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EFFECT OF DIETARY FAT ON THE INDUCTION OF HEPATIC MICROSOMAL CYTOCHROME P450 ISOZYMES BY PHENOBARBITAL*†

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Abstract—Dietary polyunsaturated fatty acid is needed for optimal induction of cytochrome P450. In this study we quantitated cytochrome P450 hemoproteins in male Sprague–Dawley rats that were starved for 36 hr and then refed a fat-free diet (FF) or a diet containing 20% corn oil for 4 days. Some received phenobarbital (Pb) sodium (80 mg/kg, i.p., daily) for 3 days prior to decapitation. Microsomal cytochrome P450 levels were measured by carbon monoxide binding spectra, and the P450 isozymes separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis were quantitated by gel scanner. Cytochrome P450 PB-B was quantitated by a Western blot technique. Rats fed FF diet and administered Pb had only 21% more microsomal P450 than non-induced controls, whereas rats fed 20% corn oil diet had 59% more P450 and Pb-treated rats fed 20% corn oil diet had 181% more P450 than FF controls. Analysis of gels showed 32, 59 and 124% more P450 protein, respectively, in FF Pb, corn oil control or corn oil Pb groups than in FF controls. Cytochrome P450 PB-B was not detected in non-induced groups, but quantitation by Western blot yielded 0.32 and 0.70 nmol/mg protein, respectively, in FF Pb and corn oil Pb groups. Our findings suggest that deprivation of dietary fat reduces the total amount of cytochrome P450 hemoprotein and its inducibility by Pb through decreased P450 hemoprotein synthesis. The limiting factor(s) restricting synthesis of new cytochrome P450 hemoproteins in rats refed a diet devoid of fat may be the inability to respond to the inducer (Pb) or the paucity of utilizable fatty acids needed for synthesis of the phospholipid matrix of the endoplasmic reticulum necessary for the support and proper juxtapositioning of these protein molecules.

A source of polyenoic fatty acids is necessary for optimal activities of the microsomal mixed-function oxidase (MFO) system. Deprivation of dietary lipid results in changes in relative content of microsomal phospholipid fatty acids, and associated with these changes are decreased metabolism of various xenobiotics, decreased content of hepatic microsomal cytochrome P450 (P450) measured by carbon monoxide binding specta and decreased binding of substrates to P450 [1]. These dietary fat-related alterations in the metabolism of drugs and carcinogens have been reported for microsomes from liver [2–5] and colon [6] and by isolated perfused liver [7]. Cheng *et al.* [8], utilizing a 21-day feeding study, reported that P450 isozymes quantitated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were not altered by a diet containing 10% corn oil, and significant differences in the quantity of protein representing each of the four isozymes of P450 were not observed. Therefore, it was postulated that dietary fats may cause selective perturbations in the membrane which, in turn, alter the intramembrane positioning of P450. These alterations in P450 orientation may increase its reactivity with substrates and with carbon monoxide and thus result in an apparent increase in P450 content. Although it is clear that high polyunsaturated fatty acid intake increases carbon monoxide binding spectra and substrate metabolism, quantitative increases in P450 hemoprotein content of hepatic microsomes from rats fed a corn oil diet have not been demonstrated clearly. The present study was initiated to determine the influence of a corn oil diet on the hemoprotein levels of P450 isozymes.

Several reports suggest that maximal induction of the MFO system by phenobarbital requires a source of dietary fat [2, 8–10]. Since feeding a diet containing coconut oil (largely saturated fatty acids) failed to permit the degree of phenobarbital induction afforded by the diet containing corn oil [9], it appears that dietary polyunsaturated fatty acids have a central role in promoting maximal induction of the mixed-function oxidases by phenobarbital.

Phenobarbital administered to rats fed a fat-free diet for 4 days does not increase significantly hepatic microsomal P450 levels as measured by carbon monoxide binding spectra [2], whereas rats fed a fat-free diet for 21 days or longer respond to phenobarbital, albeit to a lesser degree than those fed a source of polyunsaturated fat [8–10]. Within 2–3 weeks compensatory mechanisms involving fatty acid

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Table 1. Diet composition*

Ingredient	Fat-free diet (%)	20% Corn oil diet (%)
Casein (vitamin free)	20.0	23.0
Corn oil	0.0	20.0
Wheat starch	45.6	21.8
Sucrose	25.0	25.0
Alphacel	5.0	5.0
dl-Methionine	0.4	0.5
Choline	0.16	0.2
AIN vitamin mix	0.82	1.0
AIN mineral mix	3.0	3.5

* Diets were prepared to obtain equal nutrient to calorie ratio (modification of O'Connor *et al.* [12]). All ingredients except sucrose were purchased from the United States Biochemical Corp., Cleveland, OH.

desaturases become operative and partially return the unsaturated to saturated fatty acid ratio of hepatic microsomal phospholipids to near normal [11]. Another objective of this study was to determine to what extent rats fed a fat-free diet for 4 days could synthesize the specific isozyme of cytochrome P450 (PB-B form) induced by phenobarbital.

