ELSEVIER

Journal of Nutritional Biochemistry 13 (2002) 403–410

THE JOURNAL OF Nutritional Biochemistry

Dietary orotic acid affects antioxidant enzyme mRNA levels and oxidative damage to lipids and proteins in rat liver

Masashi Morifuji, Yoritaka Aoyama*

Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kita-ku, Sapporo 060-8589, Japan

Received 8 August 2001; received in revised form 30 January 2002; accepted 13 February 2002

Abstract

We investigated the effects of the dietary addition of orotic acid on liver antioxidant enzymes, mRNA levels of these enzymes, and peroxidative products by comparing casein with soy protein as the source of dietary protein. Rats fed the casein diet accumulated more liver lipids than those fed the soy protein diet when orotic acid was added. The addition of orotic acid lowered both the activity of liver Cu, Zn-superoxide dismutase and the level of Cu, Zn-superoxide dismutase mRNA. The addition of orotic acid led to a significant increase in the contents of conjugated dienes and protein carbonyls in the liver. In addition, dietary soy protein protected the increase in the levels of lipids and proteins peroxide induced by orotic acid. The addition of orotic acid to the casein diet increased the activities of both serum ornithine carbamoyltransferase and alanine aminotransferase. Thus, liver damage might result from the increased superoxide anion due to the decrease in the activity of hepatic superoxide dismutase, as well as increase in the production of hepatic peroxidative products in rats fed the casein diet with orotic acid.

Keywords: Orotic acid; Casein; Soy protein; Antioxidant enzyme mRNA; Lipids peroxide; Liver damage

1. Introduction

The content of liver lipids is changed by certain dietary conditions. For example, a severe fatty liver develops in rats fed either a choline-deficient [1,2] or an orotic acid diet [3,4]. Orotic acid, an intermediate in pyrimidine biosynthesis, is normally present in bovine milk (80 mg/L) [5]. Standerfer and Handler first observed that the addition of orotic acid resulted in lipid accumulation in rat liver [3]. One of the reasons responsible for lipid accumulation in the liver induced by dietary orotic acid is the lower synthesis or transport of VLDL from liver into serum [6]. Casein has generally been used as a sole protein source in the formation of fatty liver in rats fed orotic acid [3,4]. In our previous reports, the addition of orotic acid to the casein, but not to the egg protein, the soy protein, or the wheat gluten diets, increased serum ornithine carbamoyltransferase activity [7,8], indicating that orotic acid added to the casein diet may induce liver damages.

Dietary proteins affect the response of lipid metabolism. Many studies have indicated that soy protein is associated with the reduction of cardiovascular risk since, compared with casein and other animal proteins, soy protein reduces plasma cholesterol in animal models [9–13]. The effect of soy protein on blood cholesterol concentrations in the rabbit was first reported in the 1940s [10]. Recent results have suggested that soy protein significantly lowered total and LDL cholesterol and maintained HDL cholesterol concentrations in mildly hypercholesterolemic men [13]. Indeed, soy protein has been shown showed to reduce hepatic triacylglycerol [14]. However, its role in the regulation of lipid metabolism is not clearly defined.

Much attention is focused on the involvement of oxidative stress in aging and disease. Because of their high diffusibility and reactivity, reactive oxygen species, such as superoxide anion, hydrogen peroxide, and OH radical, attack and damage the key biological structure, including lipid, protein, and DNA [15]. Reactive oxygen species have been proposed as the attacking agents on polyunsaturated fatty acids in cell membranes. Lipid peroxidation results in reversible and irreversible cell and tissue damage, and is suspected to be strongly associated with aging, cancer, arteriosclerosis, and so on [16]. Reactive oxygen species are also known to affect antioxidant enzyme activities [17].

* Corresponding author. Tel.: +81-11-706-2811; fax: +81-11-706-2504.

E-mail address: aoyama@chem.agr.hokudai.ac.jp (Y. Aoyama).

0955-2863/02/$ – see front matter
PII: S0955-2863(02)00182-1

Table 1
Composition of the experimental diets

Ingredients	Casein g/100 g	Soy protein g/100 g
Casein[a]	24.92	
Soy protein[b]		24.97
Vitamin mixture (AIN-93)[c]	1.0	1.0
Choline bitartrate[d]	0.25	0.25
Mineral mixture (AIN-93G)[c]	3.5	3.5
Soybean oil[d]	1.0	1.0
Sucrose[e]	69.33	69.28

[a] New Zealand Dairy Board (Wellington, New Zealand).
[b] Fuji Oil Co., Ltd. (Izumisano, Osaka, Japan).
[c] P.H. Reeves, F. H. Nielsen, G. C. Fahey, J Nutr 123 (1993) 1939–1951.
[d] Wako Pure Chemical Industries, Ltd. (Osaka, Japan).
[e] Nipppon Beet Sugar MFG., Co., Ltd. (Obihiro, Hokkaido, Japan).

Therefore, the assessment of the activities of superoxide dismutase, catalase, and glutathione peroxidase is of interest because these enzymes are considered to be especially involved in the defense of the cell against reactive oxygen species.

Increased generation of reactive oxygen species has been described in several animal models of fatty liver, including models of alcohol administration [18], iron-free diets [19], or choline-deficient diets [2]. There is no report about orotic acid ingestion, which clearly causes the accumulation of hepatic lipids. The aim of this study is to clarify the effect of orotic acid on antioxidant enzyme mRNA levels and on the peroxidative products in the liver by comparing casein with soy protein as the source of dietary protein. Furthermore, we investigated the relation of liver damage and peroxidative products induced by feeding orotic acid.

