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THE EFFECT OF DIETARY LIPIDS AND VITAMIN E ON LIPID PEROXIDE FORMATION, CYTOCHROME P-450 AND OXIDATIVE DEMETHYLATION IN THE ENDOPLASMIC RETICULUM

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Abstract—The effects of varying the lipid components of the diet have been studied on the cytochrome P-450 content and the rate of oxidative demethylation of aminopyrine in the liver endoplasmic reticulum. The cytochrome P-450 content and rate of oxidative demethylation (V_{max}) were lowest when a fat-free diet was fed, increased by addition of 10% lard (containing mainly saturated and mono-unsaturated fatty acids but 6% linoleic acid) and much more by addition of 10% corn oil (containing 50% linoleic acid). Following induction with phenobarbitone the rates of oxidative demethylation and cytochrome P-450 were also greatest in animals fed the corn oil diet and least in animals fed the fat-free diet. Addition of vitamin E (120 mg/kg diet) to the lard diet caused a significant increase in the rate of oxidative demethylation but the synthetic antioxidant 2,6,di-*tert*-butyl-*p*-cresol (BHT) was ineffective. The lipid peroxide content of the endoplasmic reticulum and the rate of NADPH stimulated peroxidation were much greater if the corn oil diet was fed than if the fat free diet was fed. Addition of vitamin E reduced the lipid peroxide in the endoplasmic reticulum when a lard diet was fed but BHT was ineffective. It is concluded that polyunsaturated fatty acids, primarily linoleic acid and vitamin E are essential in the diet for the content of cytochrome P-450 and the rate of oxidative demethylation to be a maximum in the endoplasmic reticulum.

Feeding a protein deficient or a lipid free diet causes an inadequate synthesis of microsomal hydroxylating enzymes and cytochrome P-450 and induction of cytochrome P-450 by phenobarbitone is much less efficient when saturated fatty acids are incorporated into the diet as the sole source of dietary lipid than when unsaturated fats are fed [1–3]. It has therefore been postulated that polyunsaturated fatty acids might play an important role in the activity of the microsomal hydroxylating system [1].

Membranes of the subcellular organelles contain large quantities of polyunsaturated fatty acids [4] and on induction with phenobarbitone linoleic acid is incorporated into the membrane increasing the percentage of this fatty acid in the phospholipids of the endoplasmic reticulum [5]. Microsomal lipid peroxidation which involves destruction of polyunsaturated fatty acids of the membrane phospholipids causes a decrease in activity of glucose-6-phosphatase [6–8], NADH–cytochrome *c* reductase [9], aminopyrine demethylase and the quantity of cytochrome P-450 [10].

Dietary vitamin E and other antioxidants could maintain the integrity of the liver microsomal membranes and unsaturated fatty acids by decreasing the microsomal lipid peroxidation. Zalkin and Tappel [11] showed that mitochondria isolated from vitamin E deficient rabbit livers contained more lipid peroxides than mitochondria prepared from animals fed a normal diet, and they postulated that vitamin E functions as a stabilizer of cellular lipids. However, it has also been suggested that the main function of vitamin E is not connected with its protection of polyunsaturated fatty acids against autoxidation [12], and that it may be involved in the actual membrane structure protecting selenide proteins [13].

MATERIALS AND METHODS

Rats. Male albino rats 6–7 weeks old, weight 120–150 g were used in all experiments. Food and water were available *ad lib.*

Diets. Purified fat free diets were prepared according to Diplock *et al.* [14], and contained 25% casein (Unigate Ltd.), 30% sucrose, 20% wheat starch (Adcol Ltd.), 10% dried yeast (Bovril Ltd.), 10% lipid and 5% Cox's salt mix. The diet was supplemented with Rovimix A $-D_3$® (Roche Ltd.), supplying 5000 i.u. vit. A and 1000 i.u. vit. D_3/kg diet.