METHODS

Chemicals. Reagents and kits for electrophoresis and immunoblotting were obtained from Bio-Rad Laboratories (Richmond, CA). All other chemicals and biologicals were purchased from the Sigma Chemical Co. (St Louis, MO).

Animals and treatment. Male Sprague–Dawley rats (100–125 g) obtained from Charles River Laboratories, Inc. (Raleigh, NC) were acclimated on a 12-hr light/dark cycle with light from 6:00 a.m. to 6:00 p.m. and fed Purina Rodent Chow 5001 and water *ad lib.* for 7 days prior to food deprivation for 36 hr. Following this period, rats were refed a diet devoid of fat or a similar diet containing 20% corn oil for 4 days (Table 1). One-half of the rats of each dietary group were administered phenobarbital sodium (80 mg/kg, i.p.) for 3 successive days beginning 1 day after refeeding the respective diet. The remaining rats were administered an equal volume of 0.9% NaCl solution and served as controls.

Preparation of microsomes and enzyme assays. Animals were decapitated between 9:00 and 10:00 a.m., and livers were removed and chilled in ice-cold buffer. Hepatic microsomal fractions were prepared from the 9000 *g* supernatant fraction, suspended in 0.1 M phosphate buffer containing 20% glycerol, 1 mM EDTA, and 1 mM dithiothreitol (DTT), and stored at −80°. The protein concentration of hepatic microsomes was determined by the method of Lowry *et al.* [13] and total hepatic microsomal cytochrome P450 levels were measured by the method of Omura and Sato [14]. Glutathione *S*-transferase activity of hepatocyte cytosol (105,000 *g* supernatant) was measured by the method of Habig *et al.* [15]. Five micrograms of hepatic microsomal protein was separated by 10% SDS–PAGE using a Mini-protein® II dual slab cell (Bio-Rad Laboratories), employing a modification of the method of Laemmli [16], and stained with Coomassie R-250. Low molecular weight standards (Bio-Rad Laboratories) were used to identify fractions by molecular weight. Each isozyme of P450 was identified by a 3,3′,5,5′-tetramethylbenzidine staining procedure for the detection of the peroxidase activity of cytochrome P450 on SDS-PAGE [17]. Wet gels were scanned with a Gilford Response® gel scanner at 557 nm in order to quantitate each band of cytochrome P450.

Western blot analysis. Microsomal proteins separated by 10% SDS–PAGE were transferred to a nitrocellulose membrane by the method of Towbin *et al.* [18] using the Mini trans-blot® electrophoretic transfer cell (Bio-Rad Laboratories).

In the Western blotting analysis utilized in this study, P450 PB-B was identified by a rabbit antibody specific for the PB-B form. Subsequently, the nitrocellulose membrane was probed with goat anti-rabbit antibody conjugated to horseradish peroxidase, and the PB-B form was detected by the horseradish peroxidase color reaction. (Rat P450 PB-B and rabbit anti-rat P450 PB-B were supplied by Dr. F. P. Guengerich, Vanderbilt University, Nashville, TN.) The amount of P450 PB-B was quantitated by a Shimazu CS-930 dual-wavelength TLC scanner at 500 nm.

Statistical analysis. The effects of dietary corn oil on the various parameters were analyzed using one-way analysis of variance. The statistical significance of difference due to experimental treatment was evaluated by the Duncan's multiple range test and Student's *t*-test, with $P < 0.05$ set at the minimum level of significance.

RESULTS

Influence of dietary corn oil and treatment with phenobarbital on liver weight, cytosolic glutathione S-transferase and total cytochrome P450 levels in refed rats. Body weight gains and terminal body weights were not significantly different between rats fed 20% corn oil diet and those fed fat-free diet (data not shown). Liver weight, as a percentage of body weight, was not significantly different between controls fed fat-free diet and those fed corn oil diet, but it

was elevated significantly as a result of phenobarbital administration in rats fed fat-free or corn oil diets (Table 2).