2. Materials and methods

2.1. Animals

Male Wistar rats (Japan SLC, Inc., Hamamatsu, Shizuoka, Japan), with an initial body weight of about 96 g were used for this experiment. The rats were housed individually in stainless-steel wire-bottomed cages in an air-condition room, kept at a temperature of approximately 23°C, with a 12-hr light/dark cycle (lights on from 8:00 AM to 8:00 PM). This study complied with the Animal Experimental Guidelines of according to the Committee of Experimental Animal Care, Hokkaido University.

2.2. Diets

The composition of the diets is shown in Table 1. Casein (80.2 g as crude protein per 100 g; New Zealand Dairy Board, Wellington, New Zealand) and soy protein (80.1 g as crude protein per 100 g; Fuji Oil Co., Ltd., Izumisano, Osaka, Japan) were used as dietary protein sources and supplemented with 20 g of protein (N × 6.25) per 100 g to the diet [20]. Each of the two diets was then supplemented with or without orotic acid (1 g/100 g of diet; Wako Pure Industries, Ltd., Osaka, Japan). Dietary changes in the contents of protein and orotic acid were compensated for by adjusting the amount of sucrose in the diet. In this experiment, we prepared the diets, which were lower in fat and fiber, and higher in sucrose than the usual rodent diets, to be formulated to facilitate liver lipid accumulation.

Rats were allowed free access to food and water for 14 days. They were sacrificed by guillotine between 9:30 and 10:30 AM. The blood was collected and allowed to clot at room temperature. Serum was separated from whole blood by centrifugation at 3,000 × *g* for 10 min at 4°C. The liver was immediately removed, weighed, and used for RNA isolation. Samples of the liver and serum were stored at −80°C until analysis.

2.3. Measurement of serum and hepatic lipids

Liver total lipids, extracted and purified according to the method of Folch *et al.* [21], were gravimetrically estimated after removing the solvent. Liver triacylglycerol [22] and cholesterol [23] were measured by enzymatic methods, and the content of phospholipids in the liver was calculated by subtracting triacylglycerol and cholesterol from total lipids. Serum triacylglycerol and cholesterol were similarly determined. Serum phospholipids were measured by enzymatic methods [24].

2.4. Preparation of tissues for measurement of hepatic enzymes

An aliquot of frozen liver was homogenized with a Potter-Elvehjem type teflon homogenizer in 0.25 M ice-cold sucrose, 5 mM EDTA (pH 8.0), and 0.5% Triton X-100. The homogenate was centrifuged at 3,000 × *g* for 20 min. The supernatant was used for superoxide dismutase (EC 1.15.1.1) activity. For the assays of catalase (EC 1.11.1.6) and glutathione peroxidase (EC 1.11.1.9) activities, liver was homogenized in 0.32 M ice-cold sucrose, 10 mM Tris-HCl (pH 7.4), and 1 mM EDTA · 2Na. The homogenate was centrifuged at 13,600 × *g* for 30 min. The supernatant was used for estimating catalase and glutathione peroxidase. Protein content was determined using bicinchoninic acid [25] with bovine serum albumin as a standard.

2.5. Liver enzyme analysis

The activity of superoxide dismutase was measured by the inhibition of nitroblue tetrazolium reduction mediated via the xanthine/xanthine oxidase-generated superoxide anions and monitored spectrophotometically at 560 nm [26]. Cu, Zn-superoxide dismutase activity was inhibited by the addition of 0.2 mM KCN to the tissue homogenates. Total activity of superoxide dismutase was measured without

KCN solution. Cu, Zn-superoxide dismutase activity was calculated by subtracting Mn-SOD activity from total activities. One unit of superoxide dismutase activity was defined as the amount of enzyme required to inhibit the rate of nitroblue tetrazolium reduction by 50%.

Catalase activity was assayed according to the method of Aebi, following the decomposition of hydrogen peroxide at 240 nm [27]. One unit of catalase activity was defined as the amount of the enzyme required to decrease in 1 μmol of hydrogen peroxide per min.

Glutathione peroxidase activity was determined by NADPH oxidation using a coupled reaction system consisting of glutathione, glutathione reductase and *t*-butyl hydroperoxide [28]. One unit of glutathione peroxidase activity was defined as the amount of enzyme needed to oxidize 1 μmol of NADPH per min.

2.6. Isolation of total RNA

Total RNA was isolated from the liver by the Chomczynki and Sacchi method [29]. The liver (approximately 0.1 g) was homogenized with 1 ml of Isogen solution (Nippon Gene Co., Ltd., Tokyo, Japan) in a homogenizer, and 0.2 ml of chloroform was then added to the homogenate. The suspension of samples was centrifuged at 20,000 $\times$ *g* for 15 min at 4°C. The aqueous phase was transferred to a fresh tube, mixed with 0.5 ml isopropanol, and centrifuged at 20,000 $\times$ *g* for 10 min at 4°C. The RNA pellet was then dissolved in 1 ml of 75% ethanol, allowed to stand for 30 min at −80°C and reprecipitated at 20,000 $\times$ *g* for 30 min at 4°C. The RNA pellet was dried, and then dissolved in 50 μl of milliQ water. The concentration of RNA was measured from the absorbance at 260 nm (the ratio at 260/280 was between 1.6 and 1.9).

