Effects of Fish Oil Feeding Alone in Combination with Clofibric Acid on Serum Levels of Triacylglycerol and Cholesterol in Rats

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Rats were fed diets prepared with soybean oil (SO), perilla oil (PO), or fish oil (FO) for 4 weeks, and some of the rats received daily s.c. injections (100 mg/kg) of *p*-chlorophenoxyisobutyric acid (clofibric acid) for 1 week before being killed. The levels of serum triacylglycerol and cholesterol were conspicuously lower in FO-fed rats than in SO-fed rats. The administration of clofibric acid further decreased serum levels of triacylglycerol and cholesterol in SO-fed rats, but not in FO-fed rats. The decreased level of these serum lipids with FO-feeding alone were comparable to those observed in SO-fed rats which had received clofibric acid. The activity of peroxisomal β-oxidation in the liver of FO-fed rats was significantly higher (2.48 times) than that of SO-fed rats. The treatment of FO-fed rats with clofibric acid caused an additional increase in activity, as compared to the SO-fed control. The activity of peroxisomal β-oxidation in SO-fed rats treated with clofibric acid was 3.74 times that of the FO-fed control. In contrast to peroxisomal β-oxidation, the activities of catalase, glutathione (GSH) peroxidase towards hydrogen peroxide and GSH reductase were not increased by clofibric acid, regardless of the type of oil ingested. Moreover, the activities of GSH *S*-transferases towards 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) were both depressed to the same level by the administration of clofibric acid, regardless of the type of dietary oil, although FO-feeding significantly increased the activity of GSH *S*-transferase towards CDNB. PO comprises essentially the same effects on the parameters tested, to a lesser extent, compared with FO. Consequently, FO-feeding can reduce the dose of clofibric acid required to lower serum lipids, which concomitantly may prevent hepatocytes from oxidative stress that might be caused by an imbalance of hydrogen peroxide metabolism due to the increased activity of peroxisomal β-oxidation and to the decrease in the activities of detoxification by GSH *S*-transferase.

Key words —— fish oil, clofibric acid, cholesterol, triacylglycerol

INTRODUCTION

It has been established that the plasma level of cholesterol is one of the most powerful risk factors related to coronary heart disease.[1] The possibility that the plasma level of triacylglycerol is an independent risk factor in the disease has been suggested.[2,3] Therefore, reducing these serum lipids is considered essential for lowering the incidence of coronary heart disease. Hypolipidaemic drugs of fibrates effectively decrease the plasma levels of both cholesterol and triacylglycerol. However, the administration of these drugs is known to increase the formation of hydrogen peroxide by inducing peroxisomal β-oxidation.[4] Oxidative stress produced by the excess generation of hydrogen peroxide might be the cause of hepatocellular carcinomas in rodents that are chronically exposed to peroxisome proliferators.[5,6] There is considerable evidence of the beneficial effects of dietary fish oil (FO), rich in (n-3) polyunsaturated fatty acids, in lowering the incidence of cardiovascular disease,[7-9] and in preventing triacylglycerol accumulation by xenobiotics in the liver.[10] These effects seems to be due to a reduction of circulating triacylglycerol and cholesterol by eicosapentaenoic acid (20 : 5 (n-3)) and/or docosahexaenoic acid (22 : 6 (n-3)), major constituents of FO. Although the dietary intake of FO increases the hepatic activity of peroxisomal β-oxidation in rodents,[11-13] the

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ability of FO to enhance peroxisomal β-oxidation is much lower than that of clofibric acid, a hypolipidaemic fibrate and peroxisome proliferator.[11] Since, in addition to hypolipidaemic effects, the polyunsaturated fatty acids of the (*n*-3) series are considered to be essential for the development and function of the retina and brain,[14] the use of fibrates in combination with the dietary intake of FO might be beneficial. However, the pharmacological and toxicological significance of the combined effects of fibrates and FO have not been established.

