



ALANINE AMINO TRANSFERASE (ALT) SPECIFIC ACTIVITIES IN LONG TERM SYSTEMIC HYPOXIC RAT BRAIN TISSUES

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ABSTRACT

Background: Brain as a very aerobic organ is sensitive to hypoxia. Energy scarcities must be overcome by gluconeogenesis, which uses alanine or lactate as starting material. The reaction is catalyzed by alanine amino transaminase (ALAT or ALT), also known as glutamate pyruvate transaminase (GPT).

Objective: To investigate whether the specific activities of alanine aminotransferase (ALT) increased in hypoxic rat brain.

Methods: This experimental study used rats exposed to systemic normobaric hypoxia during 14 days. A group of 5 rats was sacrificed in days 1, 3, 7 and 14. The specific activities of ALT were analyzed in their brains using a reaction coupled with lactate dehydrogenase (LDH) activities.

Results: The ALT specific activities in rat brain were very low. There was no significant increase of specific activities during long term hypoxia ($p > 0.05$).

Conclusion: The rat brain ALT has no role in gluconeogenesis.

Keywords : ALT, Brain, Gluconeogenesis, Hypoxia

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INTRODUCTION

Oxidation is a very important reaction for life being, in order to extract the energy and produce useful metabolites. As an electron transfer reaction, oxidation can use various compound as an electron acceptor. However, oxygen (O₂) is used when cells need a big amount of energies, which occurs in aerobic metabolism. In this type of oxidation, energy is maximally released in oxidizing high energy nutrient substrates such as glucose, fatty acids or amino acids. For this objective, O₂ transport has to be assured can attain cells, which is not always the case. Otherwise, the cell will undergo a lack of O₂ condition or hypoxia. Hypoxia is a condition, which is characterized by insufficiency of O₂ relative to the need of organism, organ or even only at tissue level.[1]

Among the organs, brain is a very aerobic one and therefore very sensitive to hypoxia.[2] Moreover, brain is practically very dependent on glucose as energy source. Hence the glucose supply has to be assured all the time.[3] One of the common amino acid which is frequently used as a started material of gluconeogenesis is alanine. By one step transamination reaction, this amino acid is immediately converted to pyruvic acid, which is ready to be integrated into glucose. The reaction is catalyzed by alanine amino transaminase (ALAT or ALT), also known as glutamate pyruvate transaminase (GPT).

We are interested to know, whether total body hypoxia for a relatively long period would affect the brain metabolism, especially the conversion of amino acid into glucose precursor, which can be used in gluconeogenesis. For realizing our aims, we use a number of male Wistar rats, placed in a normobaric hypoxic chamber for two weeks, while water and raisin are

given ad libitum. A number of rats were sacrificed periodically and specific activities of brain alanine aminotransferase (ALT) were measured accordingly.

MATERIAL AND METHODS

This was an experimental study carried out by placing rats in a normobaric hypoxia condition for 2 weeks. Twenty rats were placed in a closed hypoxic chamber, aerated with a gas mixture containing 90% N₂ and 10% O₂ and pressure 1 atmosphere directly from a gas tube. As control group, four rats were placed in usual laboratory condition. Both groups, control as well as experimental, feed with a standard diet and had access to water freely. The experimental group animals were sacrificed in 1, 3, 7 and 14 days of hypoxia treatment by decapitation. The brains were taken immediately, placed in chilled PBS pH 7.35 containing PMSF and frozen in a deep freezer (-80°C) until the time of analysis. The protocol was reviewed and agreed by an Ethical Committee (376/PT02.FK/ETIK/2009).

Animals

Twenty-four male, young adult Sprague – Dawley rats, aged 8 – 12 weeks, were obtained from Center of Veterinary Research (BALITVET), Bogor, West Java, Indonesia. All animals were adapted in our laboratory condition for 2 weeks.

Chemicals

PBS (phosphate buffered saline) 0.1 M pH 7.4 was prepared according to the usual method. The needed chemicals were the crystals of NaH₂PO₄, Na₂HPO₄, and NaCl (Merck). Antiprotease phenylmethane sulfonyl fluoride (PMSF) was purchased from Sigma. ALT kit for alanine amino transaminase determination

was from Randox and contained buffer solution, substrate, coupling enzyme, and coenzymes. Bovine serum albumin (BSA, Sigma) was used for total protein assay.

Brain tissue extract preparation

Tissues were frozen and thawed 3 times by placing the specimen in deep freezer (-80°C) and then in a water bath 37°C. At the end of the third cycle, the tissues were homogenized by an automatic tissue grinder using a pestle. Then, homogenates were centrifuged at 5000 rpm for 10 minutes, to obtain supernatant liquid for further analysis.