As shown in Table 2, the activity of the cytosolic glutathione *S*-transferase responsible for conjugating 1,2-dichloro-4-nitrobenzene was increased approximately 23% in rats fed the 20% corn oil diet. Although phenobarbital administration induced glutathione *S*-transferase 119% in rats fed the corn oil diet, it failed to induce this enzyme significantly in rats fed the diet devoid of corn oil. Diet containing corn oil fed 4 days following 36-hr food deprivation increased the concentration of cytochrome P450 to 1.6 times that of controls fed fat-free diet. Rats injected for 3 days with phenobarbital had only 21% more cytochrome P450 than their non-injected fat-free controls, whereas phenobarbital-treated rats fed 20% corn oil diet had 181% more cytochrome P450 than fat-free controls (Table 2). When calculated as nanomoles per gram liver, phenobarbital treatment induced cytochrome P450 only in rats refed the diet containing corn oil.

Separation and quantitation of cytochrome P450 hemoprotein on SDS–PAGE by gel scanner. When 5 μg of hepatic microsomal protein was separated by 10% SDS–PAGE and stained with Coomassie R-250, three discrete bands having molecular weights of 46.9, 48.2 and 50.0 kD were defined in the control groups fed fat-free and 20% corn oil diets. Two additional discrete bands having molecular weights of 51.2 and 53.0 kD were defined in phenobarbital-treated groups (Fig. 1). The molecular weight of each band was within the range of known cytochrome P450 hemoproteins, and each band was identified by 3,3′,5,5′-tetramethylbenzidine staining for the detection of peroxidase activity of cytochrome P450 on SDS–PAGE.

Representative spectrophotometric gel scans of the SDS–PAGE of hepatic microsomal fractions from control or phenobarbital-treated rats fed a fat-free or a 20% corn oil diet are presented in Fig. 2. These P450 bands were identified numerically in the order of their increasing molecular weight (P1–P5). The P4 band was identified as P450 PB-B by SDS–PAGE of purified P450 PB-B.

The spectrophotometric quantitation of microsomal proteins separated by SDS–PAGE is presented in Table 3. Absorbance of each P450 band and total peak area of intensity were measured for assessment of P450 hemoproteins. Analysis of gels showed 32, 59 and 124% more total P450 hemoprotein, respectively, in fat-free phenobarbital, corn