In this context, the present study asked the following questions: (i) whether a synergistic or additive lipid-lowering effect can be expected in the combination of FO with clofibric acid, (ii) whether the additive elevation of hepatic activity of peroxisomal β-oxidation is caused by the combined use of FO and clofibric acid, (iii) whether FO enhances the inhibitory effects of clofibric acid on the glutathione (GSH)-requiring detoxification system, and (iv) whether effects similar to those of FO can be expected with perilla oil (PO), which is rich in α-linolenic acid (18 : 3 (*n*-3)), but not rich in either 20 : 5 (*n*-3) or 22 : 6 (*n*-3).

MATERIALS AND METHODS

Materials —— Clofibric acid, bovine serum albumin (BSA) and palmitoyl-CoA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); CoA, glutathione reductase (from yeast), NAD and NADPH were from Oriental Yeast Co. (Tokyo, Japan); GSH was from Wako Pure Chemicals Ind. Co. (Osaka, Japan); and oxidized glutathione from Seikagaku Corp. (Tokyo, Japan). Fish oil concentrate (TG 25) was obtained from Maruha Co. (Tokyo, Japan).

Treatment of Animals —— Male Wistar rats were used. After acclimatization to a standard diet for 1 week, rats (weighing 135—155 g) were assigned to one of three dietary groups and were fed diets prepared with soybean oil (SO), PO or FO. To the FO diet, 1.1% of safflower oil was added to supplement linoleic acid (18 : 2 (*n*-6)) to reach the minimal requirement. These diets provided 40% of the calories from oil. The fatty acid composition of the diets is presented in Table 1. The diet contained by weight 18.8% oil, 26.69% corn starch, 1.0% α-starch, 16.0% sucrose, 5.0% cellulose, 24.5% casein, 1.0% vitamin mix, 7.0% mineral mix and 0.01% butylated hydroxytoluene (Clea, Tokyo, Japan).[11] The diets were prepared on a weekly basis and stored at −20℃ under nitrogen. The leftover food was discarded daily. Rats were housed in stainlesssteel cages at 23℃ with a 12 h dark-light cycle. Rats were fed the experimental diets for 4 weeks, and some of them were subcutaneously administered clofibric acid (sodium salt form) at a dose of 100 mg/kg once a day for 1 week before being killed. After these treatments, all animals were killed.

Table 1. Fatty Acid Composition of Diets

Fatty acid	SO	PO	FO
16 : 0	11.0	6.7	6.4
16 : 1	0.3	0.2	9.3
18 : 0	4.2	2.1	2.7
18 : 1	24.6	19.4	10.3
18 : 2 (*n*-6)	52.8	14.6	18.6
18 : 3 (*n*-3)	7.0	56.9	2.9
20 : 3 (*n*-6)			0.9
20 : 4 (*n*-6)			1.1
20 : 4 (*n*-3)			1.2
20 : 5 (*n*-3)			33.1
22 : 4 (*n*-3)			0.7
22 : 5 (*n*-3)			1.9
22 : 6 (*n*-3)			10.8

Values represent weight percent of methyl esters. The numerical designation of fatty acids indicates their chain lengths and numbers of double bonds.

Preparation of Enzyme Source —— Livers were isolated and perfused with cold 0.9% NaCl. A piece of liver was frozen in liquid nitrogen and stored at −80℃ until the assay for peroxisomal enzymes. The remaining part of the liver was homogenized in 4 vol. of 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris–HCl buffer, pH 7.4. The homogenates were centrifuged at 20000 × *g* for 20 min. The supernatant was recentrifuged under the same conditions. The supernatant obtained was centrifuged at 105000 × *g* for 60 min. The resulting supernatant was used as a cytosolic fraction. The frozen liver was thawed at 4℃ and was homogenized in 9 vol. of the sucrose solution mentioned above. The homogenates were centrifuged at 600 x *g* for 10 min to remove the nuclear fraction. The post-nuclear supernatant was used for measuring the activities of peroxisomal enzymes. Blood was collected for the separation of serum by centrifugation. The protein concentrations were determined by the method of Lowry, *et al.*[15] using BSA as a standard.

