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Effects of Ammonia on Pentylenetetrazole-Induced Seizure Threshold

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The effect of chronic perfusion of ammonia on the seizure threshold against pentylenetetrazol was studied. Ammonia plus sodium bicarbonate and saline (0.9%) was continuously administered to two groups of rats respectively. All animals were tested three times for seizure threshold, and were then decapitated and the brains removed for analysis of the amino acids. The results showed that the infusion of ammonia increased the seizure threshold, and this protective effect was accompanied by selective changes in brain glutamate and glutamine. Thus, continuous infusion of ammonia may cause an imbalance between excitatory and inhibitory systems in favor of inhibitory systems. These findings may provide insights into the basic mechanisms of seizures observed in hepatic failure, in other hyperammonemic states, and in epilepsy.

Keywords: Epilepsy; Ammonia; Amino Acids; Glutamate; Pentylenetetrazole; Models: Hepatic Encephalopathy.

INTRODUCTION

There are a number of controversial hypotheses proposed to explain hepatic encephalopathy and its related neuro-psychiatric manifestations. One of those hypotheses is based on hyperammonemia (Plum and Cooper, 1987). As indicated in some reports, the condition may even be misdiagnosed as either schizophrenic or bipolar affective disorder (Wise, 1987). Psychiatric manifestations may occur in hepatic encephalopathy, liver failure, Reye's syndrome, and severe congestive heart failure and include convulsions as well as alterations of mood and personality, cognitive impairment, ataxia, and coma (Butterworth, 1998).

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Glutamine synthetase (GS) activity plays a pivotal role in ammonia removal by brain, and its *in vivo* GS activity is kinetically limited by sub-optimal concentrations of ammonia (Stryer, 1988).

Therefore, it is possible that a particular dose and rate of ammonia perfusion may result in reduced concentration or functional availability in brain glutamate, causing a gradual decrease in neuronal excitability and in this way could result in increased seizure threshold.

Pentylenetetrazol (PTZ) has long been used to measure the seizure threshold. It is a relatively well-studied molecule in terms of its seizure provoking mechanisms. In general, it is believed that PTZ is a convulsive drug acting on gamma-aminobutyric acid-A (GABA-A) gated chloride receptors, and a kindling agent. However, one study concluded that the overall effect of PTZ should be an increase in glutamate-GABA ratio, which may contribute to the triggering of convulsions (LaCoste, *et al.*, 1988).

In this study, we have tried to reveal, for the first time, that hyperammonemia has the capacity to limit seizure threshold, i.e.: hyperammonemia is protective, and to discuss if the method used for this study could provide insights into the basic mechanisms of seizures observed in hepatic failure, in other hyperammonemic states, and in epilepsy.

MATERIALS and METHODS

Ethics approval was obtained from the Medical Faculty of the University of Marmara, Istanbul where the experiments were conducted. 2mmol/kg/h of NH_4Cl (Carlo Erba) plus 2mmol/kg/hr of sodium bicarbonate (Sigma), in order to protect the effect of pH changes on the convulsive states, was perfused continuously to one group of 9 Wistar albino rats (male, 368.2 ± 33 gr mean weight), and saline (0.9%) to the control group of 9 rats with the same age and sex (368.7 ± 44 gr mean weight) characteristics. This particular rate of ammonia was chosen on the basis of studies on the GS activity conducted by Kanamori *et al* 1993. Perfusion was carried out using a pump connected to the injectors for each animal. Administration was done continuously via the catheters implanted into the jugular veins of the rats. Animals were anesthetized with ketamine (100 mg/kg) (Sigma) plus chlorpromazine (12.5 mg/kg) (Eczacibasi / Rh ne-Poulenc) during the jugular vein catheterization. The catheters did not hinder the movements and the food intake of the rats. Seizure thresholds of both groups were measured following administration of pentylenetetrazole (PTZ) (Sigma).

Baseline epileptic thresholds were measured one day after the operation. In subsequent days, measurements were repeated at the same hour each day. Three measurements were obtained from each animal. PTZ was infused by a pump (6 ml/min) via the intrajugular catheter. This infusion method was preferred to minimize problems of systemic uptake and metabolism of the drugs. Perfusion of PTZ was stopped immediately after observing a tonic clonic convulsion, and the perfused amount up to that point was recorded in units of mg/kg. One hour after the last measurement for seizure thresholds, all of the animals were decapitated and their brains were removed for measurement of GABA, glutamate, glutamine, glycine and aspartate, as follows:

The rat brains obtained were quickly removed and the cerebellum and brainstem were separated according to the methods of Hebel and Stromberg (1976), and weighed (control group: 1.7 ± 0.08 g; ammonia perfused group: 1.7 ± 0.16 g). The brains were kept in a deep freeze (-20^{0}C) until time of analysis.

