LIVER ANTIOXIDANT ENZYMES ACTIVITIES IN CHOLINE-DEFICIENT FEEDING RATS

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ABSTRAK

Tujuan penelitian ini adalah untuk mengetahui pengaruh kolin defisiensi terhadap aktivitas enzim antiokasidant pada hati. Penelitian ini menggunakan tikus putih jenis Wistar sebagai hewan percobaan yang diberikan pakan mengandung kolin 2,5 g choline bitartrate /kg diet (CS) dan tanpa kolin (CD).

Hasil penelitian menunjukkan bahwa total lipid, triacylglicerol, colesterol, dan pospolipid pada hati meningkat dengan nyata pada CD. Kolin defisiensi (CD) menyebabkan menurunnya kandungan triacylglicerol, colesterol, pospolipid dan meningkatnya aktivitas enzim ornithine carbamoyltransferase pada serum. Aktivitas enzim superoxide dismutase, katalase, glutathione peroxidase dan kandungan α-tocoperol pada hati menurun dengan nyata pada kolin defisiensi. Menurunnya aktivitas enzim dan kandungan non-enzim antioksidan merupakan respon dari meningkatnya superoxide anion pada hati yang disebabkan oleh meningkatnya kandungan lemak hati. Disamping itu, meningkatnya aktivitas enzim ornithine carbamoyltransferase disebabkan karena meningkatnya kandungan triacylglicerol pada hati yang menandakan bahwa terjadi kerusakan hati karena kekurangan kolin.

Key words: choline; superoxide dismutase; catalase; glutathione peroxidase; ornithine carbamoyltransferase; α-tocoperol

INTRODUCTION

Choline (trimethyl-beta-hydroxyethylammonium) is a quaternary ammonium compound that is widely distributed in plants and animals. It is necessary for normal function of the mammalian organism, and also a precursor for the biosynthesis of phospholipids, essential components of all membranes. Choline is involved in methyl group metabolism and lipid transport. Choline deficiency induces lipid accumulation in the liver of rats, because choline is needed for hepatic secretion of lipoproteins (Lombardi et. al., 1966).

Lipids act as vital substrates for lipid peroxidation. One of the manifestations of membrane damage in rats fed a choline deficient diet is lipid peroxidation, which has been shown to occur both in the kidney and liver (Monserrat et.al., 1969). The increase in amount of free oxygen radicals is accompanied by a concomitant increase in cellular damage, commonly indicated by increase in lipid peroxidation product. The level of lipid

peroxidation in cell is controlled by various cellular defense mechanisms consisting of enzymatic and nonenzymatic scavenger system (Halliwell et.al., 1984). Among enzymatic and nonenzymatic defenses, superoxide dismutase, catalase, glutathione peroxidase, and α-tocoperol have been considered essential for the cell in removing oxygen radical from tissues exposed to oxidative stress (McCord et.al., 1969., and Fridovich, 1978). Therefore, this study was undertaken to verify whether choline deficiency alters antioxidant status in rat liver.

MATERIALS AND METHODS

Animal

Male Wistar strain of specific pathogen free (Japan SLC, Inc., Hamamatsu, Shizuoka Japan), and weighting approximately 65 g at the beginning of experiment were individually housed in stainless steel wire-bottom cages in an air-conditioned room at approximately 23°C. Lighting was regulated to provide equal periods of light (08:00-20:00 h) and dark (20:00-08:00 h). This study complied with the animal experimental guides according to the committee of Experimental animal care at Hokkaido University, Japan.

Diets

The compositions of diets are shown in Table 1. The choline-deficient diet had a similar composition to the choline-sufficient diet, except for the addition of 2.5 g of choline bitartrate (Wako pure Chemical Industries, Osaka, Japan) per kg of diet. The rats were divided into two groups (n = 6) which were allowed free access to the appropriate experimental diet and water for 21 days *ad libitum*.