2.7. Extraction of mRNA

150 μg of total RNA was dissolved in 50 μl of milli Q water, and then 50 μl of 2 $\times$ E solution [20 mM Tris buffer (pH 7.5) containing 2 mM EDTA and 0.2% SDS] and 100 μl of Oligotex were added, and subsequently allowed to stand for 5 min at 65°C before 20 μl of 5 M NaCl solution was added. The solution was thoroughly mixed, incubated for 10 min at 37°C and then centrifuged at 20,000 $\times$ *g* for 3 min at 4°C. The resulting precipitation was dissolved in 100 μl of washing buffer, and then centrifuged at 20,000 x *g* for 3 min at 4°C. 100 μl of milliQ water was added to the precipitation and suspended. The solution was incubated for 5 min at 65°C and centrifuged at 20,000 $\times$ *g* for 3 min at 4°C. The resulting upper solution was mixed with 40 μl of 2.5 M sodium acetate solution and 260 μl of 99.5% ethanol, and allowed to stand for 30 min at −80°C. Then the mixture was centrifuged at 20,000 $\times$ *g* for 15 min at 4°C, and 500 μl of ice cold 70% ethanol was added to the resulting precipitation and mixed. After centrifugation at 20,000 $\times$ *g* for 15 min at 4°C, the precipitation was dried for 15 min. Poly (A) RNA solution was obtained by adding 10 μl of milliQ water.

2.8. Northern blot analysis of Cu, Zn-SOD, catalase, and glutathione peroxidase mRNA

Cu, Zn-SOD, catalase, and glutathione peroxidase mRNA levels were assessed by Northern blot analysis using 3.5 μl of a poly (A) RNA solution extracted from the liver. mRNA was denatured for 5 min at 65°C in a solution containing 5 μl of formamide, 1.5 μl of a 10 $\times$ MOPS buffer (pH 7.0), and 2 μl of formaldehyde, positioned in separate lanes of 1% agarose gel that had been prepared in a 1 $\times$ MOPS buffer (pH 7.0), and electrophoresed for 90 min to 50 V, essentially according to the Thomas method [30]. After electrophoresis, the RNA was transferred to a Gene Screen filter sheet and then hybridized overnight at 68°C with digoxygenin (DIG) RNA probes, as described in the next section. After washing the blots, hybridization was visualized by exposing Fuji X-Ray film, and the bands were quantified by densitometry. After detecting β-actin mRNA, we reprobed and detected Cu, Zn-SOD, catalase, and glutathione peroxidase mRNA expression.

2.9. Synthesis of Cu, Zn-SOD, catalase, and glutathione peroxidase probes

Cu, Zn-SOD, catalase, and glutathione peroxidase cDNA fragments were obtained by RT-PCR with synthetic gene-specific primers for rat Cu, Zn-SOD (forward 20-mer, 5′-TCT CGT CTC CTT GCT TTT TG-3′; reverse 20-mer, 5′-TTT CTT CAT TTC CAC CTT TTG -3′), catalase (forward 20-mer, 5′-GGA ACC CAA TAG GAG ATA AA-3′; reverse 20-mer, 5′-CGC TGA ACA AGA AAG TAA CC 3′), and glutathione peroxidase (forward 20-mer, 5′-ACA GAA CTG ACA GCG GAT TT -3′; reverse 20-mer, 5′-GAA TGC CTT AGG GGT TGC TA-3′), respectively. Cu, Zn-SOD cDNA was inserted into pGEM-T Easy Vector (Promega). The pGEM-T Easy was linearized with *Sty I* and used as a template for Digoxigenin-labeled RNA (DIG-RNA) probes. DIG-RNA probes were made by using a DIG RNA Labeling Kit (Roche Molecular Biochemicals, France). Catalase and glutathione peroxidase probes were labeled with DIG-PCR from the RT-PCR Products [31]. The DIG-RNA probe for Cu, Zn-SOD and the DIG-DNA probes for catalase, glutathione peroxidase, and β-actin were used for the Northern blot hybridization.

2.10. Determinations of conjugated dienes and protein carbonyls

Conjugated dienes were assessed spectrophotometically at 234 nm [32]. The contents of protein carbonyls were determined spectrophotometrically by measuring the optical density between 300 and 400 nm by the 2,4-dinitrophenylhydrazine methods [33].

Table 2
Food intake, body weight gain and liver weight

	Initial body weight g	Food intake g/14 days	Body weight gain g/14 days	Liver weight g/100 g body weight
Casein	96.2 ± 1.5	239 ± 7[a]	101.8 ± 2.4[a]	6.75 ± 0.32[c]
Casein + orotic acid	96.1 ± 1.4	213 ± 6[b]	77.5 ± 1.7[c]	8.37 ± 0.21[a]
Soy protein	96.2 ± 1.7	235 ± 8[ab]	91.3 ± 5.3[ab]	5.01 ± 0.11[d]
Soy protein + orotic acid	96.1 ± 1.8	218 ± 10[ab]	81.5 ± 5.5[ac]	7.59 ± 0.16[b]
ANOVA				
Protein		NS	NS	<0.001
Orotic acid		<0.05	<0.001	<0.001
Protein × orotic acid		NS	NS	<0.05

Data represent means ± SEM for six rats.
[a,b,c,d] Means within the same vertical column that do not share a common superscript letter were significantly different: $P < 0.05$.
NS: not significant.

2.11. Serum enzymes activities

Activities of serum ornithine carbamoyltransferase (EC 2.1.3.3) [34] and alanine aminotransferase (EC 2.6.1.2) [35] were measured as described previously [7].