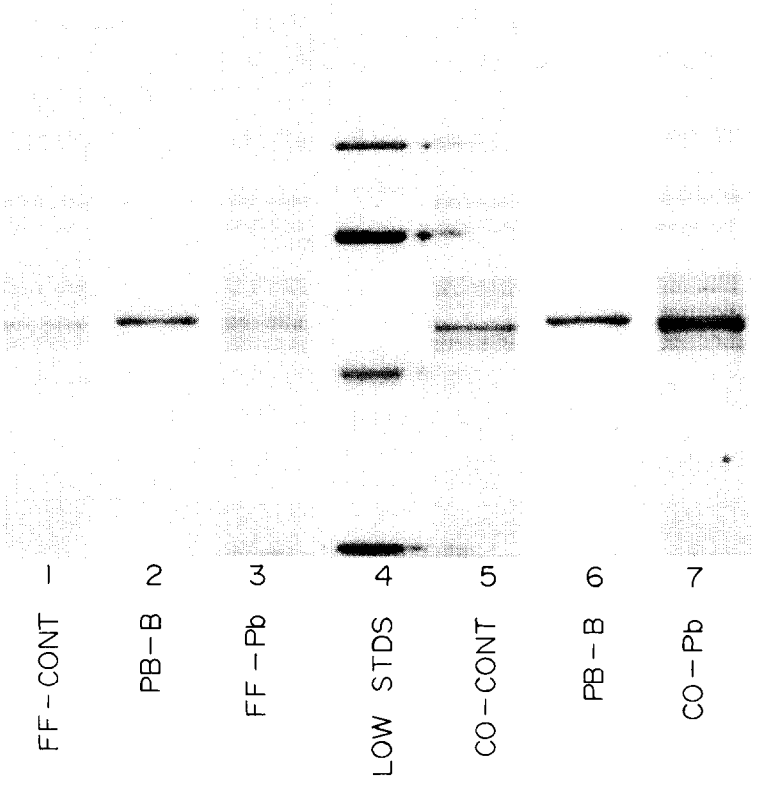

Fig. 1. Fractions of hepatic microsomal proteins separated by 10% SDS–PAGE and stained with Coomassie R-250. Rats were fasted for 36 hr prior to refeeding fat-free (FF) or 20% corn oil (CO) diet for 4 days. Some received phenobarbital (Pb) sodium (80 mg/kg, i.p.) for 3 days prior to decapitation. Key: Each 5 μg of hepatic microsomal proteins from (1) a rat fed FF diet, (3) a rat fed FF diet and administered Pb, (5) a rat fed CO diet, and (7) a rat fed CO diet and administered Pb; (2) and (6) 5 pmol of purified cytochrome P450 PB-B; (4) low molecular weight standards (Bio-Rad Laboratories) (top to bottom): phosphorylase *b* (97,400 daltons), albumin (66,200 daltons), ovalbumin (42,699 daltons), and carbonic anhydrase (31,000 daltons).

Table 2. Influence of dietary corn oil and treatment with phenobarbital (Pb) on liver weight, cytosolic glutathione *S*-transferase and cytochrome P450 levels in male rats*†

Diet	Treatment	Liver weight (g/100 g body wt)	Cytosolic glutathione *S*-transferase‡	Microsomal cytochrome P450 (nmol/mg protein)	Microsomal cytochrome P450 (nmol/g liver)
Fat-free	Control	5.19 ± 0.45[a,b]	145.2 ± 16.0[a] (100%)	0.574 ± 0.064[b] (100%)	10.70 ± 1.45[b] (100%)
	Pb	6.58 ± 0.46[c]	162.9 ± 18.5[a] (112%)	0.695 ± 0.113[b] (121%)	11.19 ± 2.73[b] (105%)
20% Corn oil	Control	4.91 ± 0.36[b]	179.3 ± 31.2[a] (123%)	0.914 ± 0.066[a] (159%)	17.85 ± 2.79[a] (167%)
	Pb	5.68 ± 0.57[a]	317.3 ± 45.7[c] (219%)	1.611 ± 0.175[c] (281%)	43.35 ± 6.55[c] (405%)

* Rats were fasted for 36 hr prior to refeeding fat-free or 20% corn oil diet for 4 days. Some received Pb sodium (80 mg/kg, i.p.) for 3 days prior to decapitation.

† Values are means ± SD for groups of 5–6 rats. Numbers in parentheses represent the percent changes, compared to a fat-free control group.

‡ Expressed in nmol 1,2-dichloro-4-nitrobenzene conjugated/mg cytosolic protein/min.

[a–c] Means which are not significantly different at $P < 0.05$ are followed by the same letter.

Table 3. Quantitative analysis of SDS–polyacrylamide gel by gel scanner*†

Diet	Treatment	Absorbance of cytochrome P450 fractions P1	P2	P3	P4	P5	Total, peaks 1, 2, 3 (unit = absorbance·mm)
Fat-free	Control	0.038 ± 0.008[a]	0.033 ± 0.007[a]	0.049 ± 0.008[b]	BD‡	BD	0.115 ± 0.019[a] (100%)
	Pb	0.051 ± 0.006[a]	0.038 ± 0.009[a]	0.058 ± 0.014[a,b]	0.058 ± 0.014[a]	0.015 ± 0.008[a]	0.151 ± 0.020[a] (132%)
20% Corn oil	Control	0.051 ± 0.005[a]	0.054 ± 0.008[c]	0.083 ± 0.016[a,c]	BD	BD	0.182 ± 0.039[a] (159%)
	PB	0.078 ± 0.021[c]	0.044 ± 0.016[a,c]	0.108 ± 0.046[c]	0.118 ± 0.052[c]	0.044 ± 0.016[c]	0.256 ± 0.104[c] (224%)

* Rats were fasted for 36 hr prior to refeeding fat-free or 20% corn oil diet for 4 days. Some received phenobarbital (Pb) sodium (80 mg/kg, i.p.) for 3 days prior to decapitation.

† Values are means ± SD for groups of 5–6 rats. Numbers in parentheses represent the percent changes of total peak area, compared to a fat-free control group.

‡ Below detection limit.

[a–c] Means which are not significantly different at $P < 0.05$ are followed by the same letter.

