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Effect of Long-Term Pyridoxine Depletion on Aspartate Aminotransferase and Pyridoxal 5'-Phosphate of Rat Liver Mitochondrial and Cytosolic Fractions

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장기간의 Pyridoxine 부족이 쥐 간의 Mitochondria 및 Cytosolic Fraction에 있는 Aspartate Aminotransferase 및 Pyridoxal 5'-Phoshate에 미치는 영향

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이유한 Sprague Dawley종의 암컷 흰쥐를 두 군으로 나누어 각각 pyridoxine이 충분한 식이(22mg/kg diet)와 pyridoxine이 부족한 식이(1.2mg/kg diet)로 성장시킨 후, 적절한 시기에 임신시켰다. 태어난 새끼가 이유할 때 결핍식이군을 둘로 나누어, 한쪽은 pyridoxine이 충분한 식이로 바꾸어준 후, 세 군의 쥐를 생후 10주까지 성장시켰다. 각 군의 새끼쥐가 각각 0, 3, 7, 10주 되었을 때, 간의 mitochondria 및 cytosolic fraction에 있는 Aspartate aminotransferase [EC 2. 6. 1. 1] activity 및 pyridoxal phosphate의 함량을 측정하였다.

결핍군의 간 aspartate aminotransterase activity는 mitochondria 및 cytosolic fraction에서 각각 대조군에 비해 유의적으로 낮았으며, 10^{-4}M pyridoxal phosphate를 첨가한 후 측정한 total enzyme activity는 거의 대조군과 비슷하였다. 3주 이후 pyridoxine이 충분한 식이로 바꾸어준 회복군에서도 대조군에 비해 유의적으로 낮았으나, 결핍군보다는 높았다. 이 두 fraction에서의 pyridoxal phosphate함량은 결핍군이 대조군보다 현저히 낮았으며, 회복군은 대조군과 비슷하였다. 이로써, 장기간에 걸친 pyridoxine결핍이 간의 aspartate aminotransferase activity 및 pyridoxal phosphate에 현저한 영향을 미치며, 이유기 이후의 식이보충에 의해서도 enzyme activity는 쉽게 회복되지 않음을 알 수 있다.

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INTRODUCTION

In general, the occurence of a primary pyridoxine deficiency is rare in the human as pyrdoxine occurs in most foodstuff. However, various conditions are known in which a relative deficiency exists either because of increased requirement during gestation and lactation, or formation of inactive complexes between this vitamin and various drugs used as oral contraceptives (1) and isoniazid (2) used as pulmonary tuberculosis. Many women use anovulatory drugs for a prolonged period of time prior to becoming pregnant and Schuster et al. (3) have established that a significant percentage of pregnant women have a relative pyridoxine deficiency.

Pyridoxine 5'-phosphate is one of the major forms of vitamin B-6 in liver. The cellular level of pyridoxal phosphate is known to influence both the activities and the contents of pyridoxal phosphate-dependent enzymes(4, 5). Two isozymes of aspartate aminotransferase, pyridoxal phosphate -dependent enzyme, are present in cells, one in the cytosol and the other in mitochondria. These two isozymes are structurally and immunologically distinct, but do not show tissue specificity within a single species (6). Okada and Hirose (7) reported that, in pyridoxine deficient rat liver, the activity of aspartate aminotransferase in liver cytosol was decresed and not restored by addition of pyridoxal phosphate to the assay medium. Not only the cytosolic enzyme but also the mitochondrial enzyme is known to show decreased activity in pyridoxine deficient rat liver(8, 9). Liver pyridoxal phosphate content was also lowered in pyridoxine deficient rats(9, 16). The majority of the pyridoxal phosphate in rat liver is localized in the cytosolic fraction and this pool of the coenzyme is preferentially depleted under conditions of vitamin B-6 deficiency. Although the effect of pyridoxine deficiency on liver aspartate aminotransferase for short time is studied by many workers(7 ~ 10), relatively little is established about the effect of long-term pyridoxine depletion on the ezyme activity and pyridoxal phosphate content of offsprings.

Accordingly, in this experiment, changes in the liver aspartate aminotransferase activity and pyridoxal content in response to long-term pyridoxine depletion were performed. As a part of this investigation, determinations were made on the aspartate aminotransferase activities and pyridoxal phosphate contents of rat liver mitochondrial and cytosolic fractions.

