Metabolomic Study on Vitamins B_1, B_2, and PP Supplementation to Improve Serum Metabolic Profiles in Mice under Acute Hypoxia Based on ^{1}H NMR Analysis[1]

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Objective To explore metabolic changes after acute hypoxia and modulating effect of vitamins B_1, B_2, and PP supplementation in mice exposed to acute hypoxia. **Methods** Fifty male Kunming mice were randomly divided into 5 groups: normal, acute hypoxia, acute hypoxia with 2, 4 and 8 time- vitamins B_1, B_2, and PP supplementation . All mice were fed with corresponding diets for two weeks and then were exposed to a simulated altitude of 6 000 meters for 8 h, except for the normal group. Nuclear magnetic resonance analysis was used to identify the changes of serum metabolic profiles. **Results** There were significant changes in some serum metabolites under induced acute hypoxia, essentially relative increase in the concentrations of lactate, sugar and lipids and decrease in ethanol. The serum levels of choline, succinate, taurine, alanine, and glutamine also increased and phosphocholine decreased in the acute hypoxia group. After vitamins B_1, B_2, and PP supplementation, all these metabolic changes gradually recovered. **Conclusion** Significant changes in serum metabolic profile were observed by metabolomics in mice exposed to acute hypoxia, and vitamins B_1, B_2, and PP supplementation proved to be beneficial to improving some metabolic pathways. It is suggested that the dietary intakes of vitamins B_1, B_2, and PP should be increased under hypoxia condition.

Key words: Vitamins B_1, B_2, and PP; Acute hypoxia; ^{1}H NMR; Mice

INTRODUCTION

Environmental challenges including lower partial pressure of oxygen, the cold, the wind, and ultraviolet are present at high altitude which make significant impact on nutritional metabolism, and physiological functions of the human body. Individuals develop symptoms, such as headache, nausea, anorexia, fatigue, insomnia, and weight loss upon arrival at high altitude[1-2]. It is suggested that the requirements for B vitamins involved in energy production be increased in order to match the enhanced energy metabolism under hypoxia conditions[3]. Our previous studies have demonstrated that a nutritional preparation containing B vitamins could significantly prolong survival time in mice exposed to acute hypoxia and improve cardiac and pulmonary functions of young adults at high altitude[4-5]. However, the underlying mechanism remains to be further investigated.

Metabolomics has been widely adopted in plant physiology, pharmacology, toxicology, and other fields. In recent years, the number of reports on its application in the field of nutriology is increasing in regard to the value of metabolites in different biofluids in nutritional metabolomics, the issues of nonnutrient chemicals and large-bowel metabolites, and the linkage of metabolomics with the wider elements of nutrigenomics[6-8]. Nuclear magnetic resonance (NMR) spectroscopy-based metabolomic strategies have been developed for generating comprehensive biochemical profiles of low-molecular-weight metabolites in biofluids that are changed in response to internal and external stimuli. Multivariate statistical analysis including data reduction and pattern recognition (PR) techniques, such as principal components analysis (PCA), can simplify NMR data processing and provide valuable information on the variation in endogenous metabolic processes[9-10]. In the present study, the metabolic changes presented in the serum of mice exposed to acute hypoxia were analyzed by using ^{1}H NMR-based metabolomic approach and the effects of dietary supplementation with vitamins B_1, B_2, and PP were investigated so as to reveal the overall metabolic changes after hypoxia exposure in mice and the modulating role played by supplemented vitamins which may help understand their actions displayed at high altitude.

[1]This research was supported by a grant from the National Natural Science Foundation of China (NO. 30872097).

