

# Riboflavin Supplementation Improves Energy Metabolism in Mice Exposed to Acute Hypoxia

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## Summary

This study investigated the effects of riboflavin on energy metabolism in hypoxic mice. Kunming mice were fed diets containing riboflavin at doses of 6, 12, 24 and 48 mg/kg, respectively for 2 weeks before exposure to a simulated altitude of 6000 m for 8 h. Changes of riboflavin status and energy metabolism were assessed biochemically. Simultaneously, a <sup>1</sup>H nuclear magnetic resonance (NMR) based metabolomic technique was used to track the changes of plasma metabolic profiling. It was found that the content of hepatic riboflavin was decreased and erythrocyte glutathione activation coefficient was elevated significantly under hypoxic condition. Meanwhile, increased plasma pyruvate, lactate,  $\beta$ -hydroxybutyrate and urea, as well as decreased plasma carnitine were observed. Riboflavin supplementation improved riboflavin status remarkably in hypoxic mice and decreased plasma levels of pyruvate, free fatty acids and  $\beta$ -hydroxybutyrate significantly. Plasma carnitine was increased in response to riboflavin supplementation. Results obtained from <sup>1</sup>H NMR analysis were basically in line with the data from biochemical assays and remarkable changes in plasma taurine, choline and some other metabolites were also indicated. It was concluded that riboflavin requirement was increased under acute hypoxic condition and riboflavin supplementation was effective in improving energy metabolism in hypoxic mice.

## Key words

Hypoxia • Riboflavin • Energy metabolism • <sup>1</sup>H NMR analysis

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## Introduction

Riboflavin is a precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), and serves as coenzymes for numerous oxidases, reductases and dehydrogenases in metabolic processes. Riboflavin deficiency results in low FMN and FAD contents in the cell and reduced activity of a series of flavin-containing enzymes (Powers 2003, Lienhart *et al.* 2013). It had been confirmed that mitochondrial  $\beta$ -oxidation was impaired significantly in response to riboflavin deficiency, because several flavin-containing enzymes, such as acyl-CoA dehydrogenase, work in the  $\beta$ -oxidation (Brady and Hoppel 1985). Riboflavin also plays a role in glutathione synthesis since FAD is a cofactor for glutathione reductase. The erythrocyte glutathione reductase activation coefficient (EGRAC) is frequently used as a sensitive maker in the assessment of riboflavin status (Hoey *et al.* 2009). In addition, riboflavin is required for methylenetetrahydrofolate reductase (MTHFR) and plays an important role in homocysteine metabolism, especially in individuals with the MTHFR 677 TT genotype (McNulty *et al.* 2006).

It has been well documented that acute hypoxia impacts significantly on nutritional status, resulting in anorexia, weight loss and altered energy metabolism (Guilland and Klepping 1985, Simon-Schnass 1992). Since energy requirement is elevated at high altitude, it is generally proposed that the requirement of the B vitamins involved in energy metabolism is also increased (Butterfield 1999). Our previous studies showed that a nutritional preparation containing B vitamins could

significantly prolong survival time in mice exposed to acute hypoxia and improve cardiac and pulmonary function of young adults at high altitude (Guo *et al.* 2004, Wang *et al.* 2005). Since riboflavin is one of B vitamins playing active roles in energy metabolism, it is important to explore its requirement at high altitude. To our knowledge, no study has been carried out experimentally so far yet to probe into the effects of riboflavin supplementation on energy metabolism under hypoxic condition.

In the present study, changes of riboflavin status were assessed in response to acute hypoxia and the effects of riboflavin supplementation were evaluated biochemically in mice. Meanwhile, a  $^1\text{H}$  nuclear magnetic resonance (NMR) based metabolomic technique was used to investigate the effects of riboflavin supplementation on plasma metabolic profiling in hypoxic mice. The purpose of this study was to investigate the changes of riboflavin status under the acute hypoxia condition and provide experimental evidences that riboflavin requirement is increased after exposure to acute hypoxia.

## Materials and Methods

### *Animal treatments*

Thirty-five male Kunming mice, weighting 14–16 g, were purchased from the Laboratory Animal Center, Academy of Military Medical Sciences (Beijing, China) and housed in a well ventilated room. Throughout the experimental period, all mice were allowed free access to food and tap water. The ambient temperature was maintained between 20 °C and 24 °C and the humidity between 40 % and 60 %. All procedures were performed in accordance with the current Chinese legislation on the care and use of laboratory animals and approved by the Department of Scientific Management of the institute.