Enzyme Assays —— Peroxisomal β-oxidation was assayed as cyanide-insensitive palmitoyl-CoA oxida-

tion, as described previously,[16] and the activity of catalase was measured according to Aebi.[17] The post-nuclear supernatant was employed as an enzyme source for these peroxisomal enzymes. Cytosolic fractions were used to assay GSH-peroxidase, GSH *S*-transferase and glutathione reductase. GSH peroxidase towards hydrogen peroxide was assayed according to Lawrence and Burk.[18] The activity of GSH *S*-transferase was determined employing CDNB and DCNB as substrates by the method of Habig, *et al.*[19] Glutathione reductase was assayed according to Carlberg and Mannervik.[20]

Measurement of Serum Lipids —— Triacylglycerol in serum was determined enzymatically using a diagnostic kit (Triglyceride E II-HA Test, Wako Pure Chemicals Ind. Co., Osaka, Japan),[21] by which the triacylglycerol concentration was corrected for free glycerol. Total cholesterol and free cholesterol in the serum were measured by enzymatic colorimetric methods with diagnostic kits (Cholesterol E-test and Free cholesterol E-test, respectively, Wako Pure Chemicals Ind. Co., Osaka, Japan).[22]

Data Analysis —— Analysis of variance was employed to test the significance of difference between dietary groups. Where differences were significant, statistical significance of the difference between any two means was determined using Sheffe's multiple range test. Statistical significance between the control and clofibric acid-treated group was determined by Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Rats were fed diets prepared with SO, PO or FO for 4 weeks, and some of the rats were administered clofibric acid at a dose of 100 mg/kg once a day for 1 week before being killed. No significant difference was observed in body weight among the six experimental groups, whereas the relative liver weights of the PO-fed and FO-fed rats were slightly higher than that of the SO-fed rats (Table 2). The administration of clofibric acid to rats produced considerable hepatomegaly, there being no significant difference in relative liver weight among the three dietary groups which received clofibric acid (Table 2).

Table 2. Effects of Dietary Oils and Clofibric Acid on Body Weight and Liver Weight

Parameters	Clofibric acid	SO	PO	FO
Body weight (g)	−	254± 9	238±18	239±22
	+	263±14	250±21	259±16
Liver weight (g)	−	12.09±0.52	12.71±1.52	12.69±1.24
	+	22.32±2.98*	20.51±2.28*	20.28±1.45*
Relative liver weight (% of body weight)	−	4.77±0.14[a]	5.36±0.26[b]	5.30±0.16[b]
	+	8.47±0.80*	8.18±0.35*	7.84±0.14*

Values represent means ± S.D. for four or five animals. Rats were fed the diets, prepared with SO, PO or FO, for 4 weeks. Some of the rats received S.C. injections once a day for 1 week before being killed. *a, b*) Differences in horizontal means without common alphabetical symbols are statistically significant. If no superscript appears, the differences of the means are not statistically significant. *, Differences are statistically significant between control groups and clofibric acid-treated groups within the groups of rats fed the same dietary oils.

Effects on Levels of Serum Lipids

The effects of dietary oils in combination with clofibric acid on the levels of serum lipids were examined (Fig. 1). Feeding rats an FO diet resulted in a marked reduction in the serum level of triacylglycerol, as compared with those fed SO or PO diets (Fig. 1A). Although the administration of clofibric acid lowered the serum triacylglycerol in SO-fed rats, no further reduction was brought about in FO-fed rats by the drug. The level of serum triacylglycerol in FO-fed rats was comparable to that of SO-fed rats treated with clofibric acid.

