Skeletal muscle fibers can be characterized by their metabolic processes, namely oxidative and glycolytic muscles, oxidative fibers primarily use oxidative phosphorylation to generate adenosine triphosphate (ATP) (Lieber and Friden, 2002). Also, oxidative fibers contain a large number of myoglobin, an oxygen-binding protein that can store oxygen and speed its delivery to mitochondria within the muscle cell. In contrast, glycolytic muscle has the less levels of myoglobin and relies on glycolysis to supply muscle energy (Lieber and Friden, 2002).

Previous study has shown that *N*-isopropyl oxamate was an effective and selective inhibitor due to the close chemical structure existing between *N*-isopropyl oxamic acid and the best substrate for HADH-isozyme II, the α-keto isocaproate (Elizondo *et al*., 2003). *N*-isopropyl oxamate has similar chemical and molecular stucture as pyruvate, as shown in Figure 2, which contributes to its affinity with LDH-C_4.

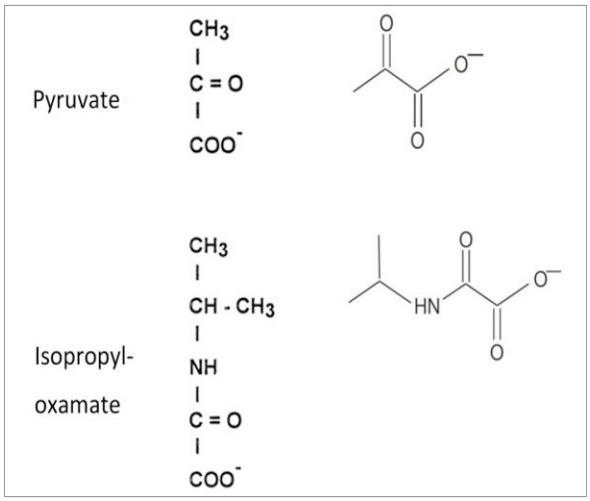

Fig. 2. Chemical and molecular structure of pyruvate and *N*-isopropyl oxamate.

In order to shed light on the expression of *Ldh-c* and its function on tolerance performance of plateau pika, in the current study, we investigated the expression level of *Ldh-c* in pika's skeletal muscle. *N*-isopropyl was used to testify the biochemical index of plateau pika skeletal muscle after LDH-C_4 was inhibited. The molecular mechanism was elucidated by measuring the LDH activity, lactate content and ATP level in pika's skeletal muscle.

MATERIALS AND METHODS

Synthesis of LDH-C_4 inhibitor N-isopropyl oxamate

Ethyl *N*-isopropyl oxamate was shaken with 50 mL NaOH for half an hour and extracted in ether. The aqueous phase was separated and acidified with HCl. Ether extraction and evaporation gave a crude product which, on mixing with light petroleum, soon became crystalline. The crystals were purified by recrystallization from chloroform (5.3 g, 81%): mp 113-114°C, ^{1}H-NMR (CDCl3) δ 1.25 (d, J=6.5 Hz, 6H), 4.07(m, 1H), 7.25 (broad s, 1H), 9.4 (broad s, 1H), IR (KBr) 3294, 2980, 1770, 1677, 1558, 1360 cm^{-1}.

Animal procedure

Plateau pikas were live-trapped at Laji Mountain in Qinghai Province, China, at an altitude of 3,850 m above sea level. The environmental temperature was 10-20°C outside. The average body weight of plateau pikas was 198±9 g. All adult animals, used in this study, were in good health and were randomly divided into 2 groups (sample size was 10 for each group) and treated as follows: i) Control Group: plateau pikas injected with 0.5 mL normal saline in each bilateral biceps femoris of hind legs; ii). Experimental Group: plateau pikas injected with 0.5 mL *N*-isopropyl oxamate in each bilateral biceps femoris of hind legs. All animals were sacrificed 30 min after injection. After experiment, all animals were anesthetized with sodium pentobarbital (5%) and then sacrificed by cervical dislocation immediately before dissection. Skeletal muscle was rapidly removed and frozen in liquid nitrogen for long-term storage. All procedures involved in the handling and care of animals were in accordance with the China Practice for the Care and Use of Laboratory Animals and were approved by the China Zoological Society (permit number: GB 14923-2010).