After being acclimatized on a basic diet (AIN-93 diet) (Reeves *et al.* 1993) for 5 days, mice were divided randomly into the normoxia control, hypoxia control and hypoxia plus riboflavin-supplemented groups. The mice in the normoxia or hypoxia control were fed continuously the basic diet, while those in three riboflavin supplemented groups received diets supplemented with riboflavin at doses of 12, 24 and 48 mg/kg, respectively. The supplemented riboflavin was 2, 4 and 8 times the level of riboflavin (6 mg/kg) contained in the basic diet. The experiment lasted 2 weeks. Finally, all mice except those in the normoxia control were exposed to a

simulated altitude of 6000 m for 8 h. The procedure was carried out in a hypoxia chamber as follows. First, all mice were elevated to 5000 m at the speed of 1000 m/min, and stopped for 3 min, and then to 6000 m at the speed of 500 m/min. After 8 h exposure at 6000 m, the mice returned to 5000 m at the speed of 500 m/min, and stopped for 3 min and then to the sea level at the speed of 1000 m/min. The blood samples were collected immediately from the orbital veins after ether anesthetization and anti-coagulated with heparin. The plasma was prepared after centrifuged at 8000g for 10 min and stored at –20 °C prior to analysis.

### *Biochemical assays*

Plasma and hepatic riboflavin was determined by high performance liquid chromatography procedures described by Wei *et al.* (2006) and Ndaw *et al.* (2000), respectively. A modified assay developed by Gu *et al.* (1982) was used for measurement of EGRAC. Plasma glucose, lactate, free fatty acids,  $\beta$ -hydroxybutyrate and urea were assayed by using corresponding assay kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), respectively. All measurements were performed strictly in line with the instructions attached.

### *$^1\text{H}$ NMR analysis*

Five plasma samples from each group were selected randomly for  $^1\text{H}$  NMR analysis. Sample preparation and NMR analysis were performed as described elsewhere with minor modifications (Lenz *et al.* 2003, Wu *et al.* 2012). Briefly, plasma samples of 180  $\mu\text{l}$  were mixed with 100  $\mu\text{l}$  of 1.0 mmol/l 3-(trimethylsilyl)-propionic-(2,2,3,3,-d4)-acid sodium (TSP) in 320  $\mu\text{l}$   $\text{D}_2\text{O}$ , shaken vigorously, and centrifuged at 13 000 $\times$ g for 10 min. The aliquots of the resulting supernatant (550  $\mu\text{l}$ ) were placed in 5 mm NMR tubes.  $\text{D}_2\text{O}$  and TSP provided the deuterium lock signal for the NMR spectrometer and chemical shift reference ( $\delta$ 0.0), respectively.

$^1\text{H}$  NMR spectra of all samples were obtained at 599.69 MHz on a Varian INOVA 600 NMR spectrometer. One-dimensional spectra were acquired using a standard NOESY pulse sequence with water suppression during a relaxation delay of 2 s and a mixing time of 150 ms. Sixty-four free reduction decays (FIDs) were collected into 64K data points with a spectral width of 7002.8 Hz, an acquisition time of 4.68 s, and a total pulse recycle delay of 6.68 s. The FIDs were multiplied

by an exponential weighting function corresponding to a line broadening of 0.5 Hz before Fourier transformation.

#### Statistical analysis

The data of biochemical assays were expressed as mean±standard deviation and evaluated by one-way ANOVA. Differences were considered significant at  $P<0.05$ .

The NMR integral data were imported into SIMCA-P (version 10.04, Umetrics Umeå, Sweden) for multivariate analysis. The Carr-Purcell-Meiboom-Gill (CPMG) data were mean-centered and Pareto-scaled, while the longitudinal eddy-current delay (LED) data

were only mean-centered prior to analysis. Principal components analysis (PCA) or partial least squares-discriminant analysis (PLS-DA) was used to detect metabolic differences between groups. In case that group separation was not satisfied, the data were preprocessed using orthogonal signal correction (OSC) to remove the variations not correlated to the group membership and further subject to PCA or PLS-DA. The results were visualized by two-dimensional score plots representing the distribution of samples, as well as the corresponding loading plots providing information on the contribution of each variable to the pattern in the scores plots.

**Table 1.** Changes of riboflavin level in plasma and liver, and EGRAC among different groups after exposed to acute hypoxia.

