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Effect of hyperammonemia on brain amino acids in young and adult ferrets*

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Summary. Effects of arginine deficiency and hyperammonemia on the brain concentrations of amino acids and urea cycle enzyme activities in young and adult ferrets were investigated. Only young ferrets developed hyperammonemia and encephalopathy immediately after consuming the arginine-free diet. Brain ornithine and citrulline concentrations in young ferrets fed arginine containing diet were significantly lower than those in adult ferrets. Compared to rats and other animals, young and adult ferrets had lower concentrations of brain glutamic acid and glutamine. Unlike in other species, brain glutamine was not elevated in young, hyperammonemic ferrets. Brain arginase and glutamate dehydrogenase activities were significantly increased in young ferrets fed arginine-free diet. Young ferrets provide a useful animal model for investigating the neurotoxicity of acute hyperammonemia.

Keywords: Amino acids – Arginine – Hyperammonemia – Ferrets
Abbreviations: ACD, Arginine-containing diet; AFD, Arginine-free diet.

Introduction

Arginine occupies an intermediate position between the dispensable and indispensable amino acids because most mammals can synthesize adequate amount of arginine for positive nitrogen balance. However recent reports indicate that a dietary arginine may be essential for young cats, dogs and ferrets and probably for other carnivorous animals (Burns et al., 1981; Czarnecki and Baker, 1984; Deshmukh and Rusk, 1989; Deshmukh et al., 1982; 1991; Morris and Rogers,

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1978; Stewart et al., 1981; Thomas and Deshmukh, 1986). We have previously reported (Deshmukh et al., 1982; 1991; Thomas and Deshmukh, 1986) that a single meal of an arginine-free diet (AFD) to juvenile ferrets produced severe hyperammonemia and encephalopathy, whereas adult ferrets did not develop hyperammonemia after ingesting an AFD.

Hyperammonemia can occur in various clinical situations such as inborn errors involving the urea cycle enzymes, organic acidemia, cirrhosis of the liver and hepatic encephalopathy. Elevated blood ammonia is toxic to the brain and leads to convulsions, coma and death. Various experimental models such as portacaval shunt and intraperitoneal injections of ammonium acetate or urease have been used to investigate the effects of acute and chronic hyperammonemia on the metabolism of amino acids in brain (Jesse et al., 1990; Semon et al., 1989). In the present paper, we have compared the brain amino acid concentrations and urea cycle enzyme activities (Fig. 1) in young and adult ferrets fed an arginine-containing diet (ACD) or AFD.

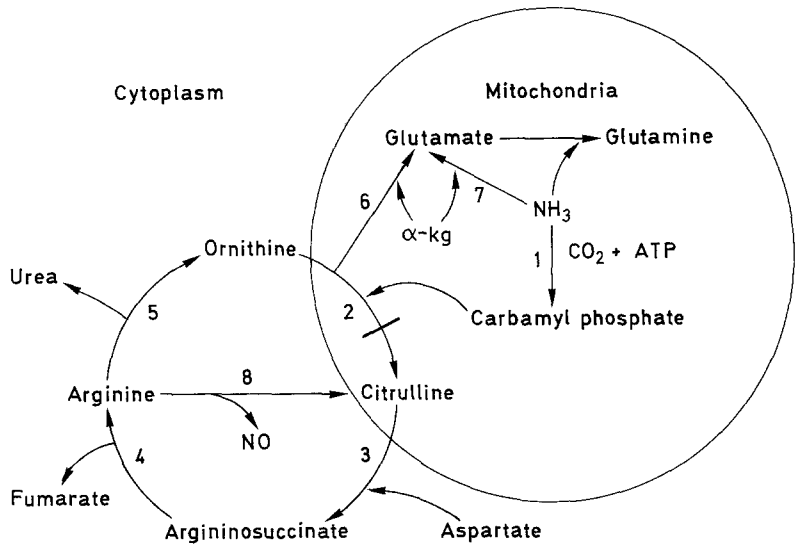

Fig. 1. Urea cycle and ammonia metabolism in brain. *1* Carbamyl phosphate synthetase; *2* Ornithine carbamyl transferase; *3* Arginino-succinate synthetase; *4* Argininosuccinase; *5* Arginase; *6* Ornithine aminotransferase; *7* Glutamate dehydrogenase; *8* Nitric oxide synthase

Materials and methods

Animals

Two-month-old (young) or eighteen-month-old (adult) male, sable-coated ferrets, vaccinated for canine distemper were purchased from Marshall Research Farm, North Rose, New York. They were housed in groups of two or three in cages with grid flooring, in an isolation room with controlled light and temperature (22–23°C, 12 hour light/dark cycle). All animal experimentation was performed with Institutional Animal Investigation Committee Approval.

