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Impact of high altitude on the hepatic fatty acid oxidation and synthesis in rats

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ABSTRACT

High altitude (HA) affects energy metabolism. The impact of acute and chronic HA acclimatization on the major metabolic pathways is still controversial. In this study, we aimed to unveil the impact of HA on the key enzymes involved in the fatty acid (FA) metabolism in liver. Rats were exposed to an altitude of 4300 m for 30 days and the expressions of two key proteins involved in FA β-oxidation (carnitine palmitoyl transferase I, CPT-I; and peroxisome proliferator-activated receptor alpha, PPARα), two proteins involved in FA synthesis (acetyl CoA carboxylase-1, ACC-1; and AMP-activated protein kinase, AMPK), as well as the total ketone body in the liver and the plasma FFAs were examined. Rats without HA exposure were used as controls. We observed that the acute exposure of rats to HA (3 days) led to a significant increase in the expressions of CPT-I and PPARα and in the total hepatic ketone body. Longer exposure (15 days) caused a marked decrease in the expression of CPT-I and PPARα. By 30 days after HA exposure, the expression levels of CPT-I and PPARα returned to the control level. The hepatic ACC-1 level showed a significant increase in rats exposed to HA for 1 and 3 days. In contrast, the hepatic level of AMPK showed a significant reduction throughout the experimental period. Plasma FFA concentrations did not show any significant changes following HA exposure. Thus, increased hepatic FA oxidation and synthesis in the early phase of HA exposure may be among the important mechanisms for the rats to respond to the hypoxic stress in order to acclimatize themselves to the stressful environments.

1. Introduction

High altitude (HA)-associated hypoxia is a significant physiological stress causing a number of metabolic changes during the acute and chronic HA acclimatization. Changes in substrate metabolism during HA exposure have attracted a wide attention [1–5]. Some studies have indicated that aerobic breakdown of carbohydrate (CHO) may be the primary source of energy turnover during acclimatization to HA. In chronic HA acclimatization, there is an increased uptake for glucose but a decreased uptake for fatty acids (FA) consumption in skeletal muscle during exercise and in heart and liver during rest [2,3,6–8]. Other studies have suggested that increased lipolysis and the resultant high blood level of fatty acids may be part of the energy source in HA [4,9–11]. In the experimental HA condition in resting rats, chronic hypoxia can enhance uptake and oxidation of FA in liver and skeletal muscle [9]. However, it was reported that under the same condition, the relative impact of FAs and CHO on metabolism was similar before and after acclimatization [12,13]. Furthermore, marked tissue-specific variations on the metabolism of the same substrate in both HA and control conditions have been reported [7,9,14].

The hepatic expression of numerous genes including those involved in glycolysis, lipid metabolism, and carbohydrate metabolism may be altered in response to acute and chronic exposure to HA [15,16]. Mitochondrial β-oxidation of FA is one of the key aerobic pathways, which starts with the translocation of activated long-chain fatty acids into the matrix of mitochondria. In β-oxidation, the long carbon chains of the FA are split into acetyl CoA which then becomes a major source of energy for the heart

Abbreviations: ACC-1, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; CHO, carbohydrate; CPT-I, carnitine palmitoyl transferase-I; FA, fatty acid; FFA, free fatty acid; HA, high altitude; LCFA, long-chain fatty acids; M-CoA, malonyl-CoA; PPARα, peroxisome proliferator-activated receptor alpha; TCA cycle, tricarboxylic acid cycle.

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and skeletal muscle. The hepatic acetyl CoA is the raw material of ketone bodies which are an important alternative fuel for the brain when the blood glucose level is low. Carnitine palmitoyl transferase-I (CPT-I), a rate-limiting enzyme in β-oxidation of FA, is a potential target for altering metabolic homeostasis under stress [17–19]. The primary function of CPT-I is to facilitate the translocation of activated long-chain FAs from the cytosol into the mitochondrial matrix for β-oxidation. It is generally accepted that FA β-oxidation is largely regulated by CPT-I [20–22]. Peroxisome proliferator-activated receptor alpha (PPARα) is a ligand-activated transcription factor and is highly expressed in the liver where it stimulates the β-oxidative degradation of FAs [23]. Activation of PPARα has been shown to affect the expression of 80–100 genes [24] and the proteins encoded by these genes are involved in all aspects of FA catabolism [23,25,26]. CPT-I is a direct downstream target for PPARα [19,27].