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0895-3988/2010
CN 11-2816/Q

MATERIALS AND METHODS

Animal Handling and Collection of Serum Samples

Fifty male Kunming mice, weighing 14-16 g (purchased from the Laboratory Animal Centre, Academy of Military Medical Sciences, Beijing), were housed in plastic animal cages. The light/dark cycles were alternated every 12 h. The ambient temperature was maintained at (25±2) ℃ and humidity was maintained between 30% to 40%. After being acclimatized to the basic diet (AIN-93 formula)[11] for 3 days, the mice were randomly assigned to 5 groups, i.e. normal control, acute hypoxia, acute hypoxia plus low, medium, and high doses of vitamin B_1, B_2, and PP supplementation groups. The supplementation groups were fed with times vitamins B_1, B_2, and PP supplemented basic diets, respectively. The content of vitamins B_1, B_2, and PP in basic diet was 5mg, 6mg, and 30mg per kilogram diet, respectively. All mice were fed the corresponding diets for two weeks. Finally, all mice except those in the normal group were exposed to a simulated altitude of 6 000 m for 8 h. The procedure was carried out in hypoxia chamber as follows: the mice were elevated to 5 000 m at the speed of 1 000 m/min, stopped for 3 min, and then to 6 000 m at the speed of 500 m/min. After 8 h exposure at 6 000 m, the mice returned to 5 000 m at the speed of 500m/min, stopped for 3 min and then to the sea level at the speed of 1 000 m/min[12]. The blood samples were collected from the orbital veins after ether anesthetization and centrifuged at 8 000×g for 10 min. The serum was taken and stored at -20 ℃ prior to analysis.

Acquisition of ^{1}H NMR Spectra

Serum samples of 180 μL were mixed with 100 μL of 1.0 mmol/L 3-(trimethylsilyl)-propionic- (2,2,3, 3,-d_4)-acid sodium (TSP) in 320 μL D_2O, shaken vigorously, and centrifuged at 13 000×g for 10 min. The aliquots of the resulting supernatant (550 μL) were placed in 5 mm NMR tubes. D_2O and TSP provided the deuterium lock signal for the NMR spectrometer and chemical shift reference (δ0.0) respectively.

High-resolution ^{1}H NMR spectra of all serum samples were obtained at 599.69 MHz on a Varian INOVA 600 NMR spectrometer. For each sample, the spectrum was acquired using 1-dimensional Carr-Purcell-Meiboom-Gill (CPMG) sequence with spectral width of 8 000Hz, 32k sampling points, acquisition time of 2s, cumulate frequency of 128, and a relaxation delay of 2s. During this course, the water peak was suppressed by a standard pulse sequence. Sixty-four free reduction decays (FIDs) were collected into 64k data points. An exponential line-broadening function of 0.5Hz was applied to the FID prior to Fourier transformation (FT).

Data Reduction and PR Analysis of ^{1}H NMR Spectra

All NMR spectra were phased and baseline corrected, and then the data were reduced to 400 integrated regions of equal width (0.01 ppm) corresponding to the region from δ4.4 to δ0.4 using the VNMR 6.1C software package (Varian, Inc., USA). The resulting table of spectral intensity information was exported into Excel, where each spectral intensity data set was normalized to unit area. The data were then exported into SIMCA-P (version 10, Umetrics AB, Umea, Sweden). Mean-centered and Pareto scaled data were used in all subsequent analysis, whereby the average value of each variable was calculated and subtracted from the data.

PCA, an unsupervised pattern recognition method, was performed to examine the dominant intrinsic variation in the data set. It was applied to the centered data to explore any clustering behavior of the samples based on intrinsic biochemical similarities. PCA facilitates the simultaneous comparison of a large number of complex objects and can be used to abstract inter-sample and inter-variable relationships within multivariate data statistically. In order to optimize the clustering of samples based purely on dietary variation rather than inter-subject differences, a spectral filter orthogonal signal correction (OSC) was applied to the data before reanalysis by PCA. OSC can generate improved models particularly where large amounts of variance not related to the property of interest are described in the data[13-14]. In this analysis, PCA, and OSC-PCA models were depicted as complementary scores plots, which displayed any intrinsic grouping of the samples based on spectral variation, and the loadings plots showed the contribution of each spectral variable to the score formation.

RESULTS

^{1}H NMR Spectroscopy of Serum

From visual inspection of the high field regions of ^{1}H NMR CPMG spectra (Fig. 1), it was found that the signals of some endogenous metabolites were changed as a consequence of acute hypoxia exposure and vitamin B_1, B_2, and PP supplementation. Compared to the normal group, there was obvious increase in the concentration of lactate (δ1.31-1.33, δ4.1-4.12) and decrease in ethanol (δ1.16-1.19, δ3.55-3.58) in the hypoxia group. After vitamin B_1, B_2 and PP supplementation, the peak intensity of lactate was decreased. In order to detect more subtle supplement-related metabolic differences, pattern recognition techniques were applied.