2.12. Statistics

Data were subjected to two-way ANOVA [36] and Duncan's multiple range test [37]. Differences between groups were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Food intake and body weight gain

The amount of food consumed was almost the same between rats fed the casein and those fed the soy protein diets. The addition of orotic acid to the casein diet decreased food intake, but that added to the soy protein diet had no effect (Table 2).

The body weight gain of rats fed the casein diet was not significantly different from that of rats fed the soy protein diet. The addition of orotic acid to the casein diet lowered body weight gain, but that added to the soy protein diet had no effect (Table 2).

3.2. Liver weight

Whether rats were fed or not fed orotic acid, the liver weight of the soy protein group was significantly lighter than that of the casein group. The addition of orotic acid to each diet markedly enlarged the liver (Table 2).

3.3. Liver lipids

The contents of triacylglycerol, cholesterol, and phospholipids were significantly higher in rats fed the casein diet as compared with those fed the soy protein diet when orotic acid was not added. The addition of orotic acid to each diet increased the contents of liver triacylglycerol, cholesterol, and phospholipids (Table 3).

Table 3
Liver and serum lipids

	Liver			Serum		
	Triacylglycerol μ mol/g liver	Cholesterol	Phospholipids	Triacylglycerol mmol/L	Cholesterol	Phospholipids
Casein	50.5 ± 2.6[c]	10.3 ± 0.6[c]	113 ± 10[c]	1.98 ± 0.16[a]	3.14 ± 0.10[a]	3.00 ± 0.15[a]
Casein + orotic acid	105 ± 6[a]	17.0 ± 0.8[a]	274 ± 14[a]	0.320 ± 0.051[c]	0.803 ± 0.054[d]	0.836 ± 0.088[d]
Soy protein	10.2 ± 0.8[d]	5.48 ± 0.10[d]	46.8 ± 1.8[d]	1.81 ± 0.17[a]	2.03 ± 0.05[b]	2.09 ± 0.05[b]
Soy protein + orotic acid	82.0 ± 4.5[b]	13.9 ± 0.4[b]	234 ± 32[b]	0.613 ± 0.106[b]	1.34 ± 0.14[c]	1.04 ± 0.12[c]
ANOVA						
Protein	<0.001	<0.001	<0.001	NS	<0.001	<0.001
Orotic acid	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Protein × orotic acid	NS	NS	NS	NS	<0.001	<0.001

Data represent means ± SEM for six rats.
[a,b,c,d] Means within the same vertical column that do not share a common superscript letter were significantly different: $P < 0.05$.
NS: not significant.

Table 4
Liver antioxidant enzyme activities and mRNA levels

	Cu, Zn-superoxide dismutase		Catalase		Glutathione peroxidase	
	Activity U/mg protein	mRNA arbitrary unit	Activity U/mg protein	mRNA arbitrary unit	Activity mU/mg protein	mRNA arbitrary unit
Casein	4.97 ± 0.36^{b}	100 ± 15^{a}	521 ± 9^{ab}	100 ± 5^{ab}	327 ± 17^{b}	100 ± 11
Casein + orotic acid	3.40 ± 0.30^{c}	64 ± 6^{bc}	469 ± 15^{b}	77 ± 8^{b}	402 ± 30^{a}	108 ± 9
Soy protein	6.51 ± 0.30^{a}	91 ± 14^{ab}	534 ± 28^{a}	119 ± 16^{a}	317 ± 14^{a}	119 ± 13
Soy protein + orotic acid	4.24 ± 0.23^{bc}	54 ± 5^{c}	487 ± 13^{b}	89 ± 2^{ab}	396 ± 18^{a}	103 ± 10
ANOVA						
Protein	<0.001	NS	NS	NS	NS	NS
Orotic acid	<0.001	<0.001	<0.01	<0.01	<0.01	NS
Protein × orotic acid	NS	NS	NS	NS	NS	NS

Data represent means ± SEM for six rats.
[a,b,c] Means within the same vertical column that do not share a common superscript letter were significantly different: $P < 0.05$.
The ratio of the Cu, Zn-superoxide dismutase, catalase, and glutathione peroxidase mRNA/β-actin mRNA intensities were used to evaluate the relative levels and are shown as a percentage of the level in the casein group.
NS: not significant.

3.4. Serum lipids

In rats not fed orotic acid, the levels of cholesterol and phospholipids were higher in the casein group than in the soy protein group, while triacylglycerol was similar. Addition of orotic acid to either the casein or the soy protein diet significantly lowered the levels of triacylglycerol, cholesterol, and phospholipids (Table 3).

3.5. Liver antioxidant enzyme activities

Cu, Zn-superoxide dismutase activity was higher in the soy protein group than in the casein group. The addition of orotic acid to either the casein or the soy protein diet significantly decreased the Cu, Zn-superoxide dismutase activity. Mn-superoxide dismutase activity was not affected by the diets (data not shown).

The addition of orotic acid to either the casein or the soy protein diet increased the activity of glutathione peroxidase, whereas when it was added to the soy protein diet but not to the casein diet, the catalase activity decreased (Table 4).

3.6. mRNA levels of Cu, Zn-superoxide dismutase, catalase and glutathione peroxidase in liver

The addition of orotic acid to either the casein or the soy protein diet significantly decreased the level of Cu, Zn-superoxide dismutase mRNA. The patterns of catalase mRNA levels were similar to those of Cu, Zn-superoxide dismutase mRNA, but not significant. The level of glutathione peroxidase mRNA was not influenced by adding the orotic acid (Table 4).