Lipogenesis including *de novo* synthesis of FAs from acetyl-CoA or malonyl-CoA (M-CoA) and conversion to triglycerides (TAGs) in the liver is a critical process for energy generation and storage. Acetyl CoA carboxylase-1 (ACC-1) is a rate-limiting enzyme in FA synthesis controlling the conversion of acetyl-CoA to M-CoA, a critical precursor for biosynthesis of FAs [28]. AMP-activated protein kinase (AMPK) integrates hormonal and nutritional signals to promote energy balance by switching on catabolic pathways and switching off ATP-consuming pathways. Activation of AMPK in the liver leads to the increased fatty acid oxidation and inhibition of lipogenesis, possibly through a reduced activity of ACC-1 and M-CoA [28–30].

However, the impact of HA on the lipid metabolism of the liver is controversial. For example, it was reported that acute and chronic hypobaric hypoxia did not affect the CPT-I activities in rat liver [7], whereas in another study, the CPT-I activities in liver were significantly reduced one day after HA exposure [31]. Still in other studies, chronic exposure to HA could stimulate all aspects of lipid metabolism in rat liver except the blood and hepatic levels of FFA [9,12]. No previous studies have documented the activities of liver PPARα, AMPKα and ACC-1 during HA exposure.

In this study, we aimed to evaluate the effect of acute and chronic exposure to HA on the FA oxidation and synthesis in rats.

2. Materials and methods

2.1. Animals

Thirty-six male SD rats (body weights: 220–300 g) were kept at a density of 1 rat/cage and placed at an altitude of 400 m at 22 ± 1 °C. After 3 days of acclimatization, rats were randomly assigned into six groups respectively named H1 (n = 6, HA exposure for 1 day), H3 (n = 6, HA exposure for 3 days), H7 (n = 6, HA exposure for 7 days), H15 (n = 6, HA exposure for 15 days), H30 (n = 6, HA exposure for 30 days), and C (n = 6, no HA exposure, controls). For hypobaric hypoxic exposure, H1, H3, H7, H15 and H30 groups were air-transferred to a plateau experimental base (altitude 4300 m). Rats were housed at the same density at 22 ± 1 °C in a humidity- and light-controlled room (lights on at 06:30 h and off at 18:30 h). Rats were given the standard rodent chow and water *ad libitum*. Following overnight fasting, rats were sacrificed under anaesthesia with 10% chloral hydrate (0.4 ml/100 g body weight, i.p). Blood samples were collected from each rat into the heparinized tubes, and plasma was separated and stored at −80 °C until analysis. The liver tissues were snap-frozen in liquid nitrogen and then stored at −80 °C until analysis. The rats in Group C were anaesthetized and sacrificed on Day 1 and processed in the same manner as described above. The study was approved by the Animal Care and Use Committee of the Lanzhou University.

2.2. RNA extraction and qPCR

The relative mRNA expression levels of CPT-I, PPARα, AMPK, and ACC-1 were determined by quantitative real time polymerase chain reaction (qPCR). Briefly, total RNA was extracted from the rat livers using RNAiso Plus reagent (TaKaRa Biotechnology Co., Dalian, China). Approximately 0.5 μg of the extracted RNA was reverse transcribed into cDNA using Primescript™ reverse transcription (RT) Master Mix (TaKaRa Biotechnology Co., Dalian, China). Reverse transcription reaction was performed at 37 °C for 15 min followed by 85 °C for 5 s. qPCR was performed in a final volume of 25 μl, using the SYBR® Premix Ex Taq™ II kit (TaKaRa Biotechnology Co., Dalian, China) on a Rotor-Gene 6000 Thermal Cycler. Each 25 μl PCR reaction contains 2.0 μl of cDNA, 1.0 μl of sense primer, 1.0 μl of antisense primer, 12.5 μl of SYBR Green PCR Master Mix, and 8.5 μl of the PCR-grade water. The cycling conditions were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. Each sample was assayed in duplicate. Fold inductions were calculated using the $2^{-\Delta\Delta Ct}$ method with β-actin being the internal reference. All primers used in this study were designed and synthesized by TaKaRa Biotechnology Company (Table 1).

