SCI.REP.KYOTO PREF.UNIV.(NAT.SCI.&LIV.SCI.),NO.42,Ser.B, p.1～6(NOV.1991)

Effects of Dietary Marine Fish Oil on Free Radical-Removing Enzyme Activities and Glutathione in Rat Livers.

YOSHIHO KATAYAMA and CHIKAKO SHIMOMURA

(Received August 13,1991)

Abstracts

In order to elucidate effects of dietary marine fish oil on free radical generation and scavenging, the changes of activities of radical-removing enzymes [GST, GSH·Px, γ-GTP, SOD and xenobiotic-metabolizing enzyme (MFO)] and glutathione were examined on rat hepatic tissues. The diet consisted of 5 % (5 GM), 20 % (20 GM) wheat gluten and 20 % casein (control) as a protein source. These diets contained adequate amounts of corn oil, minerals, vitamins and corn starch as the other nutrients. In the case of 5 GM and 20 GM groups, corn oil in the diets was replaced by marine fish oil (Gadus macrocephalus).

Food intake, body weight (body weight gain) and liver weight of the 5 GM group were significantly lower than those of the 20 GM and the control groups, although there were no differences in these parameters between the 20 GM and the control groups. For total hepatic GSH contents, low protein diet group (5 GM) showed marked decrease, whereas a significant rise of hepatic GSH content was found in the 20 GM group. GST activity of the 5 GM reduced significantly, although 20 GM group activity did not differ from the control level. In the comparison with dietary corn oil and marine fish oil, there was a different evidence that marine fish oil decreased significantly GST activity in low protein diet group and reversely this oil apparently increased it in 20 % protein diet group. Enzyme activities of SOD and γ-GTP were not different between the 5 GM, 20 GM and control groups. GSH·Px activity of the 5 GM group increased significantly and MFO activity of 20 GM group decreased markedly.

Key words : GSH, GST, GSH·Px, SOD, γ-GTP, free radical, rat liver, marine fish oil.

Introduction

Effects of protein levels in diets containing corn oil on free radical-removing enzyme activities were investigated previously by us[1]. In our results, glutathione S-transferase (GST) activities decreased significantly together with decline of dietary gluten contents (control>20 G group>>10 G group>>5 G group). And then total GSH contents in livers under these nutritional conditions significantly decreased in the order of the control and 20 G group>>10 G group>>5 G group. While a positive linear relationship between total cystine consumption from diets and total hepatic GSH contents was observed, and furthermore a positive linear correlation

Laboratory of Nutritional-Physiology and Biochemistry, Department of Food Sciences and Nutrition, Kyoto Prefectural University, Shimogamo, Sakyo-ku, Kyoto 606, Japan.

between GST activities and total GSH contents in livers was also found previously.

Recently Hornstra et al[2]. describe that a diet rich in fish and fish products may be beneficial in the prevention of ishemic heart disease in human. Many investigators have focussed their interests on nutritional effects of fish and fish oil[2,3]. On the other hand, it is recently recognized that marine fish oil in which relatively large amounts of EPA and DHA are contained can be easily peroxidized, and various functions of cell membranes of biological organisms may be damaged by these lipid-peroxides.

Therefore we conducted in the present study to investigate the effects of marine fish oil in two kinds of diets (5 % and 20 % dietary protein levels) on free radical-removing enzyme activities.

Materials and Methods

Materials

Glycylglycine, L-γ-glutamyl-p-nitroanilide, cumene hydroperoxide, nitroblue tetrazolium, xanthine and xanthine oxidase (EC 1.2.3.2) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Glutathione reductase (EC 1.6.4.2), 2,4-dinitro-l-chlorobenzene and N,N,N'N'-tetramethyl-1,4-phenylenediamine were obtained from Boehringer Mannheim GmbH, Tokyo Kasei Kogyo Co. Ltd. and Aldrich Chemical Co., respectively. Glutathione reduced form and 5,5'-dithiobis (2-nitrobenzoic acid) were obtained from Nacalai tesque Co. (Kyoto). Tris(hydroxymethyl)aminomethane and bovine serum albumin, fraction V were purchased from Wako Chemicals (Osaka). Nicotinamide adenine dinucleotide phosphate reduced form (NADPH) was purchased from Oriental Yeast Co., LTD. (Tokyo).