Dietary lipid components. Corn oil (Mazola® C.P.C.), or lard, free from antioxidants, obtained from Unigate Ltd. was added as the dietary lipid to give a final concentration of 10% lipid. 2,6 di-*tert*-butyl-*p*-cresol (BHT) (British Drug Houses Ltd.) (250 mg/kg diet), and D-α-tocopherol (Sigma) (120 mg/kg diet) were added to the diet after mixing them into the lard. The diets were analysed for lipid peroxides using an iodometric method [15]. The aldehyde breakdown products of the dietary lipids were also determined by the thiobarbituric acid method [16]. The iodine numbers of the dietary lipids were estimated according to Wij's method [17]. The mixed tocopherol content of the dietary lipid fractions was estimated by saponification in the presence of an antioxidant β-hydroxyacetanilide, and removal of interfering substances by column chromatography [18]. Columns were prepared with purified MFC Fullers earth (Hop-

kins and Williams) and stannous chloride. The tocopherol was eluted with benzene and estimated by the Emmerie–Engel reaction [19].

Determination of fatty acid composition of dietary lipids. The fatty acid composition of the dietary lipids was determined by gas–liquid chromatography. The lipids were methylated with boron fluoride–methanol complex [20], and an internal standard, arachidic acid, was added to each lipid, so that the percent methylation and recovery could be calculated. The fatty acid methyl esters were separated on a column of 10% polyethylene glycol adipate on chromosorb W80-100 mesh using nitrogen gas as a carrier in a Pye 104 Series g.l.c. chromatograph with a flame ionisation detector.

Phenobarbitone. Induction of drug metabolising enzymes after 21 days on special diets was achieved by giving the animals 1 mg/ml sodium phenobarbitone in their drinking water [21].

Tissue samples. Each rat was killed by cervical fracture, the liver removed, rinsed in ice-cold 0·25 M sucrose, blotted dry, and weighed. A 5-g sample of each liver was finely chopped and homogenised for 1 min in ice-cold 0·25 M sucrose using a Potter–Elvehjem homogeniser. The 10% homogenate was then centrifuged for 25 min at 9000*g* in a refrigerated MSE 18. The supernatant was decanted and the microsomal fraction recovered by precipitation with a calcium salt [22]. The supernatant was diluted 1:5 v/v with 12·5 mM sucrose containing 8 mM $CaCl_2$ pH 7·5, mixed well and centrifuged for 10 min at 30*g* av. The microsomal pellet was resuspended in 125 mM KCl (0·5 g liver/ml) to make a 50% microsomal fraction.

Assays. Aminopyrine demethylase activity was assayed by the method described by Wills [23]. Cytochrome P-450 was measured by the method of Omura and Sato [24], using a Carey 15 recording spectrophotometer. The microsomal fraction was diluted with phosphate buffer 0·1 M Na_2HPO_4 NaH_2PO_4 pH 7·4 so that it contained 2–3 mg protein/ml.

The lipid peroxide concentration in the microsomal fraction was measured by determination of the malonaldehyde equivalent in the tissue using the thiobarbituric acid method. Microsomal NADPH dependent lipid peroxidation was determined according to Wills [25]. TBA reactive materials were measured after incubation for 20 min and the results expressed in nmoles malondialdehyde formed /mg protein per min. The extinction coefficient for malondialdehyde in the thiobarbituric acid reaction was taken to be $1{\cdot}56 \times 10^5 cm^2 m^{-1}$ [25].

Table 1. Fatty acid analysis of dietary lipids

		% Total fatty acids	
		Lard	Corn oil
Lauric acid	(14:0)	—	1·1
Palmitic acid	(16:0)	31·4	12·9
Palmitoleic acid	(16:1)	2·4	—
Stearic acid	(18:0)	12·6	2·4
Oleic acid	(18:1)	46·3	33·0
Linoleic acid	(18:2)	5·9	49·9
Minor fatty acids		1·4	0·7

RESULTS

Diet analysis. The fatty acid composition of the lard and corn oil are shown in Table 1 and the relative quantities of vitamin E, lipid peroxide and aldehyde breakdown products of peroxidation in each diet used are shown in Table 2.