2.3. Protein extraction, SDS–polyacrylamide gel electrophoresis and Western blots

The relative protein expression of CPT-I, PPARα, AMPKα, and ACC-1 was measured by Western blots. Frozen liver samples (50–100 μg) were homogenized by manual grinding at 4 °C in 200 μl of RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with 1 mM PMSF. Insoluble material was removed by centrifugation for 10 min at 12,000*g* at 4 °C. The protein concentration in the supernatant was determined by BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). Approximately 50 μg of the extracted protein sample from each animal was denatured in loading buffer, separated by 10% sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS–PAGE) at 30 mA constant current, and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk in tris-buffered saline (TBS) containing 0.1% Tween (TBST) for 2 h at room temperature. Membranes were incubated with each of the primary antibodies (1:500) in TBST overnight at 4 °C. Rabbit anti-β-actin (1:1000) was used to detect

Table 1
Primer sequences used in qPCR amplifications.

Target	Sequences	Size (bp)	GenBank accession no.
CPT-I			
S	5′-AGGTCGGAAGCCCATGTTGTA-3′	138	NM031559.2
AS	5′-GCTGTCATGCGCTGGAAGTC-3′		
PPARα			
S	5′-GGCAATGCACTGAACATCGAG-3′	112	NM013196.1
AS	5′-GCCGAATAGTTCGCCGAAAG-3′		
ACC-1			
S	5′-CAATCCTCGGCACATGGAGA-3′	149	NM022193.1
AS	5′-GCTCAGCCAAGCGGATGTAGA-3′		
AMPK			
S	5′-CGGCAAGGAGCACAAGATCA-3′	131	NM022627.1
AS	5′-TGGGCTTCACAGAATCATCCAA-3′		
β-Actin			
S	5′-CATCCGTAAAGACCTCTATGCCAAC-3′	97	NM007393.3
AS	5′-ATGGAGCCACCGATCCACA-3′		

S: sense
AS: anti-sense

β-actin as the loading control. The membranes were then washed three times each for 5 min with TBST, and incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000) for 2 h at room temperature. After two 10-min washes with TBST and one 10-min wash with PBS, the signals were detected using the enhanced chemiluminescence (ECL) substrate (Beyotime Institute of Biotechnology, Haimen, China) and imaged using an ImageQuant 350 Imaging System (GE Healthcare Bio-Sciences Corp., Piscataway, USA). All antibodies were purchased from BIOGOT Technology Co. (Nanjing, China). The Western blot bands were analyzed by Image-pro plus analysis software Version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA), and expressed as the relative integrated intensity compared to that of the β-actin of the same sample.

2.4. Measurement of total ketone body in the liver by ELISA

A small piece of the frozen liver tissue was homogenized in PBS (PH7.2–7.4) on ice by manual grinding. Insoluble material was removed by centrifugation for 15 min at 2000 rpm at 4 °C. The concentration of the total ketone body in the supernatant was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The hepatic total ketone body was expressed as micrograms per gram tissue (μg/g tissue).

2.5. Measurement of plasma free fatty acids (FFAs) by spectrophotometry

Plasma FFAs concentration was assayed by spectrophotometry using an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The absorbance at 440 nm was recorded and the concentration of FFAs was expressed as micromoles per liter (μmol/L).