Table 1. Composition of the diets.

	5GM	20GM	Control
Protein	5%Gluten[a]	20%Gluten[a]	20%Casein[b]
Oil	5%Marine fish oil[h]	5%Marine fish oil[h]	5%Corn oil[c]
Salt mixture[d]	5%	5%	5%
Vitamin mixture[e]	1%	1%	1%
Choline chloride[f]	0.2%	0.2%	0.2%
Corn starch[g]	83.8%	68.8%	68.8%
Total	100%	100%	100%

a : G-G gluten, Glico Nutritional Co., b : vitamin free casein, Oriental Yeast Co. Ltd., c : Corn salad oil, Ajinomoto Co. Inc., d : Oriental Yeast Co. Ltd. (composition : $CaHPO_4 \cdot 2H_2O$, 0.43 g, KH_2PO_4,34.31 g, NaCl, 25.06 g, Fe-citrate, 0.623 g, $MgSO_4 \cdot 7H_2O$, 9.98 g, $ZnCl_2$, 0.02 g, $MnSO_4$ 4~5 H_2O,0.121 g, $CuSO_4 \cdot 5H_2O$, 0.156 g, KI, 0.0005 g, $CaCO_3$, 29.29 g, $(NH_4)_6Mo_7O_{24}H_2O$, 0.0025 g, Total 99.993 g) e : Oriental Yeast Co. Ltd., (composition : vitamin A acetate 5×10^5IU, 93.2 mg(46,600 IU), vitamin D 4×10^7IU, 0.5825 mg(23,300 IU), vitamin E acetate, 1,200.0 mg, vitamin K_3, 6.0 mg, vitamin B_1 hydrochloride, 59.0 mg vitamin B_2, 59.0 mg, vitamin B_6 hydrochloride, 29.0 mg, vitamin B_{12}, 0.2 mg, vitamin C, 588.0 mg, D-biotin, 1.0 mg, folic acid, 2.0 mg, calcium pantothenate, 235.0 mg, nicotinic acid, 294.0 mg, inositol, 1,176.0 mg, filled up to 100 g with lactose), f : Sigma Chemical Co.(St. Louis, U.S.A.) 100 g of choline chloride was dissolved into 50% ethanol to a final volume of 200 ml, g : Amicol C, Nichiden Chemical Co.(Osaka), h : Marine fish oil, Riken Vitamin Co.

Diets

Three kinds of diets were prepared as indicated in Table 1. These diets consisted of 5 % (5 GM) and 20 % (20 GM) wheat gluten and 20 % casein (control) as a protein source. These diets contained adequate amounts of corn oil, minerals, vitamins and corn starch as the other nutrients. In the case of 5 GM and 20 GM groups, however, corn oil was replaced by marine fish oil (*Gadus macrocephalus*).

Animals and dietary treatments

Four-week old male rats of Sprague-Dawley Crj-CD strain (Charles River Japan Inc., Atsugi), weighing 70 to 80 g were housed individually in stainless steel cages in a temperature-controlled room (22±2℃, 64±12 % of relative humidity) with a 12-hr cycle of light and dark (lights on at 8 : 00). The rats were given a commercial diet (MF, produced by Oriental Yeast Co. Ltd., Tokyo) for 8 days prior to the experiment, and were divided into 3 groups of 5 to 10 rats. The initial body weights of rats were 128 to 157 g. The diet of each group and distilled water were given *ad libitum* to the rats for 24 to 25 weeks. The body weight and food intake for each rat were usually measured at 10 : 00 to 12 : 00 AM every other day.

Procedures for enzyme preparations and enzyme assay.

The rats were sacrificed by bleeding via abdominal aorta under light diethyl ether anesthesia. The liver tissues removed immediately after bleeding, were weighed and stored at −80℃ until the enzyme assay. The following procedure for enzyme samples was carried out at 4℃.

(1) Glutathione S-transferase (GST) [EC 2.5.1.18]

An approximately 0.5 g liver tissue sample was homogenized in 2.5 ml of 0.1 M potassium phosphate buffer (pH 6.5) with a Potter-Elvehjem glass homogenizer for 2 min. The homogenate was centrifuged for 1 hr at 10,000 Xg at 4℃ and GST activity was measured spectrophotometrically for the supernatant by the method of Habig and Jacoby[4,5] and Mannervik and Guthenberg[6]. The specific activity was expressed as k_1/min/mg protein.