Total protein measurement

The brain supernatant subjected to total protein analysis, which was measured by Warburg methods.[4] In principle, the absorbance of samples and protein standard solutions is measured at 280 nm.[5] A solution of BSA in PBS with concentration 1 mg/mL was used as a stock standard solution. A number of dilutions in PBS of the stock standard solution, ranges from 25 mg, 50 mg, 100 mg, 200 mg, 300 mg, 400 mg and 500 mg/mL respectively were used to construct a standard curve.

ALT assay

The enzyme activities assay was performed according to the guidance in the kit manual, which is based on lactate dehydrogenase (LDH) coupled method as described by Whitaker.[6] Briefly, ALT catalyzes the conversion of L-alanine to pyruvate in the presence of α -oxoglutarate. The pyruvate is reduced into lactate by NADH in the presence of LDH. The reagent mixture containing the powder of α -oxoglutarate, NADH, and L-alanine was dissolved in 100 mL buffer at the time of the assay. Then 100 mL of supernatant was pipetted into a cuvette placed in a spectrophotometer, followed by 1 mL of

the reagent mixture. The difference of optical densities between first minute and fourth minutes were read at 365 nm. As a blank, 100 mL of ddH₂O was used in the place of 100 mL of supernatant. As in protein determination, all measurements were done in Duplo. The ALT activities were calculated using the following formula:

$$U/L = 3235 \times DA_{365}/\text{minute}$$

Specific activities of ALT, i.e total activities of the enzyme/mg protein were calculated by dividing values of unit/mL with mg of total protein in the same samples.

Data treatment

All of the experimental data were analyzed statistically using IBM SPSS Statistics 20.0 for Windows. As usual, normality and homogeneity data will be examined. If it is normal and homogeneous, the analysis will be continued by Anova. If the result is significant, the analysis will be continued with a post hoc test. Otherwise, if the data is not normally distributed and/or not homogeneous, the data will be converted to logarithmic form and the normality and homogeneity will be examined. If both are still not normally distributed and not homogeneous, then the analysis will be performed non-parametrically.

RESULTS

Brain protein content

Total brain protein, expressed as mg of protein/brain weight, are presented in table 1. At a glance, it seems that there is a tendency of increase of brain protein contents, with the highest value in day 3. However, Anova statistical analysis shows

that the difference is not significant ($p>0,05$).

Table 1. Means of rat brain protein during 14 days of normobaric hypoxia the experiment (mg protein/g brain weight)

Group	Mean $\pm$ SD	P value*
Control normoxia group	5.407 $\pm$ 1.406	P > 0.05
D1 normobaric hypoxia group	6.783 $\pm$ 1.676	
D3 normobaric hypoxia group	6.972 $\pm$ 1.728	
D7 normobaric hypoxia group	6.530 $\pm$ 0.745	
D14 normobaric hypoxia group	6.550 $\pm$ 1.837	

*Anova Test

ALT specific activities

Specific activities of brain ALT are expressed as U/mg brain protein. Statistical analysis indicated that the distribution of data is not normal nor the data homogenous. Conversion of all data to log form give the same results. It was decided to analyze the data in the non-parametric way. Consequently, the data cannot be represented by mean and should be expressed by median. Accordingly, the standard deviation should be changed with minimum-maximum range. The results are presented in Table 2.

The specific activities of rat brain ALT were very low. There is an impression that ALT specific activities in brain increase when the duration of exposure prolonged. However, nonparametric statistical analysis indicated that the differences are not significant ($p>0.05$).

Tabel 2. Brain ALT specific activities (U/mg protein)

Group	Median (Min-Max)	P value*
Control normoxia group	0.0108 (0.0081-0.0129)	P > 0.05
D1 normobaric hypoxia group	0.0108 (0.0081-0.0722)	
D3 normobaric hypoxia group	0.0119 (0.0065-0.0739)	
D7 normobaric hypoxia group	0.0156 (0.0065-0.0388)	
D14 normobaric hypoxia group	0.0178 (0.0075-0.0431)	