Table 1. Composition of diets (g/kg)

Composition	Choline-sufficient	Choline-deficient
Casein (vitamin-free)[1]	140	140
Vitamin mixture (AIN-93G)[2]	10	10
Choline bitartrate[1]	2.5	-
Mineral mixture (AIN-93G)[2]	35	35
Soybean oil[1]	10	10
Lard[3]	190	190
Sucrose[4]	612.5	615

1. Wako Chemical Industries Co. Ltd., Osaka, Japan
2. Reeves, P.H., Nielsen, F.H., and Fathy, G.C., J. Nutr., 123, 1939-1951 (1993).
3. Katayama Chemical Industries, Ltd., Osaka, Japan.
4. Nippon Beet Sugar MGF., Co., Ltd., Obihiro, Hokkaido, Japan.

Plasma and Tissue Collection

After feeding for 21 days, the rats were killed by decapitation and blood samples were collected. The livers were immediately removed, weighed, and frozen by dropping into liquid nitrogen. Serum was prepared from the blood samples by centrifuging at 1000 x g for 10 min. Samples of liver and serum were stored in -80°C until assay were performed.

Analytical Procedures

Lipids in the liver and serum. Hepatic total lipids were extracted and purified according to the method of Floch et al., (1957) the liver lipids being gravimetrically estimated after removing the solvent. Triacylglycerol (Nagele et.al., 1985) and cholesterol (Siedel et.al., 1983) in the liver were estimated by enzymatic methods, respectively. Phospholipids in the liver were calculated by the method of difference [total lipids–(triacylglycerol and cholesterol)]. Serum triacylglycerol (Nagele et.al., 1985), cholesterol (Siedel et.al., 1983) and phospholipids (phospholipids containing choline) (Takayama et.al., 1977) were measured by enzymatic methods, respectively.

Serum Ornithine Carbamoyltransferase. Serum ornithine carbamoyltransferase activity was estimated by determining the amount of citruline produce according to the method of Oshita et. al. (1976). One unit activity was defined as one micromole of citrulline produced per liter of serum per minut at 37^{o}C.

Superoxide Dismustase. Samples of liver were homogenized using a Potter-Elvehjem type Teflon homogenizer. The homogenizing buffer consisted of 0.25 M sucrose, 0.5 mM EDTA, and 0.5% Triton x-100 (pH 8.0). Homogenates were centrifuged at 3,000 x g for 20 min, and the resulting supernatant was used for measuring the activity of superoxide dismutase. Superoxide dismutase (EC 1.15.1.1) was measured by the inhibition of nitroblue tetrazolium reduction mediated by the xanthine/xanthine oxidase-generated superoxide anions and monitored spectrophotometrically at 560 nm (McCord et. al., 1969). Mn-superoxide dismutase was inhibited by the addition of 0.2 nM KCN to the tissue homogenates. Total activity of Superoxide dismutase was measured without KCN solution. Cu, Zn- superoxide dismutase activity was calculated from the differences subtracting the activity of Mn-suproxide dismutase from total superoxide dismutase activity. One unit activity was defined as amount of enzyme required inhibiting the rate of nitroblue tetrazolium reduction by 50%.

Catalase. Catalase (EC 1.11.1.6) activity was determined by following reduction of hydrogen peroksidase as describe by Aeibi et. al. (1974). The samples of liver were homogenized using a Potter-Elvehjem type Teflon homogenizer. The homogenizing buffer consisted of 320 mM sucrose, 1mM EDTA, and 10 mM Tris-HCl (pH 7.4). Homogenates were centrifuged at 13,600 x g for 30 min. Using the resulting supernatant fraction, catalase activity was measured spectrophotometrically at 240 nm and one unit activity was defined as amount of enzyme, which liberates half the peroxide oxygen from a hydrogen peroxide solution per minute.

Gluthathione peroxidase. Gluthathione peroxidase (EC 1.11.1.9) activity was measured as the reduction of NADPH at 340 nm by the method of Paglia (1967) and one unit activity was expressed as amount of enzyme necessary to reduce one micromole of NADPH per minute. Samples of liver were homogenized using a Potter-Elvehjem type Teflon homogenizer. The homogenizing buffer consisted of 50 mM Tris-HCl, 5 mM EDTA, and 1 mM 2- mercaptoethanol. Homogenates were centrifuged at 10,000 x g for 20 min at 2 – 8 ^{o}C. The resulting supernatants were used for measuring gluthathione peroxidase activity. One unit activity was defined as the amount of enzyme necessary to reduce one micromole of NADPH per minute.