Diet

Water and stock diet (cat chow, Ralston Purina Co., St. Louis, MO) were provided *ad libitum*. A synthetic diet containing free amino acids (4.5 g of nitrogen per 100 g of diet), vitamins, corn starch, sucrose and salt mixture was prepared as described previously (Morris and Rogers, 1978) except that corn oil was used instead of turkey fat. An AFD was prepared by substituting alanine for an isonitrogenous amount of arginine. The slight increase of alanine required was compensated for by decreasing the carbohydrate content.

Experimental design

In the first set of experiments (Table 1), 5 young and 5 adult ferrets were fasted for 16 hours and 3 ml of blood were collected by cardiac puncture from the lightly anaesthetized animals into chilled heparinized tubes (Vacutainer) and centrifuged at 8000 × g for 15 minutes in a refrigerated centrifuge. An aliquot of the plasma was stored at −20°C. Animals were sacrificed by cervical dislocation. Brains were removed, frozen in liquid nitrogen and

Table 1. Effect of arginine-containing diet versus fasting on amino acids in brains of young and adult ferrets

Amino acid μmol/g	2-months-old		18-months-old	
	Fasting	ACD	Fasting	ACD
Indispensable amino acids				
Histidine	4.84 ± 0.09	3.48 ± 0.17[a]	2.40 ± 0.16[b]	2.23 ± 0.05[b]
Isoleucine	0.04 ± 0.005	0.06 ± 0.004	0.04 ± 0.004	0.05 ± 0.005
Leucine	0.08 ± 0.004	0.10 ± 0.005	0.08 ± 0.004	0.08 ± 0.006
Lysine	0.08 ± 0.004	0.14 ± 0.005[a]	0.11 ± 0.005	0.15 ± 0.005
Methionine	0.05 ± 0.008	0.07 ± 0.005	0.04 ± 0.004	0.04 ± 0.004
Phenylalanine	0.08 ± 0.004	0.08 ± 0.017	0.07 ± 0.005	0.09 ± 0.008
Threonine	0.31 ± 0.005	0.45 ± 0.03	0.17 ± 0.01	0.24 ± 0.03[b]
Valine	0.14 ± 0.005	0.18 ± 0.005	0.11 ± 0.01	0.13 ± 0.005
Dispensable amino acids				
Alanine	0.61 ± 0.02	0.70 ± 0.01	0.62 ± 0.003	0.81 ± 0.04
Arginine	0.17 ± 0.005	0.19 ± 0.01	0.15 ± 0.005	0.20 ± 0.01
Aspartic acid	1.44 ± 0.01	1.49 ± 0.01	1.35 ± 0.01	1.37 ± 0.03
Aspargine	0.11 ± 0.005	0.13 ± 0.005	0.09 ± 0.003	0.11 ± 0.005
Citrulline	0.16 ± 0.02	0.16 ± 0.01	0.15 ± 0.01	0.25 ± 0.04[b]
Glutamic acid	2.75 ± 0.05	2.84 ± 0.03	2.57 ± 0.03	2.68 ± 0.05
Glutamine	0.99 ± 0.03	1.26 ± 0.06[a]	0.86 ± 0.03	1.04 ± 0.06[a]
Glycine	0.66 ± 0.01	0.76 ± 0.02	0.70 ± 0.03	0.76 ± 0.04
GABA	1.46 ± 0.03	1.51 ± 0.02	1.48 ± 0.003	1.50 ± 0.04
Ornithine	0.30 ± 0.05	1.27 ± 0.05[a]	2.06 ± 0.16[b]	2.86 ± 0.16[b]
Serine	0.78 ± 0.03	0.90 ± 0.08	0.72 ± 0.03	0.67 ± 0.03[a]
Taurine	1.99 ± 0.02	2.04 ± 0.02	1.68 ± 0.004[b]	1.80 ± 0.01[b]
Tyrosine	0.07 ± 0.004	0.08 ± 0.007	0.08 ± 0.007	0.07 ± 0.005

ACD Arginine-containing diet; Values are means ± SEM (n = 5). [a] Indicates statistically significant difference ($p < 0.05$) when compared with fasting in the same age group; [b] Indicates statistically significant difference when compared with 2-month-old ferrets in the same diet group

stored at −20°C. In the second set of experiments, 5 young and 5 adult ferrets were fasted overnight and fed, *ad libitum*, either the ACD or AFD. Three hours after feeding the diet, 3 ml blood were collected and processed as described above. After collecting blood, the animals were sacrificed. Brains were removed, frozen in liquid nitrogen and stored at −20°C.

