

行政院國家科學委員會專題研究計畫成果報告評估表

計畫主持人自我評估表

評審委員複評表

*計畫主持人請於專題計畫結束時詳填本自評表，並隨同專題研究成果報告一併送本會評審

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隨函附上專題研究成果報告乙份，原計畫申請書及成果報告評估表等資料，敬請撥冗惠予審查，並請於 月 日前擲還本評估表及附件；本會各委員及職員均負不對外洩露案件內容之責任，亦請對外合密。非常感謝您的協助。
國科會敬 啟 年 月 日

NSC 89-2320-B-060-022 飲食油脂量與科類
NSC 89-2320-B-060-069 影响肝癌细胞生长
計畫編號 NSC 88-2314-B-060-007 計畫名稱 乳劑之探討

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計畫主持人簽名： 陳以文 90年10月21日

評審委員簽名： 年 月 日

中文摘要

本計畫目標主要探討魚油與玉米油對經過 diethylnitrosamine initiation/phenobarbital promotion 處理後的 Sprague-Dawley 大鼠其肝前癌細胞生成之影響。每組有 12 隻大鼠，出生 24 小時後以 diethylnitrosamine 誘發前癌細胞產生(15 mg/kg)。離奶後，他們分別接受含有 13.5%魚油與 1.5%玉米油以及 15%玉米油 24 週。二種飲食皆含有 500 ppm phenobarbital 以作為腫瘤促進劑。餵食魚油大鼠相較於餵食玉米油大鼠有顯著較重的肝臟及脾臟。不同飲食油脂顯著影響肝臟磷脂質脂肪酸組成。相較於餵食玉米油，大鼠餵食魚油有顯著較低肝臟維生素E及PGE₂濃度但是卻有顯著較高脂肪過氧化情形產生。餵食魚油大鼠有顯著較高抗氧化酵素活性(GSH reductase, GST)。除了PGST-positive 前癌細胞，其他前癌細胞指標在魚油組顯著低於玉米油組。分析 Pearson 相關係數，發現前癌細胞與PGE₂有顯著正相關，但與脂質過氧化、GSH/(GSH+GSSG)比率、GSH reductase 和 GST 活性卻呈現負相關。本實驗研究發現相較於玉米油，魚油顯著抑制前癌細胞生成。其中參與機制可能包括魚油抑制PGE₂生成、提升GSH-相關抗氧化能力和增加脂肪過氧化能力。

關鍵字：魚油、玉米油、前癌細胞、前列腺素 E₂、脂質過氧化

Comparison of the Effect of Fish Oil and Corn Oil on Chemical-Induced Hepatic Enzyme-Altered Foci in Rats

**Yuh-Jane Ko, Chong-Kuei Li, Chu-Chyn Ou, Jer-Yuh Liu,
Wea-Lung Lin, and Haw-Wen Chen**

Department of Nutrition, Institute of Biochemistry, Chung Shan
Medical College, and Department of Pathology, Chung Shan Medical
College and Chung Shan Memorial Hospital,
Taichung, Taiwan 40203

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Comparison of the Effect of Fish Oil and Corn Oil on Chemical-Induced Hepatic Enzyme-Altered Foci in Rats

Yuh-Jane Ko,[†] Chong-Kuei Lii,[†] Chu-Chyn Ou,[†] Jer-Yuh Liu,[‡] Wea-Lung Lin,[§] and Haw-Wen Chen^{*†}

Department of Nutrition, Institute of Biochemistry, Chung Shan Medical College, and Department of Pathology, Chung Shan Medical College and Chung Shan Memorial Hospital, Taichung, Taiwan 40203

The effects of fish oil and corn oil diets on diethylnitrosamine initiation/phenobarbital promotion of hepatic enzyme-altered foci in female Sprague–Dawley rats were investigated. Groups of 12 rats were initiated with diethylnitrosamine (15 mg/kg) at 24 h of age. After weaning, they received diets containing either 13.5% fish oil plus 1.5% corn oil or 15% corn oil for 24 weeks. Rats fed fish oil had significantly greater liver weight, relative liver weight, spleen weight, and relative spleen weight than rats fed corn oil ($p < 0.05$). Hepatic phospholipid fatty-acid profile was significantly affected by the type of dietary lipid. The rats fed fish oil had significantly greater hepatic phospholipid 20:5 and 22:6 than rats fed corn oil; in contrast, the rats fed corn oil had significantly greater hepatic phospholipid 18:2 and 20:4 than rats fed fish oil ($p < 0.05$). Rats fed fish oil had significantly lower hepatic vitamin E and PGE₂ content but significantly greater hepatic lipid peroxidation than rats fed corn oil ($p < 0.05$). The hepatic levels of antioxidant enzymes (GSH reductase and GST) were significantly greater in rats fed fish oil than in rats fed corn oil ($p < 0.05$). Except for PGST-positive foci (foci area/tissue area), all the other foci parameters (GGT-positive foci area/tissue area, GGT-positive foci no./cm², GGT-positive foci no./cm³, PGST-positive foci no./cm², and PGST-positive foci no./cm³) measured in the fish oil group were 10–30% of those in the corn oil group ($p < 0.05$). Analyses of Pearson correlation coefficient revealed a positive correlation between hepatic GGT- or PGST-positive foci number (no./cm²) and PGE₂ content ($r = 0.66$, $P = 0.01$; $r = 0.56$, $P = 0.02$, respectively) but a negative correlation between GGT- and PGST-positive foci (no./cm²) and lipid peroxidation ($r = -0.8$, $P = 0.0006$; $r = -0.58$, $P = 0.01$, respectively), GSH/(GSH + GSSG) ratio ($r = -0.61$, $P = 0.05$; $r = -0.4$, $P = 0.14$, respectively), GSH reductase ($r = -0.75$, $P = 0.002$; $r = -0.53$, $P = 0.02$, respectively), and GST activities ($r = -0.65$, $P = 0.01$; $r = -0.44$, $P = 0.07$, respectively). Similar correlation between foci number (no./cm³) and PGE₂, lipid peroxidation, GSH/(GSH + GSSG) ratio, GSH reductase, and GST activities were obtained. The results of this study show that dietary fish oil significantly inhibited hepatic enzyme-altered foci formation compared with corn oil in rats. These results suggest that the possible mechanisms involved in this process are the stimulation of hepatic detoxification system, changes in membrane composition, inhibition of PGE₂ synthesis, the enhancement of GSH-related antioxidant capacity, and the enhancement of lipid peroxidation by fish oil.

Keywords: Lipids; enzyme-altered foci; PGE₂; detoxification system; antioxidant capacity; lipid peroxidation

INTRODUCTION

In both human epidemiological studies and laboratory animal studies, dietary fat has been shown to influence the development of cancer at different sites. Type of dietary fat is an important determinant in cancer chemoprevention. Lipids rich in n-3 polyunsaturated fatty acids (PUFA) are potential candidates to decrease cancer in several organs (Reddy and Maruyama, 1986; Jurkowski and Cave, 1985; O'Conner et al., 1989). Fish oil is a fat from fish and has an appreciable amount of

n-3 PUFA, namely, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), but a small amount of n-6 PUFA. In studies on Alaskan and Greenland Eskimos, the consumption of large amounts of marine products with high levels of n-3 PUFA in the diets were found and this was suggested to be the main reason for a low incidence of colon cancer (Blot et al., 1975; Bang et al., 1976). However, n-6 PUFA which consists of mainly linoleic acid, has been shown to promote the development of tumors in colon, mammary gland, and pancreas in animal studies (Reddy and Maruyama, 1986; Jurkowski and Cave, 1985; O'Conner et al., 1989; Onogi et al., 1996; Komaki et al., 1996). In our previous study (Chen et al., 1997a), type of dietary fat was shown to affect hepatic enzyme-altered foci formation after diethylnitrosamine (DEN) initiation and phenobarbital (PB) promotion in female Sprague–Dawley rats. However, the underlying mechanism of this effect is not known.

* To whom correspondence should be addressed. Department of Nutrition, Chung Shan Medical College, No 110, Sec. 1, Chien Kuo N. Rd., Taichung, Taiwan 40203. Telephone: 886-4-473-0022, Ext. 1747. Fax: 886-4-473-9030. E-mail: Hawwen@mercury.csmc.edu.tw.

[†] Department of Nutrition, Chung Shan Medical College.

[‡] Institute of Biochemistry, Chung Shan Medical College.

[§] Department of Pathology, Chung Shan Medical College and Chung Shan Memorial Hospital.

Liver carcinogenesis is a multistage process that includes initiation and promotion (Faber, 1984; Pitot and Sirica, 1980). After initiation by a single administration of the representative complete carcinogen DEN (Goldsworthy and Pitot, 1985), the hepatopromoter PB has been shown to increase foci and neoplasms in rats (Pitot et al., 1977). Hepatic enzyme-altered foci can reflect clonal development from single initiated hepatocytes (Peraino et al., 1984; Weinberg et al., 1987; Goldsworthy et al., 1986), but no known histochemical markers can exactly assess the relationship between foci and tumors, and none of the markers invariably appears in all tumors (Peraino et al., 1984).