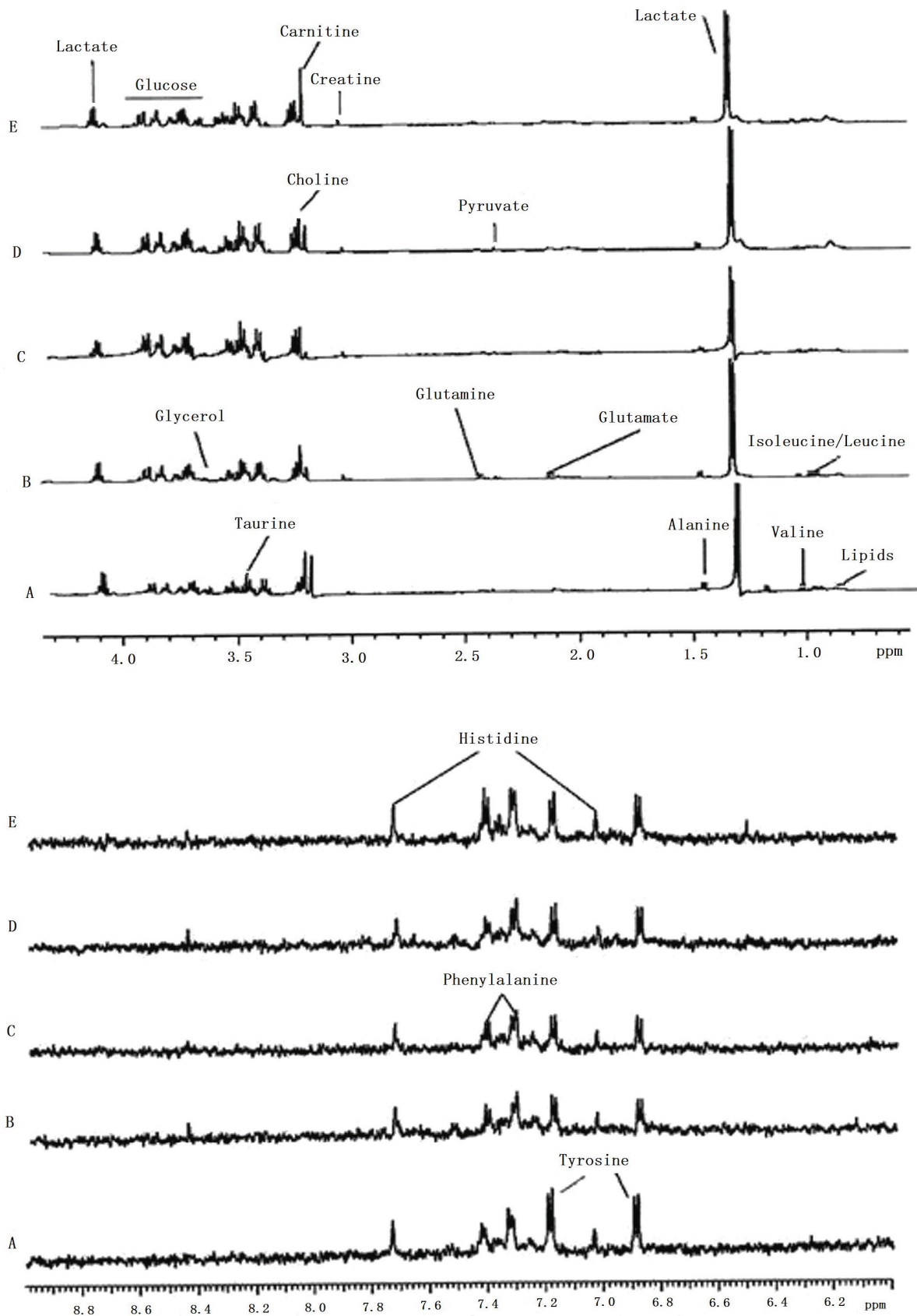
Groups	Plasma riboflavin ( $\mu\text{mol/l}$ )	Liver riboflavin ( $\text{mg}/100\text{ g FW}$ )	EGRAC
Normoxia	124.6±16.2	0.35±0.12	1.02±0.03
Hypoxia	127.7±17.4	0.19±0.04**	1.06±0.02*
Hypoxia plus 2 times R	118.8±22.3	0.23±0.10*	1.04±0.03
Hypoxia plus 4 times R	117.0±9.9	0.20±0.04*	1.04±0.04
Hypoxia plus 8 times R	141.4±26.8	0.25±0.07	1.03±0.02

Values are mean±SD, n=7. R: riboflavin. EGRAC: erythrocyte glutathione reductase activation coefficient. Significant difference from the normoxia: \*  $p<0.05$ , \*\*  $p<0.01$

**Table 2.** Changes of plasma glucose, pyruvate, lactate, free fatty acids,  $\beta$ -hydroxybutyrate and carnitine levels among different groups after exposed to acute hypoxia.