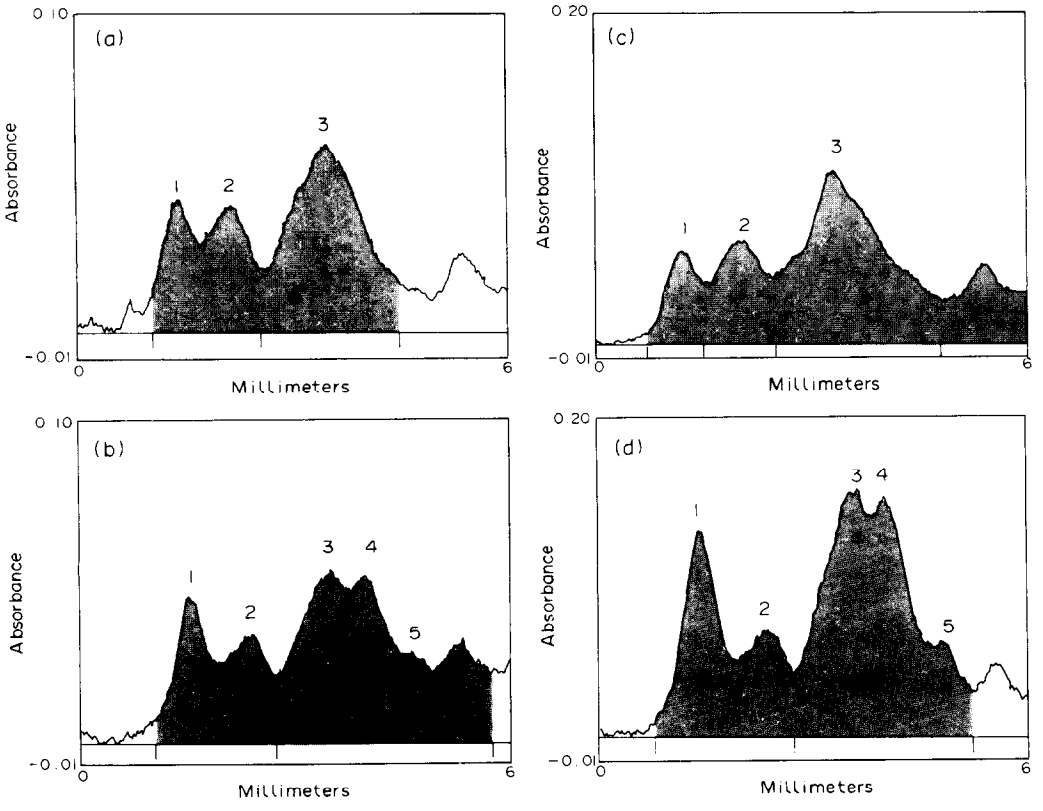

Fig. 2. Spectrophotometric gel scans of the SDS–PAGE of 5 μg of hepatic microsomal fractions from (a) a rat fed fat-free diet; (b) a rat fed fat-free diet and administered phenobarbital sodium (Pb); (c) a rat fed 20% corn oil diet; and (d) a rat fed 20% corn oil diet and administered Pb. Rats were fasted for 36 hr prior to refeeding fat-free or 20% corn oil diet for 4 days. Some received Pb (80 mg/kg, i.p.) for 3 days prior to decapitation. Five micrograms of hepatic microsomal protein was separated by 10% SDS–PAGE and each isozyme of cytochrome P-450 was identified by a 3,3',5,5'-tetramethylbenzidine staining procedure for the detection of the peroxidase activity of cytochrome P450 on SDS–PAGE. These isozymes stained with Coomassie R-250 were scanned by a Gilford Response® gel scanner and identified numerically in the order of their increasing molecular weight.

oil control or corn oil phenobarbital groups than in fat-free controls. Phenobarbital induced two new isozymes of cytochrome P450 which were below the level of detection in untreated rats and enhanced significantly the level of a constitutive form having a molecular weight of 46.9 kD. Corn oil increased the concentration of the three constitutive isozymes of cytochrome P450 measured in these assays (46.9, 48.2, and 50.0 kD).

Table 4. Quantitation of P450 PB-B by western blot analysis*†

Diet	Treatment	Content of P450 PB-B (nmol/mg protein)
Fat-free	Control	BD‡
	Pb	0.321 ± 0.049
20% Corn oil	Control	BD
	Pb	0.697 ± 0.060§

* Rats were fasted for 36 hr prior to refeeding fat-free or 20% corn oil diet for 4 days. Some received phenobarbital (Pb) sodium (80 mg/kg, i.p.) for 3 days prior to decapitation.