Inhibition of prostaglandin (PG) synthesis by PG-synthesis inhibitors is reported to suppress experimental chemical carcinogenesis in colon (Mernett, 1992), mammary (McCormick et al., 1985), pancreas (Takahashi et al., 1990), bladder (Shibata et al., 1993), and liver (Denda et al., 1989; Denda et al., 1994). Furthermore, PGs have been found to increase DNA synthesis in cultured hepatocytes (Hong and Glauert, 1996; Refsnes et al., 1994; Skouteris et al., 1988). High levels of PGs have been found in several animal and human tumors, particularly those of the E series which have been shown to be closely related to cell proliferation and tumor growth and to suppress immune responsiveness (Karmali, 1985). PGE₂ has been considered to play an important role in both human basal and squamous cell skin carcinomas because elevated levels of PGE₂ in skin carcinomas are associated with an aggressive growth pattern (Vanderveen et al., 1986). PGs of the 2 series are derived from dietary n-6 lipids. In contrast, PGs of the 3 series are derived from dietary n-3 lipids. The formation of 2-series PGs and related compounds from dietary n-6 lipids is catalyzed by cyclooxygenase (De Vries et al., 1992). EPA, an n-3 PUFA present in fish oil, can compete with arachidonic acid (AA) for cyclooxygenase and, thus, decrease the production of AA-derived compounds (Samuelsson et al., 1987). Compared with n-6 lipids, n-3 lipids have been shown to inhibit tumor development (Reddy and Maruyama, 1986; Jurkowski and Cave, 1985; O'Connor et al., 1989). The anticarcinogenic contribution of n-3 lipids may be ascribed to their inhibition of 2-series PG synthesis. In addition to PGs, lipid peroxidation has been speculated to be implicated in carcinogenesis as well. Slater (1988) found that experimental liver tumors express increased antioxidant capacity and a decreased potential for lipid peroxidation, and this condition has been suggested to favor the neoplastic growth of initiated cells (Dormandy and Wickens, 1988; Cerutti et al., 1988).

The present study investigated the effect of n-3 and n-6 lipids on hepatocarcinogenesis in terms of hepatic enzyme-altered foci formation in a DEN-initiation/PB-promotion model, and the possible mechanisms involved in this process. Two commonly used end points for the detection of preneoplastic hepatic lesions were used, the expression of the placental form of glutathione S-transferase (PGST) and γ -glutamyl transpeptidase (GGT).

MATERIALS AND METHODS

Animals and Diets. Pregnant Sprague-Dawley rats were purchased from the National Animal Breeding and Research Center (Taipei, Taiwan). To investigate the different experimental conditions on foci and tumor generation, Peraino et al. (1984) devised an experimental hepatocarcinogenesis pro-

Table 1. Composition of Experimental Diets

ingredient	fish oil (%)	corn oil (%)
corn oil ^a	1.5	15.0
fish oil ^b	13.5	
casein ^c	15.0	15.0
dextrose ^c	15.0	15.0
corn starch	44.95	44.95
cellulose ^c	5.0	5.0
AIN76 vitamin mixture ^c	1.0	1.0
AIN76 mineral mixture ^c	3.5	3.5
choline ^c	0.2	0.2
DL-methionine ^c	0.3	0.3
phenobarbital ^d	0.05	0.05

^a From CPC Intl Inc. (Englewood, NJ). ^b From Tama Biochemical Co. (Tokyo, Japan). ^c From Teklad (Madison, WI). ^d From Sigma Chemical (St. Louis, MO).

Table 2. Fatty Acid Composition of Dietary Fats

fatty acid	fish oil (%)	corn oil (%)
14:0	8.02	0.07
16:0	9.75	10.37
16:1	9.87	0.08
18:0	3.64	1.96
18:1n-9	7.75	26.29
18:2n-6	1.48	60.48
20:0	0.53	0.4
18:3n-3	1.04	
20:1	0.09	0.35
20:2	0.25	
20:3	0.19	
22:0	0.33	
20:4n-6	1.99	
22:1	0.06	
20:5n-3	37.01	
24:1	0.13	
22:6n-3	17.87	

col that produced a rapid induction of maximal yields of foci and tumors with minimal carcinogen treatment. In this study, we used the experimental protocol of Peraino et al. (1984). Female pups were initiated with 15 mg/kg DEN in phosphate buffered saline (pH 7.0) at 24 h of age by intraperitoneal injection. Twenty-four weaning rats were randomly assigned to two experimental diets and there were 12 rats in each group. The experimental diets were nutritionally complete and provided 30% of energy as fat (Table 1). Fish oil was obtained from Tama Biochemical Co. (Tokyo, Japan) and corn oil was obtained from CPC Intl. Inc. (Englewood, NJ). The α -tocopherol content of fish oil was 49 tocopherol equiv (TE)/100 g and that of corn oil was 17 TE/100 g. The two groups of rats were provided ad libitum access to feed and tap water for 24 weeks.

Plasma Preparation. After 24 weeks of feeding, the rats were fasted overnight and killed by an overdose of CO₂. Blood for the PGE₂ assay was drawn from the jugular vein. Nine parts of blood were added to one part of the anticoagulant (50 mM EDTA). For the endogenous PGE₂ assay, anticoagulant containing 0.7 mg/mL indomethacin was used. The blood was put into a centrifuge tube, and the tube was gently inverted. Plasma was obtained by centrifugation of blood at 1500g for 5 min. Plasma was removed after centrifugation and stored at -80 °C for later analysis.

Lipid and Liver Phospholipid Fatty-Acid Profile Assay. The fatty acid composition of fish oil and corn oil was analyzed by gas chromatography (Table 2). Liver lipids were extracted according to the method of Folch et al. (1957). Total phospholipids were isolated from the lipid extracts by thin-layer chromatography as previously described by Chen et al. (1997). Fatty-acid analysis was performed as previously described by Lepage and Roy (1986). Fatty-acid methyl esters were quantified by gas chromatography using a 30-m fused-silica column with an internal diameter of 0.25 mm (Supelco, Bellefonte, PA). Peaks were identified by comparing retention times with those of authentic fatty-acid methyl ester standards (Alltech, Deerfield, IL). The percentage of each fatty acid was determined by integration of the peak areas.

Liver α -Tocopherol Level and Lipid Peroxidation Assays. Hepatic α -tocopherol concentration was determined as described by Catignani and Bieri (1983) with some modification (Lii et al., 1997). Briefly, 50 μ L of an internal standard (α -tocopheryl acetate in ethanol) and hepatic homogenate (100 μ L taken from 0.1 g liver:1 mL of 50 mM potassium phosphate buffer, pH 7.0) were mixed by vortexing for 1 min. To extract the lipid, 200 μ L of HPLC-grade hexane was added and the suspension was mixed for an additional 1 min. Phases were separated by centrifugation at 550g for 2 min, and the hexane layer was withdrawn and evaporated under nitrogen. The residue was then redissolved in 50 μ L of filtered HPLC-grade methanol by mixing, and 20 μ L of the mixture was injected into an HPLC. The HPLC column was 3.9 mm \times 30 cm stainless steel packed with micro Bondapak C-18. The detector wavelength was 290 nm, with a sensitivity of 0.01 absorbance unit full scale. The solvent was 100% HPLC-grade methanol, and the flow rate was 1.2 mL/min.

Liver lipid peroxidation was measured by assaying thiobarbituric acid reactive substances (TBARS) using a modification of the procedure described by Fraga et al. (1988). Briefly, liver samples were homogenized in 50 mM potassium phosphate buffer (pH 7.4). To the liver homogenate were added 0.5 mL of 3% sodium dodecyl sulfate, 2 mL of 0.1 N HCl, 0.3 mL of 10% phosphotungstic acid, and 1 mL of 0.7% 2-thiobarbituric acid. The mixture was heated in boiling water for 30 min, and TBARS was extracted into 5 mL of 1-butanol. After centrifugation, the fluorescence of the butanol layer was measured at 515 nm excitation and 555 nm emission in a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan). The values were expressed in nmol/g liver. A malondialdehyde standard curve was also prepared, using 1,1,3,3-tetramethoxypropane.

Hepatic Antioxidant Enzyme Activity Assays. Hepatic cytosolic and microsomal fractions of rats were prepared by differential centrifugation (Huang et al., 1988). Hepatic cytosolic GSH peroxidase activity was determined spectrophotometrically with a coupled procedure using H_2O_2 as the substrate (Lawrence and Burk, 1976). Hepatic cytosolic GSH reductase activity was measured as described by Bellomo et al. (1987). Hepatic GST activity was determined by the method of Habig et al. (1974). Samples and reference cuvettes were read for five minutes in a dual-beam spectrophotometer set at 340 nm. Activity was expressed as nanomoles of 1-chloro-2,4-dinitrobenzene conjugate formed per milligram of protein per minute. Protein content was determined by the method of Lowry et al. (1951).