Groups	Glucose ( $\text{mmol/l}$ )	Pyruvate ( $\mu\text{mol/l}$ )	Lactate ( $\text{mmol/l}$ )	Free fatty acids ( $\mu\text{mol/l}$ )	$\beta$ -Hydroxy- butyrate ( $\text{nmol/l}$ )	Carnitine ( $\mu\text{mol/l}$ )	Urea ( $\text{mmol/l}$ )
Normoxia	13.55 ±2.75	0.39 ±0.11	3.70 ±2.82	859.04 ±390.27	4.31 ±0.36	9.28 ±3.04	5.16 ±0.90
Hypoxia	14.03 ±2.79	0.57 ±0.07**	6.35 ±2.01*	973.91 ±259.39	5.38 ±0.51**	2.82 ±1.89*	9.15 ±2.65**
Hypoxia plus 2 times R	14.13 ±1.12	0.59 ±0.05**	5.37 ±4.05*	693.88 ±292.34	5.21 ±0.26**	2.10 ±0.52*	9.04 ±1.68**
Hypoxia plus 4 times R	12.51 ±1.72	0.52 ±0.09*	7.82 ±2.42**	788.72 ±339.81	4.96 ±0.16**	4.92 ±1.31	9.50 ±2.75**
Hypoxia plus 8 times R	12.63 ±1.23	0.48 ±0.05*	7.39 ±1.44**	372.43 ±127.28###	4.93 ±0.05**#	6.86 ±1.22	9.40 ±1.71**

Values are mean ± SD, n=7. R: riboflavin. Significant difference from the normoxia: \*  $p<0.05$ , \*\*  $p<0.01$ . Significant difference from the hypoxia: #  $p<0.05$ , ##  $p<0.01$



**Fig. 1.** Representative  $^1\text{H}$  NMR spectra of plasma samples. The vertical scale in the aromatic region (bottom) was magnified four times compared with that in the aliphatic region (top). **A:** normoxia control; **B:** hypoxia control; **C:** hypoxia plus 2 times riboflavin-supplemented group; **D:** hypoxia plus 4 times riboflavin-supplemented group; **E:** hypoxia plus 8 times riboflavin-supplemented group

## Results

### Riboflavin status

As indicated in Table 1, plasma riboflavin level did not change significantly in mice after exposure to acute hypoxia compared to the normoxia control. Supplementation of 8 times riboflavin increased plasma riboflavin level slightly without statistic significance.

A significant decrease in hepatic content of riboflavin was observed in the acute hypoxia group ( $P < 0.01$ ). Riboflavin supplementation prevented the decline in hepatic content of riboflavin after acute hypoxia treatment. The acute hypoxia plus 8 times riboflavin supplemented group was not different statistically from the normoxia control in the content of hepatic riboflavin (Table 1).

After exposure to acute hypoxia, EGRAC was raised significantly ( $P < 0.05$ ) as compared to the normoxia control. A decrease trend was noted in EGRAC in response to 2 times, 4 times and 8 times riboflavin supplementation in mice treated with acute hypoxia, indicating an improved riboflavin status (Table 1).

### Plasma levels of glucose, pyruvate, lactate, free fatty acids, $\beta$ -hydroxybutyrate, carnitine and urea

Compared to the normoxia group, plasma glucose did not change significantly in mice treated with acute hypoxia. No significant change was also found for plasma glucose after riboflavin supplementation. However, plasma pyruvate was increased remarkably after acute hypoxia exposure ( $P < 0.01$ ) and decreased in response to riboflavin supplementation in a dose dependent manner. On the other hand, plasma lactate was increased significantly after acute hypoxia exposure ( $P < 0.05$ ), and more increase was noted after riboflavin treatment (Table 2).