Amino acid analysis

Portions of brain (cerebral cortex) were pulverized in liquid nitrogen and 10% (w/v) homogenate was prepared in 3% (w/v) sulphosalicylic acid. Amino acids in brain were determined using high performance liquid chromatography (Waters Associates) with gradient programmer and an integrator using a C-18 reverse phase column as described previously (Portoles et al., 1985).

Ammonia assay

Brain samples were homogenized (10%, w/v) in chilled perchloric acid (1 M). Homogenates were centrifuged at 10,000 × g for 15 min at 0–2°C, the supernatants were neutralized to pH 7.0 with 1 N sodium hydroxide. Ammonia in the supernatants and plasma was assayed by glutamate dehydrogenase reaction (Mondzack et al., 1965). Ammonia-free water was used as a blank and also for preparing all reagents.

Enzyme assays

Brain samples were homogenized (10%) in distilled water and divided into two portions. For assaying mitochondrial enzymes, Triton X-100 (0.1%) was added to one portion of the homogenate. The homogenates were centrifuged at 5,000 × g for 15 min. The activities of following enzymes in the supernatant were determined at 37°C: Arginase (Tarrab et al., 1974) (EC 3.5.3.1.), ornithine-oxo-acid aminotransferase (Peraino and Pitot, 1963) (EC 2.6.1.13), argininosuccinate synthetase (Nuzum and Snodgrass, 1975) (EC 6.3.4.5.), argininosuccinase (Nuzum and Snodgrass, 1975) (EC 4.3.2.1.) and glutamate dehydrogenase (Schmidt, 1974) (EC 1.4.3.1.). Proteins were measured by Lowry's method (Lawry et al., 1951).

Statistical analysis

In order to investigate the effect of diets, values in ACD and AFD groups were compared in each age-group. In order to investigate the age-dependent changes, values in young ferrets were compared with adult ferrets in each diet group. Student's test was used to calculate the statistical difference. P values < 0.05 were considered statistically significant.

Results

Young ferrets that were food deprived for 16 hours had significantly lower concentration of brain ornithine and higher concentrations of histidine and taurine than adult ferrets after an identical treatment (Table 1). Although brain ornithine concentration increased significantly in young ferrets following an ACD, the levels remained much lower than that in adult ferrets (Table 1). Brain citrulline concentrations increased significantly following ACD in adult but not in young ferrets. Brain arginine concentrations were identical in both age groups of ferrets fed ACD.

Young ferrets fed AFD became hyperactive two hours after ingesting the diet. Within 30 minutes of the onset of hyperactivity, they became prostrate

and subsequently developed coma. Ferrets exhibited typical signs of hyperammonemia such as irritability, seizures and coma. Bleeding through the nose and sialorrhea was seen in a few hyperammonemic ferrets. The blood and brain samples taken after feeding were timed to coincide with the development of these symptoms. Adult ferrets did not develop hyperammonemia after eating an AFD. Control ferrets fed ACD in both age groups did not develop hyperammonemia. No significant difference was observed in the amount of food intake between ACD and AFD groups. Ferrets in both age groups consumed about 15–20 g of diet (Thomas and Deshmukh, 1986) (0.7 to 0.9 g of nitrogen).

There were no significant differences in the plasma ammonia values between young and adult ferrets fed ACD (59 ± 5 vs 47.4 ± 6.2 μmol/l, mean ± SEM, n = 5). However, a 37 fold increase in plasma ammonia (2,195 ± 102 μmol/l, mean ± SEM, n = S) was seen in young ferrets at 3 hours after feeding AFD. In

Table 2. Amino acids and ammonia concentrations in brains of young and adult ferrets fed arginine-containing or arginine-free meals