EXPERIMENT

1) Animals and Diets

Weanling female Sprague Dawley rats weighing 50-60 g supplied by Animal Breeding Laboratory of Seoul National University, were used in this experiment. After adaptation with normal chow diet for 3 days, rats were divided into two groups, the control and the deficient. The control group was fed a pyridoxine-sufficient diet (control diet) which contained 22mg pyridoxine · HCl /kg diet and the deficient group was fed a pyridoxine -deficient diet containing 1.2mg pyridoxine ·HCl /kg diet. The composition of the experimental diet is given in Table 1. Rats were housed in plexiglas cages, temperature and humidity were kept 20 ± 1℃, and 55 ± 1 % respectively. Light and darkness were also controlled(7:00-19:00). And food and water provided ad. libitum.

When female rats grew approximately 180-200 g in weight, they were mated with males of the same strain, and after pregnancy rats were individually housed. After the pups were born and weaned, deficient group was subdivided into two groups. One(supplemented group) switched to control diet and the other(deficient group)continued the same deficient diet throughout.

On the second day after birth, pups from each dam were counted, weighed, and randomly reduced to 8. After weaning, only the males were used for observation and biochemical analysis in order to eliminate the sexual difference. At the age of

Table 1. Composition of experimental diet (g/100g diet)

	Pyridoxine -sufficient	Pyridoxine -deficient
Vitamin-free casein (1)	20.0	20.0
Corn starch	58.5	58.5
Corn oil	5.0	5.0
Beef tallow	5.0	5.0
Salt mixture (2)	4.0	4.0
Cellulose (3)	5.0	5.0
Vitamin mixture $-B_6$ (4)	–	2.08
$+B_6$ (5)	2.2	0.12
DL-Methionine	0.3	0.3

(1) ICN Nutritional Biochemicals, Cleveland, Ohio, USA.
(2) AIN Mineral Mixture 76, ICN
(3) ICN
(4) Vitamin fortification mixture, without B_6, ICN.
(5) Vitamin fortification mixture, ICN.

0, 3, 7, 10 weeks, 5 pups of each group were randomly chosen and starved for 8 hr, they were sacrificed by decapitation. Livers were immediately removed, frozen on dry ice and stored at −20℃ until use. Serum was also collected.

2) Biochemical Analysis

Rat liver was homogenized in ice-cold 0.1 M potassium phosphate buffer, pH 7.4, with or without 10^{-4} M pyridoxal phosphate using a motor-driven homogenizer with teflon pestle and was centrifuged at 10,000 × g for 10 min. All centrifugations were performed at 4℃. Some of the supernatant was used for the determination of liver protein content. Transfered the supernants to another centrifuge tubes, suspended the mitochondrial pellets thoroughly in a few ml of homogenization buffer and wash twice by centrifugation for 10 min each at 10,000 × g. Then resuspended the washed mitochondria in a minimal volume of buffer, they were used for determinations of mitochondrial aspartate aminotransferase activity and pyridoxal phosphate contents. For the preparrions of cytosolic fractions, the 100,000 × g supernatants recentrifuged at 100,000 × g for 1 hr., then the resulting supernatants were used.

Aspartate aminotransferase activity was determined by the method of Reitman and Frankel (11). Liver cytosolic and mitochondrial fractions prepared by phosphate buffer with or without pyridoxal phosphate were used for determination. Preincubation was performed at 37℃ for 60 min. Activity measured in the presence of pyridoxal phosphate is defined as "total enzyme activity"; that measured in the absence of pyridoxal phosphate as "holoenzyme activity"; and the numerical difference between the two as "apoenzyme level". The ratio (activity-pyridoxal phosphate)/(activity+pyridoxal phosphate) expressed as a percentage was used as a measure of the proportion of enzyme existing as holoenyme. Serum aspartate aminotransferase activity was also measured.

Pyridoxal phosphate content was measured according to the method of Wada and Snell (12). Liver cytosolic and mitochondrial fractions prepared by phosphate buffer without pyridoxal phosphate were used. Serum pyridoxal phosphate contents were also measured. For measurement of pyridoxal phosphate content, the protein was precipitated with cold 10% trichloroacetic acid, standed for 20 min., and centrifuged at 3,000 × g for 10 min. Protein was determined by the metnod of Lowry et al. (13) with bovine serum albumin as standard.