*Kruskall Wallis Test

DISCUSSION

Metabolically, the brain is the most active organ in the body. It needs a large amount of energy to maintain its functions and its integrity.[7] Hence, brain is a very aerobic organ, which consumed about 20% of total inspired oxygen. Brain depends almost absolutely on glucose as fuel.[3] Glucose is completely oxidized into CO₂ and H₂O, and releases a large amount of energy are fixed as ATP. Hypoxia, therefore, has a severe consequence for the brain, which, if it can not be overcome in a short time, will be fatal for this organ. There are several ways for organism to overcome hypoxia conditions. The acute phase of hypoxia (first seconds and minute) is usually overcome by physiological mechanisms. The hypoxia itself has a direct action on blood vessels, it causes vasodilation on cerebral and heart muscle blood vessels, which increases tissue perfusion. Hypoxia regulates also blood vessels via chemoreceptors in carotid and aortic bodies, which exerts its influence through neural system.[8] After the acute period, organism survival in hypoxia relies

on gene expression dependent mechanism, which is mostly undertaken by Hypoxia-Inducible Factors (HIFs), a group of three different transcription factors regulating a number of proteins needed in order to face the chronic hypoxia.[9] In general, all proteins which are regulated by HIFs are essential for surviving in a conditioned lack of oxygen. The proteins which are regulated by HIF can be grouped into proteins control red blood cell production and vascular system, proteins control the energy metabolism, the protein control cell development and the proteins control homeostasis and extracellular matrix integrities.[10]

Our study is a part of a larger research on normobaric hypoxia, in which internal organs of the experimental rats, under chronic normobaric hypoxia, were investigated. It was reported that during the experiment, the animals underwent hypoxia.[11] Under this condition, the HIF-1 increased practically in every organ such as liver [12], kidney [13], stomach [14] and heart [15]. Hence, undoubtedly the brain of hypoxic rats undergoes hypoxia too. In the lack of oxygen, cell cannot oxidize glucose completely into water and carbon dioxide and will release lesser energy. To meet the need for energy on the same level, cell should take up higher number of glucose, which can lead to hypoglycemia. To maintain the glucose at the minimal tolerable level, organism will synthesize glucose from non-carbohydrate compounds, known as gluconeogenesis. For this objective, organism uses protein, after degraded into amino acids, as raw material. One of the common amino acid which is frequently used as a started material of gluconeogenesis is alanine. By one step transamination reaction, this amino acid is immediately converted to pyruvic acid, which is ready to be integrated into

glucose. The reaction is catalyzed by alanine amino transaminase (ALAT or ALT), also known as glutamate pyruvate transaminase (GPT).

ALT is distributed in various organs, and depending on the animal, higher specific activities are found in heart, liver, and kidney for sea lion, harbor seal and elephant seal.[16] The authors did not report ALT in brain. It is not clear, whether the activities in brain are nil or simply the authors did not measure. However, ALT was found in very low concentrations in rat brain and kidney.[17] In their report, the number of ALT is not expressed in usual way as activities, but in the weight unit, which is reported as 50 ng enzymes/mg protein lysate. In our investigation, we found the activities of ALT in brain tissue homogenate. We also found that the activities were very low. As seen in Table 2, it seems that the brain ALT specific activities tended to increase during the experiment. However, nonparametric statistical analysis indicates that the increase is not significant ($p>0.05$).

Almost all glucogenic amino acids can be converted to glucose, except leucine and lysine. Alanine and glutamine have the most important role, both are mobilized immediately from blood soluble protein (albumin) and from skeletal muscle.[18] Among both amino acids, alanine is much more important, because this is the eminent amino acid transported from muscle to the liver during physical activities and also in calories deficiency.[19] The glucose alanine cycle, which is also known as the Cahill cycle, is also important to assure glucose homeostasis in health as well as in disease.[20] For catalyzing this reaction, the role of alanine aminotransferase is very crucial, because it converts directly the alanine into pyruvic acid, which can

directly be integrated into gluconeogenesis pathway to produce glucose. Liver, kidney and small intestine are known as organs capable to carry out gluconeogenesis. However, liver is always the main place for gluconeogenesis, as it can synthesize the glucose from alanine (Cahill cycle) or lactate (Cori cycle). Recently, it was also reported that astrocyte in brain is able to perform gluconeogenesis, a process that is crucial for the survival neuron cell in the hypoxia condition. However, astrocyte gluconeogenesis uses lactate instead of alanine.[21] From this point of view, it is considered that specific activities of ALT in brain are very low because brain can synthesize glucose from lactate, therefore the increase of its activity during the hypoxia period was not significant compared to normal oxygen living rats.

CONCLUSION

In conclusion, systemic hypoxia for a relatively long period affects various organs, which try to overcome the scarcity of energy by gluconeogenesis, itself can use alanine and/or lactate as starting substrates. If the alanine acts as starting compound, the involved cell needs ALT to convert the alanine to pyruvate. As the brain has very low ALT specific activities, it can be said that hypoxic brain depended on liver gluconeogenesis for the glucose supply and on the local gluconeogenesis which starts from lactate.

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