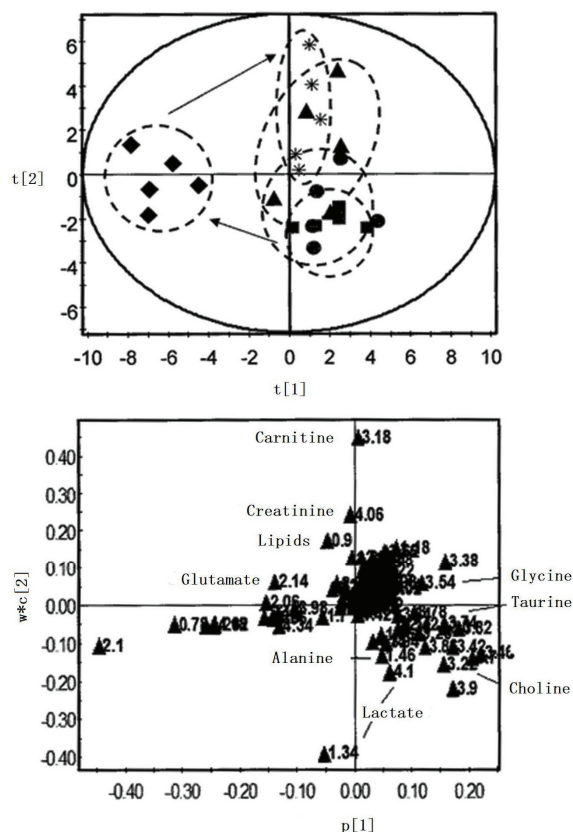
Plasma free fatty acids did not change significantly in mice subjected to acute hypoxia. When riboflavin supplementation was increased to 8 times, plasma free fatty acids were decreased significantly as compared to the normoxia control ( $P < 0.05$ ). Plasma  $\beta$ -hydroxybutyrate was increased significantly ( $P < 0.01$ ) after acute hypoxia exposure and responded inversely to riboflavin supplementation. Moreover, plasma carnitine was decreased significantly in mice after exposure to acute hypoxia ( $P < 0.05$ ). Riboflavin treatment increased plasma carnitine in a dose dependent manner. No significant difference was found in plasma carnitine level between the normoxia group and acute hypoxia plus

8 times riboflavin supplemented group (Table 2).

Plasma urea was significantly increased after acute hypoxia treatment ( $P < 0.01$ ). No significant change was observed after riboflavin supplementation (Table 2).

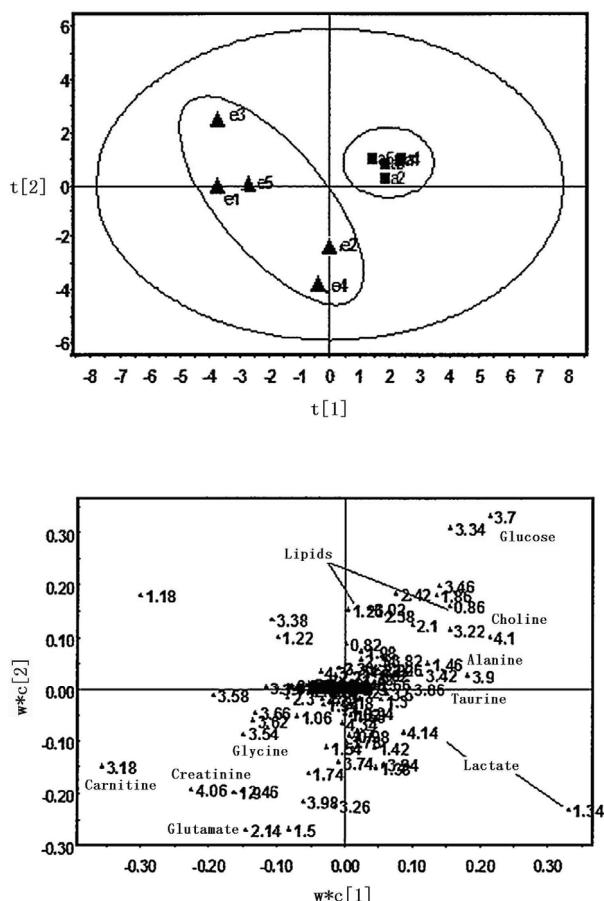
### Plasma $^1\text{H}$ NMR spectra

In the aliphatic regions (0.8–4.5 ppm, Fig. 1 top) of the  $^1\text{H}$  NMR spectra, it was found by visual inspection that signals of some endogenous metabolite changed in response to acute hypoxia exposure and riboflavin treatment. Compared to the normoxia group, there was an obvious decrease in carnitine level in the acute hypoxia group and a recovery was noted upon riboflavin supplementation. In the aromatic region (6.6–8.6 ppm, Fig. 1 bottom), visual changes was also observed for some amino acids, such as histidine, tyrosine and phenylalanine.