3) Chemicals and Instrument

Bovine serum albumin, pyridoxal 5'-phophate and pyruvic acid were purchased from Sigma chemical co.; α-ketoglutarate from Merck; and L-aspartic acid from Wako Pure Chemical Industries, LTD. Pye Unicam SP 6-400 Spectrophometer was used to obtain absorbance data and centrifugation was performed with Hitachi 55P-72 Ultracentrifuge.

4) Statistics

Student's t-test was used to compare significant difference between the control and the deficient groups.

RESULTS

1) Animal Conditions

The body and liver weights and liver protein levels of offspring rats in different group were compared(Table 2). The body and liver weights of deficient group (DD) were similar to control group (CC) at birth, but significantly lower at 10 weeks. But those of supplemented group (DC) that switched to control diet at 3 weeks postpartum were not significantly different from control group at 7 and 10 weeks of age. Ratios of liver weight to body weight of deficient group was similar to or even higher than those of control group. There were no significant differences in liver protein content among groups at the different developmental stages, except 3 and 7 week-old deficient rats. Okada et al. (4) reported that amino acid contents in the liver from pyridoxine-deficient rats changed in various degrees, owing to impairment of amino acids metabolism, while the rate of protein synthesis in the deficient rat liver was maintained at nomal levels.

During the lactation period and thereafter, physical appearances of the deficient rats were not greatly different from those of the controls in color and thickness of hair. And no sign of deficiency symptom such as tremor, rapid movement, and convulsion were noticed in deficient dams and their pups.

2) Mitochondrial and Cytosolic Aspartate Aminotransferase Activity in Rat Liver

Table 2. Effect of long-term pyridoxine depletion on growth, liver weights, and liver protein contents of offspring rats

age (weeks)	group	body weight (g)	liver weight (g)	liver weight % of body weight	protein content (mg/g liver)
0	CC	6.67 ± 0.39	0.22 ± 0.04	3.30	64.17 ± 15.04
	DD	6.72 ± 0.34	0.23 ± 0.03	3.42	63.80 ± 16.19
3	CC	67.23 ± 4.74	2.57 ± 0.19	3.82	137.50 ± 7.78
	DD	60.85 ± 4.39*	2.40 ± 0.25	3.94	116.97 ± 19.97*
7	CC	258.10 ± 29.54	9.42 ± 1.24	3.65	146.67 ± 20.68
	DC	274.58 ± 8.06	11.43 ± 0.82	4.16	135.30 ± 22.45
	DD	238.34 ± 12.90++	9.18 ± 0.67++	3.85	118.80 ± 19.01*
10	CC	411.46 ± 33.91	14.57 ± 1.93	3.54	153.27 ± 16.62
	DC	414.83 ± 13.08	16.60 ± 1.41	4.00	147.77 ± 11.37
	DD	358.84 ± 42.04*+	11.90 ± 1.81*, ++	3.32	137.50 ± 15.26

values are means ± SD for 5 rats.
CC : control
DD : deficient throughout
DC : deficient group supplemented at 3 weeks postpartum
* : $p < 0.05$ significantly different from control group(CC)
+ : $p < 0.05$ " " supplemented group(DC)
++ : $p < 0.01$ " " "

Table 3. Effect of long-term pyridoxine depletion on aspartate aminotransferase activity on liver mitochondrial and cytosolic fraction of offspring rats[a] (Units / g Liver)

age (weeks)	fraction	group	− PAL − P[b] (Holoenzyme activity)	+ PAL − P (Total enzyme activity)	% of Holoenzyme
0	Mitochondria	CC	3256.0 ± 11.3	4570.7 ± 270.4	71.2
		DD	3052.1 ± 116.0**	4454.0 ± 60.9	68.5
	Cytosol	CC	3386.7 ± 76.3	5276.1 ± 71.2	64.2
		DD	3448.0 ± 33.9	5389.3 ± 82.2	64.0
3	Mitochondria	CC	2950.3 ± 8.5	3552.0 ± 14.9	83.1
		DD	2942.1 ± 14.0	3708.0 ± 33.9	79.3
	Cytosol	CC	4369.3 ± 185.7	5449.3 ± 118.1	80.2
		DD	3933.3 ± 153.7**	5469.3 ± 49.9	71.9
7	Mitochondria	CC	2960.7 ± 33.9	3488.0 ± 80.1	84.9
		DC	2922.3 ± 8.5*	3589.3 ± 23.2	81.4
		DD	2916.2 ± 10.1*	3393.3 ± 70.5*,++	85.9
	Cytosol	CC	4012.0 ± 53.9	5309.3 ± 33.9	75.6
		DC	3916.0 ± 74.8*	5362.6 ± 67 9	73.0
		DD	3815.3 ± 36.5**,+	5296.2 ± 12.3	66.4
10	Mitochondria	CC	3102.3 ± 65.0	3296.0 ± 68.4	94.1
		DC	3032.1 ± 22.6*	3397.3 ± 132.4	89.3
		DD	2964.0 ± 28.3**,++	3548.0 ± 67.5	83.5
	Cytosol	CC	4361.3 ± 45.7	5342.7 ± 9.4	81.6
		DC	4056.0 ± 16.3*	5109.3 ± 18.0**	79.4
		DD	3769.3 ± 92.8**,+	5189.4 ± 18.8**	72.6