† Values are means ± SEM for groups of 5–6 rats.

‡ Below detection limit.

§ Significantly different from corresponding treatment of fat-free groups at $P < 0.01$.

Effect of dietary corn oil on P450 PB-B induction. Representative Western blots of hepatic microsomal P450 PB-B from control or phenobarbital-treated rats are shown in Fig. 3. Each nitrocellulose membrane contained purified standard P450 PB-B (0.5 to 4 pmol) [19] and one microsomal sample from each treatment group. These were separated simultaneously by SDS–PAGE and treated with rabbit anti-rat P450 PB-B, thus allowing direct quantitation with the standard. As shown in Fig. 3, anti-P450 PB-B IgG crossreacted with a minor phenobarbital-induced form of P450. Integration of the resulting peaks by TLC scanner indicated that staining of horseradish peroxidase was directly proportional to the amount of microsomal protein or purified antigen applied to each well (correlation coefficient is >0.99).

Constitutive cytochrome P450 PB-B was not detectable in the absence of inducer. Feeding corn

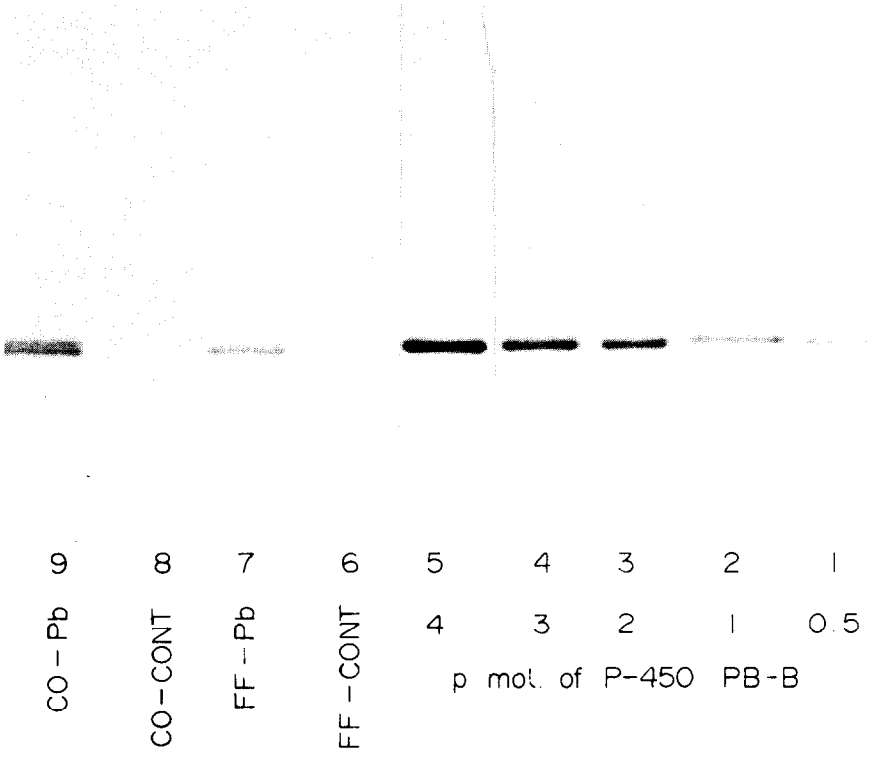

Fig. 3. Effect of dietary fat on the Western blot analysis of hepatic microsomal cytochrome P450 PB-B from control or phenobarbital (Pb) treated rats. Rats were fasted for 36 hr prior to refeeding fat-free (FF) or 20% corn oil (CO) diet for 4 days. Some received Pb sodium (80 mg/kg, i.p.) for 3 days prior to decapitation. SDS–PAGE (10%) was performed on 0.5, 1, 2, 3, and 4 pmol of P450 PB-B, and 2.5 μg of hepatic microsomal proteins from (6) a rat fed FF diet; (7) a rat fed FF diet and administered Pb; (8) a rat fed CO diet; (9) a rat fed CO diet and administered Pb. The nitrocellulose membrane was treated with rabbit anti-rat P450 PB-B and probed with goat anti-rabbit antibody conjugated to horseradish peroxidase.

oil enhanced the induction of cytochrome P450 PB-B hemoprotein to levels more than two times those of rats fed the fat-free diet (Table 4).