The serum levels of total cholesterol in FO-fed and PO-fed rats were lower than that of the SO-fed rats, with a greater extent of reduction in the FO-fed rats (Fig. 1B). The levels of both free cholesterol and cholesterol ester were lower in the FO-fed rats than in either SO-fed or PO-fed rats (Fig. 1C and D). Although the administration of clofibric acid to SO-fed and PO-fed rats conspicuously decreased the serum levels of total cholesterol and cholesterol ester, no further reduction in serum cholesterol was brought about by the treatment of FO-fed rats with clofibric acid (Fig. 1B and D). Consequently, the serum level of total cholesterol in FO-fed rats was comparable to that of the SO-fed or PO-fed rats which were administered clofibric acid. Clofibric acid did not substantially affect the level of free cholesterol, regardless of the type of dietary oil (Fig. 1C).

Effects on Peroxisomal β-Oxidation and Hydrogen Peroxide Detoxification in the Liver

The effects of dietary oils on peroxisomal

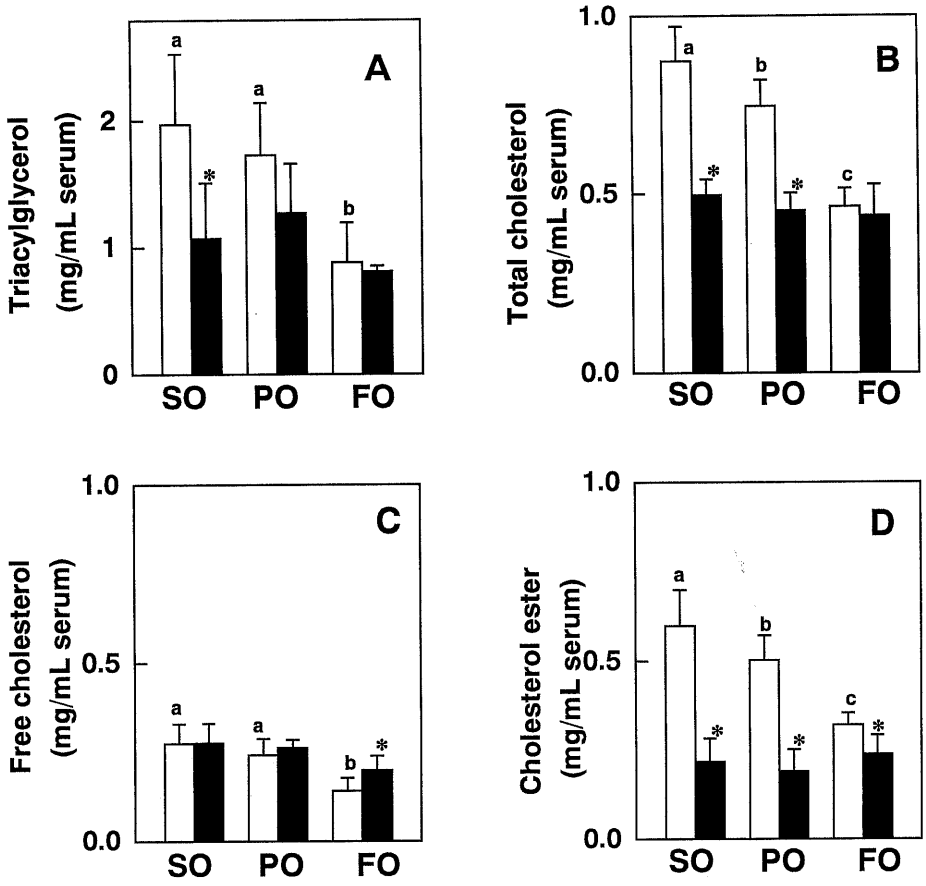

Fig. 1. Effects of Dietary Oils and Clofibric Acid on Serum Levels of Triacylglycerol and Cholesterol

Values represent means ± S.D. for four or eleven animals. The treatments of rats were the same as in Table 2. A, triacylglycerol; B, total cholesterol; C, free cholesterol; D, cholesterol ester. Open bars, control; closed bars, clofibric acid-treated. Differences in the means of three control groups and in three clofibric acid-treated groups without common alphabetical symbols are statistically significant. If no alphabetical symbol appears, the difference of the means was not statistically significant. *, Differences are statistically significant between control groups and clofibric acid-treated groups within the groups of rats fed the same dietary oils.