2.6. Statistical analysis

All data are presented as means ± SD. The data were analyzed using one-way ANOVA with Tukey's test or Tamhane's T2 test for pairwise comparisons between the means. SPSS (version 17.0) and GraphPad softwares (version 5) were used for all statistical analysis. A $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Expressions of CPT-I, PPARα, ACC-1, and AMPK

To evaluate the impact of the HA on the hepatic fatty acid oxidation, we measured the expression of two important enzymes CPT-I and PPARα in rat liver. As shown in Fig. 1A and B, compared to the unexposed rats, there was a significant acute increase in the

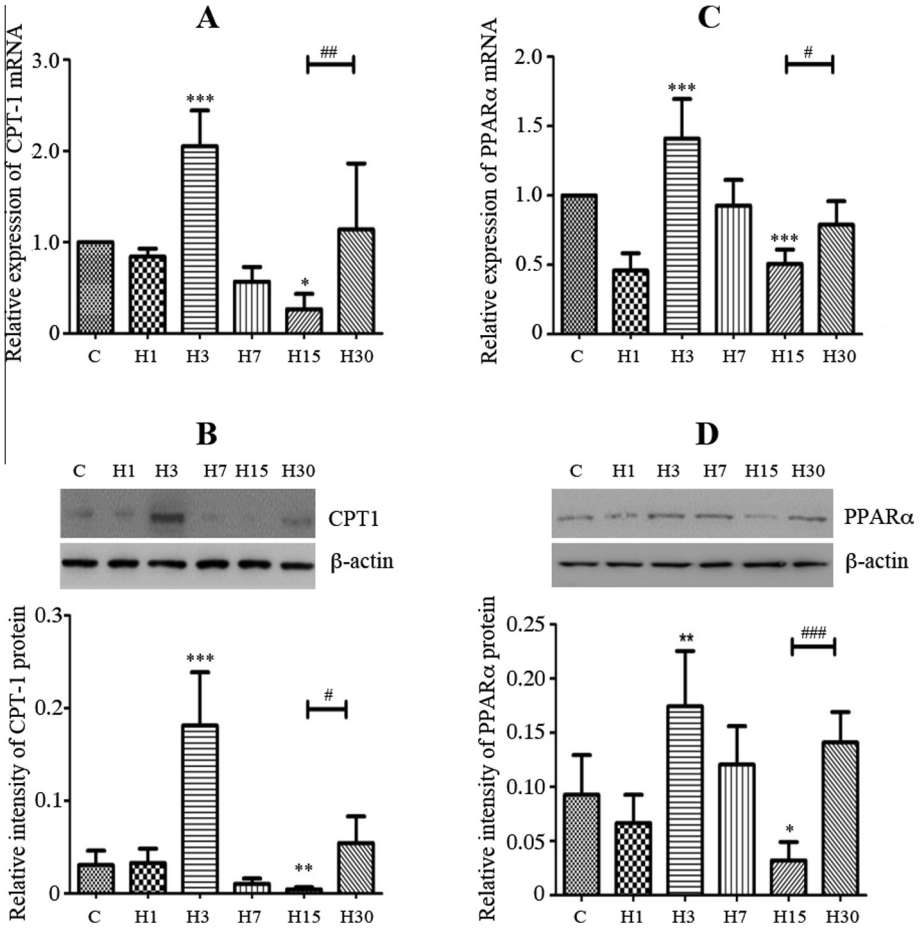

Fig. 1. Relative expression of CPT-I mRNA (A) and CPT-I protein (B), PPARα mRNA (C) and PPARα protein (D) in rats exposed HA for various durations. The expression of mRNA was measured by qPCR and the protein expression by Western blot. Control group: rats were not exposed to HA ($n = 6$). H1, rats were exposed to HA for 1 day ($n = 5$); H3, rats were exposed to HA for 3 days ($n = 5$); H7, rats were exposed to HA for 7 days ($n = 5$); H15, rats were exposed to HA for 15 days ($n = 5$); H30, rats were exposed to HA for 30 days ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, all compared to controls. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, all compared to H15.

expression of CPT-I mRNA and CPT-I protein 3 days following HA exposure (H3) (1 vs 2.05 ± 0.39, and 0.18 ± 0.058 vs 0.031 ± 0.016, respectively, *all* $p < 0.001$). After 7 days (H7), the mRNA and protein expression of CPT-1 declined and reduced to a significant lower level at day 15 (H15) (1 vs 0.27 ± 0.17, $p < 0.05$; 0.031 ± 0.016 vs 0.0047 ± 0.0021, $p < 0.01$, respectively). After 30 days (H30), both mRNA and protein levels of CPT-I returned to a significantly higher level than those of H15 (1.14 ± 0.73 vs 0.27 ± 0.17, $p < 0.01$; 0.055 ± 0.029 vs 0.0047 ± 0.0021, $p < 0.05$, respectively) but similar to that of the control.

