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EFFECT OF DIETARY FAT ON THE *IN VITRO* HEPATOTOXICITY OF PARACETAMOL

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Abstract—In the present study, we have examined the effect of dietary fat on paracetamol-induced liver injury in an *in vitro* rat liver slice model. Rats were fed, for 7–10 days, diets containing either butter or polyunsaturated vegetable margarine, two fat sources commonly consumed in the human diet. Liver slices were then exposed to paracetamol for 2 hr and further incubated for 4 hr without paracetamol. Cell damage in the slices was quantified at 6 hr by measuring leakage of lactate dehydrogenase, increase in water content and potassium loss. Covalent binding of radioactive paracetamol to liver and the membrane fatty acid composition of the liver were also measured. Liver slices from rats fed butter diets were significantly more sensitive to the toxic effects of paracetamol than those from margarine fed rats. The membrane lipid composition of the livers also reflected the differing fatty acid content of the two diets.

Two recent reports have proposed revised estimates of the amounts of nutrients required for a balanced diet [1, 2]. One of the more striking recommendations in both reports was for a considerable reduction in total fat consumption, with particular emphasis on reducing saturated fat intake. In recent years, total dietary fat intake in the U.K. (expressed as % total calories) has remained relatively constant at 38–40% [3]. In the last decade there has been an increase in the ratio of polyunsaturated to saturated fat in the diet, although this trend now appears to be levelling out [3].

Recommendations for decreasing fat consumption were prompted mainly by studies suggesting a positive correlation between high dietary fat intake and the incidence of coronary heart disease, and cancer of the breast and large bowel (summarized in Ref. 1). However, there has been little consideration of other consequences to health of changing dietary fat intake, such as altered metabolism and toxicity of the many foreign compounds to which we are continuously exposed in our environment. The cytochrome P450-dependent microsomal mixed function oxidase (MFO†) system plays an important role in the metabolism of many of these foreign compounds, and the effects of dietary fat on P450 dependent xenobiotic metabolism are well documented [4–10] and have been reviewed recently [11, 12].

Effects of dietary fat on xenobiotic metabolism should be considered both in terms of the total quantity of fat ingested and its fatty acid composition. Generally, deprivation of dietary lipids results in decreased metabolism of various xenobiotics and a decreased content of hepatic cytochrome P450 [5]. Both constitutive and inducible levels of P450 may be affected by dietary fat.

Recent evidence shows a particular requirement for polyunsaturated fatty acid (PUFA) for maximal activity of MFO activity [4, 6–10]. It has been widely reported that the fatty acid composition of cellular membranes and in particular the PUFA content of the microsomal fraction is highly dependent on the fat composition of the diet. An important consequence of this with regard to xenobiotic metabolism is that changes in the PUFA content of the endoplasmic reticulum may alter membrane conformation and fluidity, resulting in changes in the activity of membrane bound MFOs [7, 13, 14].

In the present study, we have examined the effect of different types of dietary fat on paracetamol hepatotoxicity and liver membrane fatty acid composition, using an *in vitro* rat liver slice model. Rats were fed two fat sources which are common components of the human diet, either butter, a highly saturated animal fat (68.7% saturated fat), or a polyunsaturated vegetable margarine (50.7% unsaturated fat). The fat content of the diets was 10% by weight (20.2% total calories).

Paracetamol hepatotoxicity has been studied extensively, and the initial metabolic events which cause toxicity are well documented. The initial phase of paracetamol metabolism involves formation of glucuronide and sulphate conjugates which are safe, excretable products. A small fraction of paracetamol is converted by the microsomal MFO system to the reactive metabolite *N*-acetyl-*p*-benzoquinoneimine. In overdose, the normal detoxification pathway of this metabolite via glutathione is saturated and the reactive metabolite can attack cell components leading to cell death. It is these subsequent mechanisms that are not fully understood.

By studying the effect of dietary fat on membrane fatty acid composition and subsequent paracetamol toxicity, we hope to gain further understanding of

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† Abbreviations: PUFA, polyunsaturated fatty acid; MFO, mixed function oxidase; PUM, polyunsaturated vegetable margarine; LDH, lactate dehydrogenase; TCA, trichloroacetic acid.

the sequence of events leading to cell death, and how these events may be affected by changes in dietary habit.