β-oxidation in the liver were examined (Table 3). The activities of peroxisomal β-oxidation of PO-fed and FO-fed rats were 1.63 and 2.48 times higher, respectively, than that of the SO-fed rats. The administration of clofibric acid to rats in these three diverse dietary conditions markedly induced peroxisomal β-oxidation (SO-fed, 9.28 times; PO-fed, 6.15 times; FO-fed, 4.65 times). The activity of FO-fed rats which received clofibric acid was 11.55 times greater than the SO-fed control, with this value being comparable to the sum (11.76 times) of the increase in the FO-fed control (2.48 times) and the SO-fed rats which received clofibric acid (9.28 times). A similar relation was found in the PO-fed and clofibric acid-treated rats, although there was no significant difference in the elevated activities by clofibric acid between the SO-fed and PO-fed rats. The activity of SO-fed rats which were treated with clofibric acid was 3.74 times higher than that of FO-fed rats.

Although catalase activity was little influenced by the difference in oil type intake, FO-feeding lowered the activity of GSH peroxidase towards hydrogen peroxide by 30%, compared with the levels of the SO-fed and PO-fed controls (Table 3). The treatment of rats with clofibric acid only slightly changed the activities of either catalase or GSH peroxidase, regardless of kinds of dietary oils ingested (Table 3).

The activity of glutathione reductase in the liver of FO-fed rats was slightly higher than that in either SO-fed or PO-fed rats; the administration of clofibric acid slightly decreased the activities of SO-fed and PO-fed rats (Table 3). Consequently, the activity of glutathione reductase in

Table 3. Effects of Dietary Oils and Clofibric Acid on Peroxisomal β-Oxidation and Detoxification of Hydrogen Peroxide in the Liver

Enzymes	Clofibric acid	SO	PO	FO
Peroxisomal	−	4.73±0.44[a)]	7.71±0.22[b)]	11.74±0.91[c)]
β-oxidation[1]	+	43.90±2.93*[a)]	47.38±3.66*[a,b)]	54.64±4.98*[b)]
Catalase[2]	−	17.52±1.86	17.16±1.56	16.98±1.44
	+	18.90±1.32	19.26±3.24	19.20±1.86
GSH peroxidase[1]	−	111.0±21.8[a)]	111.8±19.0[a)]	77.1±14.9[b)]
(with H_2O_2)	+	166.1±44.2*	135.3±20.6	110.4±27.3
Glutathione	−	95.4±3.2[a)]	91.7±3.0[a)]	122.0±7.0[b)]
reductase[1]	+	87.7±3.9*[a)]	89.7±2.3[a)]	102.0±5.8*[b)]

Values represent means ± S.D. for four or five rats. The treatments of animals were the same as in Table 2. *a, b, c)* Differences in horizontal means without common alphabetical symbols are statistically significant. If no superscript appears, the differences of the means are not statistically significant. *, Differences are statistically significant between control groups and clofibric acid-treated groups within the groups of rats fed the same dietary oils.
1 nmol/min/mg protein.
2 1/min/mg protein.

the liver of FO-fed rats was 1.4 times that of the SO-fed rats which received clofibric acid (Table 3).

Effects on GSH *S*-Transferase

The activity of GSH *S*-transferase towards CDNB in the liver of FO-fed rats was 1.3 times that of the SO-fed or PO-fed rats (Fig. 2A). Upon the administration of clofibric acid, the activities were markedly decreased to a similar level among the three dietary groups (Fig. 2A). The activity of GSH *S*-transferase towards DCNB was strikingly reduced to approximately 40% of the control by the treatment of rats with clofibric acid (Fig. 2B). Consequently, the activities of GSH *S*-transferase towards CDNB and DCNB in FO-fed rats were 2.5 and 2.8 times, respectively, higher than those of SO-fed rats treated with clofibric acid.