α-Tocoperol. Samples of liver were cut into small pieces and weighed about 100 mg. Then, immediately added with 1% NaCl solution in the tubes used for saponification. An amount of 6% ethanolic pyrogallol and 60% KOH was added, and the mixture was

saponified at 70°C for 60 min. NaCl solution 1 % was added and the whole mixture was extracted with 10% ethyl acetate in *n*-hexane. After centrifugation at 3,000 rpm for 5 min, the *n*-hexane layer was collected and added with decane/hexane mixture (5 : 95, v/v), then was evaporated. α-Tocoperol was measured by HPLC equipped with fluorescence detector with excitation at 297 nm and emission at 327 nm, using 2,2,5,7,8-penthamethyl-6-chromanol (Wako Chemical Industries Co.Ltd., Osaka, Japan) as an internal standard according to the method of Ueda et.al. (1987). Nucleosil $5NH_2$ was used for the analytical column and for the mobile phase *n*-hexane/2-propanol (97:3, v/v) was used, with a flow rate of 2 ml/min.

Protein. The protein content was determined using bicinchoninic acid (Smith et. al., 1985) with bovine serum albumin as the standard.

Statistical Analysis

Significance of differences between the Choline-sufficient and the choline-deficient groups were determined by Student *t*-test (Snedecor et. al., 1989) Values of $P<0.05$ were considered to be significant.

RESULTS

The food intake, body weight gain and liver weight are shown in Table 2. The food intake and body weight gain were no significantly different between the choline-sufficient and choline-deficient groups, though the liver weight of the rats fed on the choline-deficient diet was significantly heavier than that of rats fed on the Choline-sufficient diet.

The liver lipid contents are shown in Table 3. The contents of total lipids, triacylglycerol, cholesterol, and phospholipids were significantly increased in liver of the rats fed on the choline-deficient diet. Lipids levels and ornithine carbamoyltransferase activity in the serum are shown in Table 4. The serum levels of triacylglycerol, cholesterol, and phospholipids were significantly reduced by the choline deficiency. Feeding the choline-deficient diet increased the activity of ornithine carbamoyltransferase in the serum.

The activities of superoxide dismutases, catalase , and glutathione peroxidase in liver are shown in Table 5. Total superoxide dismutase and Cu, Zn- superoxide dismutase activities in liver significantly decreased after feeding the choline-deficient diet. No differences were observed on Mn-superoxide dismutase activity. Choline deficiency also reduced the activities of glutathione peroxidase and catalase in liver. α-Tocoperol level decreased in the liver of rats fed on the choline-deficient diet.

Table 2. Food intake, body weight gain, and liver weight of rats fed on either choline-sufficient or choline-deficient diets.

Diets	Food intake (g/21 days)	Body wt. gain (g/21 days)	Liver wt. (g/100 g of body wt.
Choline-sufficient	226 ± 6 a	88.8 ± 2.8 a	4.87 ± 0.07 b
Choline-deficient	230 ± 8 a	80.9 ± 3.7 a	8.64 ± 0.18 a

Data represent means ± SEM for six rats

a,b Means within the same vertical column that do not share a common letter were significantly different ($P<0.05$).

Table 3. The liver lipid contents of rats fed on either choline-sufficient or choline – deficient diets.

Diets	Total lipids (mg/g of liver)	Triacylglycerol (mg/g of liver)	Cholesterol (mg/g of liver)	Phospholipids (mg/g of liver)
Choline-sufficient	58.4 ± 1.2 b	18.3 ± 0.8 b	3.45 ± 0.06 b	36.6 ± 0.7 b
Choline-deficient	347 ± 9.0 a	144 ± 5.0 a	3.91 ± 0.17 a	199 ± 6.0 a

Data represent means ± SEM for six rats
a,b Means within the same vertical column that do not share a common letter were significantly different (*P<0.05*).

Table 4. Lipids levels and ornithine carbamoyltransferase activity in the serum of rats fed on either choline-sufficient or choline-deficient diets.