PPARα, another important molecule involved in fatty acid oxidation, showed the similar trend to CPT-1 (Fig. 1C and D). Compared to control rats, there were a significant increase in the hepatic expression of PPARα mRNA and protein in rats exposed to HA for 3 days (H3) (1 vs 1.4 ± 0.28, $p < 0.001$; 0.17 ± 0.015 vs 0.093 ± 0.037, $p < 0.01$, respectively). Both mRNA and protein levels declined to a significantly lower level by 15 days (H15) (1 vs 0.51 ± 0.1, $p < 0.001$; 0.093 ± 0.037 vs 0.032 ± 0.017, $p < 0.05$, respectively) and returned to a level that is higher than that of H15 (0.79 ± 0.17 vs 0.51 ± 0.1, $p < 0.05$; 0.141 ± 0.028 vs 0.032 ± 0.017, $p < 0.001$, respectively) but similar to that of the control.

In order to determine the impact of HA on fatty acid synthesis, we measured the expression of ACC-1 and AMPK in response to HA exposure. As shown in Fig. 2A and B, compared to controls, there was an acute significant increase in the mRNA and protein expression of ACC-1 at days 1 (H1) and 3 (H3) following HA exposure (4.01 ± 1.04 vs 1.0, and 2.65 ± 0.61 vs 1.0 for ACC-1 mRNA, respectively, all $p < 0.001$; 0.567 ± 0.091 vs 0.101 ± 0.037, and 0.494 ± 0.088 vs 0.101 ± 0.037 for ACC-1 protein, respectively, all $p < 0.001$). No significant changes were observed for the mRNA and protein expression of ACC-1 at other time points.

In contrast to the changes to ACC-1, rats exposed to HA exhibited a significant reduction in the expression of AMPK both at the mRNA (Fig. 2C) and protein (Fig. 2D) levels. By day 30, there was a very minor restoration of AMPK at both mRNA and protein levels.

3.2. Concentration of total ketone body in the liver

Compared to control rats, there was a significant increase in the concentration of total ketone body in the liver in animals exposed to HA for 3 days (H3) (20.1 ± 1.13 vs 16.7 ± 0.625, $p < 0.01$). No significant changes were found in other time points (Fig. 3A).

3.3. Plasma free fatty acid (FFA) concentration

In all rats exposed to HA, the plasma FFA concentration did not show any significant change as opposed to the control animals (Fig. 3B).