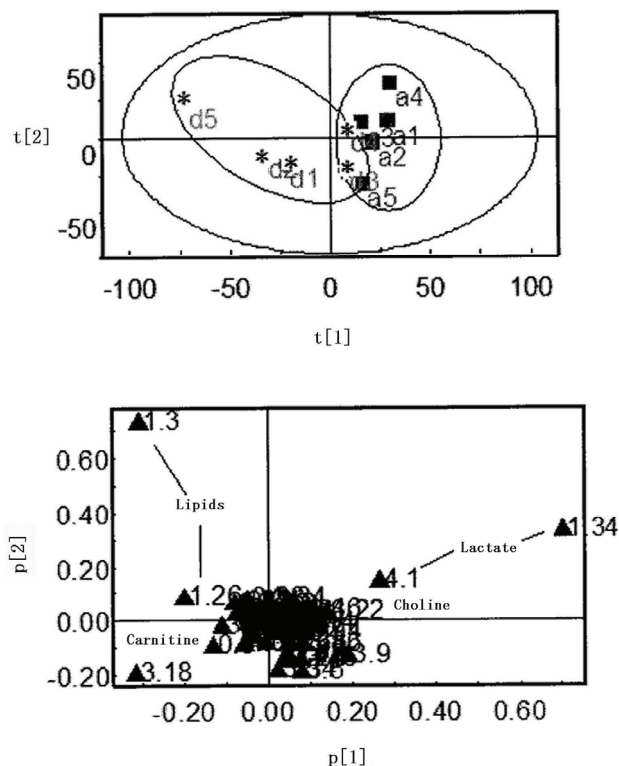


**Fig. 2.** Scores plot (**top**) and loadings plot (**bottom**) derived from  $^1\text{H}$  NMR spectra of plasma samples after orthogonal signal correction (OSC) and partial least squares-discriminant analysis (PLS-DA). In the scores plot, each point represents a single mouse plasma sample and  $t[1]$ ,  $t[2]$  indicate the scores on principal component 1 and 2, respectively. The metabolites responsible for the variance in the scores plot are indicated in the loadings plot by their distance from the origin and  $p[1]$ ,  $w*c[2]$  represent the weights on principal component 1 and 2, respectively.  $\blacktriangle$ : normoxia control;  $\blacksquare$ : hypoxia control;  $\bullet$ : hypoxia plus 2 times riboflavin-supplemented group;  $\blacklozenge$ : hypoxia plus 4 times riboflavin-supplemented group;  $*$ : hypoxia plus 4 times riboflavin-supplemented group

In order to detect more subtle metabolic differences, pattern recognition techniques were applied. The PCA scores plot of pc1 versus pc2 of all plasma samples from five groups showed that five clusters were presented, indicating that the metabolic trajectory of the acute hypoxia group moved away from the normoxia group. Meanwhile, the metabolic trajectory of the 4 times riboflavin supplemented group was clearly separated from the acute hypoxia group. When 8 times riboflavin was supplemented, the metabolic trajectory moved close to the normoxia group (Fig. 2 top). The corresponding loadings plot revealed that the separation between different groups was mainly attributed to the changes of following metabolites: lipids ( $\delta 0.9$ ), lactate ( $\delta 1.34$ ,  $4.1$ ), alanine ( $\delta 1.46$ ), glutamate ( $\delta 2.14$ ), choline ( $\delta 3.22$ ), taurine ( $\delta 3.42$ ), carnitine ( $\delta 3.18$ ), glycine ( $\delta 3.54$ ) and creatinine ( $\delta 4.06$ ) (Fig. 2 bottom).



**Fig. 3.** Scores plot (**top**) and loadings plot (**bottom**) derived from  $^1\text{H}$  NMR spectra of plasma samples from the normoxia (▲) and hypoxia (■) groups after partial least squares-discriminant analysis (PLS-DA). In the scores plot, each point represents a single mouse plasma sample and  $t[1]$ ,  $t[2]$  indicate the scores on principal component 1 and 2, respectively. The metabolites responsible for the variance in the scores plot are indicated in the loadings plot by their distance from the origin and  $w*c[1]$ ,  $w*c[2]$  represent the weights on principal component 1 and 2, respectively.



**Fig. 4.** Scores plot (**top**) and loadings plot (**bottom**) derived from  $^1\text{H}$  NMR spectra of plasma samples from the hypoxia (■) and hypoxia plus 8 times riboflavin supplemented (\*) groups after principal components analysis (PCA). In the scores plot, each point represents a single mouse plasma sample and  $t[1]$ ,  $t[2]$  indicate the scores on principal component 1 and 2, respectively. The metabolites responsible for the variance in the scores plot are indicated in the loadings plot by their distance from the origin and  $p[1]$ ,  $p[2]$  represent the weights on principal component 1 and 2, respectively.