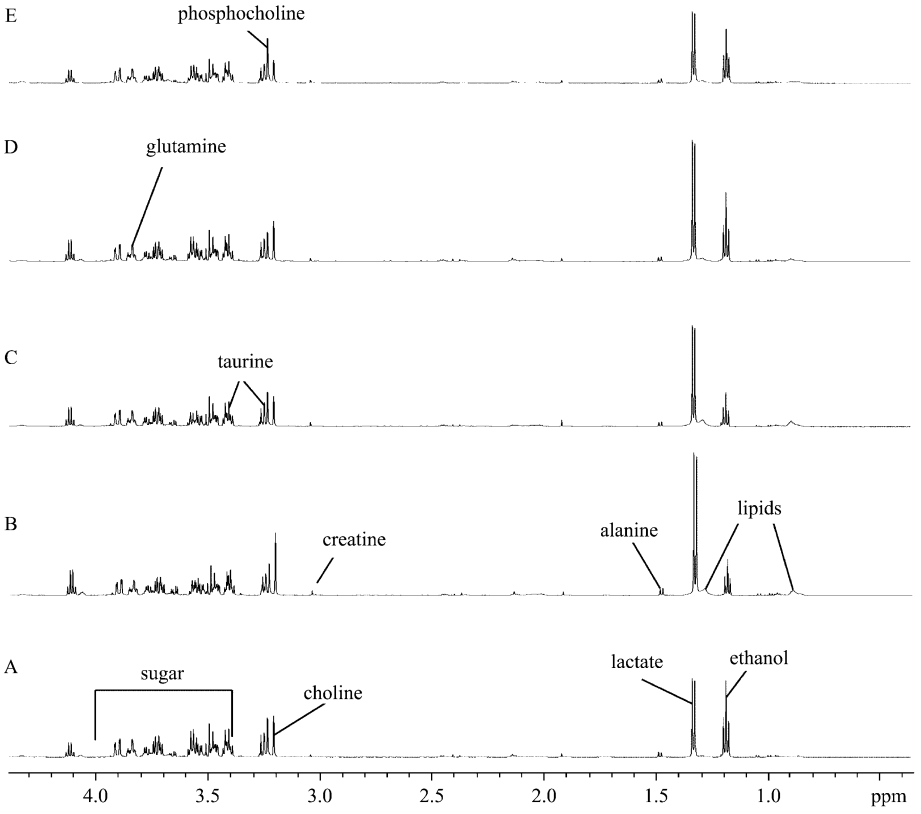

FIG. 1. Representative 600MHz ^{1}H NMR spectra of serum, control (A), acute hypoxia (B), acute hypoxia plus 2 time-vitamins B_1, B_2, and PP(C), acute hypoxia plus 4 time- vitamins B_1, B_2 and PP(D), acute hypoxia plus 8 time-vitamins B_1, B_2, and PP (E).

Chemometric Analysis of ^{1}H NMR Spectra

The OSC-PCA scores plot of pc1 versus pc2 of all serum samples from five groups showed that five clear clusters were observed, indicating that the metabolic trajectory of the hypoxia group moved away from the normal group, whereas in the vitamins B_1, B_2 and PP supplementation groups it returned to normal gradually (Fig. 2 top). The corresponding loadings plot revealed that the separation between different groups was mainly attributed to the changes of following endogenous metabolites: lipids (δ0.8-0.9, δ1.2-1.29), lactate (δ1.31-1.33, δ4.1-4.12), ethanol (δ1.16-1.19, δ3.55-3.58), *O*-acetyl-glycoprotein (δ2.13, 2.14), taurine (δ3.25, 3.42), sugar (δ3.4-4.0), and glutamine (δ3.76-3.78) (Fig. 2 bottom).