3.7. The contents of conjugated dienes and protein carbonyls

In rats fed the diet without orotic acid, the content of conjugated dienes was higher in the casein group than in the soy protein group. The addition of orotic acid to either the casein or the soy protein diet led to a significant increase in the level of conjugated dienes. When orotic acid was not added, feeding of the soy protein diet caused a significant decrease in the content of protein carbonyls as compared with the casein diet. Feeding orotic acid caused a marked increase in the content of protein carbonyls. However, the content of the protein carbonyls in the casein group was not different from that of the soy protein group when orotic acid was added to the diets (Table 5).

3.8. Serum enzyme activities

The activities of both ornithine carbamoyltransferase and alanine aminotransferase in serum were similar between rats fed the casein and the soy protein diets when orotic acid was not added. The addition of orotic acid to the casein diet but not to the soy protein diet markedly increased the activities of both ornithine carbamoyltransferase and alanine aminotransferase (Table 6).

Table 5
Liver conjugated dienes and protein carbonyls

	Conjugated dienes arbitrary unit	Protein carbonyls nmol/mg protein
Casein	100 ± 2^{c}	1.39 ± 0.14^{b}
Casein + orotic acid	206 ± 6^{a}	2.01 ± 0.23^{a}
Soy protein	71 ± 3^{d}	0.838 ± 0.031^{c}
Soy protein + orotic acid	126 ± 9^{b}	1.73 ± 0.13^{ab}
ANOVA		
Protein	<0.001	<0.05
Orotic acid	<0.001	<0.001
Protein × orotic acid	<0.001	NS

Data represent means ± SEM for six rats.
[a,b,c] Means within the same vertical column that do not share a common superscript letter were significantly different: $P < 0.05$.
NS: not significant.

Table 6
Serum ornithine carbamoyltransferase (OCT) and alanine aminotransferase (ALT) activities

	OCT IU/L	ALT Kermen unit
Casein	3.83 ± 0.25^{b}	30.03 ± 1.3^{b}
Casein + orotic acid	13.2 ± 2.6^{a}	52.8 ± 5.8^{a}
Soy protein	3.30 ± 0.12^{b}	3.32 ± 5.2^{b}
Soy protein + orotic acid	4.98 ± 0.67^{b}	33.8 ± 1.9^{b}
ANOVA		
Protein	<0.01	<0.05
Orotic acid	<0.001	<0.01
Protein × orotic acid	<0.01	<0.01

Data represent means ± SEM for six rats.
[a,b] Means within the same vertical column that do not share a common superscript letter were significantly different: $P < 0.05$.
NS: not significant.

4. Discussion

The aim of this study is to clarify the effects of orotic acid on hepatic antioxidant enzyme activities, their mRNA levels, and the contents of conjugated dienes and protein carbonyls by comparing casein with soy protein as the source of dietary protein.

In this study, we reconfirmed that the contents of liver triacylglycerol, cholesterol and phospholipids increased in rats fed orotic acid (Table 3), as previously reported [7,8]. Orotic acid diets decreased the levels of serum lipids. Upon feeding casein and soy protein diets supplemented with orotic acid the serum triacylglycerol level was significantly decreased by 84% and 66%, respectively. Orotic acid selectively blocks the secretion by the liver of the very low density lipoprotein (VLDL) containing apo lipoprotein B [6]. This is not due to the inhibition of apo lipoprotein B synthesis, but to a modification to the packing of apo lipoprotein B with lipids at the trans-golgi compartment. Moreover, in an early report, an orotic acid diet inhibited the glycation of apo lipoprotein B [38]. However, this mechanism was completely unclear. Thus, an orotic acid diet blocks the transportation of VLDL from the liver to the serum, leading to the accumulation of lipids in the liver.

Furthermore, the dietary protein type affected the lipid metabolism response. This study also showed soy protein was more effective in decreasing the levels of cholesterol and phospholipids in serum, and that it lowered the contents of liver lipids (Table 3). In general, the intake of plant protein compared with animal protein showed the hypocholesterolmic effects in various animal species, including humans [7–13]. Two explanations for the different effects of plant and animal protein have been offered. One is based on a difference in the physicochemical properties of dietary proteins and luminal digestion products. Plant proteins suppress the intestinal absorption or reabsorption of cholesterol and bile acids, and thereby decrease the pool size of cholesterol within the body. Another is based on the differences in the amino acid composition of dietary proteins. The cystine, arginine, and glycine levels are markedly lower, and the lysine level is higher in the casein than in the soy protein, suggesting that the difference in the amino acid components might account for the lipid accumulation in the liver. However, the manner in which amino acid composition exerts its effects is not fully understood.

We confirmed for the first time that the addition of orotic acid decreased the hepatic Cu, Zn-superoxide dismutase activity (Table 4) as previously described [8]. Superoxide dismutase catalyzes the dismutation of superoxide anion into hydrogen peroxide. Therefore, it is possible that dietary orotic acid caused the increase in superoxide anion in the liver. One possible pathway by which dietary orotic acid induced the superoxide anion might exist. In previous reports, orotic acid ingestion decreased the hepatic ATP levels and increased the urinary output of uric acid [39]. In addition, fatty liver caused by orotic acid can be overcome by feeding alloprinol, which is an inhibitor of xanthine oxidase [40]. Xanthine oxidase contributes to the main generation source of superoxide anion [41]. In several reports, a dose of allopurinol improves the xanthine oxidase-derived oxidative stress in the liver [42,43]. Thus, orotic acid feeding might multiply superoxide production in the liver through exhausting the adenine nucleotide and activating the xanthine oxidase pathway.