RNA extraction and quantification of Ldh-c mRNA level by real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen Corp, USA). RNA concentration and purity were assessed by UV spectrophotometry (1.8 <A260/A280<2.0). RNA integrity was checked using electrophoresis. Reverse transcription reaction was carried out starting from 4 μg of total RNA using the First Strand cDNA Synthesis kit (Thermo Scientific, USA). To make standard curves, 1 μL of first-strand cDNA was amplified with Premix Ex Taq Version Kit (TaKaRa BIO Inc., Japan), and quantification of PCR products was checked for plotting standard curves. The initial product concentration was set at 1 and standard curves were generated using a ten-fold serial dilution ranging from 1 to 10^{-7}.

Real-time PCR was performed using the SYBR Premix Ex Taq™ II (TaKaRa BIO Inc., Japan) protocol on BIO-RAD Connect real-time PCR detection system with cycling conditions of 95°C for 3 min, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. *β*-actin was used as an internal control. The PCR primers for *Ldh-c*

and *β*-actin were designed as follows: *Ldh-c*: forward, 5'-TATCGAGAATCTGATCGCAGAAGAC-3' and reverse, 5'-GGGCAAGTTCATCAGCCAAATCC-3', the amplicon length was 130 bp. *β*-actin: forward, 5'-CTCTTCCAGCCCTCCTTCTT-3' and reverse 5'-AGGTCCTTACGGATCTCCAC-3', the amplicon length was 98 bp. The *Ldh-c* mRNA level was normalized with *β*-actin mRNA to compensate for variations in initial RNA amounts. Normalization was carried out by dividing the logarithmic value of *Ldh-c* by the logarithmic value of *β*-actin.

Western blot analysis

Total cellular proteins were extracted by RIPA lysis buffer containing protein inhibitors, BCA protein assay kit (Pierce Biotechnology, USA) was used to assess protein concentration. Proteins were separated by SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane (0.22 μm). After blocking the non-specific binding sites for 2 h with 5% non-fat milk, the membranes were incubated with a rabbit monoclonal antibody against LDHC (SIGMA-ALDRICH, USA, at 1:4,000 dilution) or GAPDH (Genetex, USA, at 1:5,000 dilution) at 4°C overnight. The membranes were then washed with TBST (Tris-Buffered Saline with Tween-20) for six times at room temperature for 10 min. After washing, the target protein was probed with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Santa Cruz, USA, at 1:6,000 dilution) at 37˚C for 2 h. After ten times washing, the bound antibody was detected by chemiluminescence with the ECL Detection Reagent (Pierce Biotechnology, USA) (Zhang *et al*., 2015; Jin *et al*., 2016).

Assay of LDH activities, LD contents and ATP levels

The samples of skeletal muscle were homogenized on ice as a 1:9 (*W/V*) dilution in 0.9% physiological saline. The homogenate was centrifuged at 15,000 r/min at 4°C for 10 min, and the supernatant was collected. The total protein concentration, LDH activity and content of lactic acid (LD) were determined using commercially available assay kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). The amount of ATP was measured by the luciferin-luciferase method (John, 1970) following the protocol of ATP detection kit (Beyotime, China). The luminescence from a 100 μL sample was assayed in a luminometer (Promega, GloMax 20/20, USA) together with 100 μL ATP detection buffer from the ATP detection kit. Standard curves were also generated and the protein concentration of each sample was determined using the BCA Protein assay (Pierce, USA).

Data analysis

All values were expressed as mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA) and Duncan's test using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was considered statistically significant.

RESULTS

mRNA and protein level of Ldh-c expression

Figure 3 shows the mRNA and protein levels of Ldh-c, examined by qRT-PCR and Western blot assays, in skeletal muscle of plateau pika. The relative expression levels of *Ldh-c* mRNA and protein were 0.80±0.06 and 0.98±0.18, respectively.

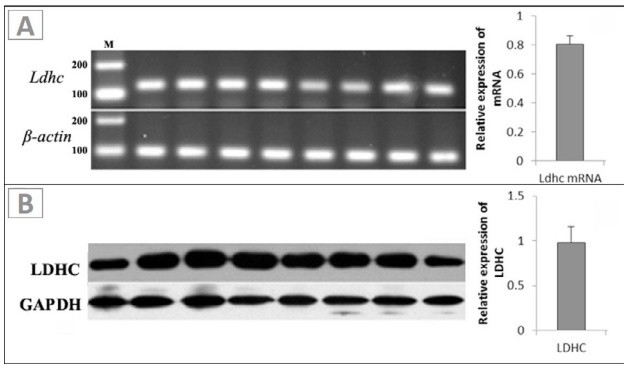

Fig. 3. The expression level of *Ldh-c* in plateau pika skeletal muscle. **A,** The expression level of *Ldh-c* mRNA in plateau pika skeletal muscle. M: marker; **B,** The expression level of LDH-C protein in plateau pika skeletal muscle. Mean ± SD, $n = 8$.