Amino acid μmol/g	2-months-old ferrets		18-months-old ferrets	
	ACD	AFD	ACD	AFD
Indispensable amino acids				
Histidine	3.48 ± 0.17	2.69 ± 0.11[a]	2.23 ± 0.05[b]	1.74 ± 0.10[ab]
Isoleucine	0.06 ± 0.004	0.06 ± 0.006	0.05 ± 0.005	0.04 ± 0.004
Leucine	0.10 ± 0.005	0.08 ± 0.007	0.08 ± 0.006	0.08 ± 0.006
Lysine	0.14 ± 0.005	0.12 ± 0.01	0.15 ± 0.005	0.16 ± 0.005
Methionine	0.07 ± 0.005	0.06 ± 0.004	0.04 ± 0.004	0.05 ± 0.008
Phenylalanine	0.08 ± 0.017	0.08 ± 0.004	0.09 ± 0.008	0.08 ± 0.004
Threonine	0.45 ± 0.03	0.45 ± 0.04	0.24 ± 0.03[b]	0.26 ± 0.03[b]
Valine	0.18 ± 0.005	0.16 ± 0.005	0.13 ± 0.005	0.14 ± 0.01
Dispensable amino acids				
Alanine	0.70 ± 0.01	0.68 ± 0.03	0.81 ± 0.04	0.74 ± 0.02
Arginine	0.19 ± 0.009	0.18 ± 0.01	0.20 ± 0.01	0.18 ± 0.02
Aspartic acid	1.49 ± 0.01	1.46 ± 0.02	1.37 ± 0.03	1.41 ± 0.02
Aspargine	0.13 ± 0.005	0.14 ± 0.005	0.11 ± 0.005	0.12 ± 0.005
Citrulline	0.16 ± 0.013	0.16 ± 0.01	0.25 ± 0.02[b]	0.28 ± 0.04[b]
Glutamic acid	2.84 ± 0.03	2.73 ± 0.03	2.68 ± 0.05	2.61 ± 0.02
Glutamine	1.26 ± 0.06	1.21 ± 0.06	1.04 ± 0.06	0.92 ± 0.02[b]
Glycine	0.76 ± 0.02	0.74 ± 0.03	0.76 ± 0.04	0.87 ± 0.05
GABA	1.51 ± 0.02	1.44 ± 0.03	1.50 ± 0.04	1.47 ± 0.02
Ornithine	1.27 ± 0.05	1.56 ± 0.08[a]	2.86 ± 0.16[b]	3.09 ± 0.07[b]
Serine	0.90 ± 0.08	0.91 ± 0.07	0.67 ± 0.03	0.61 ± 0.02[b]
Taurine	2.04 ± 0.02	2.05 ± 0.02	1.80 ± 0.01[b]	1.76 ± 0.02[b]
Tyrosine	0.08 ± 0.007	0.06 ± 0.06	0.07 ± 0.005	0.07 ± 0.005
Ammonia	1.4 ± 0.09	7.3 ± 0.8[a]	1.7 ± 0.09	1.6 ± 1.3

ACD Arginine-containing diet, *AFD* Argine-free diet, Values are means ± SEM (n = 5). [a] Indicates statistically significant difference ($p < 0.05$) when compared with ACD in the same age group; [b] Indicates statistically significant difference when compared with 2-month-old ferrets in the same diet group

adult ferrets, there were no changes in plasma ammonia following AFD. Brain ammonia concentrations in young ferret fed AFD were significantly higher than in those fed ACD, whereas there was no significant difference in the brain ammonia concentrations in adult ferrets fed ACD and AFD (Table 2). Brain ammonia concentrations in young ferrets fed ACD were comparable to those in similarly treated adult ferrets.

In young as well as adult ferrets fed AFD, histidine concentrations were significantly lower than in those fed ACD (Table 2). Ornithine concentrations in young ferrets fed AFD were significantly higher than in those fed ACD. There were no significant changes in any other amino acid due to AFD treatment in either age groups. However, the concentrations of ornithine and citrulline in young ferrets fed either ACD or AFD remained significantly lower than those in adult ferrets in the same diet group.

Compared to young ferrets, adult ferrets fed ACD had higher levels of arginase, argininosuccinate synthetase and glutamate dehydrogenase activities. In young ferrets, the activities of arginase and glutamate dehydrogenase were significantly increased following an AFD treatment whereas similar increase was not seen in adult ferrets (Table 3).