Furthermore, catalase and glutathione peroxidase immediately detoxify hydrogen peroxide and convert lipid hydroperoxides to nontoxic alcohol. The addition of orotic acid significantly decreased hepatic catalase activity (Table 4). On the other hand, glutathione peroxidase showed higher activity (Table 4), and it seemed to be induced in orotic acid-fed rats without stimulation by hydrogen peroxide, which is produced by superoxide dismutase.

The addition of orotic acid to either the casein or the soy protein diet decreased both Cu, Zn-superoxide dismutase and catalase mRNA levels (Table 4). There were parallel changes between their activities and mRNA levels in the liver, respectively (Cu, Zn-superoxide dismutase: $n = 24$, $r = 0.60$, $p < 0.01$, catalase: $n = 24$, $r = 0.75$, $p < 0.001$). Such close parallelism strongly suggests that the expression of these enzymes is regulated by transcriptional control. Indeed, previous reports have also identified transcriptional control over antioxidant enzyme expression [44,45], and genetic regulatory elements and promoter sequences for antioxidant enzyme genes have been recognized [46,47]. However, the mechanism by which this control could operate is not known.

The addition of orotic acid had no significant effect on the glutathione peroxidase mRNA level (Table 4). There was no correlation between the enzyme activity and the mRNA level ($n = 24$, $r = 0.34$, $p = 0.10$). No increase in the glutathione peroxidase mRNA level could be explained by the instability of mRNA during storage. Furthermore, the evidence of post-transcriptional controls has previously been presented for antioxidant enzymes [48,49]. Therefore, it is possible that post-transcriptional controls, which cause

the enzyme activity change to be different from the mRNA level change, might be operating.

The marked increase in both conjugated dienes and protein carbonyls was observed by orotic acid-feeding (Table 5). A significant negative correlation was found between liver superoxide dismutase and conjugated dienes (n = 24, $r = -0.75$, $p < 0.001$), and the liver superoxide dismutase mRNA level and conjugated dienes (n = 24, $r = -0.48$, $p < 0.05$). Therefore, the decrease in liver superoxide dismutase activity and the mRNA level induced by the orotic acid might relate to the accumulation of lipid peroxide.

Indeed, dietary soy protein had a significant positive effect on Cu, Zn-superoxide dismutase activity, which might also have contributed to the lower levels of conjugated dienes and protein carbonyls in rats fed soy protein (Tables 4 and 5). The presence of variable amounts of biologically active components in soy protein, such as isoflavones, has the antioxidant effect of scavenging free radicals *in vitro* [50]. Soy protein used in this study contains 143 mg isoflavones/100 g soy protein, composed (in weight %) of 15.2% genistein, 52.1% genistin, 8.1% daizein, 24.5% daizin [51]. Wei et al. reported that isoflavones decreased xanthine oxidase-induced superoxide production in an HL-60 cell line [52]. Tasai and Huang also showed that the soy protein diet, compared with the ethanol-extracted soy protein diet (low in isoflavones), significantly decreased hepatic thiobarbituric acid-reactive substances in hamsters [53]. Therefore, the content of isoflavones in soy protein might have a beneficial effect on the antioxidant system in this study.

An increase in the activity of serum alanine aminotransferase is one of the signs of liver damage [35]. However, this enzyme is widely distributed not only in the liver, but also in the extrahepatic organs. On the other hand, ornithine carbamoyltransferase is specifically localized to the liver [54]. Therefore, estimating the activity of the latter enzyme in serum is considered to be more appropriate in demonstrating liver damage. Orotic acid added to the casein diet but not to the soy protein diet increased the activities of ornithine carbamoyltransferase and alanine aminotransferase in serum (Table 6). Thus, the addition of orotic acid to the casein diet but not to the soy protein diet might cause liver damage. In addition, hepatic superoxide dismutase activity was negatively correlated with serum ornithine carbamoyltransferase activity (n = 24, $r = -0.67$, $p < 0.001$), and a positive correlation was found between conjugated dienes and serum ornithine carbamoyltransferase activity (n = 24, $r = 0.75$, $p < 0.001$). Thus, these results clearly showed that a significant increase in serum ornithine carbamoyltransferase activity might result from the increased superoxide anion due to the diminished activity of hepatic superoxide dismutase and to the increased production of hepatic peroxidative products in rats fed the casein diet with orotic acid. Thus, a significant increase in peroxidative products might cause the liver damage.

Overall results indicated that the addition of dietary orotic acid to the diet containing casein as the protein source induced not only marked lipid accumulation in the liver, but also the decreased activity of liver superoxide dismutase, i.e., a possible increase in superoxide anion. Reactive oxygen species attack hepatic lipids, and induce the production of lipid peroxide. It was thought that lipid peroxide, either directly or indirectly, is responsible for much of the oxidative stress that damages living cells. Therefore, when the diet contained orotic acid, feeding the casein diet caused a massive increase in the contents of peroxidation products. Thus, the oxidative damage to lipids and protein might contribute to liver damage.

References

[1] B. Lombardi, G. Ugazio, A.N. Raick, Choline-deficiency fatty liver: Relation of plasma phospholipids to liver triglycerides, Am J Physiol 210 (1966) 31–36.

[2] I. Grattagliano, G. Vendemiale, P. Caraceni, M. Domenicali, B. Nardo, A. Cavallari, F. Trevisani, M. Bernardi, E. Altomare, Starvation impairs antioxidant defense in fatty livers of rats fed a choline-deficient diet, J Nutr 130 (2000) 2131–2136.

[3] S.B. Standerfer, P. Handler, Fatty liver induced by orotic acid feeding, Proc Soc Exp Biol Med 90 (1955) 270–271.