The PCA scores plot (Fig. 3 top) of pc1 versus pc2 showed a clustering of serum samples related to the normal and hypoxia groups. The corresponding loadings plot suggested that the separation of metabolic pattern of the two groups was due to the increased concentrations of lipids (δ0.8-0.9,1.2-1.29), lactate (δ1.31-1.33, 4.1-4.12), alanine (δ1.47, 1.48), succinate (δ2.4), choline (δ3.20), taurine (δ3.25, 3.42), sugar (δ3.4-4.0), glutamine (δ3.76-3.78), and decreased concentrations of ethanol (δ1.16-1.19, 3.55-3.58) and phosphocholine (δ3.22, 3.23) in the hypoxia group (Fig. 3 bottom).

The OSC-PCA scores plot of pc1 versus pc2 of the hypoxia group and 2 time- vitamin B_1, B_2, PP supplementation groups indicated that two groups were well separated (Fig. 4 top). The corresponding loadings plot showed that increased concentrations of lipids (δ0.8-0.9, 1.2-1.29), ethanol (δ1.16-1.19, 3.55-3.58), succinate (δ2.4), and glutamine (δ3.76-3.78) and decreased concentrations of lactate (δ1.31-1.33, 4.1-4.12), creatine (δ3.03), choline (δ3.20), and sugar (δ3.4-4.0) in the 2 time – vitamin supplementation group contributed most to the separated clustering of the two groups (Fig. 4 bottom).

The PCA scores plot of pc1 versus pc2 derived from the serum samples of the hypoxia and 4 time-vitamins B_1, B_2, and PP supplementation groups also revealed a clear separation (Fig. 5 top). From the corresponding loadings plot, the increase in the concentration of ethanol (δ1.16-1.19, 3.55-3.58) and

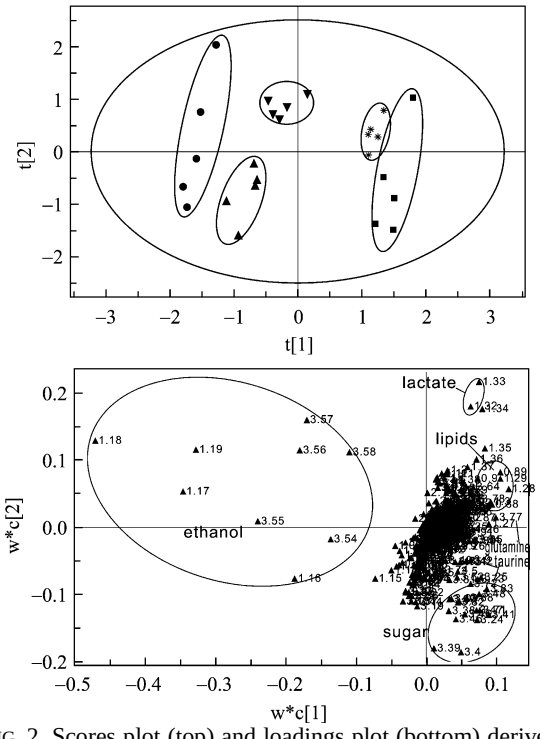

FIG. 2. Scores plot (top) and loadings plot (bottom) derived from ^{1}H NMR analysis of serum samples from control(●), acute hypoxia (■), acute hypoxia plus 2 time-vitamins B_1, B_2, and PP (*), acute hypoxia plus 4 time-vitamins B_1, B_2, and PP(▼), acute hypoxia plus 8 time-vitamins B_1, B_2, and PP (▲).