Table 3. Urea cycle enzyme activities (*n*mol/min/mg protein) in brains of young and adult ferrets fed arginine containing or arginine-free diet

Enzyme	2-mo-old ferrets fed		18-mo-old ferrets fed	
	ACD	AFD	ACD	AFD
Arginase	4.5 ± 0.2	7.5 ± 0.4[a]	5.8 ± 0.5[b]	6.9 ± 0.3
Ornithine aminotransferase	3.6 ± 0.2	3.4 ± 0.3	3.6 ± 0.2	3.5 ± 0.2
Argininosuccinase	1.0 ± 0.1	0.8 ± 0.1	1.3 ± 0.1	0.8 ± 0.1
Argininosuccinate synthetase	0.5 ± 0.1	0.7 ± 0.1	2.4 ± 0.8[b]	1.3 ± 0.3
Glutamate dehydrogenase	76 ± 1.4	96 ± 3.6[a]	112 ± 7.6[b]	102 ± 6.1
Protein (mg/g)	105 ± 3.6	88.8 ± 1.5[a]	80.4 ± 1.4[b]	83.0 ± 3.0

ACD Arginine-containing diet, *AFD* Arginine-free diet, Values are means ± SEM (n = 5) [a] Indicates statistically significant difference ($p < 0.05$) when compared with ACD in the same age group, [b] Indicates statistically significant difference as compared with 2-month-old ferrets in the same diet group

Discussion

Recent studies suggest that brain glutamine plays an important role in the pathogenesis of hyperammonemia. Takahashi et al. (1991) reported that inhibition of brain glutamine accumulation prevents the cerebral edema in hyperammonemic rats. Hawkins and Jessey (1991) demonstrated that hyperammonemic rats without increase in brain glutamine behaves normally, indicating that the deleterious effect of chronic hyperammonemia commences with the synthesis of glutamine. Our results do not support this hypothesis because brain glutamine concentration was not altered due to AFD-induced

hyperammonemia in young ferrets (Table 2). The discrepancy may be due to differences in the mode of induction of hyperammonemia or due to species specific differences.

Brain glutamate and glutamine concentrations in young ferrets were significantly lower than those seen in other species (Kamata et al., 1980; Semon et al., 1989). Glutamine synthetase is the only mechanism by which brain can detoxify ammonia because the brain tissue lacks the complete urea cycle (Jones et al., 1961). However, in our study, brain glutamine concentrations in hyperammonemic ferrets were not significantly different from those in control animals (Table 2). These results are in agreement with the hypothesis that under hyperammonemic conditions, cerebral glutamine synthetase may not be efficient in removing blood ammonia (Cooper et al., 1985).

Brain arginine concentrations in young ferrets fed either ACD or AFD were comparable to those in adult ferrets. Brain tissue contains significant amount of arginase activity (Sadasivudu and Indira, 1986). Ornithine formed from arginine can either be decarboxylated to form putrescine or transaminated into glutamic acid by the action of ornithine aminotransferase. The activity of ornithine aminotransferase was not affected due to hyperammonemia (Table 3). However, compared to rats, ferrets fed ACD had very high concentrations of brain ornithine and low concentrations of glutamic acid suggesting that the conversion of ornithine into glutamate may be altered in ferrets.

Rogers and Phang (1985) reported that the activity of pyrroline-5-carboxylate synthase in the intestinal mucosa of cats was very low as compared to that of rats. They concluded that the limitation or lack of *de novo* synthesis of ornithine from glutamate in cats may be the metabolic basis for the rapid onset of hyperammonemia following an arginine-free meal. According to Stewart et al. (1981), low levels of hepatic ornithine are probably responsible for making cats susceptible to hyperammonemia following an arginine-free diet. This explanation appears unlikely because although both young and adult ferrets had low levels of hepatic ornithine, only young ferrets became hyperammonemic after ingesting an AFD (Deshmukh et al., 1991).

Our observation that young ferrets fed AFD had higher glutamate dehydrogenase activity (Table 3) is consistent with previous reports of increased brain glutamate dehydrogenase activity in hyperammonemic conditions (Benjamin, 1983; Norenberg, 1977). In adult ferrets, AFD treatment did not alter the glutamate dehydrogenase activity indicating that the increased activity in young ferrets was probably due to hyperammonemia rather than due to arginine deficiency. Increased glutamate dehydrogenase activity signifies increased synthesis of glutamate from α-ketoglutarate.

The mechanism by which ammonia causes neurotoxicity has not been completely understood. Ferrets provide a useful animal model for studying the effects of acute hyperammonemia because hyperammonemia can be rapidly induced in young ferrets by simple dietary manipulations. It is important to note that the amino acid values in the present study are from the cerebral cortex obtained three hours after feeding the specified diet. The three hour period was selected because plasma ammonia concentrations and the severity of sickness was maximum at that time. An analysis of amino acids in different regions of the brain at

various time intervals following AFD may help to clarify the mechanism of hyperammonemia induced by arginine-free diet in young ferrets.

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