[4] J.Y. Cha, Y. Mameda, K. Yamamoto, K. Oogami, T. Yanagita, Association between hepatic triacylglycerol accumulation induced by administering orotic acid and enhanced phosphatidate phosphohydrolase activity in rats, Biosci Biotechnol Biochem 62 (1999) 508–513.

[5] B.L. Larson, H.M. Hegarty, Orotic acid in milks of various species and commercial dairy products, J Dairy Sci 62 (1979) 1641–1644.

[6] A.-M. Hebbachi, M.C.L. Seelaender, P.W. Baker, G.F. Gibbons, Decreased secretion of very-low-density lipoprotein triacylglycerol and apoliprotein B is associated with decreased intracellular triacylglycerol lipolysis in hepatocytes derived from rats fed orotic acid or n-3 fatty acids, Biochem J 325 (1997) 711–719.

[7] Y. Aoyama, M. Wada, Supplementation of orotic acid to the casein, but not to egg protein, soy protein, or wheat gluten diets increases serum ornithine carbamoyltransferase activity, J Nutr Biochem 11 (2000) 306–310.

[8] Y. Aoyama, M. Wada, M. Morifuji, Orotic acid added to the casein, but not to egg protein, soy protein, or wheat gluten diets increases 1, 2-diacylglycerol level and lowers superoxide dismutase activities in rat liver, Biosci Biotechnol Biochem 65 (2001), 2166–2173.

[9] K.K. Carroll, E.M. Kurowska, Soy consumption and cholesterol reduction: Review of animal and human studies, J Nutr 125 (Suppl. 3) (1995) S594–S597.

[10] D. Meeker, H.D. Kesten, Effect of high protein diets on experimental atherosclerosism of rabbits, Arch Pathol 31 (1940) 147–162.

[11] D. Kritchevsky, Dietary protein and experimental atherosclerosis, Ann N Y Acad Sci 676 (1993) 180–187.

[12] M. Sugano, K. Koba, Dietary protein and lipid metabolism: a multifunctional effect, Ann N Y Acad Sci 676 (1993) 215–222.

[13] S. Teixeira, S.M. Potter, H.-J. Weigel, S. Hannum, J.W. Erdman, C.M. Hasler, Dose-dependent effects of soy protein in hypercholesterolemic men, FASEB J 12 (1998) A237.

[14] N. Iritani, K. Nagashima, H. Fukuda, A. Katsurada, T. Tanaka, Effects of dietary proteins on lipogenic enzymes in rat liver, J Nutr 116 (1986) 190–197.

[15] A.T. Diplock, J.-L. Charleux, G. Crazier-Willi, F.J. Kok, C. Rice-Evans, M. Roberfroid, W. Stahl, J. Vina-Ribes, Functional food science and defense against reactive oxygen species, Br J Nutr 80 (Suppl. 1) (1998) S77–S112.