Hepatic GSH Status Assay. Frozen liver tissue was used to determine the GSH redox status. GSH and GSSG were determined by HPLC as described by Reed et al. (1980) with some modification (Lii and Huang, 1997).

Plasma PGE₂ Analysis. The plasma PGE₂ content was analyzed by radioimmunoassay (New England Nuclear Corp., Boston, MA) according to the manufacturer's instructions.

Hepatic Enzyme-Altered Foci Analysis. The largest lobes of the rat liver were cut into 1-cm thick slices, frozen on dry ice, and stored at -80°C . Frozen liver slices were further sliced into 10- μ m serial sections for PGST- and GGT- positive focus assays. PGST-positive foci were visualized by immunohistochemical methods as described by Hendrich et al. (1991). Rabbit anti-PGST antiserum was kindly provided by Dr. Hendrich (Iowa State University, IA). PGST-positive foci were detected with a Vectastain ABC/peroxidase immunoassay kit (Vector Laboratories, Burlingame, CA). For color development, aminoethylcarbazole (AEC kit, Vector Laboratories, Burlingame, CA) was used as the substrate for peroxidase.

GGT-positive foci assays were performed according to the method described by Rutenburg et al. (1969). Briefly, the 10- μ m liver sections were air-dried, submerged in freshly prepared γ -glutamyl-4-methoxy-2-naphthylamine solution for 15 min at room temperature, washed in 0.85% saline solution for 2 min, and stabilized with 0.1 M CuSO_4 for 2 min, washed in deionized water, and air-dried. Finally, we added glycerol gelatin and put the sections under glass covers. The sizes and numbers of GGT- and PGST-positive foci were quantified under a microscope with Leica Q500MC software (Germany).

Table 3. Food Intake, Body Weight Gain, Liver Weight, Relative Liver Weight, Spleen Weight, and Relative Spleen Weight of Rats Treated with DEN/PB and Fed Fish Oil or Corn Oil for 24 Weeks^a

	fish oil ^{b,c}	corn oil ^{b,c}
food intake (g)	3067 \pm 163	3261 \pm 248
body weight gain (g)	274 \pm 35	271 \pm 41
liver weight (g)	14.4 \pm 1.6*	11.1 \pm 1.6
liver weight/body weight (%)	4.3 \pm 0.3*	3.4 \pm 0.4
spleen weight (g)	1.0 \pm 0.2*	0.8 \pm 0.2
spleen weight/body weight (%)	0.3 \pm 0.1*	0.2 \pm 0.1

^a Female pups are initiated with DEN (15 mg/kg) at 24 h of age. There are 12 rats in each group. Food intake is measured twice weekly. ^b Values are means \pm SD. ^c Groups not sharing a symbol (*) are significantly different ($p < 0.05$).

Table 4. Hepatic Phospholipid Fatty Acid Profile of Rats Treated with DEN/PB and Fed Fish Oil or Corn Oil for 24 Weeks^a

fatty acid	fish oil (%) ^{b,c}	corn oil (%) ^{b,c}
16:0	11.9 \pm 1.5	10.3 \pm 1.5
16:1	1.5 \pm 0.3	1.3 \pm 0.4
18:0	33.1 \pm 1.5	36.3 \pm 1.9*
18:1	1.8 \pm 0.3	2.0 \pm 0.3
18:2n-6	3.5 \pm 0.7	5.4 \pm 1.0*
20:0	8.7 \pm 1.2	8.9 \pm 2.4
18:3n-3	3.7 \pm 0.9	3.5 \pm 1.6
20:4n-6	17.1 \pm 1.4	27.8 \pm 3.3*
20:5n-3	4.7 \pm 2.1*	ND
22:6	14.0 \pm 1.8*	4.6 \pm 1.0

^a Female pups are initiated with DEN (15 mg/kg) at 24 h of age. There are 12 rats in each group. Food intake is measured twice weekly. ^b Values are means \pm SD. ^c Groups not sharing a symbol (*) are significantly different ($p < 0.05$).

The foci were recognized only if their foci diameter was larger than 0.25 mm.

Statistical Analysis. All analyses were conducted in duplicate for each sample. Data were analyzed by using analysis of variance (ANOVA) (SAS Institute, Cary, NC). Student's *t*-test was used to evaluate the significance between means of treatment. Pearson correlation coefficients were also calculated. A value of $p < 0.05$ was taken to be statistically significant.

RESULTS

Food intake and body weight gain of rats fed fish oil or corn oil were not significantly different. Liver weight and relative liver weight were significantly greater in rats fed fish oil than in rats fed corn oil ($p < 0.05$) (Table 3). Spleen weight and relative spleen weight were significantly greater, as well, in rats fed fish oil than in rats fed corn oil ($p < 0.05$) (Table 3).

The hepatic phospholipid fatty-acid profile reflected the source of dietary lipids. Hepatic phospholipid 16:0, 16:1, 18:1, 20:0, and 18:3 n-3 were not significantly different in rats fed either fish oil or corn oil (Table 4). However, rats fed fish oil had significantly greater hepatic phospholipid 20:5 n-3 and 22:6 n-3 than rats fed corn oil ($p < 0.05$). In contrast, rats fed corn oil had significantly greater hepatic phospholipid 18:0, 18:2 n-6 and 20:4 n-6 than rats fed fish oil ($p < 0.05$) (Table 4).

The liver vitamin E content of rats was significantly affected by the source of dietary lipids. Rats fed fish oil had significantly lower liver vitamin E content than rats fed corn oil; liver lipid peroxidation was significantly greater in rats fed fish oil than in rats fed corn oil ($p < 0.05$) (Table 5). Liver GSH content was not significantly different in rats fed either fish oil or corn oil, but liver GSH/(GSH + GSSG) ratio, which reflects the redox

Table 5. Liver Vitamin E Content, Lipid Peroxidation, GSH Status, GSH Reductase, GSH Peroxidase, GST Activities, and Plasma PGE₂ Level of Rats Treated with DEN/PB and Fed Fish Oil or Corn Oil for 24 Weeks^a

	fish oil ^{b,c}	corn oil ^{b,c}
vitamin E ($\mu\text{g/g}$)	7.7 \pm 2.3	17.0 \pm 5.9*
TBARS (nmol/g)	199.5 \pm 10.8*	51.0 \pm 12.1
GSH (nmol/mg protein)	23.8 \pm 2.8	20.3 \pm 4.5
GSH/(GSH + GSSG)	0.93 \pm 0.01*	0.88 \pm 0.02
GSH reductase (nmol/min/mg protein)	75.8 \pm 6.9*	59.2 \pm 6.8
GSH peroxidase (nmol/min/mg protein)	832 \pm 80	843 \pm 180
GST (nmol/min/mg protein)	3504 \pm 642*	2607 \pm 476
PGE ₂ (ng/mL)	4.9 \pm 1.1	7.3 \pm 1.4*

^a Female pups are initiated with DEN (15 mg/kg) at 24 h of age. There are 12 rats in each group. Food intake is measured twice weekly. ^b Values are means \pm SD. ^c Groups not sharing a symbol (*) are significantly different ($p < 0.05$).

Table 6. Hepatic GGT- and PGST-Positive Foci of Rats Treated with DEN/PB and Fed Fish Oil or Corn Oil for 24 Weeks^a

	fish oil ^{b,c}	corn oil ^{b,c}
GGT-positive foci		
foci area/tissue area (%)	0.14 \pm 0.14	0.75 \pm 0.52*
foci no./cm ²	1.5 \pm 0.7	7.3 \pm 3.1*
foci no./cm ³	21.7 \pm 13.8	122.7 \pm 47.4*
PGST-positive foci		
foci area/tissue area (%)	0.58 \pm 0.64	1.11 \pm 1.16
foci no./cm ²	2.6 \pm 2.6	8.7 \pm 5.8*
foci no./cm ³	17.8 \pm 12.9	141.4 \pm 86.9*

^a Female pups are initiated with DEN (15 mg/kg) at 24 h of age. There are 12 rats in each group. Food intake is measured twice weekly. ^b Values are means \pm SD. ^c Groups not sharing a symbol (*) are significantly different ($p < 0.05$).

status of tissues, was significantly greater in rats fed fish oil than in rats fed corn oil ($p < 0.05$) (Table 5). Liver GSH reductase and GST activities were significantly greater in rats fed fish oil than in rats fed corn oil ($p < 0.05$) (Table 5). Dietary lipid source had no effect on hepatic GSH peroxidase activity. However, plasma PGE₂ level was significantly lower in rats fed fish oil than in rats fed corn oil ($p < 0.05$) (Table 5).

Dietary lipid source had a significant effect on hepatic enzyme-altered foci formation. Rats fed fish oil developed significantly fewer GGT-positive foci than rats fed corn oil ($p < 0.05$) (Table 6). The parameters of hepatic enzyme-altered foci measured included foci area/tissue area (%) and foci number (no./cm² or cm³). Dietary lipid source had no effect on the PGST-positive foci area as a percentage of tissue area, however, rats fed fish oil had a significantly lower PGST-positive foci number (no./cm² or cm³) than rats fed corn oil ($p < 0.05$) (Table 6).

