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Supplementing Bovine Milk Immunoglobulin G Prevents Rats Fed on a Vitamin E-Deficient Diet from Developing Peroxidation Stress

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Key Words

Milk whey · Immunoglobulin G · Lipoperoxidation · Catalase · Superoxide dismutase · Glutathione peroxidase

Abstract

Background/Aims: Bovine milk whey, which mainly includes β-lactoglobulin (58%), α-lactalbumin (22%) and immunoglobulin G (IgG; 11%), is a source of a wide range of proteins with a useful nutritional and physiological function. We previously demonstrated antiperoxidative effects of whey-derived α-lactalbumin and β-lactoglobulin in rats. The aim of the present study was to investigate the role of the IgG in antiperoxidative properties. ***Methods:*** Four-week-old, male Sprague-Dawley rats were fed a vitamin E-deficient diet (control) or a diet supplemented with 2.5% of an IgG-rich fraction for 4 weeks. Different antiperoxidative parameters were measured in blood and liver from the rats. ***Results:*** In comparison to the rats fed the control diet, those fed the IgG-containing diet were resistant to peroxidation stress. The IgG diet resulted in a decreased concentration of thiobarbituric acid-reactive substances in red blood cells (RBC) and plasma, and increased activities of catalase, superoxide dismutase and glutathione peroxidase in the RBC and liver. The plasma density (d) < 1.063 g/ml lipoproteins from the IgG group were more resistant against the induced lipid peroxidation. ***Conclusion:*** These results demonstrate antiperoxidative properties of IgG. The underlying mechanism(s) remain to be determined.

Introduction

Our previous studies showed that an antiperoxidative effect of fermented bovine milk products by different kinds of lactic acid bacteria and bifidobacteria was specifically attributed to the whey protein content [1]. The bovine milk whey contains about 20% of the total milk protein. The average composition of the whey protein is as follows: 58% β-lactoglobulin, 22% α-lactalbumin, 11% immunogloublin G (IgG), 7% serum albumin, 2% IgA and traces of lactoferrin [2]. Our previous study also showed that whey protein isolate, compared with casein, suppressed peroxidation stress in rats fed a low vitamin E-containing diet [3]. Furthermore, this whey protein effect could not be simply replaced by an amino acid mixture of a similar composition, suggesting the importance of the protein structure or the order in which peptides or amino acids are released during the digestion of whey proteins [3]. In fact, supplementing α-lactalbumin or β-lactoglobulin, in comparison with bovine milk casein, resulted in an antiperoxidative action in rats fed a low vitamin E-containing diet. These antiperoxidative properties of α-

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Table 1. Composition of experimental diets (g/kg diet)

Ingredients	VE-sufficient control	VE-deficient	
		control	IgG
Casein	200	200	175
IgG-rich fraction	–	–	25
Safflower oil	97.7	–	–
VE-stripped safflower oil	–	97.7	97.7
Butter oil	2.3	2.3	–
Vitamin mixture	10	–	–
VE-free vitamin mixture	–	10	10
Mineral mixture	35	35	35
Choline bitartrate	2	2	2
DL-Methionine	3	3	3
Cellulose	5	5	5
α-Corn starch	15	15	15
Sucrose	450	450	452.3

VE = Vitamin E.

lactalbumin and β-lactoglobulin might be attributed to the amounts of sulfur-containing amino acids in α-lactalbumin and γ-glutamylcysteine residue in β-lactoglobulin, respectively [4]. Because those are used as a precursor for glutathione (GSH) synthesis in various tissues [4]. Recently, many reports have focused on isolation and application of bovine milk whey proteins as a physiologically functional food ingredient [5–7], especially in cancer prevention [8–11]. In the present study, we investigated the antiperoxidative effect of whey IgG in rats fed a vitamin E-deficient diet.

Materials and Methods

Animals and Diet

Four-week-old, male Sprague-Dawley rats (Seiwa Experimental Animals, Fukuoka, Japan) were acclimatized on a commercially available nonpurified diet (NMF, Oriental Yeast, Tokyo, Japan) for 1 week. Then, the rats were divided into three dietary groups, 6 rats each, and given free access to the AIN-76™-based purified diets [9] as shown in table 1.