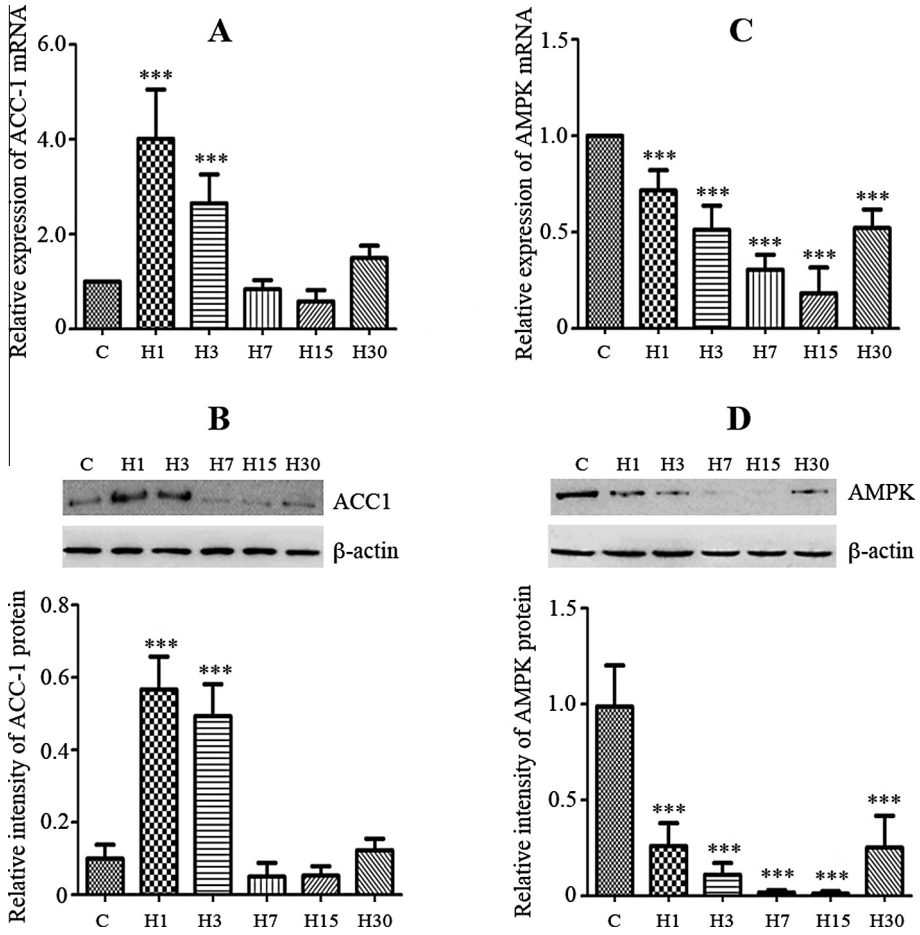

Fig. 2. Relative expression of ACC-1 mRNA (A) and ACC-1 protein (B), AMPK mRNA (C) and AMPK protein (D) in rats exposed HA for various durations. The expression of mRNA was measured by qPCR and the protein expression by Western blot. Control group: rats were not exposed to HA ($n = 6$). H1, rats were exposed to HA for 1 day ($n = 6$); H3, rats were exposed to HA for 3 days ($n = 6$); H7, rats were exposed to HA for 7 days ($n = 6$); H15, rats were exposed to HA for 15 days ($n = 6$); H30, rats were exposed to HA for 30 days ($n = 6$). ***$p < 0.001$, compared to controls.

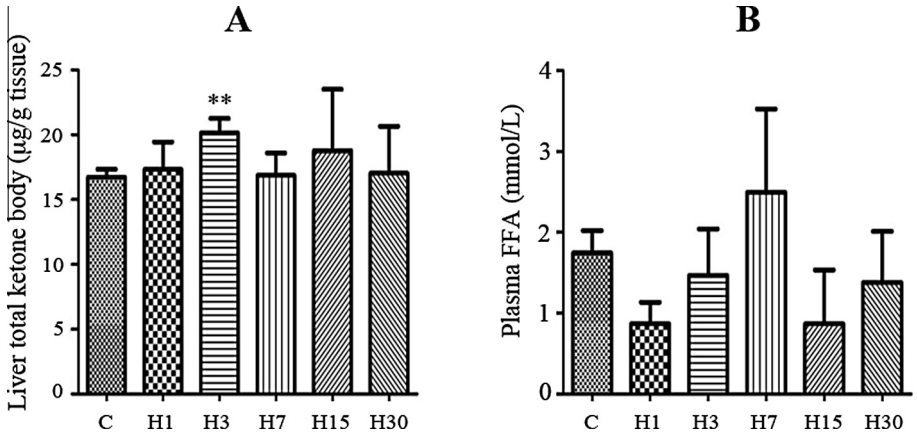

Fig. 3. Total ketone body concentrations in the rat livers (A) was measured by ELISA in control rats (no HA exposure, $n = 6$) and rats exposed to HA ($n = 6$). The plasma concentration of free fatty acid (FFA) (B) was measured by spectrophotometry ($n = 6$). $^{**}p < 0.01$, compared to controls. All values are expressed as mean ± SD.