Amino acid extracts from the tissue samples were done at room temperature. Tissue samples were treated in ultrasonifier (Vir Sonic 300 Virtis) with a mixture of 4 ml ethanol (Merck) and 0.1 HCl (Merck) [1:1] for 30 seconds, and then pellets and supernatants were separated following a 20 min of centrifugation (4^{0}C, 8000 rpm). Analytic columns [Shodex (4.6-250 mm) C_{18}-5B stainless steel (A01281)] and front columns [Waters μ Bondapak C_{18} Guard Pak Insert] were used for High Pressure Liquid Chromatography (HPLC). Mixture ratios of Solvent-A [20mM potassium acetate (Sigma) (pH 5.5)] and Solvent-B [Methanol (HPLC grade, Carlo Erba)] in the mobile phase of HPLC with the constant flow rate of 1.2 ml/min at times 0, 20, 25, 28 and 40 min were 80-20%, 30-70%, 30-70%, 80-20% and 80-20% respectively. 10nM stock solutions of D-aspartic acid (Sigma), L-glutamic acid (Sigma), L-glutamine (Sigma), glycine (Merck) and GABA (Sigma) in 0.1 N HCl were prepared. Then serial solutions diluted at 0.05, 0.1, 0.2 and 0.4 nM respectively from these stock solutions were prepared. 2mg o-phthalaldehyde (OPA) (Sigma) was dissolved in a solution of 100 μl ethanol, 10μl β-mercaptoethanol (β-MPE) (Merck), and 890μl 0.1M sodium tetraborate (Merck) [pH9.1].

80 μl 0.1N HCl, 80μl sodium tetraborate and 20μl OPA - β-MPE solutions were added to 20 μl of diluted standard amino acid solution and the mixture was mixed in a Vortex for 60 sec and then centrifuged (60 sec, 10,000 rpm). Using a Hamilton microinjector, 20μl of this mixture was taken for chromatography. In the same way, 20μl of brain homogenates were obtained, derivatization was done and chromatography was applied to these 20μl volumes.

Peaks and retention times from the chromatograms of the brain extracts were compared to standard amino acids, and the values of the same corresponding peaks were measured using Baseline 810 software (Waters). Amino acid concentrations expressed in mol/g wet tissue were calculated.

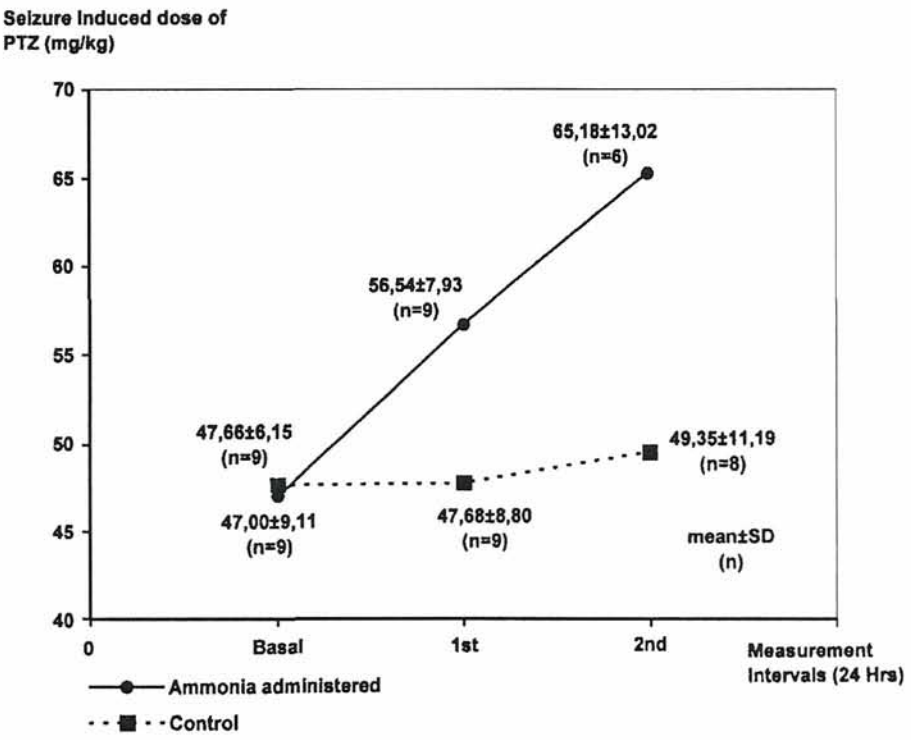

Figure 1. Effect of ammonia perfusion on PTZ-induced seizure threshold on the rats. Depicts the trends of seizure thresholds of both groups of rats (ammonia administered group is shown in solid line, and the control group is shown in dotted line) as a function of time. Statistically significant difference is found between the slopes of the two groups ($F=6,53$; $p=0,016$).