Diets	Total lipids (mg/dl)	Triacylglycerol (mg/ dl)	Cholesterol (mg/ dl)	Ornithine Carbamoyltransferase (U/L)
Choline-sufficient	342 ± 31 a	82.0 ± 4.4 a	233 ± 15 a	4.27 ± 0.59 b
Choline-deficient	143 ± 14 b	67.5 ± 3.7 b	163 ± 9.0 b	17.6 ± 3.4 a

Data represent means ± SEM for six rats
a,b Means within the same vertical column that do not share a common letter were significantly different (*P<0.05*).

Tabel 5. The liver antioxidant enzymes activities and α-tocoperol of rats fed on either choline-sufficient or choline-deficient diets.

	Choline-sufficient	Choline-deficient
Superoxide dismutase (SOD)		
- Total SOD (U/mg protein)	47.9 ± 2.8 a	30.4 ± 3.1 b
- Cu, Zn-SOD (U/mg protein)	40.4 ± 2.8 a	24.8 ± 3.8 b
- Mn- SOD (U/mg protein)	7.52 ± 1.47 a	5.61 ± 1.45 a
Catalase (mU/mg protein)	1.22 ± 0.06 a	5.61 ± 1.45 b
Glutathione peroxidase (U/mg protein)	0.295 ± 0.015 a	0.238 ± 0.006 b
α-tocoperol	0.675 ± 0.085 a	0.316 ± 0.024 b

Data represent means ± SEM for six rats
a,b Means within the same horizontal column that do not share a common letter were significantly different (*P<0.05*).

DISCUSSION

The result of this study clearly shown that choline deficiency caused an accumulation of lipids in the liver (Table 3). The total lipids in the liver of rats fed the choline-deficient diet were significantly higher than those of rats fed the basal diet. Choline is required for hepatic secretion of lipoproteins. Lipoprotein are produced from lipids and the protein in the endoplasmic reticulum and golgi apparatus in the liver, and then transported into the serum as very low density lipoprotein (VLDL) which contain high amount of triacylglycerol. Hepatic secretion of VLDL into blood requires active phosphatidylcholine biosynthesis (Yao et.al., 1988), and in the choline deficiency, the diminished capacity of liver cells to synthesis new phosphatidylcholine molecules

resulted in accumulation of triacylglycerol in the liver. In fact, serum triacylglycerol content was significantly lower in the rats fed the choline-deficient diet (Table 4).

Ornithine carbamoyltransferase activity was significantly increased in serum of rats fed the choline deficient diet (Table 4). Ornithine carbamoyltransferase is specially located in the liver with low values in other tissues (Jones et.al., 1961). In mammals, ornithine carbamoyltransferase is a mitocondrial enzyme that catalyses a step in the urea cycle, that detoxifies ammonia. This process occurs almost exclusively in the liver of mammalian cells. The release of such a mitochondrial enzyme from the liver may have arisen from hepatic necrosis and also be associated with a specific form of liver disease.

The antioxidant defense system is composed mainly of three enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. The superoxide dismutases convert superoxide anion into H_2O_2 and O_2. Catalase catalyzes the dismutation of H_2O_2, forming as neutral product O_2 and H_2O. Gluthathione peroxidase catalyzes the reductive destruction of hydrogen and lipid hydroperoxides, using gluthatione as an electron donor (Harris, 1992). A significant decline in the level of liver superoxide dismutase, catalase, glutathione peroxidase and α-tocopherol (Table 5) of rats fed the choline-deficient diet was observed in this study. The decreased in enzymatic and non-enzymatic antioxidant protection might induce the elevation in lipid peroxidation level (Miyasawa et.al., 1984). The increase in amount of free oxygen radicals accompanied by a concomitant with increased in cellular damage, which commonly indicated by increase in lipid peroxidation product. Normally, membranes are probably being exposed regularly to low amount of free radicals, this may be protected by some defensive mechanism. Such mechanism the availability of phosphatidylcholine that has been reported to favor the breakdown of lipid peroxides, may be induced by free radicals (Miyasawa et.al., 1984).

CONCLUSION

The decrease enzymatic and nonenzymatic antioxidant contents might be responsible for the increase in superoxide anion in the liver. In addition, the increased in serum ornithine carbamoyltransferase activity might be due to the increased in triacylglycerol in the liver which associated with liver damage induced by choline deficiency.

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