The correlation coefficients between GGT- and PGST-positive foci number and plasma PGE₂, liver vitamin E, lipid peroxidation, GSH/(GSH + GSSG) ratio, GSH

reductase, and GST activities were calculated. These results are shown in Table 7.

DISCUSSION

In the present study, we did not include the groups of rats without PB treatment. The reason was that in our previous study we found the high-fish-oil diet can inhibit GGT-positive foci formation compared to the high-corn-oil diet only in the presence of both DEN and PB (Chen et al., 1997a).

In this study, dietary lipid source had no effect on the growth of rats (Table 3). However, rats fed fish oil had significantly greater liver weight, relative liver weight, spleen weight, and relative spleen weight (Table 3). Significantly greater liver weights and relative liver weights of rats fed fish oil compared with those of rats fed corn oil were also found in our previous study. This led us to suggest that fish oil can exert a stimulatory effect on hepatic microsomal enzymes (Chen et al., 1996). In another previous study, we found that fish oil significantly increased hepatic microsomal cytochrome P-450 2B1 and 2E1 activities in comparison with those of corn oil (Chen et al., 1997b). There is evidence that long-chain fatty-acids-caused hepatomegaly partly accounted by their PPAR ligand property, and subsequently leads to increase in cytochrome P450 4A activity. These evidences suggest that the induction of peroxisome, mitochondria, and endoplasmic reticulum proliferation partly accounts for the enlargement of liver (Reddy et al., 1996; Gibson and Lake, 1993). Taken together, these results suggest that fish oil's effect on hepatic enzyme-altered foci may occur through its modulation of the hepatic detoxification system and lead to reduced promotion effect of PB. The main metabolic pathway of PB is hydroxylation and subsequent glucuronidation, and both the hydroxylate and glucuronidate forms of PB are inactive metabolites (Battino et al., 1995). In the present study, rats fed fish oil had significantly greater spleen weight and relative spleen weight compared to those of rats fed corn oil ($p < 0.05$). This result is in agreement with the finding of Blok et al. (1996) who showed that this increase was largely due to macrophage accumulation in the spleen.

In the present study, dietary lipid significantly affected the hepatic phospholipid fatty-acid profile of rats (Table 4). These results are consistent with those of our previous studies (Chen et al., 1997a,b). Changes in membrane composition and inhibition of intercellular communication by PB have been suggested to be responsible for its promoter function (Williams, 1981). The possibility that the effect of dietary lipid on hepatic enzyme-altered foci is through its action on membrane composition cannot be excluded. Levels and activities of liver antioxidants, antioxidant enzymes, lipid peroxidation, and plasma PGE₂ were significantly affected

Table 7. Correlation Coefficients between Foci Number and Plasma PGE₂, Liver Vitamin E, Lipid Peroxidation, GSH/(GSH + GSSG) Ratio, GSH Reductase, and GST Activities of Rats Treated with DEN/PB and Fed Fish Oil or Corn Oil for 24 Weeks

	PGE ₂		Vitamin E		TBARS		GSH/(GSH + GSSG)		GSH reductase		GST	
	r	P	r	P	r	P	r	P	r	P	r	P
GGT-positive												
foci no./cm ²	0.66	0.01	0.57	0.04	-0.80	0.0006	-0.61	0.05	-0.75	0.002	-0.65	0.01
foci no./cm ³	0.66	0.01	0.49	0.09	-0.82	0.0003	-0.62	0.04	-0.75	0.002	-0.65	0.01
PGST-positive												
foci no./cm ²	0.56	0.02	0.62	0.007	-0.58	0.01	-0.40	0.14	-0.53	0.02	-0.44	0.07
foci no./cm ³	0.64	0.004	0.72	0.001	-0.72	0.0008	-0.53	0.04	-0.65	0.004	-0.52	0.03

by dietary lipids (Table 5). Rats fed fish oil had significantly lower hepatic vitamin E content than rats fed corn oil ($p < 0.05$). In contrast, liver lipid peroxidation (TBARS) was significantly greater in rats fed fish oil than in rats fed corn oil. These results are in agreement with those of Chen et al. (1997a) and Cho and Choi (1994), and suggest that the more double bonds in fish oil compared to corn oil causes the rats to consume more vitamin E. Also, the greater lipid peroxidation potential of fish oil compared to that of corn oil results from the higher number of double bonds in fish oil. In this study, rats fed fish oil had greater hepatic GSH content than rats fed corn oil, however, the difference was not significant. In addition, the hepatic GSH/total GSH ratio was significantly greater in rats fed fish oil than in rats fed corn oil ($p < 0.05$). Both of these observations may have been due to the significantly greater hepatic GSH reductase activity in rats fed fish oil than in rats fed corn oil ($p < 0.05$). GSH reductase is responsible for the conversion of oxidized GSH to reduced GSH. In this study, rats fed fish oil also had significantly greater hepatic GST activity than rats fed corn oil ($p < 0.05$). These increases in the activities of antioxidant enzymes (e.g., GSH reductase and GST) may imply that fish oil is an oxidative stress-causing factor (decreased hepatic vitamin E content and increased lipid peroxidation). Rats fed fish oil had significantly lower hepatic PGE₂ levels than rats fed corn oil ($p < 0.05$). This was probably due to the decreased supply of the dietary precursor (n-6 PUFA) for PGE₂ synthesis in the fish oil group. The data of Tables 4 and 5 were obtained from whole liver homogenate and we thought these changes occur in the foci as well.

Except for the PGST-positive foci measured as foci area/tissue area (%), all the other parameters measured were significantly greater in rats fed corn oil than in rats fed fish oil (Table 6). These results support previous observations in animals that intake of n-6 PUFA promotes the development of tumors in colon, mammary gland, pancreas, and liver compared to intake of n-3 PUFA (Reddy and Maruyama, 1986; Jurkowski and Cave, 1985; O'Conner et al., 1989; Chen et al., 1997a; Sugie et al., 1995; Rahman et al., 1999). In other words, n-3 PUFA can inhibit tumor promotion compared to n-6 PUFA. To determine the possible mechanisms involved in fish oil inhibition of hepatic enzyme-altered foci, we analyzed the Pearson's correlation coefficients between hepatic enzyme-altered foci, plasma PGE₂ level, hepatic vitamin E content, lipid peroxidation, GSH/(GSH + GSSG) ratio, GSH reductase and GST activities, respectively (Table 7). Most of the correlation coefficients were found to be statistically significant. The positive correlation found between hepatic enzyme-altered foci and PGE₂ supports the promotion role of PG in tumor development in various organs in animals, and inhibition of PG synthesis can suppress experimental chemical carcinogenesis (Mernett, 1992; McCormick et al., 1985; Takahashi et al., 1990; Shibata et al., 1993; Denda et al., 1989; Denda et al., 1994). The positive correlation found between hepatic enzyme-altered foci and vitamin E, and the negative correlation between hepatic enzyme-altered foci and lipid peroxidation and GSH-related antioxidant capacity (GSH/(GSH + GSSG), GSH reductase, and GST activities), implies that increased oxidative stress can reduce the susceptibility of animals to tumor promotion. The increases in GSH-related antioxidant capacity in the fish oil group com-

pared to those in corn oil group in this study may have been due to the oxidative stress exerted by the fish oil and then enhanced the GSH-related antioxidant capacity to defend against the oxidative stress. Dormandy and Wickens (1988) and Cerutti et al. (1988) proposed that the increased antioxidant capacity and decreased lipid peroxidation conditions favored the neoplastic growth of initiated cell. The liver weight and relative liver weight were significantly greater in rats fed fish oil than in rats fed corn oil (Table 3). In addition to the stimulatory effect on hepatic microsomal enzymes by fish oil, the other possibilities may include the greater number of Kupffer cells found in nontransformed liver than in hepatic enzyme-altered foci as suggested by Janossy et al. (1986). In this study, the fish oil group had significantly fewer hepatic enzyme-altered foci than the corn oil group.

In summary, fish oil significantly inhibited hepatic enzyme-altered foci formation compared to corn oil. The possible mechanisms involved in this process include stimulation of the hepatic detoxification system, changes in membrane composition, inhibition of PGE₂ synthesis, enhancement of GSH-related antioxidant capacity, and increased lipid peroxidation by fish oil in the presence of both DEN and PB.

ABBREVIATIONS USED

DEN, diethylnitrosamine; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GGT, γ -glutamyl transpeptidase; PB, phenobarbital; PG, prostaglandins; PGST, placental form of glutathione S-transferase; PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric acid reactive substances.