RESULTS

Due to a tortion and occlusion of the catheters, one animal from the control group and three from the ammonia perfused group were excluded.

Results were analyzed by repeated measurements of ANOVA with the within-subject factor seizure threshold in mg/kg PTZ (three levels: baseline, first, and second measurement), and a between subjects factor (control vs. ammonia perfused group).

Table 1: Effects of ammonia treatment on brain amino acid concentrations in PTZ-tested animals.

AMINO ACIDS	GROUP	N	Amino Acid Concentrations (μmol/g wt) (mean–SD)
GABA	control	8	1.99±0.43
	ammonia perfused	6	1.89±0.40
Aspartate	control	8	1.75±0.33
	ammonia perfused	6	1.78±0.28
Glutamate	control	8	3.90±1.04
	ammonia perfused	6	4.23±1.00
Glycine	control	8	1.32±0.10
	ammonia perfused	6	1.27±0.23
Glutamine	control	8	2.74±2.31
	ammonia perfused	6	5.49±1.68 *

* p = 0,03 compared to control.

Greenhouse-Geisser Epsilon were used to adjust degrees of freedom for the averaged results. In comparison with the control animals, the ammonia perfused animals showed statistically significant increases in their seizure thresholds against pentylenetetrazole ($F=6.53$, $p=0.016$), which were further dependent on the time of PTZ challenge (Figure 1). Multivariate regression analysis was conducted in order to determine which amino acid(s) was (were) the main predictor(s) of the increase of the seizure threshold. The amino acid concentrations in the brains of both groups in terms of mol/g wet tissue (wt) and the group statistics belonging to the biochemical analysis results are shown in Table 1. Multivariate regression analysis with the stepwise selection method (probability of F to entry criterion was 0.05; probability of F to remove criterion was 0.1) showed that before decapitation, the main predictors of the level of epileptic threshold (Y) were the decrease of glutamate (X_1) (β_1= -9.38, $p=0.0001$) and the increase of glutamine (X_2) (β_2=6.43, $p=0.0000$). The statistical model summary was as follows: $F=42.12$, $p=0.000$, $R=0.92$, $R^2=0.85$, Adjusted $R^2=0.83$. The dependent variable included in the model was the last measurement of seizure threshold before decapitation. Partial regression plots and linear graphics of two independent variables (glutamate and glutamine) against the dependent variable of seizure threshold are shown in Figure 2.

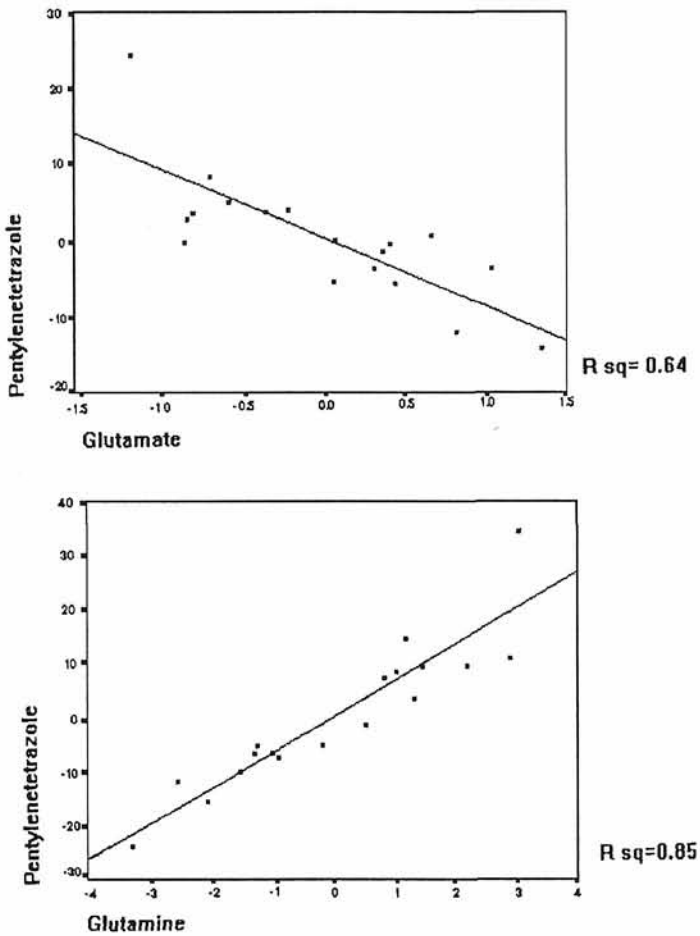

Figure 2. Partial regression plots dependent variable: Measurement values of the last epileptic threshold. Depicts the plots of the residuals of Y (the last seizure threshold measured in units of mg/kg pentylenetetrazole) and X (glutamate and glutamine in units of mol/g wet tissue).