Further analysis was carried out either between the normoxia group and acute hypoxia group or between the acute hypoxia group and 8 times riboflavin supplemented group. The PCA scores plot of pc1 versus pc2 showed two clusters of plasma samples related to the normoxia and acute hypoxia groups (Fig. 3 top). The corresponding loadings plot suggested that the separation of metabolic pattern of the two groups was due to the changes of following metabolites: lipids ( $\delta 0.9$ ), lactate ( $\delta 1.34$ ,  $4.1$ ), alanine ( $\delta 1.46$ ), glutamate ( $\delta 2.14$ ), pyruvate ( $\delta 2.38$ ), glutamine ( $\delta 2.42$ ), glucose ( $\delta 3.0$ - $4.0$ ), carnitine ( $\delta 3.18$ ), choline ( $\delta 3.22$ ), taurine ( $\delta 3.42$ ) and glycine ( $\delta 3.54$ ) (Fig. 3 bottom). The PCA scores plot of pc1 versus pc2 derived from the acute hypoxia and 8 times riboflavin supplemented groups indicated that two groups were separated (Fig. 4 top). The corresponding loadings plot suggested that the separation of metabolic pattern of the two groups was due to the changes of following metabolites: lipids ( $\delta 0.9$ ), lactate ( $\delta 1.34$ ,  $4.1$ ), carnitine ( $\delta 3.18$ ) and choline ( $\delta 3.22$ ) (Fig. 4 bottom).



## Discussion

In the present study, riboflavin status was assessed by the change of riboflavin content in plasma and liver, as well as EGRAC, a sensitive biochemical marker for riboflavin status (Hoey *et al.* 2009). It was found that plasma riboflavin content did not change significantly in response to acute hypoxia exposure, while the content of hepatic riboflavin was decreased significantly. It seems likely that plasma riboflavin level remains stable after acute hypoxia treatment partially *via* a compensatory process, in which more riboflavin is released into the bloodstream from the liver. However, EGRAC was elevated, suggesting that riboflavin status was compromised and more riboflavin was needed under acute hypoxia condition. This is in agreement with the results reported previously by Jiang *et al.* (1998), in which they demonstrated that riboflavin requirement was increased based on urinary riboflavin excretion in young volunteers exposed to the high altitude.

A large body of evidence indicates that hypoxia exposure leads to a disruption of energy metabolism, which is attributed to sympathetic activation at the early phase of exposure (Rafacho *et al.* 2013, Jun *et al.* 2012, Reinke *et al.* 2011). In the present study, it was noted that glucose metabolism switched from aerobic oxidation to anaerobic glycolysis as evidenced by increased production of pyruvate and lactate after acute hypoxia treatment. Increased plasma urea level suggested that protein catabolism was also enhanced. The elevated plasma level of  $\beta$ -hydroxybutyrate indicated that the ketogenesis was increased under acute hypoxia condition, which was resulted from both free fatty acid mobilization from white adipose tissue and decreased fatty acid oxidation (Jun *et al.* 2012). In addition, lower plasma carnitine level was detected after hypoxia exposure and this could be attributed to enhanced formation of acylcarnitines as demonstrated previously by Bruder and Raff (2010) in acute hypoxia treated young rats.

Riboflavin supplementation improved riboflavin status remarkably in hypoxic mice, especially in the hypoxia plus 8 times riboflavin supplemented group. Both plasma and hepatic contents of riboflavin were increased, whereas EGRAC was decreased significantly as compared to the hypoxia control. Meanwhile, plasma levels of pyruvate, free fatty acids and  $\beta$ -hydroxybutyrate were decreased significantly after riboflavin supplementation, suggesting that energy metabolism was partially improved. It is understandable because

riboflavin in the form of FAD is a cofactor required by two critical enzymes in energy metabolism, i.e. pyruvate dehydrogenase and acyl-CoA dehydrogenase, which catalyze the decarboxylation of pyruvate and the first step in the  $\beta$ -oxidation of fatty acids, respectively (Powers 2003, Lienhart *et al.* 2013). Moreover, plasma carnitine was increased in response to riboflavin supplementation in a dose dependent manner. It is possible under acute hypoxic condition that the activity of acyl-CoA dehydrogenase is increased in response to riboflavin supplementation because it is sensitive to riboflavin status as reported previously by others (Hoppel *et al.* 1979, Sakurai *et al.* 1982, Ross and Hoppel 1987). As a result, the  $\beta$ -oxidation of fatty acids under acute hypoxia is restored to some extent and more acyl CoA is switched into  $\beta$ -oxidation. Therefore, more carnitine can be liberated from acylcarnitines during this process and recycled into the cytosol. It should be pointed out that carnitine not only is required for the transport of long-chain fatty acids from the cytosol into the mitochondria, but also acts as an antioxidant and is effective in reducing hypoxia-induced oxidative stress and delaying muscle fatigue in rats as demonstrated by Dutta *et al.* (2008). Therefore, the protective action of carnitine against hypoxic damages, especially on mitochondria in hypoxic condition is worthy to be further investigated in detail.