Vitamin E and BHT protected the lard against peroxidation and although vitamin E was much more efficient in causing a reduction of dietary lipid peroxides both antioxidants reduced the concentration of the aldehyde breakdown products to an equal extent. Despite the high content of unsaturated fatty acids in corn oil, with an iodine number of 100, the dietary lipid peroxide concentration remained low owing to the presence of a high concentration of naturally occurring vitamin E in the oil.

Effect of dietary lipid on cytochrome P-450 and oxidative demethylation. The effects of dietary lipids upon microsomal aminopyrine demethylase activity and cytochrome P-450 are shown in Table 3. The levels of cytochrome P-450 and activity of aminopyrine demethylase were both lower after feeding the fat-free diet than after feeding the lard diet, and highest rates of oxidative demethylation and levels of cytochrome P-450 were observed after feeding the corn oil diet.

The increases in activity of oxidative demethylation of aminopyrine and cytochrome P-450 concentration in the microsomal fraction were not caused by changes in the protein content of the fractions. These were not significantly different after feeding the three diets before induction, and after induction the protein content of the microsomal fraction of the group fed corn oil was slightly greater than that of the group fed the fat free diet (Table 3). These results therefore provide clear evidence that dietary lipid is essential

Table 2. Vitamin E, lipid peroxide and aldehyde content of diets and iodine numbers of dietary lipids

Diet	Lipid peroxide m-moles/kg diet	Malonaldehyde equiv. (μmoles/kg diet)	Vitamin E content (mg/kg diet)	Dietary lipid iodine number
Fat-free	0·39	1·15	0	0
Lard	1·07	60·3	2·25	64
Lard + vit. E*	0·26	19·1	120·0	64
Lard + BHT†	0·92	19·6	2·25	64
Corn oil	0·27	144·6	101·0	100

* 120 mg D-α-tocopherol/100 g lard or /kg diet.
† 250 mg BHT/100 g lard or /kg diet.

Table 3. The effect of dietary lipids on microsomal protein, aminopyrine demethylase activity and levels of cytochrome P-450

		Diet		
		Fat free	10% Lard	10% Corn oil
Cytochrome P-450 nmoles/mg prot.	basic	0·36 ± 0·01	0·47 ± 0·01	0·62 ± 0·03
	induced	0·62 ± 0·02	1·15 ± 0·06	1·48 ± 0·26
Aminopyrine demethylase K_m(mM)	basic	0·44 ± 0·01	0·49 ± 0·02	0·55 ± 0·02
	induced	0·34 ± 0·01	0·41 ± 0·01	0·46 ± 0·02
Aminopyrine demethylase V_{max} (nmoles/min/mg prot)	basic	2·91 ± 0·43	4·45 ± 0·01	6·06 ± 0·28
	induced	6·39 ± 0·27	10·95 ± 0·74	13·20 ± 0·78
Microsomal protein concentration (mg prot/g liver)	basic	19·4 ± 1·3	20·2 ± 1·5	19·8 ± 0·7
	induced	24·8 ± 1·2	26·9 ± 3·8	28·7 ± 0·7

Results are expressed as the mean ± S.E.M.
All diets were fed for 21 days before induction with phenobarbitone which was given in the drinking water for a further 10 days.

for maximum activity of detoxication enzymes in the liver.

Effects of dietary antioxidants on oxidative demethylation activity and levels of cytochrome P-450. If polyunsaturated fatty acids are important for enzymes of the hydroxylating system then addition of antioxidants which protect these polyunsaturated fatty acids might be valuable. To show the effect of antioxidants the lard diet was supplemented with vitamin E. Table 4 shows that vitamin E increased the rate of oxidative demethylation and the level of cytochrome P-450 from those of the lard diet to values similar to those observed after feeding the corn oil diet. However the supplementation of the lard diet with the antioxidant, BHT, had much less effect than the vitamin E.