N-isopropyl oxamate as LDH-C_4 inhibitor

N-isopropyl oxamate is more specific inhibitor for LDH-C_4 than *N*-propyl oxamate at concentrations up to 0.1 mmol/L (Wang *et al*., 2015). When pikas were injected with 1 mL of 1 mol/L *N*-isopropyl oxamate in hind legs for 30 min, HPLC analysis showed the inhibitor concentration was 0.08 mmol/L in the blood (Wang *et al*., 2015). While at this concentration, LDH-C_4 was inhibited by 70%, the LDH-A_4 and LDH-B_4 were only inhibited by about 5% (Wang *et al*., 2015). Therefore, *N*-isopropyl oxamate was an optimal inhibitor to study the function of LDH-C_4 in glycolysis of plateau pikas.

LDH activities, LD contents and ATP levels in the skeletal muscle

Figure 4 shows LDH activities 10.01±0.59 U/mg, and 6.35±0.51 U/mg in skeletal muscle of control and inhibitor groups, respectively. LD contents in skeletal muscle were

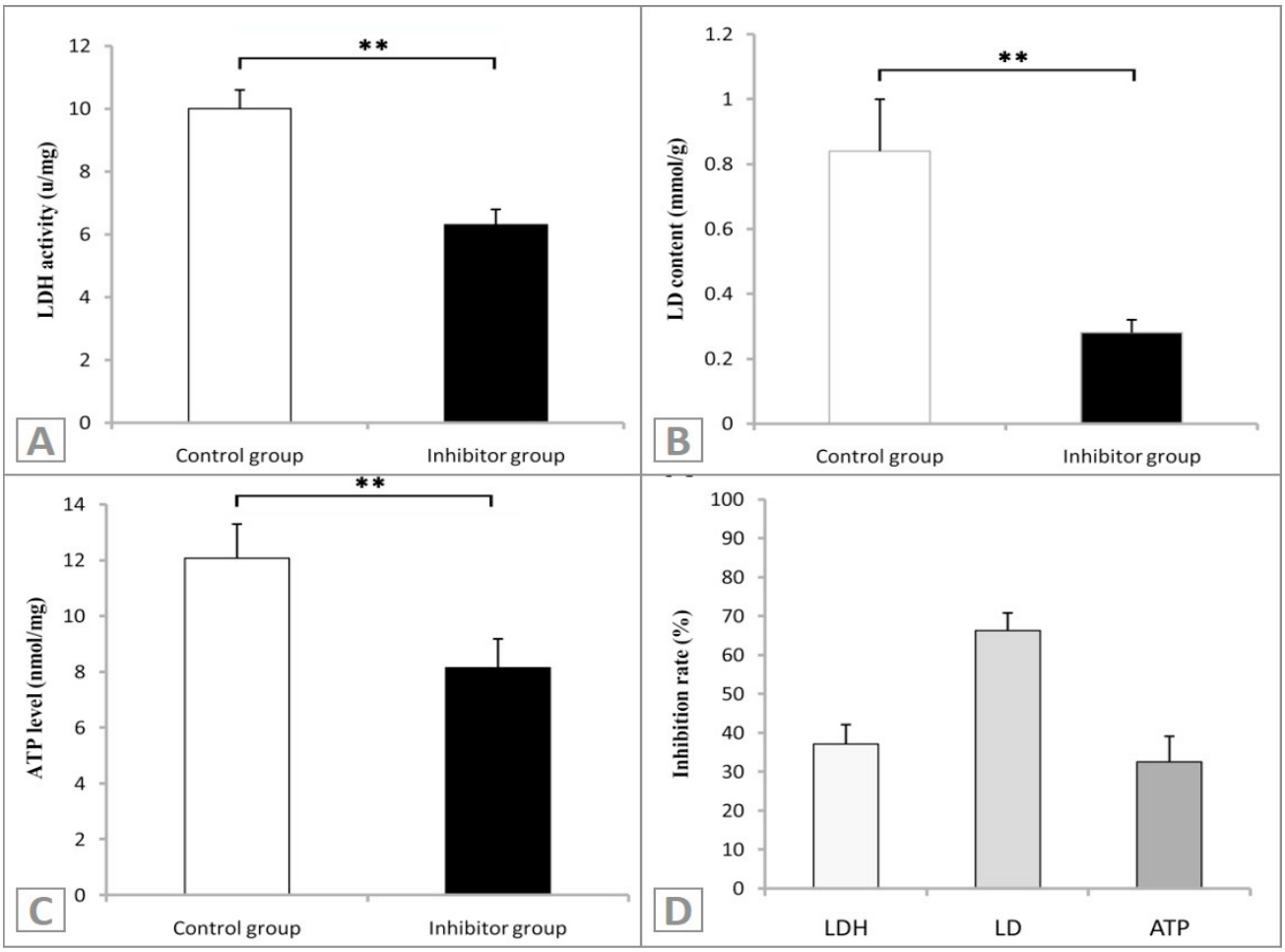

Fig. 4. Comparison of LDH, LD, ATP and their inhibition rates of plateau pika skeletal muscle with treatment. **A,** LDH activities in the skeletal muscle of plateau pika with treatment; **B,** LD contents in the skeletal muscle of plateau pika with treatment; **C,** ATP levels in the skeletal muscle of plateau pika with treatment; **D,** Inhibition rates of LDH, LD and ATP in the skeletal muscle with treatment. All data were expressed as mean±SD; sample size was 8 for each group. $^{**}p<0.01$.

0.85±0.17 mmol/ g, and 0.29±0.05 mmol/g, in control group and inhibitor group, respectively. ATP levels in skeletal muscle were 12.06±1.23 nmol/mg and 8.15±1.03 nmol/mg in control group and inhibitor group, respectively. The inhibition rates of LDH, LD and ATP in the skeletal muscle were 37%, 66%, and 32%, respectively (** $p<0.01$).

DISCUSSION