Metabolomics is one of new “omics” disciplines and defined as “the quantitative measurement of metabolic responses to pathophysiological stimuli or genetic modification” (Nicholson *et al.* 1999). It has been proved to be efficient in probing endogenous metabolic perturbations induced by various endogenous and exogenous stimuli. Two major techniques, NMR and gas chromatography or liquid chromatography/mass spectrometry, are currently applied in metabolomic analysis. The multivariate data analysis methods, such as PCA and PLS-DA are frequently used to simplify metabolomic data processing in developing the metabolic profiles in response to different treatments (Smolinska *et al.* 2012, Wu *et al.* 2012, Lenz *et al.* 2003). In the present study, a  $^1\text{H}$  NMR based metabolomic technique was used to investigate the effects of acute hypoxia and riboflavin supplementation on plasma metabolic profiling in hypoxic mice. The results obtained are basically consistent with the data from biochemical assays and confirm that acute hypoxia induces a significant change in plasma metabolic profiles in mice and an improvement is demonstrated upon riboflavin supplementation. For example, plasma levels of pyruvate, lactate and lipids

were found to be increased, whereas carnitine decreased in response to acute hypoxia. Riboflavin intervention decreased plasma pyruvate level and increased carnitine level. It was also noted that plasma levels of some amino acids were changed after acute hypoxic treatment and riboflavin supplementation. It is explainable because the metabolism of some amino acids, such as alanine, glycine and glutamate, is closely related to the glucose homeostasis and detectable changes will be expected upon exposure to acute hypoxia and riboflavin supplementation (Sakami and Harrington 1963, Hue 1987). However, an obvious increase was also noted for plasma taurine after acute hypoxic exposure in this study. It is known that taurine acts as a cellular osmoregulator, as well as an antioxidant *in vivo* (Schaffer *et al.* 2000, Green *et al.* 1991). Previously, al-Bekairi (1989) also demonstrated that hypoxia caused a considerable increase in plasma and brain taurine in rats. It had been shown that hypoxia induced an osmotic stress and a large amount of taurine was released from the heart, which would contribute to the increase in plasma taurine (Schaffer *et al.* 2002). After riboflavin supplementation, increase in plasma taurine was reduced, implying that cellular osmotic stress was partially ameliorated. Furthermore, an increase of plasma choline was detected after acute hypoxia and a decrease noted after riboflavin supplementation. Jenden and Scremin also demonstrated that hypoxia induced an increase in arterial blood choline, which was resulted from parallel decreases in choline production and clearance. They postulated that increased blood choline level might be helpful in preventing the cerebral loss of choline under acute hypoxia condition (Jenden and Scremin 1995, Scremin and Jenden 1992).

In conclusion, this study demonstrates that riboflavin requirement is increased under acute hypoxia condition and riboflavin supplementation is effective partially in improving energy metabolism in hypoxic mice. The <sup>1</sup>H NMR based metabolomic analysis further provides supportive data. Meanwhile, more information related to the changes of other metabolites is also

obtained. Although a number of important biochemical markers related to energy metabolism in plasma were determined, we are aware that the current study is limited. The levels of some critical metabolites and activities of flavin-dependent enzymes, such as pyruvate dehydrogenase and acyl-CoA dehydrogenase, are not measured in different tissues, though supportive data are available in literature. Further studies are warranted to investigate the effects of riboflavin on the activities of critical enzymes and metabolites involved in energy metabolism in different tissues and elucidate underlying molecular mechanisms in acute hypoxia.

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

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### Abbreviations

ANOVA: analysis of variance  
 CPMG: Carr-Purcell-Meiboom-Gill  
 EGRAC: erythrocyte glutathione activation coefficient  
 FAD: flavin adenine dinucleotide  
 FIDs: free reduction decays  
 FMN: flavin mononucleotide  
 FW: fresh weight  
 LED: longitudinal eddy-current delay  
 MTHFR: methylenetetrahydrofolate reductase  
 NMR: nuclear magnetic resonance  
 OSC: orthogonal signal correction  
 PCA: principal components analysis  
 PLS-DA: partial least squares-discriminant analysis  
 TSP: 3-(trimethylsilyl)-propionic-(2,2,3, 3,-d4)-acid sodium

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