DISCUSSION

In the present study, the effects of dietary manipulation with SO, PO and FO in combination with the administration of clofibric acid were examined with regard to the effects on serum levels of lipids. In accordance with the previous study,[11] FO-feeding reduced serum levels of both triacylglycerol and total cholesterol more markedly than SO-feeding. The reduced levels of triacylglycerol and total cholesterol by FO-

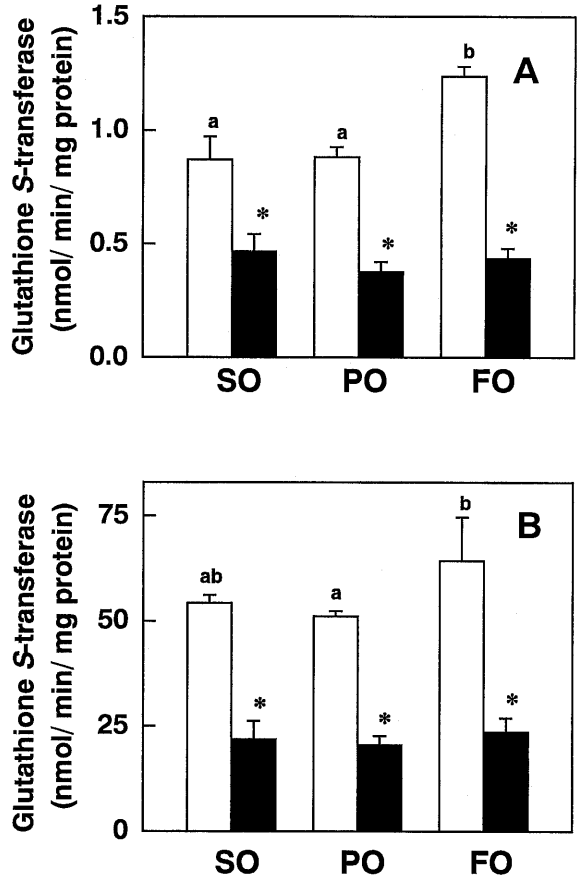

Fig. 2. Effects of Dietary Oils and Clofibric Acid on GSH *S*-Trasnferases in Livers

Values represent means ± S.D. for four or five animals. The treatments of rats were the same as in Table 2. A, GSH *S*-transferase towards CDNB; B, GSH *S*-transferase towards DCNB. Open bars, control; closed bars, clofibric acid-treated. Differences in the means of three control groups and in three clofibric acid-treated groups without common alphabetical symbols are statistically significant. If no alphabetical symbol appears, the difference of the means was not statistically significant. *, Differences are statistically significant between control groups and clofibric acid-treated groups within the groups of rats fed the same dietary oils.

feeding were comparable to the levels which were achieved in SO-fed rats by daily administration of clofibric acid at a dose of 100 mg/kg for 1 week. However, no potentiation in the reduction of the serum levels of lipids was observed by the combination of FO-feeding and clofibric acid treatment, indicative that a mechanism similar to that by which clofibric acid lowers serum lipids might operate for FO, and that the hypolipidaemic action of the drug depends on the type of dietary oil supplemented. Although inconsistent results were reported with regard to the extent of the reduction of serum cholesterol by FO,[11,12,23] the discrepancy seems to be attributable to differences in the concentrations of FO in diets and/or the duration of dietary treatments of

animals. Our previous study[11] suggested a reciprocal relationship between the hepatic content of 22 : 6 (*n*-3) and the serum level of cholesterol.