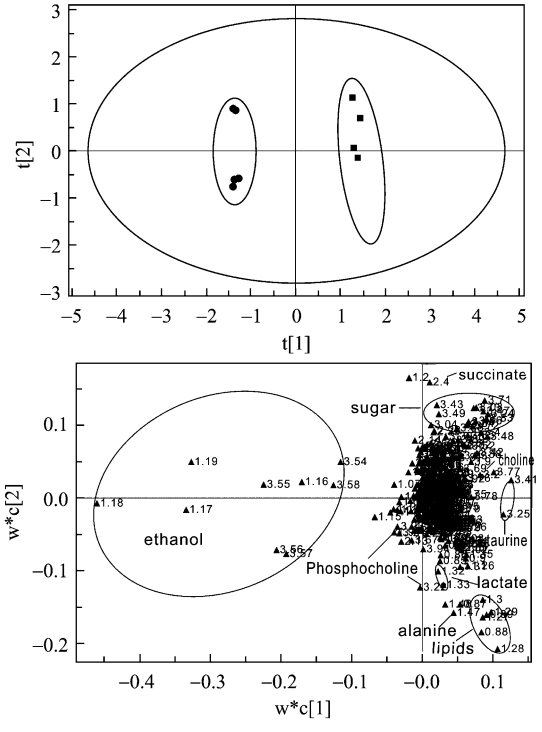

FIG. 3. Scores plot (top) and loadings plot (bottom) derived from ^{1}H NMR analysis of serum samples from acute hypoxia (■) and control(●).

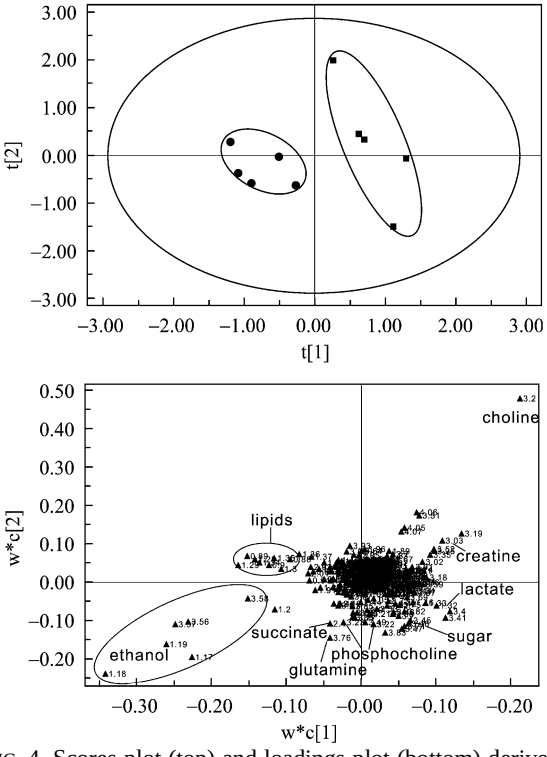

FIG. 4. Scores plot (top) and loadings plot (bottom) derived from ^{1}H NMR analysis of serum samples from acute hypoxia (■) and acute hypoxia plus 2 time-vitamins B_1, B_2, and PP (●).

decrease in the concentrations of lactate (δ1.31-1.33, 4.1-4.12), succinate (δ2.4), choline (δ3.20), phosphocholine (δ3.22, 3.23), and sugar (δ3.4-4.0) were observed in the 4 time- vitamin B_1, B_2, PP supplementation group (Fig. 5 bottom).

The PCA scores plot of pc1 versus pc2 obtained from the serum samples of the hypoxia and 8 time-vitamin B_1, B_2 and PP supplementation groups also displayed an evident separation (Fig. 6 top). The differences existing between the two groups were mainly generated from increased concentrations of ethanol (δ1.16-1.19, 3.55-3.58), and decreased concentrations of lactate (δ1.31-1.33, 4.1-4.12), lipids (δ0.8-0.9, 1.2-1.29), succinate (δ2.4), choline (δ3.20), glutamine (3.76-3.78), and sugar (3.4-4.0) in the 8 time- vitamin B_1, B_2, and PP supplementation group (Fig. 6 bottom).