In order to create conditions that increase the cellular oxidative stress of the rats, the experimental diet contained vitamin E-stripped safflower oil and a vitamin E-free vitamin mixture [3]. The vitamin E-deficient diet (control diet) therefore contained about 15 mg α-tocopherol per kg diet, which was 20% less than the vitamin E-sufficient diet (about 75 mg vitamin E/kg diet). A vitamin E-sufficient diet was also prepared as a positive control. To the vitamin E-deficient diet 2.5% bovine milk-derived IgG-rich fraction (12.5% of total proteins; Snow Brand Milk Products, Saitama, Japan) was added and it was referred to as IgG diet. Since the IgG-rich fraction contained 86% protein and 9.2% fat, an equivalent amount of milk fat, as butter oil, was added to the control diets at the expense of safflower oil. The diets contained about 4,100 kcal/kg diet. The rats were housed individually in a temperature-controlled room at 22–25 °C with a 12-hour light:dark cycle and fed the purified diets for 4 weeks. Food and nonionized water were freely available throughout the experimental period. The body weight and food intake were recorded every other day.

After food had been removed for 10 h, the rats were killed by withdrawing blood from the abdominal aorta under light diethyl ether anesthesia. Blood was collected in tubes to which heparin had been added and centrifuged at 1,000 *g* for 10 min to separate plasma and red blood cells (RBC). The erythrocyte lysates (20%, v/v) were prepared by lysing aliquots of the washed RBC with deionized water [12]. The liver was excised, and the mitochondria were isolated from a portion as already described [13]. The remainder of the liver as well as the portions of the RBC lysates and plasma were kept at −70 °C until needed for analysis. This experiment was carried out according to the guidelines for animal experiments of the Faculty of Agriculture and Graduate Course at Kyushu University and Law No. 105 and Notification No. 6 of the government of Japan.

Preparation of Plasma Lipoproteins and Oxidative Modification of the d < 1.063 g/ml Lipoprotein Fractions

Pooled plasma from 2 rats was used to prepare plasma lipoproteins according to the method described previously [14]. Fresh plasma containing 2.69 mmol/l of Na_2-EDTA was adjusted for density (d) to 1.063 g/ml with KBr. Two lipoprotein fractions (d < 1.063 g/ml and d > 1.063 g/ml) were separated by spinning at 1.6×10^6 *g* for 18 h at 4 °C in an ultracentrifuge (Beckman, Palo Alto, Calif., USA). The upper layer containing lipoproteins of d < 1.063 g/ml was harvested by tube slicing and extensively dialyzed overnight against phosphate-buffered saline containing 10 μmol/l of Na_2-EDTA. Transition metal ion-dependent peroxidation of the plasma lipoproteins of d < 1.063 g/ml was induced by adding 1 mmol/l ascorbic acid and 0.1 mmol/l $FeSO_4 \cdot 7\ H_2O$ in water [15]. Incubation was carried out in a water bath at 37 °C with constant shaking. The samples were removed at appropriate intervals and the oxidation reaction was terminated by adding a final concentration of 20 μmol/l butylated hydroxytoluene and 2 mmol/l Na_2-EDTA. Lipid peroxidation was evaluated by the thiobarbituric acid (TBA) assay [16].

Analysis

Blood samples were analyzed for hemoglobin by the cyanomethemoglobin method (Cyanomethemoglobin Test, Wako Pure Chemicals, Osaka, Japan). The in vitro hemolysis test was performed according to the methods described by Mino et al. [17]. Freshly prepared RBC were treated with chloroform-ethanol as described previously [18] and assayed for the activities of catalase (EC 1.11.1.6) [19] and superoxide dismutase (SOD, EC 1.5.1.1) [20]. RBC lysates were also analyzed for TBA-reactive substances (TBARS) as described previously [21]. Aliquots of the postmitochondrial fraction were treated with Drabkin's reagent to remove hemoglobin as described previously [22] and assayed for the activities of catalase, SOD as mentioned above and for the activity of glutathione peroxidase (GSHPX, EC 1.11.19) [23]. Mitochondrial TBARS were measured as described previously [24].