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Suppression of Altered Hepatic Foci Development by a High Fish Oil Diet Compared With a High Corn Oil Diet in Rats

Chong-Kuei Lii, Chu-Chyn Ou, Kai-Li Liu,
Jer-Yuh Liu, Wea-Lung Lin, and Haw-Wen Chen

Abstract: Effects of low corn oil, high corn oil, and high fish oil diets on altered hepatic foci development in female Sprague-Dawley rats were investigated. Rats assigned to Groups 1-4 were initiated with saline as the control and those assigned to Groups 5-7 were initiated with diethylnitrosamine (DEN 15 mg/kg) at 24 hours of age. After weaning, all rats, except those in Group 1, received 500 ppm phenobarbital (PB) in their diet as tumor promoter for three months. Altered hepatic foci development was significantly lower in DEN-initiated rats fed the high fish oil + PB diet than in DEN-initiated rats fed the high corn oil + PB diets. Liver weight and relative liver weight were significantly greater in rats fed the high fish oil + PB diet than in rats fed the other diets, and hepatic biotransformation/detoxification enzyme activities were greater in rats fed the fish oil + PB diets than in rats fed the other diets. These results suggest that the effect of a high fish oil diet on altered hepatic foci may occur through regulation of hepatic biotransformation/detoxification enzyme activities, leading to alteration in the tumor-promoting action of PB. Dietary lipid significantly affected the hepatic phospholipid fatty acid composition of rats. *n*-3 polyunsaturated fatty acids were incorporated into membrane phospholipid at the expense of *n*-6 polyunsaturated fatty acids. A high fish oil diet caused greater oxidative stress in rats, as measured by plasma vitamin E level, red blood cell glutathione status, liver lipid peroxidation, and hepatic glutathione reductase activity. Pearson's correlation analysis indicated that the foci number was negatively correlated to the liver thiobarbituric acid-reactive substance and 7-pentoxoresorufin O-dealkylase activity, and the foci area was negatively correlated to the liver thiobarbituric acid-reactive substance activity ($p < 0.05$) in rats of groups that developed foci. These results suggest that the type of dietary lipid is the more important determinant for γ -glutamyl transpeptidase-positive foci development than the amount of dietary lipid when rats consumed approximately the same amount of calories in all the dietary groups, and the underlying mechanisms may be par-

tially ascribed to the antioxidant/oxidation status and biotransformation/detoxification system of rats.

Introduction

The effects of amount and type of dietary lipid on carcinogenesis have attracted considerable attention. Human epidemiological studies and laboratory animal studies have shown these two factors to have a high correlation with cancer development at different organs. High dietary lipid intake has been shown to increase the incidence of spontaneous mammary tumors in mice (1-3), mammary tumors (induced by chemical carcinogens or hormones) in mice (4) and rats (5-7), transplantable mammary tumors in mice (8,9) and rats (10), chemically induced hepatic γ -glutamyl transpeptidase (GGT)-positive foci (11) or tumors (12) in rats, chemically induced pancreatic ductular carcinoma (13) in hamsters, and chemically induced colon tumors (14) in rats. In addition to the amount of dietary lipid, the type of dietary lipid also plays an important role in cancer development. In the carcinogen-induced mammary tumor model during the tumor promotion phase, rats fed relatively high dietary levels of fish oil consistently show inhibition of mammary tumorigenesis (15,16). Fish oil rich in *n*-3 polyunsaturated fatty acids (PUFA) given in the postinitiation (17) and the promotion phase (18) can inhibit chemical carcinogenesis in the liver. The growth of azaserine-induced putative preneoplastic atypical acinar cell nodules in rat pancreas was significantly inhibited by a 20 wt% menhaden oil diet compared with a 20 wt% corn oil diet (19). Various menhaden oil proportions (5.9%, 11.8%, or 17.6%) mixed with corn oil, leading to a total of 23.5% fat, resulted in 37-48% incidence of colonic adenocarcinomas in comparison with 81% in rats maintained on a diet with 23.5% corn oil (20).

Rat hepatocarcinogenesis is a multistage process that includes initiation, promotion, and progression (21,22). Al-

tered hepatic foci are preneoplastic lesions that develop after diethylnitrosamine (DEN) initiation and phenobarbital (PB) promotion during hepatocarcinogenesis (18) and reflect clonal development from single initiated hepatocytes (23,24). DEN is known to be a representative complete carcinogen, and PB is an effective promoter of hepatocellular carcinogenesis (25,26). In this study, the end point for altered hepatic foci was GGT expression, which is the most prevalent and one of the most widely used markers for foci (27).

Prostaglandins (PGs) are a group of biologically active compounds that can alter hepatic cell proliferation. After indomethacin administration, PG synthesis and DNA synthesis in regenerating liver were reduced (28). In cultured rat hepatocytes, PGE₂ has been found to increase DNA synthesis (29). In addition, inhibition of PG synthesis has been reported to suppress chemical carcinogenesis in various organs (e.g., colon, breast, pancreas, and liver) (30–33). Intake of n-3 lipids has also been shown to reduce chemically induced tumor development compared with n-6 lipids (15–20), and the underlying mechanism may be ascribed to the inhibition of 2-series PG synthesis by n-3 lipids (34).

The effect of n-3 lipids on antioxidant capacity and lipid peroxidation potential of tumors (35) and the possible modulation of bioactivation and detoxification systems by n-3 lipids (36) have also been reported to play important roles in chemical carcinogenesis in addition to their influence on PG synthesis. The present study examined the influence of the amount and type of dietary lipid on chemically induced altered hepatic foci development and the possible involvement of the aforementioned mechanisms.

Materials and Methods

Animals and Diets

Pregnant Sprague-Dawley rats were purchased from the National Animal Breeding and Research Center (Taipei, Taiwan). To investigate the effect of different experimental conditions on foci and tumor generation, Peraino and co-workers (24) devised an experimental hepatocarcinogenesis protocol that produced a rapid induction of maximal yields of foci and tumors with minimal carcinogen treatment. In this study, we used the experimental protocol of Peraino and co-workers. Thirty-three female pups were initiated with DEN (15 mg/kg) in phosphate-buffered saline (pH 7.0) at 24 hours of age by intraperitoneal injection and 29 female pups were intraperitoneally injected with saline as the control at the same time. After they were weaned, the rats were randomly assigned to seven experimental groups. The experimental diets were formulated on the basis of the American Institute of Nutrition standard reference diet with the modification of varying sources of carbohydrate with all diets supplying approximately the same amount of protein, minerals, vitamins, fiber, and calories (37) (Table 1). Rats in Groups 2–7 also consumed diets with 500 ppm of the tumor promoter PB. Groups 1–7 were provided ad libitum access to feed and tap water for three months.

Altered Hepatic Foci Analysis

The largest lobes of the rat liver were cut into 1-cm-thick slices, frozen on dry ice, and stored at -80°C. Frozen liver slices were further sliced into 10- μ m serial sections for GGT-positive foci assays. GGT-positive foci assays were performed according to the method described by Rutenburg and associates (38). Briefly, the 10- μ m liver sections were air-dried, submersed in freshly prepared γ -glutamyl-4-methoxy-2-naphthylamine solution for 15 minutes at room temperature, washed in 0.85% saline solution for 2 minutes, stabilized with 0.1 M CuSO₄ for 2 minutes, washed in deionized water, and air-dried. Finally, glycerol gelatin was added, and the sections were put under glass covers. The size and number of GGT-positive foci were quantified under a microscope with Leica Q500MC software (Germany). The foci were recognized only if their diameter was >0.25 mm.

Plasma Preparation

After three months of feeding, the rats were fasted overnight and killed by an overdose of CO₂. Blood for the PGE₂ assay was drawn from the jugular vein. Nine parts of blood were added to one part of the anticoagulant (50 mM EDTA). For the endogenous PGE₂ assay, anticoagulant containing 0.7 mg/ml indomethacin was used. The blood was put into a centrifuge tube, and the tube was gently inverted. Plasma was obtained by centrifugation of blood at 1,500 g for five minutes. Plasma was removed after centrifugation and stored at -80°C for later analysis.

Lipid and Liver Phospholipid Fatty Acid Profile Assay

The fatty acid composition of fish oil and corn oil was analyzed by gas chromatography (Table 2). Liver lipids were extracted according to the method of Folch and colleagues

Table 1. Composition of Diets^a

	Low Corn Oil Diet	High Corn Oil Diet	High Fish Oil Diet
Casein ^b	20.0	23.5	23.5
Dl-Methionine ^b	0.3	0.35	0.35
Cornstarch ^b	52.0	32.9	32.9
Dextrose ^b	13.0	8.32	8.32
Alphacel ^b	5.0	5.9	5.9
Corn oil ^c	5.0	23.5	3.0
Fish oil ^d	0	0	20.5
Mineral mix ^b	3.5	4.11	4.11
Vitamin mix ^b	1.0	1.18	1.18
Choline bitartrate ^b	0.2	0.24	0.24

a: Values are percentages. Experimental diets were formulated on the basis of the American Institute of Nutrition standard reference diet with the modification of varying sources of carbohydrate, with all diets supplying approximately the same amount of protein, minerals, vitamins, fiber, and calories (37).

b: From Teklad (Madison, WI).

c: From CPC (Englewood, NJ).

d: From Tama Biochemical (Tokyo, Japan).