DISCUSSION

In the present study, the results of ^{1}H NMR-based metabolomic analysis demonstrated that exposure to acute hypoxia led to significant changes of serum metabolic profiles in mice. The increased levels of serum sugar and lipids suggested an overall enhanced carbohydrate and lipid metabolism after acute hypoxia exposure. It was not surprising that aerobic oxidation of glucose was inhibited as

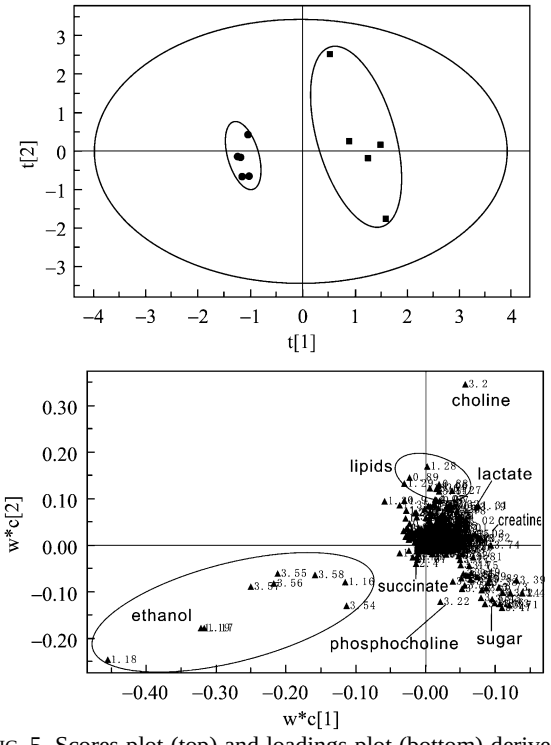

FIG. 5. Scores plot (top) and loadings plot (bottom) derived from ^{1}H NMR analysis of serum samples from acute hypoxia (■) and acute hypoxia plus 4 time-vitamins B_1, B_2, and PP(●).

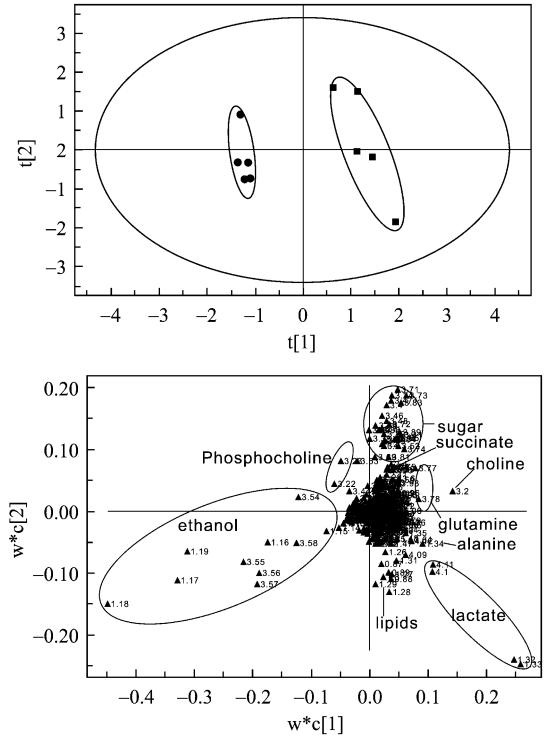

FIG. 6. Score plots (top) and loadings plot (bottom) derived from ^{1}H NMR analysis of serum samples from acute hypoxia (■) and acute hypoxia plus 8 time-vitamins B_1, B_2, and PP (●).

indicated by elevated lactate level, due to limited oxygen supply under hypoxia condition. The increase of succinate, one of key intermediates in citric acid cycle, also confirmed that the citric acid cycle was inhibited. The increased lipolysis illustrated that fat mobilization was enhanced for more energy production at high altitude[15]. Ethanol, normally negligible in blood, contributed considerably to the separating clustering in the five groups of this study. We speculated that the strong resonance of ethanol might come from ether used as anesthetic before blood collection, because ether can be transformed to ethanol *in vivo*[16]. However, acute hypoxia may hamper this transforming process.