Only very small changes in the protein content of the microsomal fraction were caused by addition of vitamin E or BHT to the lard diet before induction, and after induction vitamin E caused a small increase in protein content (Table 4).

The increase in rate of oxidative demethylation and cytochrome P-450 content of the microsomal fraction caused by vitamin E cannot therefore be explained on the basis of a change of protein concentration. BHT may be unable to reach the site of microsomal drug hydroxylation, or alternatively the antioxidant properties of vitamin E are not responsible for its effect on cytochrome P-450 and aminopyrine demethylase activity.

Effect of lipid peroxidation on microsomal aminopyrine demethylase activity and level of cytochrome P-450. The peroxidation of endogenous lipids in the endoplasmic reticulum membrane is actively stimulated by NADPH and is enzyme dependent. It involves an NADPH–cytochrome *c* oxidoreductase and the rate of lipid peroxidation depends on the activity of the NADPH-dependent electron transport chain [23, 25].

The rate of NADPH linked lipid peroxidation was lower after feeding a fat-free or a lard diet than after feeding a corn oil diet (Table 5). However, the supplementation of the lard diet with vitamin E did not

Table 4. The effect of dietary antioxidants on microsomal protein aminopyrine demethylase activity and levels of cytochrome P-450

		10% Lard* +vit. E	10% Lard† +BHT	10% Lard
Cytochrome P-450 (nmoles/mg prot.)	basic	0·64 ± 0·04	0·49 ± 0·02	0·47 ± 0·01
	induced	1·51 ± 0·07	1·06 ± 0·02	1·15 ± 0·06
Aminopyrine demethylase K_m(mM)	basic	0·43 ± 0·01	0·44 ± 0·02	0·49 ± 0·02
	induced	0·37 ± 0·01	0·38 ± 0·01	0·41 ± 0·01
Aminopyrine demethylase V_{max} (nmoles/min/mg)	basic	7·01 ± 0·44	4·8 ± 0·08	4·45 ± 0·10
	induced	13·76 ± 0·17	11·7 ± 0·31	10·95 ± 0·74
Microsomal protein concentration (mg prot./g liver)	basic	18·9 ± 0·8	17·4 ± 0·7	20·2 ± 1·5
	induced	29·9 ± 0·7	28·2 ± 1·0	26·9 ± 3·8

Results are expressed as the mean ± S.E.M.
* 120 mg D-α-tocopherol 100 g lard or kg diet.
† 250 mg BHT/100 g lard or /kg diet.

Table 5. Effect of dietary lipids on microsomal lipid peroxide and rate of lipid peroxidation

	Microsomal lipid peroxide concn (nmoles/mg prot.)		NADPH-linked lipid peroxidation (nmoles/mg prot./min)	
	Basic	Induced	Basic	Induced
Fat free	3·30 ± 0·36	2·38 ± 0·12	1·27 ± 0·09	0·70 ± 0·06
10% Lard	4·79 ± 0·21	3·76 ± 0·43	0·96 ± 0·05	0·53 ± 0·13
10% Corn oil	29·45 ± 5·34	12·30 ± 2·00	2·05 ± 0·21	2·15 ± 0·23
10% Lard + vit. E	3·61 ± 0·64	5·06 ± 0·76	0·39 ± 0·05	0·84 ± 0·05
10% Lard + BHT	5·40 ± 0·10	4·41 ± 0·67	1·13 ± 0·14	1·02 ± 0·04

Results are expressed as the mean ± S.E.M.

raise the rate of NADPH dependent lipid peroxidation as it did the content of cytochrome P-450 and aminopyrine demethylase activity, but actually reduced the rate of NADPH linked lipid peroxidation. Supplementation of the lard diet with BHT increased the lipid peroxide content and the rate of lipid peroxidation especially after phenobarbitone induction (Table 5). BHT cannot therefore be considered to exert an antioxidant effect on lipid peroxidation in the microsomal fraction as does vitamin E.