Total lipids in liver, plasma and the plasma lipoproteins d < 1.063 g/ml were extracted by the sodium dodecyl sulfate procedure [25], and their α-tocopherol content was measured by high-perfor-

Table 2. Growth parameters of rats fed the vitamin E-deficient diet containing milk IgG

Groups	Body weight gain, g	Food intake g/day	Food efficiency g gain/g food intake	Liver %
VE-sufficient				
Control	224±10	22.4±0.7	0.36±0.01	4.1±0.2
VE-deficient				
Control	224±6	21.8±0.5	0.37±0.01	3.8±0.1
IgG	211±7	22.5±0.4	0.34±0.01	3.7±0.1

Data are mean ± SE for 6 rats. VE = Vitamin E.

Table 3. Some antioxidant parameters of rats fed the vitamin E-deficient diet containing milk IgG

Parameters	VE-sufficient control	VE-deficient	
		control	IgG
Blood hemoglobin, g/dl	14.3±0.1[a]	14.3±0.2[a]	15.0±0.2[b]
RBC hemolysis inhibition, %	99.6±0.0[a]	98.8±0.2[b]	99.3±0.3[a,b]
TBARS			
RBC, nmol/g He	33.9±0.8[a]	38.1±1.5[b]	32.8±1.5[a]
Plasma, nmol/ml	12.1±0.5[a]	16.7±1.2[b]	11.4±2.0[a]
Mitochondria, nmol/g liver	13.8±1.0	16.3±1.8	15.8±0.8
α-Tocopherol			
Plasma, μg/ml	56.8±4.9[a]	24.7±1.7[b]	24.2±1.8[b]
Plasma lipoproteins, μg/ml plasma			
d < 1.063 g/ml	51.1±4.9[a]	22.0±1.8[b]	21.8±1.8[b]
d > 1.063 g/ml	5.7±0.1[a]	2.7±0.1[b]	2.4±0.1[b]
Liver, μg/g	111.4±10.8[a]	40.2±8.6[b]	42.6±4.9[b]
Glutathione, mg/g liver			
Total	0.32±0.0[a]	0.28±0.0[b]	0.30±0.0[a,b]
Reduced (GSH)	0.22±0.0	0.18±0.0	0.18±0.0
Oxidized (GSSG)	0.09±0.0	0.09±0.0	0.11±0.0

Data are mean ± SE for 6 rats per groups. Means with different superscripts are significantly different ($p < 0.05$). VE = Vitamin E.

mance liquid chromatography (Waters 600E, Japan Millipore, Tokyo, Japan) according to a method described previously [26] with a slight modification using the Zorbax SIL column (4.6 mm × 25 cm; Rockland Technologies, USA) and mobile phase mixture composed of n-hexane:dioxan:isopropanol (985:10:5, v/v). Plasma and the lipoprotein lipids were enzymatically determined with commercially available kits (Cholesterol C Test, Phospholipid B Test and Triglyceride G Test, Wako Pure Chemicals). Liver lipids were extracted by the method of Folch et al. [27] and subjected to chemical determination for cholesterol [28], triglycerides [29] and phospholipids [30]. The fatty acid composition of the total lipids in the lipoproteins of d < 1.063 g/ml was determined as described previously [31].

Statistical Analysis

All data were expressed as mean ± SE and statistical differences were determined by Duncan's multiple range test [32].

Results

Data in table 2 shows the growth parameters of rats. Diets had no significant effect on growth parameters of rats, such as body weight gain, food intake, food efficiency and relative liver weight. The daily energy intake (means ± SE) was 91.8 ± 2.8, 89.2 ± 2.2 and 91.9 ± 1.7 kcal/day for the vitamin E-sufficient, vitamin E-deficient control and IgG group, respectively.

Table 3 shows the concentration of blood hemoglobin, in vitro hemolysis of RBC, the concentration of TBARS in RBC, plasma and liver, concentrations of α-tocopherol in plasma, plasma d < 1.063 g/ml, lipoproteins and liver or concentration of glutathione in liver. The rats fed the

Table 4. Activities of catalase, SOD and GSHPX in RBC and liver of rats fed the vitamin E-deficient diet containing milk IgG

Parameters	VE-sufficient control	VE-deficient	
		control	IgG
RBC, U/g He			
Catalase	1,539±76[a]	823±55[b]	3,317±261[c]
SOD	1,863±31[a]	1,275±75[b]	1,610±48[c]
GSHPX	25.3±0.6[a]	22.2±0.8[b]	24.3±1.2[a,b]
Liver, U/g liver			
Catalase	1,325±53	1,276±69	1,198±38
SOD	1,054±13[a]	883±23[b]	1,018±14[a]
GSHPX	30.5±1.3	29.3±0.9	32.0±1.6

Data are mean ± SE for 6 rats per group. Means with different superscripts are significantly different ($p < 0.05$). VE = Vitamin E.