Existing reports show that the plateau pika has high microvascular density myoglobin content, large surface area and mitochondria number in skeletal muscle (Zhu *et al*., 2009). LDH isozymes LDH-A_4, LDH-A_3B_1, LDH-A_2B_2 (Zhu *et al*., 2009) in skeletal muscle suggest that pika skeletal muscle mainly relies on the aerobic respiration.

In our previous study we found that LDH-C_4 had a lower *K*m for pyruvate (~0.052 mmol/L) and a higher *K*m for lactate (~4.934 mmol/L) compared with LDH-A_4 and LDH-B_4 (Wang *et al*., 2016). This finding implies that LDH-C_4 has an affinity for pyruvate that is 90-fold higher than that for lactate and suggests that pyruvate turnover to lactate may be high even at large contents of endogenous or extracellular lactate *in vivo*. This finding is also supported by the finding that addition of excess lactate did not affect ATP generation in spermatozoa (Hereng *et al*., 2011).

Comparative results showing the *K*i value of N-isopropyl oxamate on murine LDH-A_4, LDH-B_4 and LDH-C_4 of 0.01 mmol/L, 0.40 mmol/L and 0.80 mmol/L (Wong *et al*., 1997) suggest that *N*-isopropyl oxamate has strong inhibitory effect on LDH-C_4 and weak effects on LDH-A_4 and LDH-B_4.

In present study, the results showed that *Ldh-c* expresses in skeletal muscle of plateau pika. The injection of 1 mol/L *N*-isopropyl oxamate in the biceps femoris of plateau pika for 30 min resulted in blood concentration of *N*-isopropyl oxamate of 0.08 mmol/L. At this concentration, *N*-isopropyl oxamate inhibited 70% LDH-C_4 activity without affecting the enzymatic activity of recombinant pika LDH-A_4 and LDH-B_4 (Wang *et al*., 2015). After experiment, LDH activity and LD and ATP content in biceps femoris of treated pikas decreased significantly compared to untreated animals, and inhibition rates of LDH, LD, and ATP by *N*-isopropyl oxamate were 37%, 66% and 32%, respectively.