Table 2. Fatty Acid Composition of Dietary Fats^a

Fatty Acid	Corn Oil	Fish Oil
14:0	0.19	7.34
16:0	10.17	11.85
16:1		7.84
18:0	3.69	1.32
18:1(n-9)	26.34	8.67
18:2(n-6)	58.27	2.93
20:0	0.66	0.28
18:3(n-3)	0.42	0.85
20:1	0.26	1.86
20:2		0.28
22:0		0.34
20:4(n-6)		3.45
20:5(n-3)		35.37
22:6(n-3)		17.61

a: Values are percentages.

(39). Total phospholipids were isolated from the lipid extracts by thin-layer chromatography, as previously described by Chen and others (18). Fatty acid analysis was performed as previously described by Lepage and Roy (40). Fatty acid methyl esters were quantified by gas chromatography using a 30-m fused silica column with an internal diameter of 0.25 mm (Supelco, Bellefonte, PA). Peaks were identified by comparing retention times with those of authentic fatty acid methyl ester standards (Sigma Chemical, St. Louis, MO). The percentage of each fatty acid was determined by integration of the peak areas.

Plasma α -Tocopherol Level and Liver Lipid Peroxidation Assays

Plasma α -tocopherol concentration was determined as described by Catignani and Bieri (41). Briefly, 50 μ l of an internal standard (α -tocopheryl acetate in ethanol) and 100 μ l of plasma were mixed by vortexing for one minute. To extract the lipid, 200 μ l of high-performance liquid chromatography (HPLC)-grade hexane was added and the suspension was mixed for an additional one minute. Phases were separated by centrifugation at 550 g for two minutes, and the hexane layer was withdrawn and evaporated under nitrogen. The residue was then redissolved in 50 μ l of filtered HPLC-grade methanol by mixing, and 20 μ l of the mixture was injected into an HPLC.

Liver lipid peroxidation was measured by assaying thiobarbituric acid-reactive substances (TBARS) using a modification of the procedure described by Fraga and co-workers (42). Briefly, liver samples were homogenized in 50 mM potassium phosphate buffer (pH 7.4). To the liver homogenate were added 0.5 ml of 3% sodium dodecyl sulfate, 2 ml of 0.1 N HCl, 0.3 ml of 10% phosphotungstic acid, and 1 ml of 0.7% 2-thiobarbituric acid. The mixture was heated in boiling water for 30 minutes, and TBARS were extracted into 5 ml of 1-butanol. After centrifugation, the fluorescence of the butanol layer was measured at 515-nm excitation and

555-nm emission in a fluorescence spectrophotometer (model F-4500, Hitachi, Tokyo, Japan). The values were expressed in nanomoles per gram of liver. A malondialdehyde standard curve was also prepared using 1,1,3,3,-tetramethoxypropane.

Plasma PGE₂ Analysis

The plasma PGE₂ content was analyzed by radioimmunoassay (New England Nuclear, Boston, MA) according to the manufacturer's instructions.

RBC Glutathione Status Assay

Freshly prepared red blood cells (RBCs) were used to determine the glutathione (GSH) redox status. GSH and oxidized GSH (GSSG) were determined by HPLC as described by Reed and colleagues (43) with some modification (44).

Hepatic Antioxidant Enzyme Activity Assays

Hepatic cytosolic and microsomal fractions of rats were prepared by differential centrifugation (45). Hepatic cytosolic GSH peroxidase activity was determined spectrophotometrically with a coupled procedure using H₂O₂ as the substrate (46). Hepatic cytosolic GSH reductase activity was measured as described by Bellomo and others (47). Protein content was determined by the method of Lowry and associates (48).

Hepatic Phase I and Phase II Enzyme Activity Assays

The activity of 7-pentoxoresorufin *O*-dealkylase (PROD) was assayed according to the method of Lubet and co-workers (49). A fluorescence spectrophotometer (model F-4500, Hitachi) was used to measure resorufin at 515-nm excitation and 555-nm emission and the entrance with exit slits set at 10 nm. Microsomal NADPH-*P*-450 reductase activity was determined by the method of Phillips and Langdon (50). Hepatic glutathione *S*-transferase (GST) activity was determined by the method of Habig and colleagues (51). Samples and reference cuvettes were read for five minutes in a dual-beam spectrophotometer set at 340 nm. Activity was expressed as nanomoles of 1-chloro-2,4-dinitrobenzene conjugate formed per milligram of protein per minute.

Statistical Analysis

All analyses were conducted in duplicate for each sample. Data were analyzed by using analysis of variance (SAS Institute, Cary, NC). Tukey's test was used to evaluate the significance between means. Pearson correlation coefficients were also calculated for GGT-positive foci vs. plasma PGE₂, vitamin E, liver phase I and II enzymes, and TBARS; $p < 0.05$ was considered statistically significant.

Table 3. Body Weight Gain, Liver Weight, and Relative Liver Weight of Rats^{a,b}

Group No.	Treatment	n	Body Wt Gain, g	Liver Wt, g	Relative Liver Wt, %
1	LCO	6	209 ± 20	7.88 ± 0.74 [‡]	2.94 ± 0.25 [‡]
2	LCO + PB	8	214 ± 24	9.27 ± 2.03 [†]	3.44 ± 0.49 ^{†,‡}
3	HCO + PB	8	214 ± 25	9.81 ± 1.47 [†]	3.62 ± 0.46 [†]
4	HFO + PB	7	217 ± 30	12.08 ± 1.91 [*]	4.39 ± 0.49 [*]
5	DEN → LCO + PB	10	207 ± 16	8.98 ± 1.11 [†]	3.38 ± 0.26 ^{†,‡}
6	DEN → HCO + PB	11	209 ± 16	9.65 ± 1.32 [†]	3.62 ± 0.34 [†]
7	DEN → HFO + PB	12	214 ± 18	12.08 ± 1.35 [*]	4.53 ± 0.51 [*]

a: Values are means ± SD. LCO, low corn oil; HCO, high corn oil; HFO, high fish oil; PB, phenobarbital; DEN, diethylnitrosamine.

b: Groups not sharing a symbol (*,†,‡) are significantly different ($p < 0.05$) by Tukey's test.

Results

Body weight gain of rats in different groups was not significantly different. Liver weight was significantly greater in rats fed the fish oil + PB diet than in rats fed the low corn oil and low corn oil and high corn oil + PB diets ($p < 0.05$). Rats fed a high corn oil + PB diet had significantly greater liver weight than rats fed a low corn oil diet ($p < 0.05$) and greater liver weight than rats fed a low corn oil + PB diet. Relative liver weight was significantly greater in rats fed a high fish oil + PB diet than in rats fed the other diets ($p < 0.05$), and the relative liver weight of rats fed low corn oil and high corn oil + PB diets was greater than that of rats fed a low corn oil diet (Table 3).

Without DEN initiation, rats developed no hepatic GGT-positive foci. In the presence of DEN initiation and PB promotion, rats fed low and high corn oil diets had significantly greater hepatic GGT-positive foci number than rats fed a high fish oil diet ($p < 0.05$), and significantly greater hepatic GGT-positive foci area was found in rats fed a high corn oil diet than in rats fed a high fish oil diet (Table 4).

The hepatic phospholipid fatty acid profile reflected the source of dietary lipids. Rats fed low corn oil and low corn oil and high corn oil + PB diets had significantly greater hepatic phospholipid 20:4(n-6) levels than rats fed a high fish oil + PB diet ($p < 0.05$). In contrast, rats fed a high fish oil + PB diet had significantly greater hepatic phospholipid 20:5(n-3) and 22:6(n-3) levels than rats fed low corn oil and low corn oil and high corn oil + PB diets ($p < 0.05$; Table 5).

The plasma vitamin E level of rats fed a high fish oil + PB diet was lower than that of rats fed the other diets. Also, rats fed a high corn oil + PB diet had a lower plasma vitamin E level than rats fed low corn oil diets with or without PB. Rats initiated with DEN and fed a high fish oil + PB diet had significantly lower plasma PGE₂ level than rats fed the other diets ($p < 0.05$). No difference in plasma PGE₂ level was found between rats fed low or high corn oil diets in the presence of PB. The RBC GSH status was greater in rats fed low and high corn oil + PB diets than in rats fed a high fish oil + PB diet. No difference in the RBC GSSG status was found among the different groups of rats. Liver lipid peroxidation was significantly greater in rats fed a high fish oil + PB diet than in rats fed low corn oil or low corn oil and high corn oil + PB diets ($p < 0.05$). Rats fed a high fish oil + PB diet had greater hepatic GSH reductase activity than rats fed the other diets, and rats not initiated with DEN and fed no PB had the lowest GSH reductase activity. Rats fed a low corn oil diet had significantly greater hepatic GSH peroxidase activity than rats fed low and high corn oil and high fish oil + PB diets ($p < 0.05$; Table 6).