4. Discussion

HA exerts multiple effects on the physiological functions of the respiratory and cardiovascular systems, but the impact of acute and chronic HA exposure on the key enzymes involved in the energy production is still a matter of debate [1,7,9,12,31]. Here, we investigated the effect of HA exposure on the expression of several rate-limiting enzymes and related transcription factors involved in FA β-oxidation and synthesis in rats. Rats were exposed to an altitude of 4300 m to create an acute (within 2 weeks) and a chronic (2–8 weeks) HA exposure model. With respect to the hepatic FA β-oxidation, we have observed a significant increase at early acute exposure (3 days), a marked decrease at early chronic HA exposure (15 days) and a significant restoration at chronic exposure (30 days). Increased mitochondrial β-oxidation of FAs in the liver leads to increased production of acetyl CoA, an important enzyme for the synthesis of FA, triglyceride and ketone body. It is possible that FA may act as an important energy fuel and a compensation for CHO during acute exposure to HA.

It is well-known that ketone body is an important alternative energy source for hepatic gluconeogenesis and brain metabolism in case of insufficient glucose supply. Meanwhile, ketone body is a reliable indicator for FA oxidation. Liver is the exclusive organ for the generation of ketone body. The increased expression of CPT-I and PPARα together with the enhanced generation of total ketone body in the HA exposed rat livers may suggest an adaptive strategy in case of limited oxygen supply to tissues, and thus undoubtedly support a critical role of liver in the acclimation to stressful events.

Exposure to HA also stimulate the expression of enzymes involved in the regulation of fatty acid synthesis. This is typically exemplified by our findings that an early acute HA exposure (1 and 3 days) could significantly increase the expression of hepatic ACC-1, a rate-limiting enzyme in FA biosynthesis [28]. Coincidentally, increased expression of CPT-I, PPARα, and ACC-1 was associated with a significant reduction in the expression of AMPK, an enzyme reported to inhibit lipogenesis through suppressing the activity of ACC-1 and M-CoA [28–30]. However, a parallel change between ACC-1 and AMPK did not exist during chronic HA exposure (15 and 30 days). Thus, it is likely that ACC-I activity is not exclusively controlled by AMPK.

Most plasma FFAs are derived from hydrolysis of adipose tissue [32]. Higher concentrations of plasma FFAs have been found in events that are associated with increased lipid metabolism such as HA related hypoxia [3,33]. However, in our study, the plasma FFA concentrations was not affected by HA exposure, a finding consistent with the published data [12]. Apparently, the plasma level of FFAs does not necessarily reflect the rate of fat oxidation.

In this study, we observed a marked reduction of the hepatic level of CPT-1 and PPARα in animals exposed to HA for longer period (15 days), followed by a recovery almost to the level of unexposed rats (by 30 days). CPT-I is a direct downstream target for PPARα [19,27], and FA oxidation consumes more oxygen than glycolysis, and CHO oxidation generate more ATP per molecule of oxygen than FA oxidation. Thus, we reasoned that the observed pattern of change in the FA metabolic enzymes indicates that a decreased FA oxidation is a more preferable and appropriate adaptive mechanism for the animals to acclimatize themselves to the HA induced hypoxic environment. Further studies to clarify the signaling pathways leading to the observed changes are warranted.

It should be noted that our findings are not in complete agreement with the data published by others. For example, Dutta et al. [31] reported that in rats exposed to acute HA (1 day), there was a significant reduction in the liver mitochondrial CPT-I, whereas slightly longer exposure (7 days) did not cause any significant changes. We speculated that such a partial discrepancy between the reported findings and our own data may reflect a difference in the experimental design. The HA exposure was simulated at an altitude of 6096 m in Dutta's study, whereas in our experiments, rats were exposed to an altitude of 4300 m. Thus, in the study by Dutta et al., rats were exposed to a much severe hypoxia, which may be more detrimental to the liver's ability to generate CPT-I. To make the issue even more complicated, Kennedy et al. [7] reported that exposure of rats to a similar HA environment (i.e., an altitude of 4300 m for 1 day) did not affect the CPT-I activity. The conflicting results among these studies not only reflect the inevitable experimental variations, but perhaps more importantly indicate the complex network controlling the enzymatic activities of lipid metabolism in response to hypoxia.

In summary, increased hepatic FA oxidation and synthesis may be among the important mechanisms for the rats to respond to the HA induced hypoxia in order to acclimatize themselves to the stressful environments. More studies are necessary to unveil the underlying molecular mechanism controlling the expressions of key enzymes involved in the FA oxidation and synthesis.

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