a : Rat liver was homogenized in ice-cold 0.1 M potassium phosphate buffer, PH 7.4 with or without 10^{-4}M PAL-P and incubated at 37℃ for 1hr.

b : PAL-P, Pyridoxal phosphate

Values are means ± SD for 5 rats.

*: $p < 0.05$ significantly different from control group (CC)

**: $p < 0.01$ " " "

+: $p < 0.05$ " " supplemented group (DC)

++: $p < 0.01$ " " " "

The aspartate aminotransferase activities in the mitochondrial and cytosolic fractions are presented in Table 3 and 4. Both the mitochondrial and cytosolic holoenzyme activities in the liver preparation were significantly lower in deficient and supplemented group than in control group. But the total enzyme activities were not significantly different from each other, only that of deficient group at 7 weeks was significantly lower than that of the others at same age. In cytosolic fractions, there were significant decrease in total enzyme activities of the deficient and supplemented group compared to control group at 10 weeks of age. Ratioss of holoenzyme activity to total activity were reduced in pyridoxine deficiency.

3) Pyridoxal Phosphate Content in Liver Mitochondrial and Cytosolic Fraction and in Serum

Pyridoxal phosphate contents of each group in liver and serum are shown in Table 5. The pyridoxal phosphatc contents of deficient group in

liver preparation were similar to those of control at birth, but were significantly lower after 3 weeks of age. Both in mitochondrial and in cytosolic fractions of liver, there were no significant differences between the control and supplemented group throughout the experimental period. In serum, deficient group exhibited a marked decrease compared to the control and supplemented group from 0 week of age. It reflects maternal pyridoxine depletion.

In this experiment, we could observed that the pyridoxal phosphate contents in liver and serum of the supplemented group were completely restored to the control levels by dietary pyridoxine supplementation after weaning.

DISCUSSIONS

As an animal develops a vitamin B-6 deficiency, alterations in the flux of metabolites through pathways utilizing vitamin B-6 containing enzymes ultimately leads to the outward clinical signs characteristic of a vitamin B-6 deficiency. Numerous biochemical lesions have been identified including impaired growth and development (14), deficits in physical and neuromotor development(15).

In the present investigation, it was demonstrated that long-term pyridoxine depletion affected growth, liver weight, aspartate aminotransferase activity, and pyridoxal phosphate level of liver mitochondrial and cytosolic fractions of offsprings. Growth, liver weight, and liver pyridoxal phosphate level

Table 4. Effect of long-term pyridoxine depletion on pyridoxal phosphate contents in liver mitochondrial and cytosolic fractions and in serum of offspring rats

age (weeks)	group	liver		serum
		mitochondral (μg/g liver)	cytosolic (μg/g liver)	(μg/ml)
0	CC	4.57 ± 2.92	17.02 ± 2.40	7.80 ± 2.15
	DD	6.85 ± 0.35	15.20 ± 0.70	3.54 ± 0.92**
3	CC	6.83 ± 0.86	6.70 ± 0.61	10.54 ± 1.46
	DD	1.47 ± 1.08**	2.03 ± 0.23**	5.81 ± 2.90**
7	CC	4.75 ± 0.35	16.37 ± 2.64	13.38 ± 0.89
	DC	4.03 ± 0.92	12.57 ± 1.78*	13.05 ± 0.61
	DD	0.73 ± 0.09**,++	5.43 ± 0.66**,++	6.61 ± 1.96**,++
10	CC	3.30 ± 0.80	10.50 ± 1.52	10.04 ± 1.16
	DC	3.20 ± 0.71	10.20 ± 1.14	10.31 ± 1.34
	DD	1.30 ± 0.72**,++	3.87 ± 1.72**,++	4.86 ± 3.19**,++

values are means ± SD for 5 rats.