DISCUSSION

These results show that both the quantity of lipid in the diet and its composition play very inportant roles in the regulation of the concentration of cytochrome P-450 in the microsomal fraction and of the rate of oxidative demethylation both before and after induction with phenobarbitone. The rate of oxidative demethylation is much greater when lard containing mainly saturated and mono-unsaturated fat (Table 1) is added to the diet but even greater if corn oil, containing a high concentration of polyunsaturated fatty acids, and especially linoleic acid, is incorporated into the diet (Table 3). It appears very likely that the dietary unsaturated lipids provide a supply of essential unsaturated fatty acids for incorporation into the membranes of the endoplasmic reticulum. It has already been shown that linoleic acid is rapidly incorporated into membrane phospholipids during phenobarbitone induction. [5].

In addition to supplying polyunsaturated fatty acids it appears that corn oil was especially effective in causing stimulation of oxidative demethylation on account of its vitamin E content. The importance of vitamin E was demonstrated by adding vitamin E to the lard diet. The addition did not change the iodine number of the unsaturated fatty acid content of the diet but caused a marked stimulation of the level of cytochrome P-450 and of the rate of oxidative demethylation as indicated by the V_{max} (Table 4).

Vitamin E could act in several ways. It could play a role as an antioxidant preventing lipid peroxide formation in the diet before feeding or in the endoplasmic reticulum protecting the unsaturated fatty acids of the membrane. Alternatively, it may play a specific role as part of the structure of the membranes of the endoplasmic reticulum and thus be essential for maximum activity of oxidative demethylation.

It is clear that addition of vitamin E to the lard diet does depress the lipid peroxide content of the diet (Table 2). Tissues from vitamin E deficient animals form much more lipid peroxide than those from normal animals and there is good evidence that vitamin E acts as stabiliser of unsaturated membrane lipids [11]. It is also established that induction of lipid peroxidation in membranes of the endoplasmic reticulum leads to a sharp fall in the activity of oxidative demethylation which is believed to be caused by a loss of membrane integrity [8]. A primary function of vitamin E may be to protect vital selenium containing proteins of the endoplasmic reticulum membrane [13].

The possible role of vitamin E was investigated by two methods—firstly, by investigating the peroxide content and peroxidation rates in the endoplasmic reticulum after feeding different diets, and secondly by replacing the vitamin E by an artificial antioxidant, BHT.

When added to the lard diet, vitamin E reduced both the peroxide content and the rate of lipid peroxidation before induction but increases were observed after induction (Table 5). It is also significant that the corn oil diet, which contained a relatively high concentration of vitamin E, also caused the formation of a high lipid peroxide content and a rapid rate of peroxidation in the endoplasmic reticulum (Table 5). BHT however added to the lard did not reduce the microsomal lipid peroxide content or the rate of lipid peroxidation and increases were observed (Table 5). These results therefore tend to support the concept that non-specific antioxidants cannot protect the unsaturated lipids of the membranes and that the vitamin E may be specifically localised in the membrane structure as suggested previously [13]. It is also significant that the feeding of phenobarbitone leads to a decrease in peroxidation rate and peroxide content in all experiments where vitamin E was not added to the diet. Phenobarbitone also appears to be able to stabilise the membranes in a manner similar to that observed for vitamin E and membrane stabilisation may be an essential prelude to induction.

These experiments therefore demonstrate that lipids, primarily polyunsaturated fatty acids, linoleic acid and vitamin E are essential in the diet for maximum activity of oxidative drug metabolism. Neither vitamin E nor polyunsaturated fat given alone is as effective as a combination of both factors. For maximum activity of the oxidative drug metabolising enzymes of the endoplasmic reticulum a delicate balance between the two factors is essential to prevent

extensive destruction of the membranes of the endoplasmic reticulum by lipid peroxidation.

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