DISCUSSION

Effects of dietary fats on specific functions of the drug-metabolizing enzymes have been studied extensively [20, 21], and their involvement in the metabolism of foreign substances including drugs, polycyclic aromatic hydrocarbons, carcinogens, and other environmental contaminants reported. However, molecular mechanisms by which dietary fat may affect these metabolizing enzymes are still unknown. The results of our study demonstrate that feeding diets containing 20% corn oil for 4 days produced greater activity of the mixed-function oxidases and higher concentrations of the constitutive forms (46.9, 48.2, 50.0 kD) of hepatic microsomal cytochrome P450 hemoproteins than measured in the endoplasmic reticulum of hepatocytes from rats fed a fat-free diet (Table 3). Constitutive P450 PB-B was not detectable in control rats fed fat-free or 20% corn oil diets.

Previous investigators have suggested that phenobarbital enhances the mRNA level of P450 PB-B by increasing the rate of transcription [22, 23]. Our findings indicate that little induction of P450 PB-B hemoprotein by phenobarbital occurs in the absence of dietary fat, whereas this induction is amplified two times in rats fed dietary corn oil. Thus, deprivation of dietary fat not only decreases the total amount of microsomal P450 hemoprotein but reduces its inducibility by phenobarbital. This reduced level of microsomal cytochrome P450 may be due to a paucity of fatty acids needed for synthesis of the phospholipid matrix of endoplasmic reticulum necessary for the support and proper juxtapositioning of these protein molecules. Alternatively, synthesis of new cytochrome P450 hemoproteins in rats refed a diet devoid of fat may be restricted by the inability to respond to the signal generated by the inducer, phenobarbital.

The amount of total microsomal proteins per gram liver, which is normally induced by phenobarbital, was not increased in the absence of dietary polyunsaturated fatty acid, whereas phenobarbital significantly induced the synthesis of new microsomal proteins in the presence of dietary fat (data not shown). Although cytosolic glutathione *S*-transferase has been shown to be inducible by phenobarbital [24], the induction of this soluble enzyme by phenobarbital occurred only in the presence of dietary corn oil (Table 2). These findings suggest that polyunsaturated fatty acids affect some signal

involved in the *de novo* synthesis of proteins including P450 hemoprotein, possibly through alteration of responsiveness through protein kinase C [25].

Although the induction of P450 PB-B by phenobarbital is clearly illustrated, the underlying mechanisms and the receptor for phenobarbital are still unknown. Active protein kinase C is believed to phosphorylate a specific protein that binds to the genome and represses the genes for P450 PB-B [26]. Recently, phenobarbital was shown to inhibit protein kinase C by occupying the diacylyglycerol activating site [25]. Inhibition of this site appears to reduce the activation of the specific PB-B repressor protein, thus causing significant elevation of cytochrome P450 in the presence of phenobarbital [26].

The results of our study suggest that in rats fed adequate polyunsaturated fatty acids, such as linoleate, phospholipid synthesis provides the optimum structural integrity of the endoplasmic reticulum for positioning the various enzymes including cytochrome P450. In the absence of these polyunsaturated fatty acids, endoplasmic reticulum changes [1] may limit the amount and activity of these enzymes. Thus, lower concentrations of cytochrome P450 are observed in animals fed fat-free diet than in those fed adequate polyunsaturated fatty acid.

The unresponsiveness of animals fed the diet devoid of polyunsaturated fatty acids to the inductive effects of phenobarbital may be related to a decreased efficiency of phenobarbital to inhibit protein kinase C. In the animals fed fat-free diet, increased activity of phospholipases (A and C) may elevate levels of intracellular fatty acids needed for essential cellular processes and thus provide increased concentrations of diacylglycerol. Since phenobarbital competes with diacylglycerol for protein kinase C [25], this inhibition may be compromised severely in animals acutely fed fat-free diet. Without efficient inhibition of kinase C by phenobarbital, little or no induction occurs. The diacylglycerol derived from membrane phospholipids has various fatty acids of different chain length depending upon the fatty acid make-up of the membrane. Those having a preponderance of unsaturated fatty acids are most efficient in activating protein kinase C [27]. The results of our study would support this mechanism if the level of highly reactive diacylglycerol is increased in animals fed the fatty acid deficient diet.

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