In addition to beneficial hypolipidaemic effects, fibrates are known to have undesirable activity in inducing peroxisome proliferation.[4-6] The present study showed that clofibric acid markedly induced peroxisomal β-oxidation in the three dietary conditions, and an additional increase in the activity was observed by the treatment of FO-fed rats with clofibric acid, as compared with the SO-fed control. The previous study[24] showed a similar additional increase in the activity of peroxisomal β-oxidation by the combined treatment of rats with partially hydrogenated marine oil and clofibrate. Since clofibrate increases the hepatic content of mRNA specific for peroxisomal acyl-CoA oxidase[25] and partially hydrogenated marine oil seems to enhance the activity by reducing the rate of acyl-CoA oxidase degradation,[26] a similar mechanism for partially hydrogenated marine oil might operate to increase the activity of peroxisomal β-oxidation by FO-feeding. However, a recent study[27] provided evidence that polyunsaturated fatty acids activate a peroxisome proliferator-activated receptor, as did Wy 14643, a potent peroxisome proliferator, whereas no significant difference was reported in the efficiency to activate the receptor between (*n*-6) fatty acids and (*n*-3) fatty acids. Therefore, further study is required to elucidate the detailed mechanism by which FO additionally increased the activity of peroxisomal β-oxidation.

The activities of catalase and GSH peroxidase, which are considered to be involved in the detoxification of hydrogen peroxide, were not substantially elevated by clofibric acid, without regard to dietary manipulation with oils. It is possible, therefore, that the administration of clofibric acid enhances the generation of hydrogen peroxide which exceeds the ability of the detoxifying system to scavenge, leading to an increase in oxidative stress for hepatocytes, and finally to hepatocellular carcinomas.[5,6] On the other hand, compared with SO-feeding, FO-feeding significantly increased the activity of peroxisomal β-oxidation and slightly depressed GSH peroxidase. Nevertheless, the increased activity of peroxisomal β-oxidation by FO-feeding alone was approximately one-fourth that of rats fed the SO diet and received clofibric acid, and the levels of serum cholesterol and triacylglycerol were the same as those of the animals under these two different treatments. Consequently, these results strongly indicate that FO intake causes a reduction of serum lipids without producing a substantial imbalance in the metabolism of hydrogen peroxide, and, therefore, avoids oxidative stress in the liver. In contrast to clofibrate, FO is not considered to increase the incidence of carcinoma.[28]

Clofibrate has been shown to depress the hepatic activity of GSH *S*-transferase,[29,30] and FO-feeding has been demonstrated to enhance the activity of GSH *S*-transferase towards CDNB.[12] The present study confirmed that clofibric acid strongly depressed the activities of GSH *S*-transferase towards both CDNB and DCNB, regardless of the differences in dietary oils supplemented. Since FO-feeding increased the activity of GSH *S*-transferase towards CDNB 1.3 times, compared with that of SO-fed control, the elevated activity in FO-fed rats was 2.5 times greater than that of the SO-fed rats which received clofibric acid. Similarly, the activity of GSH *S*-transferase towards DCNB in the liver of FO-fed rats was 2.8 times higher than that of the SO-fed and clofibric acid-treated rats.

PO comprises 18 : 3 (*n*-3), but neither 20 : 5 (*n*-3) or 22 : 6 (*n*-3), and 18 : 3 (*n*-3) can be converted to the latter fatty acids in liver.[31] Nevertheless, the hypocholesterolemic effect of PO was much less than that of FO. In contrast to FO, this effect of PO was potentiated by clofibric acid. Accordingly, there seems to be no merit in choosing PO instead of FO for the purpose of lowering serum lipids, although the beneficial effects of PO-feeding on neural systems should be noted.[32,33]

Finally, the present study showed that FO-feeding reduced both triacylglycerol and cholesterol in the serum of rats, with efficiency comparable to that of the treatment of SO-fed rats with clofibric acid. Moreover, the risk produced by a FO-caused imbalance in hydrogen peroxide metabolism and inhibitory effects on GSH *S*-transferases may be lower than that by clofibric acid. Accordingly, the present results suggest the pharmacological merit of dietary FO intake to reduce the dose of clofibrate necessary for lowering serum lipids, and also toxicological merit to prevent the liver from impairment of its detoxification systems caused by the drug.

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