*: $p < 0.05$ significantly different from control group(DC)
**: $p < 0.01$ " " "
+: $p < 0.05$ " " supplemented group(DC)
++: $p < 0.01$ " " "

were readily recovered by dietary supplementation after weaning, whereas liver aspartate aminotransferase activity in both mitochondrial and cytosolic fractions were not nearly restored.

The holoenzyme activities of aspartate aminotransferase of deficient rats were lower than the controls in the liver mitochondrial and cytosolic fractions. The enzyme activities in cytosolic fractions were more affected than those in mitochondrial fractions by dietary depletion. However, after addition of pyridoxal phosphate to these enzyme preparations, the transaminase activities were almost completely restored to normal, indicating that the transaminase molecule is present as the active apoenzyme. Okada and Hirose (7) reported that aspartate aminotransferase activity in liver preparations, after incubation with 10^{-4} M pyridoxal phosphate, was only partially restored whereas the enzyme activity of kidney preparation was completely restored.

Vitamin B-6 dependent enzymes respond differently to a vitamin B-6 deficiency, i.e., the transaminase activity is reduced while phosphorylase activity remains relatively constant (16). It has been reported that the rate of synthesis of the cytosolic aspartate aminotransferase increases in pyridoxine deficient rat livers, while that of the mitochondrial aspartate aminotransferase is very similar in deficient and control rats (17). The pyridoxal phosphate may be a modulator of cytosolic aspartate aminotransferase in rat livers. The cytosolic aspartate aminotransferase activity in rat liver is also known to be induced by gluconeogenic conditions such as a high protein diet and administration of hydrocortisone (18). Thus, the increase in the rate of synthesis of the cytosolic aspartate aminotransferase in pyridoxine deficient rat liver is thought to be related to an increase in the functional state of the glucocorticoid hormone(17).

By pyridoxine supplementation after weaning, despite of increased level of pyridoxal phosphate in rat liver, the reduced aspartate aminotransferase activities of supplemented rats were not readily recovered. Kuroda et al. (19) suggested that the existence of change of the enzyme molecule in pyridoxine deficient rat liver. The ratios of holoenzyme activity to total activity were reduced in pyridoxine deficient rats and these results are consistent with those of Okada et al. (20).

Liver pyridoxal phosphate contents in mitochondrial and cytosolic fractions at different developmental stages were lower in pyridoxine deficient rats. This finding confirms the observations of previous workers (9). Besides in liver, Coburn et al. (10) showed that pyridoxal phosphate concentrations in plasma, muscle, adrenal glands and thymus of vitamin B-6 deficient rats were significantly lower than those of controls. These reductions were completely restored by dietary pyridoxine supplementation after weaning.

These results suggest that there is a quantitative and a qualitative changs of aspartate aminotransferase and pyridoxal phosphate in liver mitochondrial and cytosolic fraction by long-term pyridoxine deficiency and these reductions can partially recovered by dietary pyridoxine supplementation after weaning.

SUMMARY

Weanling female Sprague Dawley rats were fed diets containing 22mg pyridoxine · HCl/kg diet (control diet) and 1.2mg pyridoxine · HCl/kg diet (deficient diet). One control group and one deficient group were fed their diet throughout growth, gestation and lactation. After the pups were born and weaned, the deficient group was divided into two groups. One switched to control diet(supplemented group) and the other continued the same deficient diet(deficient group) until 10 week-old. The liver mitochondrial and cytosolic aspartate aminotransferase activity and pyridoxal phosphate content were determined in offspring rats.

The aspartate aminotransferase activities in both

liver mitochondrial and cytosolic fractions of deficient group were significantly lower than those of controls, but there were no significant differences between two groups after addition of 10^{-4}M pyridoxal phosphate to the medium. By pyridoxine supplementation after weaning, the reduced aspartate aminotransferase activities were only partially restored to control levels. The pyridoxal phosphate content of deficient group in liver mitochondrial and cytosolic fractions were also significantly different from those of controls, but readily restored by dietary supplementation.

These results suggest that there is a quantitative and a qualitative changes of aspartate aminotransferase and pyridoxal phosphate in liver mitochondrial and cytosolic fraction by long-term pyridoxine deficiency and these reductions can partially recovered by dietary pyridoxine supplementation after weaning.

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