PB administration enhanced hepatic PROD activity of rats. Rats fed a high fish oil + PB diet had significantly greater hepatic PROD activity than rats fed low and high corn oil + PB diets. Also, rats fed a high fish oil + PB diet had greater hepatic NADPH-P-450 reductase activity than rats fed the other diets. PB administration significantly enhanced hepatic GST activity ($p < 0.05$). Rats initiated with DEN and fed a high fish oil + PB diet had the greatest

Table 4. Hepatic GGT-Positive Foci Number and Area of Rats^{a,b}

Group No.	Treatment	n	Foci, no./cm ²	Area Occupied by Foci, mm ² /cm ²
1	LCO	6	0 ± 0 [†]	0 ± 0 [†]
2	LCO + PB	8	0 ± 0 [†]	0 ± 0 [†]
3	HCO + PB	8	0 ± 0 [†]	0 ± 0 [†]
4	HFO + PB	7	0 ± 0 [†]	0 ± 0 [†]
5	DEN → LCO + PB	10	8.10 ± 5.32 [*]	0.37 ± 0.29 ^{*,†}
6	DEN → HCO + PB	11	10.75 ± 4.25 [*]	0.54 ± 0.31 [*]
7	DEN → HFO + PB	12	2.67 ± 3.23 [†]	0.14 ± 0.17 ^{†,‡}

a: Values are means ± SD. GGT, γ -glutamyl transpeptidase.

b: Groups not sharing a symbol (*,†,‡) are significantly different ($p < 0.05$) by Tukey's test.

Table 5. Hepatic Phospholipid Fatty Acid Profile of Rats^{a,b}

Group No.	Treatment	n	Fatty Acid, %									
			14:0	16:0	16:1(n-7)	18:0	18:1(n-9)	18:2(n-6)	18:3(n-3)	20:4(n-6)	20:5(n-3)	22:6(n-3)
1	LCO	6	1.51 ± 0.74	22.50 ± 3.90*	0.60 ± 0.23*†	35.42 ± 1.96	8.72 ± 4.34	4.87 ± 1.43*†	0.10 ± 0.15	22.05 ± 7.52*	0 ± 0†	4.25 ± 1.85†
2	LCO + PB	8	1.91 ± 1.16	17.75 ± 4.40*†	0.51 ± 0.41*†	36.86 ± 2.79	8.89 ± 3.49	6.12 ± 1.83*†	0.05 ± 0.14	24.35 ± 5.89*	0.24 ± 0.64†	3.47 ± 0.64†
3	HCO + PB	8	1.47 ± 0.55	18.87 ± 3.89*†	0.37 ± 0.20†	37.24 ± 2.91	8.49 ± 3.92	5.90 ± 1.14*†	0.23 ± 0.34	23.86 ± 4.79*	0.29 ± 0.58†	4.21 ± 1.36†
4	HFO + PB	7	1.40 ± 0.88	19.83 ± 4.46*†	0.80 ± 0.17*	36.83 ± 1.54	8.21 ± 3.78	4.38 ± 1.96†	0.20 ± 0.21	11.01 ± 2.32†	8.55 ± 3.78*	8.79 ± 3.04*
5	DEN → LCO + PB	10	1.25 ± 0.75	19.78 ± 2.66*†	0.66 ± 0.19*†	36.14 ± 3.08	7.51 ± 3.56	6.31 ± 1.27*†	0.04 ± 0.09	26.62 ± 4.55*	0.02 ± 0.06†	3.77 ± 1.13†
6	DEN → HCO + PB	11	1.83 ± 1.49	15.23 ± 2.93†	0.34 ± 0.40†	35.62 ± 2.50	6.86 ± 4.49	7.05 ± 1.69*	0 ± 0	26.27 ± 5.36*	0 ± 0†	5.19 ± 1.64†
7	DEN → HFO + PB	12	2.25 ± 1.43	20.80 ± 3.71*	0.64 ± 0.26*†	35.25 ± 1.93	9.47 ± 3.51	5.76 ± 1.91*†	0.12 ± 0.23	12.22 ± 5.57†	4.42 ± 5.03†	9.07 ± 2.49*

a: Values are means ± SD.

b: Groups not sharing a symbol (*,†,‡) are significantly different ($p < 0.05$) by Tukey's test.

Table 6. Plasma Vitamin E and PGE₂ Levels, RBC GSH Status, Liver Lipid Peroxidation, and GSH Reductase and GSH Peroxidase Activities of Rats in Different Dietary Groups^{a,b}

Group No.	Treatment	n	Vitamin E, µg/ml	PGE ₂ , ng/ml	GSH, nmol/mg protein	GSSG, nmol/mg protein	TBARS, nmol/g	GSH Reductase, nmol NADPH/min/mg protein	GSH Peroxidase, nmol NADPH/min/mg protein
1	LCO	6	11.16 ± 1.27*	8.17 ± 1.12*	6.26 ± 2.34**†	0.04 ± 0.07	80 ± 26†	22.04 ± 3.51†	526 ± 69*
2	LCO + PB	8	10.04 ± 1.99**††	7.00 ± 1.00**†	7.67 ± 1.31*	0.02 ± 0.02	69 ± 18†	26.39 ± 4.26††	347 ± 116†
3	HCO + PB	8	9.44 ± 2.51**††	6.53 ± 1.32**†	6.83 ± 1.45**†	0.02 ± 0.02	67 ± 20†	26.63 ± 3.48††	310 ± 77††
4	HFO + PB	7	8.41 ± 1.61††	5.20 ± 1.26††	5.05 ± 0.97†	0.01 ± 0.02	159 ± 58*	30.68 ± 4.47**†	286 ± 64††
5	DEN → LCO + PB	10	10.83 ± 1.93**†	6.46 ± 1.00**†	7.46 ± 0.93*	0.04 ± 0.04	65 ± 26†	30.17 ± 4.00**†	366 ± 77†
6	DEN → HCO + PB	11	8.89 ± 1.66**††	6.78 ± 0.98**†	6.53 ± 1.43**†	0.04 ± 0.04	78 ± 20†	27.87 ± 4.30††	286 ± 73††
7	DEN → HFO + PB	12	7.54 ± 1.47†	4.90 ± 1.51†	5.13 ± 0.94†	0.05 ± 0.04	223 ± 106*	34.84 ± 4.21*	236 ± 46†

a: Values are means ± SD. PGE₂, prostaglandin E₂; GSH, glutathione; GSSG, oxidized GSH; TBARS, thiobarbituric acid-reactive substances.

b: Groups not sharing a symbol (*,†,††) are significantly different (p < 0.05) by Tukey's test.

Table 7. Hepatic Phase I and Phase II Enzyme Activity of Rats^{a,b}

Group No.	Treatment	n	PROD, pmol/min/mg protein	NADPH-P-450 Reductase, nmol/min/mg protein	GST, nmol/min/mg protein
1	LCO	6	3.83 ± 1.83 [‡]	117 ± 21 [‡]	1,050 ± 293 [‡]
2	LCO + PB	8	51.14 ± 35.06 ^{‡,§}	156 ± 32 ^{‡,§}	2,019 ± 391 [‡]
3	HCO + PB	8	118 ± 41.78 [‡]	217 ± 60 ^{‡,‡}	2,080 ± 327 [‡]
4	HFO + PB	7	218.33 ± 144.06 ^{*,†}	312 ± 90 [*]	2,518 ± 600 ^{*,†}
5	DEN → LCO + PB	10	120.90 ± 48.71 [‡]	218 ± 49 ^{‡,‡}	2,198 ± 440 [‡]
6	DEN → HCO + PB	11	131.78 ± 37.54 ^{‡,‡}	211 ± 56 ^{‡,‡}	2,299 ± 652 [‡]
7	DEN → HFO + PB	12	239.91 ± 64.27 [*]	264 ± 54 ^{*,†}	3,152 ± 515 [*]

a: Values are means ± SD. PROD, 7-pentoxoresorufin O-dealkylase; GST, glutathione S-transferase.

b: Groups not sharing a symbol (*, †, ‡, §) are significantly different ($p < 0.05$) by Tukey's test.

hepatic GST activity. Rats fed a high fish oil + PB diet also had greater hepatic GST activity than rats fed low and high corn oil + PB diets (Table 7).

The correlation coefficients between GGT-positive foci number and area and plasma PGE₂, vitamin E and liver TBARS, GST, glutathione reductase, glutathione peroxidase, PROD, and NADPH-P-450 reductase activities were calculated in all groups combined, groups that developed foci (Groups 5-7), and individual groups. The only significant correlation coefficients were found between foci number and liver TBARS ($r = -0.47$, $p = 0.0048$) and PROD activity ($r = -0.44$, $p = 0.0162$) and foci area and liver TBARS ($r = -0.37$, $p = 0.0318$) in rats of groups that developed foci (Groups 5-7). The correlation coefficients between foci development and other parameters were not significant in rats of all groups combined, groups that developed foci, or individual groups.