The extraordinary function of pika *Ldh-c* was also due to its amino composition. We had compared the amino sequence comparison of pika LDHA, LDHB and LDHC with other mammalian species. As shown in Figure 5, Pika (*Ochotona curzoniae*) *Ldh-a* (HQ704676) has 95.20%, 96.10%, 94.60% and 94.30% amino homologous respectively, to that of *Rattus norvegicus*, *Mus musculus*, *Bos taurus* and *Homo sapiens* (Fig. 5A); *Ldh-b* (HQ704677) has 98.80%, 97.30%, 97.05% and 96.50%, respectively (Fig. 5B); *Ldh-c* (HQ704678) had 87.70%, 74.70%, 75.30% and 87.30%, respectively (Fig. 5C). These results show that pika LDHC amino homologous to the LDHC of other species is significant lower than LDHA and LDHB, suggesting that the kinetic properties of LDH-C_4 are also related to its amino acid composition. In addition, Pika LDHA and LDHB have 77% and 72% amino homology with LDHC (Fig. 5D). LDHC shares more in common with LDHA than LDHB. This theory is proved by that *Ldh-c.* arose from a second independent gene duplication event, by duplication of the *Ldh-a* during mammalian evolution (Li *et al*., 1983, 2002; Millan *et al*., 1987). In addition, based on the amino acid constitution of pika LDHC subunit, we found that the pika LDH-C_4 contains 26 and 20 more residues of isoleucine than LDH-A_4 and LDH-B_4. Isoleucine residues in pika LDH-C_4 form part of the hydrophobic region present only at the active site of pika LDH-C_4 (Wang *et al*., 2015). It seems that this hydrophobic region allows the enzyme to discriminate between *N*-isopropyl oxamate α-keto and α-hydroxy acids with different side chains, facilitating, through hydrophobic interactions, the proper binding of those substrates as *N*-isopropyl oxamate with nonpolar side chains and rejecting those with polar side chains.

CONCLUSIONS

Collectively, our results suggest that decreased ATP in skeletal muscle of plateau pika in hypoxic environment is due to inhibition LDH-C_4 activity. Pika have reduced oxygen dependence and enhanced adaptation to hypoxic environments due to increased anaerobic glycolysis by LDH-C_4 in skeletal muscle since this is the role of LDH-A_4 in most species on plain land environment.

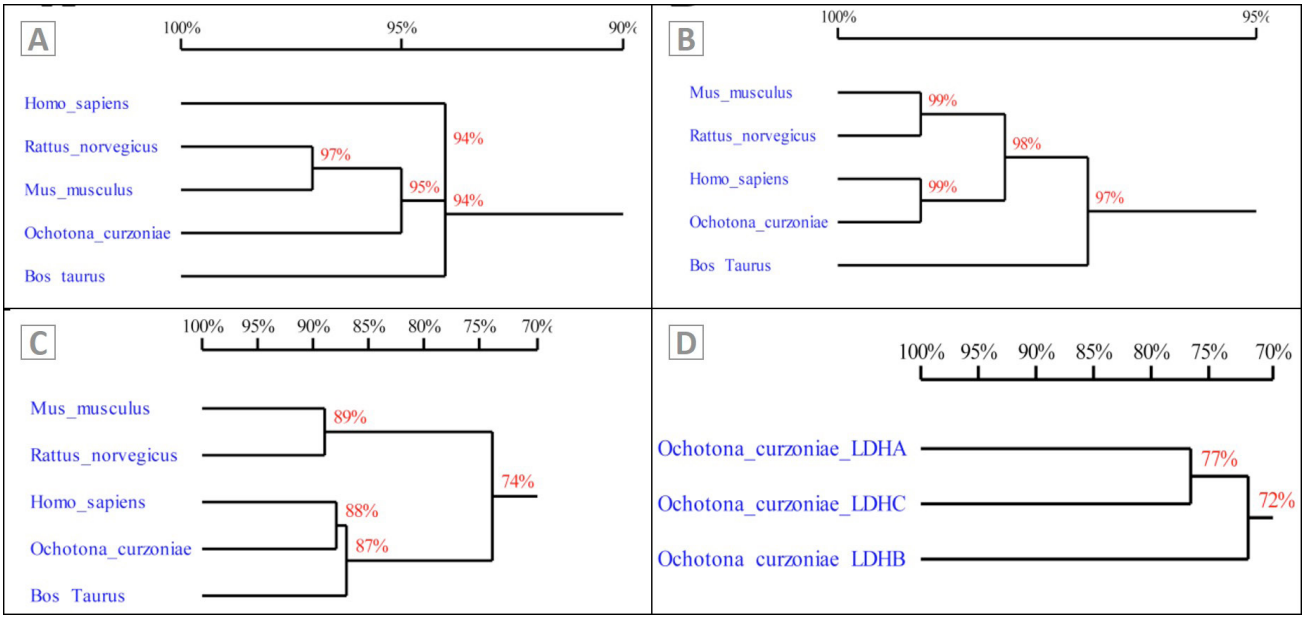

Fig. 5. Amino sequence comparison of LDHA **(A)**, LDHB **(B)** and LDHC **(C)** of amino homologous tree of *Rattus norvegicus*, *Mus musculus*, *Bos taurus*, *Homo sapiens* and *Ochotona curzoniae.* **D,** Amino homologous comparison of pika LDHA, LDHB and LDHC.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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