Table 5. Plasma, plasma d < 1.063 g/ml lipoproteins and liver lipids of rats fed the vitamin E-deficient diet containing milk IgG

Parameters	VE-sufficient control	VE-deficient	
		control	IgG
Plasma, mg/dl			
Total cholesterol (A)	86.8±2.3	85.8±5.3	73.1±6.0
HDL cholesterol (B)	52.2±0.3[a]	54.7±0.5[b]	49.7±0.8[c]
B/A	0.64±0.1	0.61±0.1	0.69±0.1
Triglycerides	182±21	167±20	142±20
Phospholipids	165±3.1	164±6.8	148±7.3
Plasma lipoproteins (d < 1.063 mg/ml), μg/ml plasma			
Total cholesterol	30.4±0.1[a]	29.7±0.6[a]	25.1±0.1[b]
Triglycerides	138±1.7[a]	114±1.2[b]	114±1.7[b]
Phospholipids	43.6±0.43[a]	38.3±0.52[b]	35.3±0.35[c]
Liver, mg/g			
Total cholesterol	9.31±1.1	7.92±0.2	8.76±0.6
Triglycerides	38.3±8.9	26.3±3.3	24.0±3.0
Phospholipids	8.53±0.2[a]	9.51±0.3[b]	9.27±0.3[a]

Data are mean ± SE for 6 rats per group. Means with different superscripts are significantly different ($p < 0.05$). VE = Vitamin E.

IgG diet had a slightly higher concentration of blood hemoglobin compared to the other groups. Feeding rats the vitamin E-deficient diet increased tissue peroxidation stress, as reflected by increasing the in vitro hemolysis of RBC, increased concentrations of TBARS in RBC and plasma or decreased concentrations of α-tocopherol in plasma and liver. On the other hand, the IgG diet partially protected the RBC and plasma from peroxidation stress, as reflected by a reduction of TBARS in these tissues. The IgG diet, however, did not prevent the reduction of α-tocopherol concentrations in plasma and livers of the rats. Table 3 also shows the concentrations of glutathione in the livers. The vitamin E deficiency tended to lower the total glutathione content in the liver, although there was no significant difference between the vitamin E-deficient control and the IgG group.

Table 4 shows the activities of catalase, SOD and GSHPX in RBC and livers of the rats. As a result of peroxidation stress, the rats fed the vitamin E-deficient diet, compared to those fed an adequate amount of vitamin E, had a significant reduction of the activities of catalase, SOD and GSHPX in RBC and the activity of SOD in the liver. Supplementing the milk IgG-rich fraction restored the activities of these enzymes to the same level as in the rats fed the vitamin E-sufficient diet.

Table 5 shows the concentration of lipids in plasma and liver of the rats. The IgG diet showed a hypolipidemic effect and resulted in lower lipid concentrations in plasma and plasma d < 1.063 g/ml lipoprotein fraction, although it was not always significant. However, liver lipids remained unaffected.

Figure 1 shows the in vitro time course transition metal ion-mediated peroxidation of plasma d < 1.063 g/ml lipoprotein fractions. Their lipid compositions are shown in table 5. There was no significant difference in the fatty acid composition between the dietary groups. The lipoproteins from the rats fed the vitamin E-sufficient diet were resistant to the peroxidation stress and the TBARS values increased very slowly throughout the 12-hour incubation with ferrous ions. On the other hand, the concentration of TBARS increased rapidly in the lipoproteins from rats fed the vitamin E-deficient diets. The lipoproteins from the IgG diet group were more resistant to the peroxidation stress than those from the vitamin E-deficient control group.