Discussion

In this study, different levels and types of dietary lipid did not affect the body weight gain of rats regardless of whether the rats were initiated with DEN or promoted with PB. This may have been due to the approximately equal amount of calories in the different diets. Therefore, the suppressive effect of a high fish oil diet on hepatic GGT-positive foci development compared with high corn oil diet was not related to the caloric intake. Significantly greater liver weight and relative liver weight were found in rats fed a high fish oil + PB diet than in rats fed the other diets. A similar result was found in our previous study, which showed that 14% fish oil feeding caused significantly greater liver weight and relative liver weight than the same amount of corn oil feeding in spontaneously hypertensive rats (52). Rats fed diets with 500 ppm PB had significantly greater liver weight than rats fed no PB, and the liver-enlarging effect of PB is known to be related to hypertrophy and/or hyperplasia of hepatocytes (53). We also found that dietary fish oil increases the microsomal protein content compared with dietary corn oil in rats (52). In another previous study, we investigated the effect of different types of dietary lipid on hepatic cytochrome P-450

activity and found that fish oil exhibited a greater stimulatory effect on hepatic phase I and II detoxification enzyme systems than corn oil (54). These findings suggest that the effect of fish oil on altered hepatic foci may occur through its influence on PB bioactivation to reduce the tumor-promoting action of PB.

Rats not initiated with DEN developed no hepatic GGT-positive foci (Table 4), and the results demonstrate the role of DEN as a complete carcinogen (25). Rats initiated with DEN and fed different amounts and types of dietary lipid with 500 ppm PB developed hepatic GGT-positive foci. In our previous study (18), the effect of different dietary lipids on altered hepatic foci was only found in the presence of both DEN and PB. Therefore, in the present study, we only investigated the role of amount and type of dietary lipid on altered hepatic foci in the presence of both factors. In the present study, it seems that the type of dietary lipid was a more important determinant of the development of hepatic GGT-positive foci than the amount of dietary lipid when the diets had approximately the same caloric content. These findings suggest that a certain amount of n-6 PUFA is necessary for chemically induced altered hepatic foci development (55,56). Hopkins and Carroll (55) found that rats treated with dimethylbenz[a]anthracene and maintained on a diet with 17 wt% saturated fat in combination with 3 wt% n-6 dietary lipid had mammary tumor development similar to that of rats maintained on a 20 wt% n-6 dietary lipid. Ritskes-Hoitinga and co-workers (56) demonstrated that a dose-response relationship between linoleic acid concentration and mammary tumor incidence or latency period was not observed when Balb/c mice were fed the mouse mammary tumor virus strain diets with 36 or 16 en% fat, and there were four levels of linoleic acid in each group, e.g., 2, 3, 6, and 10 en%.

In the present study, the hepatic phospholipid fatty acid profile reflected the dietary source, and a similar result was found in our previous studies (18,54). Alteration in cell membrane composition can be caused by dietary fat and can affect membrane physical-chemical properties including fluidity, which may modulate membrane-associated physiological processes (57). The liver tumor-promoting action of PB has been described for its alteration of the membrane com-

position and interference with the transmission of regulatory factors from normal to spontaneously altered cells (58). It is possible that the effect of dietary lipid on altered hepatic foci occurs through its influence on cell membrane composition and then affects the capability of PB to inhibit intercellular communication.

In the present study, we also assayed antioxidants, antioxidant enzymes, and PGE₂. The antioxidant results are consistent with those of Chen and colleagues (18) and suggest that fish oil has greater lipid peroxidation potential and that more vitamin E was utilized by rats fed a high fish oil diet. The observation of Dormandy and Wickens (59) and Cerutti and associates (60) suggested that increased antioxidant capacity and decreased lipid peroxidation conditions favor neoplastic growth of initiated cells. The lower plasma vitamin E level and significantly greater liver lipid peroxidation in the fish oil group suggest that the growth of neoplastic cells may be influenced by the cellular antioxidant capacity and lipid peroxidation potential. The lower RBC GSH content of rats fed the high fish oil diet suggests that more GSH was utilized to compensate for the greater oxidative stress caused by the high fish oil diet. The activities of two GSH-related antioxidant enzymes were determined in the present study. GSH reductase is responsible for the regeneration of GSH from GSSG. The greater GSH reductase activity in rats fed a high fish oil + PB diet in the present study may have been due to the lower RBC GSH status, and the enhancement of this activity can compensate for the lower RBC GSH, which was due to the greater utilization by rats fed a high fish oil + PB diet. Rats fed a high fish oil + PB diet had lower hepatic GSH peroxidase activity than rats fed the other diets. In addition to GSH peroxidase, GST, a conjugating biotransformation enzyme, also possesses GSH peroxidase activity, i.e., the selenium-independent GSH peroxidase. Sheen and others (61) suggested that selenium-dependent GSH peroxidase and selenium-independent GSH peroxidase constitute a constant pool, and they may have a complementary effect on each other. In the present study, rats fed a high fish oil + PB diet had greater hepatic GST activity than rats fed the other diets (Table 7). These results were consistent with the hypothesis proposed by Sheen and others that selenium-dependent GSH peroxidase and selenium-independent GSH peroxidase are complementary with each other. Dormandy and Wickens and Cerutti and associates proposed that increased antioxidant capacity and decreased lipid peroxidation conditions favor neoplastic growth of initiated cells. The antioxidant/oxidation status of rats may be partially, if not totally, responsible for the effect of different types of dietary lipid on hepatic GGT-positive foci formation. The significantly lower plasma PGE₂ level of rats fed a fish oil diet was due to the lower levels of dietary precursor for PGE₂ synthesis, e.g., linoleic acid. The fatty acid composition of dietary corn oil and fish oil was analyzed, and results are shown in Table 2. The linoleic acid content of corn oil was 58%, and that of fish oil was 2.9%. The difference in linoleic acid between these two oils was ~20-fold. In previous studies (15-20), results showed that intake of n-3 lipids

inhibited chemically induced tumor development compared with n-6 lipids, and the inhibition of 2-series PG synthesis by n-3 lipids was described as a possible mechanism for this inhibition (34). PGs have been reported to play an important role in chemical carcinogenesis (30-33), and the promoting action of PB has been suspected to be related to the increased PG synthesis (62). However, results of a previous study (60) and the present study showed that PB administration did not increase PGE₂ content in liver or plasma, as would be expected, but instead significantly decreased PGE₂ content. The results exclude the possibility that the PB-promoting action occurs through the PGE₂ pathway. Peebles and Glauert (62) proposed that the promoting action of PB may be *via* the activation of the transcription factor nuclear factor- κ B, which regulates cell proliferation and apoptosis in the liver (63,64). Eicosanoid inhibitors such as aspirin and sodium salicylate have also been shown to inhibit the activation of the transcription factor nuclear factor- κ B (65).

The role of hepatic bioactivation and biotransformation systems in chemical hepatocarcinogenesis was determined in the present study. Rats fed diets containing 500 ppm PB had greater hepatic PROD activity than rats fed a diet without PB (Table 7). These results are consistent with those of Chen and co-workers (54), and it is well known that PB can induce hepatic cytochrome IIB activity. We also found that rats fed a fish oil + PB diet had greater hepatic PROD activity than rats fed the other diets, and similar results were also found in our previous study (54). We suspect that specific fatty acids such as eicosapentaenoic acid and docosahexaenoic acid in fish oil may be responsible for the modulation of PROD expression induced by PB. The hepatic phase II enzyme GST was induced by PB administration, and the activity was greater in rats fed a fish oil + PB diet than in rats fed the other diets. In addition to PB administration, hepatic phase I and phase II enzyme activity was also affected by the amount and type of dietary lipid. These results suggest that the effect of dietary lipid on hepatic GGT-positive foci may occur through regulation of hepatic phase I and phase II enzyme activity and lead to changes in the promoting action of PB in different groups. To investigate the underlying mechanisms responsible for the inhibitory effect of a high fish oil diet on hepatic GGT-positive foci compared with a high corn oil diet, the Pearson correlation coefficients were evaluated between foci development and plasma PGE₂, vitamin E, liver TBARS, GST, GSH reductase, GSH peroxidase, PROD, and NADPH-P-450 reductase activities, respectively. Results showed significant correlation coefficients only between foci number and liver TBARS and PROD activity and between foci area and liver TBARS in rats that developed foci.

On the basis of the results, the underlying mechanisms responsible for the inhibitory effect of a high fish oil diet on hepatic GGT-positive foci compared with a high corn oil diet may be partially ascribed to the antioxidant/oxidation status and biotransformation/detoxification system of rats. In conclusion, the type of dietary lipid is a more important determinant for GGT-positive foci development than the amount of dietary lipid.

Acknowledgments and Notes

This work was supported by the National Science Council of Taiwan, ROC, under Contract NSC 89-2320-B-040-022. Address correspondence to Dr. H.-W. Chen, Dept. of Nutrition, Chung Shan Medical College, Taichung, Taiwan 40203. E-mail: Hawwen@mercury.csmc.edu.tw.

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