The factor analysis with extraction method of principle component analysis and varimax rotation with Kaiser normalization was run to see if it was possible to group the amino acids which fit into the theoretical grouping of excitatory and inhibitory classification. The analysis successfully grouped the amino acids as excitatory (glutamate and aspartate: factor loadings were 0.78 and 0.95 respectively) and inhibitory (GABA and glycine: factor loadings were 0.95 and 0.85 respectively). Two factors explained the 89% of the total variance. This indicates the validity of the conclusions regarding inhibitory/excitatory balance.

There were no statistically significant differences found by student's T test between the control group and the ammonia perfused group for the independent mean values of the amino acids. Glutamine was an exception, which showed a statistically significant increase in the group of ammonia perfused animals (t=-2.46, p=0.03, 95% confidence interval for difference: -5.183 to -0.311). This test was conducted with the amino acid results obtained from the animals that survived until the end of the study.

DISCUSSION

The findings of the present study reveal, for the first time, that particular dose and rate of ammonia perfusion increases the seizure threshold to pentylenetetrazole in a linear fashion.

The reason for using PTZ was that, as is indicated before, it is a relatively well studied molecule in terms of its seizure provoking mechanisms. It has been considered as a kindling agent, and repeated administration is expected to decrease seizure threshold. Thus the increase of PTZ-induced seizure threshold due to ammonia perfusion strongly supports the reliability of the finding and validates the chosen method for seizure provocation. Besides, PTZ-induced changes in brain amino-acid content has been found just the opposite of our findings i.e. repeated administration of PTZ leads to statistically significant increase of excitatory amino acids in different parts of the brain. Despite the fact that there is no contradictory finding with regard to its general effect on the amino acid pool in the brain, it seems further analysis using different experimental and statistical models is needed to reach a proper conclusion on this regard (Sejima *et al.*, 1997). Shortly, this study shows that given rate of ammonia appears to be able to overcome the PTZ induced epilepsy prone biochemical changes as well.

In this study, univariate tests showed that only a significant change for glutamine is observed by student's T test. Indeed, by its nature, any statement about relationship between a dependent variable (i.e. seizure threshold) and independent variable (i.e. amino acid) of the data collected for this study is contingent upon the other variables (i.e. other amino acids) in the equation. Therefore, in order to see whether the biochemical data tell us something valuable, which might be helpful to explain the increase of seizure threshold due to ammonia, in building our model, we used multivariate approaches. However, univariate statistics have their own practical value. At least, they help to enlighten the way of main effect of a variable on a dynamic system. As in the case of our study, ammonia seems to have an effect on the glutamine increasing mechanisms. But, we can only understand on the basis of multivariate analysis how this leads an increase of epileptic threshold, and which parameter(s) is the predictor of this functional change. It is understood from the point of this mathematical model that when all the parameters are taken together, a decrease in glutamate would accompany increases in seizure threshold. That is, multiple linear regression analysis summarized the data as well as highlighted the relationships among the variables. The method also helped to predict the seizure thresholds, indicating that the predictors were increase of glutamine and decrease of glutamate in the sense of partial regression (i.e. on the basis of adjusted data obtained by regression analysis) (Fig. 2).

The results demonstrate that the protective effect of ammonia on PTZ induced convulsions is accompanied by increased glutamine with no changes in excitatory, or inhibitory amino acids. On the other hand, brain glutamate concentrations were predictors of the increase in PTZ-induced seizure threshold of ammonia treated animals.

This could be explained by biochemical mechanisms such as the activity changes of GS, or the direct effect of ammonia both on inhibitory (via inhibiton of the postsynaptic potential and/or on the excitatory postsynaptic glutamate receptors) (Raabe. 1987; Szerb and Butterworth, 1992).

However, it is clear that, further studies are required in order to more fully elucidate the mechanisms of action whereby ammonia is protective in PTZ-induced seizures.

CONCLUSION

The findings from the present study reveal, for the first time, that hyperammonemia has the capacity to limit seizure threshold, ie: hyperammonemia is protective. Thus, continuous infusion of ammonia may cause an imbalance between excitatory and inhibitory systems in favor of inhibitory systems. However, it remains to be determined whether this protective effect applies to other convulsive states.

The model used for this study may also be a good candidate to search for the other hepatic encephalopathy and hyperammonemia related convulsive state as well as the other neuro-psychiatric manifestations.

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