[16] R.G. Cutler, Genetic stability and oxidative stress, in: I. Emerit, B. Chance (Eds.), Free Radicals and aging, Birkhauser Verlag, Basel, Switzerland, 1992, pp. 31–46.
[17] P.M. Huang, L.H. Chen, Y. Osio, D.A. Cohen, Effects of diet composition on liver antioxidant defense and detoxification enzymes in mice with murine AIDS, Nutr Res 14 (1994) 1841–1851.
[18] C.S. Lieber, Biochemical and molecular basis of alcohol-induced injury to liver and other tissues, N Engl J Med 320 (1989) 1353–1354.
[19] M. Uehara, H. Chiba, H. Mogi, K. Suzuki, S. Goto, Induction of increased phosphatidylcholine hydroperoxide by an iron-deficient diet in rats, J Nutr Biochem 8 (1997) 385–391.
[20] Experimental Animal Nutrition Committee of the American Institute of Nutrition, Guidelines for describing diets for experimental animals, J Nutr 117 (1987) 16–17.
[21] J. Folch, M. Lees, G.H. Slone-Stanley, A simple method for the isolation and purification of total lipides for animal tissues, J Biol Chem 226 (1957) 497–509.
[22] U. Nagele, A.-W. Wahlefeld, J. Ziegenhorn, Lipids, fatty acids and derivatives. Triglycerides. Calorimetric method, in: H. U. Bergmeyer, (Ed.-in-chief), Methods in Enzymatic Analysis 3rd ed., VCH Publishers, Deerfield Beach, Florida, 1985, Vol. 8, pp. 12–18.
[23] J. Siedel, E.O. Nagele, J. Ziegenhorn, A.-W. Wahlefeld, Reagent for the enzymatic determination of serum total cholesterol with improved lipolytic efficiency, Clin Chem 29 (1983) 1075–1080.
[24] M. Takayama, S. Itoh, T. Nagasaki, I. Tanimizu, A new enzymatic method for the determination of serum choline-containing phospholipids, Clin Chim Acta 79 (1977) 93–98.
[25] P.K. Smith, R.H. Krohn, G.H. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal Biochem 150 (1985) 76–85.
[26] C. Beauchamp, I. Fridovich, Superoxide dismutase improved assays and an assay applicable to acrylamide gels, Anal Biochem 44 (1971) 276–287.
[27] H. Aebi, Catalase in vitro, Methods Enzymol 105 (1984) 121–126.
[28] D.E. Paglia, W.N. Valentine, Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase, J Lab Clin Med 70 (1967) 158–169.
[29] P. Chomozynski, N. Sacchi, N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction, Anal Biochem 162 (1987) 156–159.
[30] P.S. Thomas, Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose, Proc Natl Acad Sci USA 77 (1980) 5201–5205.
[31] R. Seibi, H.J. Holtke, R. Runger, A. Meindl, H. G. Zachau, R. Rasshofer, M. Roggendorf, H. Wolf, N. Arnold, J. Wienberg, Nonradioactive labeling and detection of nucleic acids. III. Applications of the digoxigenin system, Biol Chem Hoppe Seyler 371 (1990) 939–951.
[32] P.O. Recknagel, E.A. Glende, Spectrophotometric method of lipid conjugated dienes, Methods Enzymol 105 (1984) 331–337.
[33] R.L. Levine, D. Garland, C.N. Oliver, A. Amici, I. Climent, A.G. Lenz, B.W. Ahn, S. Shantiel, E.R. Stadtman, Determination of carbonyl content in oxidatively modified proteins, Methods Enzymol 186 (1990) 464–478.
[34] M. Ohshita, H. Takeda, Y. Kamiyama, K. Ozawa, I. Honjo, A direct method for the estimation of ornithine carbamoyltransferase activity in serum, Clin Chim Acta 67 (1976) 145–152.
[35] F. Wroblewski, J.S. La due, Serum glutamic pyruvic transaminase in cardiac and hepatic disease, Proc Soc Exp Biol Med 91 (1956) 569–571.
[36] G.W. Snedecor, W.G. Cochran, Two-way classification, in: Statistical Methods, 8th ed., The Iowa State University Press, Ames, Iowa, 1989, pp. 254–272.
[37] D.B. Duncan, Multiple range and multiple F tests, Biometrics 11 (1955) 1–42.
[38] L.A. Pottenger, G.S. Getz, Serum lipoprotein accumulation in the livers of orotic acid-fed rats, J Lipid Res 12 (1971) 450–459.
[39] D.S.R. Sarmae, H. Sidransky, Studies on orotic acid fatty liver in rats: factors influencing the induction of fatty liver, J Nutr 98 (1969) 33–40.
[40] H.G. Windmueller, L.H. Von Euler, Prevention of orotic acid-induced fatty liver with allopurinol, Proc Soc Exp Biol Med 136 (1971) 98–101.
[41] J.M. McCord, Oxygen-derived free radicals in postischemic tissue injury, N Engl J Med 312 (1985) 159–163.
[42] W. Karwinski, O. Soreide, Allopurinol improves scavenging ability of the liver after aschemia/reperfusion injury, Liver 17 (1997) 139–143.
[43] J. Vina, A. Gimeno, J. Sastre, C. Desco, M. Asensi, F.V. Pallardo, A. Cuesta, J.A. Ferrero, L.S. Terada, J.E. Repine, Mechanism of free radical production in exhaustive exercise in humans and rats; role of xanthine oxidase and protection by allopurinol, IUBMB Life 49 (2000) 39–44.
[44] S.J. Seo, H.T. Kum, G. Cho, H.M. Rho, G. Jung, Sp1 and C/EBP-related factor regulate the transcription of human Cu/Zn SOD gene, Gene 17 (1996) 177–185.
[45] K. Sato, K. Ito, H. Kohara, Y. Yamaguchi, K. Adachi, H. Endo, Negative regulation of catalase gene expression in hepatoma cells, Mol Cell Biol 12 (1992) 2525–2533.
[46] A.K. Jaiswal, Antioxidant response element, Biochem Pharmacol 48 (1994) 439–444.
[47] J. O'Prey, S. Ramsay, I. Chambers, P.R. Harrison, Transcriptional up-regulation of the mouse cytosolic glutathione peroxidase gene in erythroid cells is due to a tissue-specific 3′ enhancer containing functionally important CACC/GT motifs and binding sites for GATA and Ets transcription factors, Mol Cell Biol 13 (1993) 6290–6303.
[48] L.B. Clerch, A. Wright, D. Massaro, Dinucleotide-binding site of bovine liver catalase mimics a catalase mRNA-binding protein domain, Am J Physiol 270 (1996) L790–L794.
[49] Y.-S. Ho, M.S. Dey, J.D. Crapo, Antioxidant enzyme expression in rat lungs during hyperoxia, Am J Physiol 270 (1996) L810–L818.
[50] J.M. Hodgson, K.D. Croft, I.B. Puddey, T.A. Mori, J.J. Beilin, Soybean isoflavonoids and their metabolic products inhibit in vitro lipoprotein oxidation in serum, J Nutr Biochem 7 (1996) 664–669.
[51] W. Ni, S. Yoshida, Y. Tsuda, K. Nagao, M. Sato, K. Imaizumi, Ethanol-extracted soy protein isolate results in elevation of serum cholesterol in exogenously hypercholesterolemic rats, Lipids 34 (1999) 713–716.
[52] H. Wei, R. Bowen, Q. Cai, S. Barnes, Y. Wang, Antioxidant and antipromotional effects of the soybean isoflavone Genistein, Proc Soc Exp Biol Med 208 (1995) 124–130.
[53] P.-J. Tasai, P.-C. Huang, Effects of isoflavones containing soy protein isolate compared with fish protein on serum lipids and susceptibility of low density lipoprotein and liver lipids to in vitro oxidation in hamsters, J Nutr Biochem 10 (1999) 631–637.
[54] Y. Wakabayashi, The glutamate crossway, in: L. A. Cynober (Ed.), Amino Acid Metabolism and Therapy in Health and Nutritional Disease, CRC Press, New York, NY, 1995, pp. 89–98.