Discussion

The antioxidative properties of bovine milk whey IgG were examined in rats fed a vitamin E-deficient diet. In normal animal cells, there is an appropriate balance between prooxidant and antioxidant. This balance would be shifted toward the prooxidant if the levels of antioxidants were diminished by feeding a vitamin E-deficient diet. In the present study, rats fed the vitamin E-deficient control diet had a lower concentration of vitamin E in the

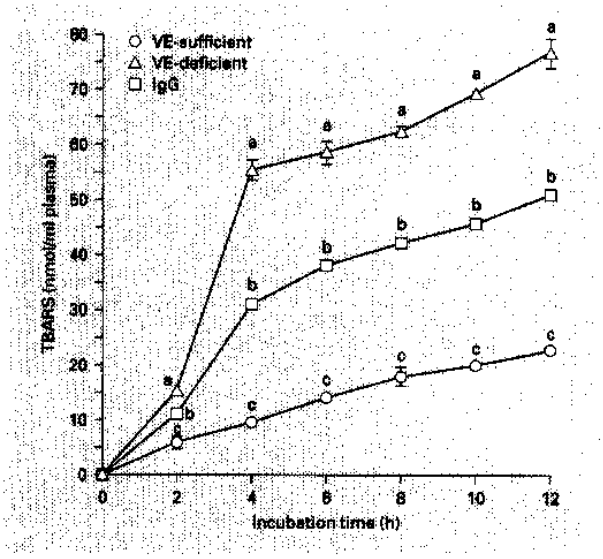

Fig. 1. In vitro transition metal ion peroxidation of plasma d < 1.063 g/ml lipoprotein fractions of rats fed the vitamin E (VE)-deficient diet containing milk IgG. Each point and bar show means ± SE of three independently pooled plasma samples. Different superscript letters show significant differences at $p < 0.05$.

plasma and liver by about 50%, increased concentrations of TBARS in RBC and plasma, or reduced activities of the antioxidative enzymes in the RBC and liver. However, this diet was found to exert an antioxidant effect when supplemented with 2.5% of milk IgG. The IgG diet increased the activities of catalase and SOD in the RBC and SOD in the liver, lowered TBARS in the plasma and RBC and delayed the in vitro peroxidation of plasma d < 1.063 g/ml lipoprotein fraction by transition metal iron.

The resistance of plasma lipoproteins from the IgG diet against peroxidation stress did not appear to be attributed to its vitamin E content (table 3), lipid composition (table 5) or polyunsaturated fatty acid content (data not shown). It has been shown that the length of the lag period before the acceleration of the peroxidation in human LDL by Cu^{2+} is not related to the vitamin E concentration of LDL, but is correlated with the LDL content of peroxide [33, 34]. Therefore, it is likely that dietary supplementation of IgG lowered the in vitro formation of lipid peroxides under conditions where dietary vitamin E is deficient.

Bounous et al. [4] also attributed the anticarcinogenic effect of whey to its content of γ-glutamylcysteine residue. They hypothesized that the glutathione-promoting activity of dietary whey protein concentrate is dependent on the glutamylcysteine groups located in serum albumin, β-lactoglobulin and possibly IgG. In the present study, feeding the vitamin E-deficient diet to rats decreased the concentration of total glutathione in the liver. However, supplementation of the diet with the IgG-rich fraction tended to have no prominent effect on the level of the glutathione. Furthermore, no data is available in the literature regarding the presence of γ-glutamylcysteine residue in bovine milk IgG or a specific biological action of the dietary IgG in the free radical stress-related disorders.

However, several studies suggested that bovine milk IgG might have the potential to modulate immune responses in vivo [35–37], thereby preventing inflammatory reactions that cause a series of oxidative reactions [38–41]. Reilly et al. [42] suggested that proteolytic enzymes in the stomach and intestine initially degrade antibodies or IgG to Fab and Fc fragments, and these fragments retain some of their neutralizing activity locally in the gastrointestinal tract, thereby preventing gastrointestinal infections caused by enteric pathogens such as *Escherichia coli*. Therefore, it remains to be determined whether dietary IgG could potentiate an antioxidative action through the intestinal mucosa.

In summary, our study shows that milk whey IgG exhibited an antioxidative effect and specifically participated in the overall observed antiperoxidative action of milk whey.

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