(2) γ-glutamyl transpeptidase (γ-GTP) [EC 2.3.2.2]

The enzyme preparation was prepared from the 10,000 Xg precipitate in GST enzyme preparation above described. The precipitate was re-homogenized for 2 min within 5 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 1 % TritonX-100, and the consequent homogenate was centrifuged for 10 min at 100 Xg at 4℃. The γ-GTP activities were determined for this supernatant by the method of Meister et al[7]. The specific activity was expressed as micromoles of p-nitroaniline released/min/mg enzyme protein.

(3) Glutathione peroxidase (GSH·Px) [EC 1.11.1.9]

The GSH·Px activity was assayed by the method of Tappel[8]. About 0.5 g of liver tissue samples was homogenized in 4.0 ml of 10 mM potassium phosphate buffer (pH 7.0) for 2 min, and the homogenate was centrifuged at 13,000 Xg for 15 min and the supernatant was then recentrifuged at 27,000 Xg for 30 min. GSH·Px activity was determined for the final supernatant. The specific activity in this assay was expressed as μmol of NADPH oxidized/min/mg enzyme protein.

(4) Mixed function oxidase system (MFO)

About 0.5 g of rat liver tissue samples was homogenized in ice-cold sucrose solution (0.35 M sucrose containing 0.035 M $KHCO_3$, 0.025 M KCl, 0.004 M $MgCl_2$ and 0.02 M K_2HPO_4). Homogenate was centrifuged at 1,000 Xg for 10 min and supernatant was then recentrifuged at 12,000 Xg for 15 min. The microsomal fraction was obtained by ultracentrifugation of the 12,000 Xg supernatant for 60 min at 105,000 Xg with a Hitachi 65 P automatic preparative ultracentrifuge. The final precipitates suspended in 2 ml of 0.1 M Tris-HCl buffer (pH 7.5) were used as microsomal fraction samples. For the enzyme assay according to the modified method described by O'Brien and Rahimutula[9], the reaction mixture consisted of 2.6 ml of 0.1 M Tris-HCl buffer (pH 7.5) containing lmM EDTA, 0.2 ml of microsomal fraction and 0.1 ml of each 6 mM tetramethyl-phenylenediamine (TMPD) and 15 mM cumene hydroperoxide. The rate of TMPD oxidation was monitored spectrophotometrically at 610 nm in the first one minute of the reaction by measuring the formation of Wurster's blue free radical.

(5) Superoxide dismutase (SOD) [EC 1.15.1.1]

The assay of SOD activity was performed by the method of Imanari et al[10] and Mc Cord et al[11]. The enzyme solution was obtained from the 105,000 Xg supernatant in MFO enzyme preparation. A unit of SOD activity

was defined as that of enzyme required to produce 50 % inhibition of the rate of NBT reduction.

Biochemical assay

Protein content of enzyme preparation was determined by the method of Lowry et al[12]. The assay of total glutathione was performed by the method of Taniguchi et al[13,14]. and Tietze[15]. GSH contents were expressed as μmol glutathione/g liver tissue.

Statistical analysis

Each value represents mean±half range of confidence interval (confidence limit) at $p<0.05$. Statistical analysis was performed by the paired t test for difference between means. Level of significance was set at $p<0.05$.

Results and Discussion

Body weights and body weight gains

Body weights and body weight gains in parentheses of the 5 GM and 20 GM groups were 163±17 g (20±13 g) and 525±63 g (382±54 g) at 24 weeks, respectively and 165±16 g (23±13 g) for the 5 GM group at 25 weeks. In the comparison of these values between 5 GM and 20 GM groups, the 20 GM (normal protein level) group was significantly higher than that of 5 GM (low protein level) group. When compared with the 5 G and 20 G groups in previous results[1], these values at either experimental period were the almost same whether corn oil in the diet was replaced by marine fish oil or not.

Food intake

Each rat of the control group fed on 18.3±1.4 g of the diet per day, and food intakes per rat of the 5 GM and 20 GM groups were 8.6±1.1 g and 17.2±2.1 g, respectively. Food intake of the 5 GM group was significantly less than those of the 20 GM and the control groups. There was, however, no difference in food intake between the 20 GM and the control.