It was observed that serum choline increased and phosphocholine decreased in mice after acute hypoxia exposure. Choline is synthesized from serine and methionine and serves as a precursor for acetylcholine and membrane phosphatidylcholine. Choline also plays an important role in structural integrity and signaling process for cell membranes and is a major source for methyl groups[17]. The increased serum choline level is possibly a compensatory response of the body in preventing the loss of choline in brain after exposure to acute hypoxia[18]. Weiss *et al*. found that in most animals and cell strains, exogenous cytidine diphosphate-choline (CDP-choline) could reduce the hypoxia induced injury[16]. Klein *et al*. reported that decreased choline concentration in brain could be effectively prevented via the blood choline homeostatic mechanisms[20]. However choline can also be generated from degradation of phosphorylcholine under catalysis by phosphatidases. In the hypoxia environment, the activity of phosphatidase A2 was enhanced in the rat blood, which contributed partly to the increased serum level of choline[21]. Phosphocholine is a precursor for phosphorylcholine, which is an essential component in membrane structure. In the early stage of acute hypoxia exposure, both the number of cellular mitochondria and the surface area of mitochondria membranes were increased for adaptation[22]. Therefore, the decreased serum level of phosphocholine might be associated with the augmented utilization of phosphorylcholine under hypoxia condition.

Taurine is an end product of the metabolic pathway of sulfur-containing amino acids[23]. The elevated serum level of taurine in this study indicated that the metabolism of sulfur containing amino acids was accelerated under acute hypoxia. The biological significances and the mechanism involved are needed to be explored further.

After supplementation of vitamins B_1, B_2, and PP, the metabolic profile gradually returned to that of the

normal group, possibly via their biochemical functions *in vivo*. It is not surprising that the biochemical reactions using thiamine pyrophosphate (TPP)[24] as cofactor will be improved after vitamin B_1 supplementation. For example, the production of lactate under hypoxia condition could be reduced by increased oxidative decarboxylation of pyruvate after vitamin B_1 supplementation in this study. Fumagalli *et al*. demonstrated that after intravenous injection of 100mg TPP to normal subjects exposed to hypoxia, the respiratory function and the oxygen utilization were greatly improved[25]. Supplementation of vitamin B_2 may also improve the aerobic metabolism and the function of electron transport chains in mitochondria by enhancing the activity of these oxidative enzymes including FMN or FAD as coenzyme under hypoxia conditions, and then resulted in decreased lactate production as observed in this study. The decrease of succinate also indicated that the citric acid cycle was improved after vitamin B_2 supplementation through FAD, the coenzyme for succinate dehydrogenase. Vitamin PP can be transformed to NAD^+, NADH, $NADP^+$, NADPH, and acts as coenzymes for many dehydrogenases involved in redox reactions *in vivo*[26-27]. It was found that the lifetime of free and protein-bound NADH decreased with hypoxia in cell culture and tissue slices[28]. Kinnula *et al.* also reported that the marked reduction of cytostolic free NAD in the liver and free NAD pool in the mitochondria occurred under acute hypoxia[29]. Supplementation of vitamin PP, therefore, can meet the increased needs under hypoxia condition. From the data discussed above, it is confirmed that the recommended intake of vitamins B_1, B_2 and PP should be increased at high altitude.

We also found remarkable changes in serum levels of choline, phosphocholine, and glutamine after supplementation of vitamins B_1, B_2, and PP. However, it is rather difficult to provide detailed explanation based on the results available in this study. Further studies are clearly needed to probe into the metabolic changes including key enzymes activities in related biochemical pathways.

In conclusion, the significant metabolic changes were demonstrated after acute hypoxia exposure in mice and could be improved remarkably by supplementation of vitamins B_1, B_2, and PP. To our knowledge, this is the first report on application of ^{1}H NMR-based metabolomic approach in confirming the beneficial effects of vitamin B_1, B_2, and PP supplementation after hypoxia exposure. It is indicated that the actions displayed by vitamins B_1, B_2, and PP may be achieved through various biochemical pathways of energy metabolism. The data presented in this study also provide new evidences that dietary supplementation with vitamins B_1, B_2 and PP should be increased under acute hypoxia condition. Future studies should be directed to the changes of enzymatic activities and their gene expression under acute hypoxia and to the elucidation of the mechanism of vitamins B_1, B_2, and PP in relieving hypoxia damages.

ACKNOWLEDGEMENT

The assistance provided by Professor Xian-Zhong YAN and Dr Bo SUN from the National Center of Biomedical Analysis, Beijing in ^{1}H NMR data analysis is highly appreciated.

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(*Received November* 20, 2009 *Accepted June* 9, 2010)