Liver weight (4.10±0.64 g) of the 5 GM group decreased much more than those of the 20 GM and the control groups, although there was no difference between the 20 GM (12.8±2.0 g) and the control (11.8±1.5 g) groups.

GSH contents in the liver tissues

Total glutathione contents in liver tissues are shown in Table 2. GSH content of the 5 GM group decreased more markedly than those of the 20 GM and the control groups. While feeding of normal protein level diet

Table 2. The total glutathione contents in rat livers.

	(μmol GSHeq/g of liver)
	total GSH
5GM	1.08±0.38 **
20GM	6.37±0.55 *
Control	5.00±0.83

Each value represents the mean ± half range of confidence interval (confidence limit) $p<0.05$.
* Significant difference from the control group at 95% level.
** Significant difference from the 20GM group at 95% level.

containing marine fish oil (20 GM group) resulted in a significant but weak rise of hepatic GSH content as compared with the control. When compared with 5 G and 20 G groups in the previous data[1], the value of the 5 GM group was significantly smaller than that (1.62±0.20) of the 5 G group, but the value of 20 GM group was apparently larger than that (5.12±1.19) of the 20 G group. Thus there was different evidence that lower hepatic GSH content due to feeding of marine fish oil was observed in low protein diet group and reversely higher hepatic GSH content due to feeding of the same oil was obtained in normal protein level diet group.

Activities of GST, γ-GTP, GSH·Px, SOD and MFO.

Average activities of these enzymes are indicated in Table 3.

Table 3.
Activities of Glutathione S-Transferase, γ-Glutamyl transpeptidase, Glutathione Peroxidase, Superoxide Dismutase and Microsomal Mixed-Function Oxidase in the Livers of the Rats fed Various Diets.

	5GM	20GM	Control group
Glutathione S-Transferase[1]	0.076±0.006 *	0.333±0.048	0.346±0.058
γ-Glutamyl Transpeptidase[2]	0.827±0.732	0.123±0.046	0.183±0.112
Glutathione Peroxidase[3]	0.336±0.135 *	0.217±0.038	0.180±0.038
Superoxide Dismutase[4]	1.70±1.10	1,08±0.49	1.58±0.20
The Microsomal Mixed-function[5] Oxidase system (Peroxidase activity)	24.82±5.19	19.30±3.37 *	31.76±8.05

1 : k_1 ($min^{-1}mg^{-1}$)
2 : Specific activity ($\mu M^{-1}min^{-1}mg^{-1}$)
3 : Specific activity (μmol of NADPH oxidized per min mg^{-1})
4 : Specific activity (units per mg)
5 : Specific activity ($\mu M^{-1}min^{-1}mg^{-1}$)
* Significant difference from the control group at 95% level.

GST activities

It had been certified previously that the first-order equation was applied to this GST reaction under the analytical conditions. Thus specific activity was expressed as k_1/min/mg protein. GST activity of 5 GM group showed significant reduction as compared with the control. For 20 GM group the activity revealed somewhat low level as compared with the control, although the difference between this group and the control was not significant. When compared with the previous results (5 G and 20 G dietary groups containing corn oil), the activity of the former group (5 GM) was apparently lower than that (0.110±0.011) of 5 G group. Whereas the latter group (20 GM) showed somewhat higher level as compared with 20 G group (0.230±0.039).

SOD activities

As shown in Table 3 there were no differences in the SOD activities between 5 GM or 20 GM and the control groups.

γ-GTP activity

γ-GTP activity of 5 GM group tended to be higher as compared with the control. There was, however, no significant difference between the 5 GM and the control groups. The activity of 20 GM group had an almost equal level to the normal.

GSH·Px activities

Glutathione peroxidase activity of 5 GM group increased significantly as compared with the control. While the activity tended to be higher in the liver of rats fed 20 GM diet, although this difference from the control was not significant.

MFO activities.

MFO activity of 5 GM group had a tendency to decrease and 20 GM group showed a significantly lower activity as compared with the control.

From these data above described, body weight (body weight gain), food intake and liver weight may be mainly influenced by differences of protein levels in diets rather than by species of dietary oil.

There was a different evidence that total hepatic GSH content significantly decreased in low protein diet group (5 GM), whereas 20 GM group showed a significant rise of hepatic GSH content. Thus it was demon-

strated that effects of feeding of marine fish oil on rat hepatic GSH content were modified by differences of protein levels in diets. Furthermore GST activity of 20 GM group was the almost same as the control level, but this group showed significant increase as compared with 20 G group containing dietary corn oil[1]. Namely, when protein in diets is at normal level (20 %), it is suggested that feeding of marine fish oil may produce more beneficial effects on the cellular GSH and GST activity rather than feeding of corn oil does. GSH·Px activities had a tendency to increase as compared with the control. Igarashi et al[16], however, have reported that GSH·Px activity occur a decrease by feeding of cod liver oil in comparison with corn oil. Their data are inconsistent with our present results. Our results may reflect the possibility that for purpose of protection from free radical damages, the elevation of GSH·Px activity may compensate for reduction of GST activity resulted from feeding of low gluten diet containing marine fish oil.

It is well known that SOD can be easily induced under an environment of high pressure oxygen or by treatments of free-radicals[17,18]. From our results, however, SOD activity was the almost same as the control level. It is suggested that SOD may not be induced by feeding of marine fish oil consisted of polyunsaturated fatty acids.

Acknowledgements

We wish to thank Riken Vitamin Co. for providing with marine fish oil and Glico Nutritional Co. for providing with G-G gluten and also members of co-laboratory for their technical assistance.

References

1) Katayama, Y. and Shimomura, C. *Abstracts of the 14th International Congress of Nutrition (in Seoul, Korea)* p 722 (1989).
2) Houwelingen, A.C.V., Kester, A.D.M. and Hornstra, G. : *Nutri. Res.* **9**, 1187~1196 (1989).
3) Jen, K-L, Alexander, M. Zhong, S., Rose, K., Lin, P.K.H. and Kasim, S.E. : *Nutri. Res.* **9**, 1217~1228 (1989).
4) Habig, WH, and Jakoby, WB. : *Methods in Enzymol. Jakoby, WB ed. Academic Press, New York.* **77**, 218~231 (1981).
5) Habig, WH, and Jakoby, WB. : *Methods in Enzymol. Jakoby WB ed. Academic Press, New York.* **77**, 398~405 (1981).
6) Mannervik, B. and Guthenberg, C. : *Methods in Enzymol. Jakoby WB ed. Academic Press, New York.* **77**, 231~235 (1981).
7) Meister, A., Tate, S.S. and Griffith, O.W. : *Methods in Enzymol. Jakoby, WB ed. Academic Press, New York.* **77**, 237~253 (1981).
8) Tappel, A.L. : *Methods in Enzymol. Fleischer, S. and Packer L. ed. Academic Press, New York.* **LII**, 506~513 (1978).
9) O'Brien, P.J. and Rahimutula, A.D. : *Methods in Enzymol. Fleischer S. and Packer L. ed. Academic Press, New York.* **LII**, 407~412 (1978).
10) Imanari, T., Hirota, M. and Miyazaki, M. : *Igaku no Ayumi.* **101**, 496~497 (1977) (in Japanese).
11) Mc Cord, J.M. and Fridovich, I. : *J. Biol. Chem.* **244**, 6049~6055 (1969).
12) Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. : *J. Biol. Chem.* **193**, 265~275 (1951).
13) Taniguchi, N., Tsukada, Y., Mukuo, K. and Hirai, H. : *Gann.* **65**, 381~387 (1974).
14) Taniguchi, N., Tsukada, Y. and Hirai, H. : *Biochim. Biophys. Acta.* **354**, 161~167 (1974).
15) Tietze F. : *Anal. Biochem.* **27**, 502~522 (1969).
16) Igarashi, O. and Nakano, I. : *Kasankashishitsu to Seitai (Lipid peroxide in biological organisms) Uchiyama, M., Matsuo, M. and Sagai, M. ed. Japan Sci. Soci. Press, Tokyo.* 80~97 (1987).
17) Yam, J., Frank, L. and Roberts, R.J. : *Pediatr. Res.* **12**, 115~119 (1978).
18) Frank, L and Massaro, D. : *Am. J. Med.* **69**, 117~126 (1980).
SCI.REP.KYOTO PREF.UNIV.(NAT.SCI.&LIV.SCI.),NO.42,Ser.B, p.1~6(NOV.1991)