MATERIALS AND METHODS

Animals and slices. Male Wistar rats (OLAC, Bicester, U.K.) weighing 120–200 g were fed for 7–10 days a semi-purified diet containing 10% fat (w/w) as either butter or polyunsaturated vegetable margarine (PUM). The diet was based on cornflour with 15% casein, plus vitamins and minerals [15]. Phenobarbitone solution (0.1%) was provided *ad lib.* as the sole source of drinking water. Phenobarbitone was given for at least 5 days prior to killing. Vitamin E (5 mg α-tocopherol acetate in two drops of olive oil) was given by mouth 12–24 hr before killing. Rats were exsanguinated under anaesthesia using fentanyl citrate (0.01 mg/kg, i.m.) and diazepam (2.5 mg/kg, i.p.) (Janssen, Wantage, U.K.). The livers were rapidly removed and slices of 0.3 mm thickness or less (approximately 100 mg) were cut by hand on a Stadie–Riggs stage with a long razor blade (A. H. Thomas Co., Philadelphia). Slices were put into 25 mL Erlenmeyer flasks containing 5 mL of Ringer solution ±5 mM paracetamol (Ringer composition; NaCl 125 mM, KCl 6 mM, $MgSO_4$ 1.2 mM, NaH_2PO4 1 mM, $CaCl_2$ 1 mM, glucose 10 mM, Hepes buffer 15 mM, pH 7.4 at 37°, as described previously [15]). Flasks were incubated at 37°, under oxygen, in a shaking water bath (90 strokes/min). After 2 hr the slices were taken out of the first flask and reincubated in fresh, paracetamol-free Ringer solution for a further 4 hr.

Chemicals. Paracetamol and all other reagents used were purchased from the Sigma Chemical Co. (Poole, U.K.) or BDH Ltd (Poole, U.K.) and were of analytical grade. [^{14}C]Paracetamol, labelled in ring C-3 and C-5 position, was from Amersham International (Amersham, U.K.).

Measurement of enzyme leakage, K^+ and water content. Injury was assessed by measuring leakage of lactate dehydrogenase (LDH) from the slice into the incubation medium, loss of potassium from the slice, and an increase in its water content [16]. LDH released into the Ringer solution was expressed as a percentage of the amount of enzyme originally present in the slice before incubation [16].

In some experiments, isocitrate dehydrogenase leakage was also measured and gave essentially the same results. For measurement of water and potassium content, slices were removed from the incubation medium, blotted and weighed. They were then dried at 90° overnight and weighed again. Water content was expressed as mg water per mg dry weight of liver slice. Potassium content was measured in an HCl extract of the dried piece of liver, by atomic absorbtion, as described previously, and expressed as nmoles K^+ per mg dry weight of liver slice [16].

Measurement of covalent binding of radiolabelled paracetamol. In the present study the radiochemical purity of [^{14}C]paracetamol was assessed to be 99.9% after purification by HPLC [17].

Liver slices were incubated in Ringer paracetamol for 2 hr, as described above, but with the addition of 1 μCi of [^{14}C]paracetamol. Slices were removed from the incubation medium, rinsed in cold 0.15 M sodium chloride, blotted, weighed and then homogenized in 10 mL of 10% trichloroacetic acid (TCA) containing 1 mM "cold" paracetamol. The homogenate was centrifuged at 2500 rpm for 10 min and the supernatant discarded. The pellet was resuspended and then washed twice in 10 mL of 10% TCA. The TCA pellet was then washed in 10 mL of 80% methanol (v/v aqueous). The final pellet was digested in 2 mL of 1 M NaOH at 37° overnight. Radioactivity in 200 μL of the digested pellet was determined by liquid scintillation counting in a Packard Tri-carb system. Protein in the digest was determined by the method of Lowry *et al.* [18].

Analysis of membrane phospholipid fatty acids. Rats were fed their respective diets for 7–10 days, then killed as described above. The liver was removed and chilled in ice-cold saline. A 10% homogenate in 0.15 M KCl/25 mM Tris was prepared and centrifuged at 10,000 *g* at 4° for 10 min, and the resulting supernatant centrifuged at 100,000 *g* for 1 hr at 0–4°. The microsomal pellet was resuspended in 0.05 M phosphate buffer pH 7.4, to give 4 mg microsomal protein/mL.

Lipid analysis. The lipid extraction was based on a method by Bligh and Dyer [19]. One millilitre of microsomal suspension was vortexed with 5 mL methanol containing 0.01% butylated hydroxytoluene. After addition of 2.5 mL of chloroform the mixture was centrifuged at 2000 rpm for 5 min. The supernatant was decanted off, mixed with 5 mL chloroform:water (1:1) and the phases separated by centrifugation. The chloroform layer was evaporated under nitrogen, 1 mL boron trifluoride (14% in methanol) added, and the sample left overnight at room temperature. Five millilitres hexane:water (1:1) was added and mixed, and the hexane layer containing the fatty acid methyl esters evaporated under nitrogen and reconstituted in 20 μL hexane. One microlitre was injected onto an Omegawax 320 fused silica capillary column (30 m × 0.32 mm, 0.25 μm film thickness, Supelco, Saffron Walden, U.K.) in a Hewlett Packard 5730 gas chromatograph with F.I.D. detection. Chromatographic conditions were as follows: injection temperature 200°; detector temperature 250°; carrier gas nitrogen (5–6 mL/min). The column was temperature programmed for 8 min at 150°, then 4°/min to 220° and finally 8 min at 220°.

Statistical analysis. All results are given as means ± SD. The statistical significance of the differences between the two treatment groups was determined by the Student's *t*-test.

RESULTS

Liver slices from rats fed a polyunsaturated fat source in the diet were less susceptible to the toxic effects of paracetamol than slices from rats fed a saturated fat source.

Liver slices—water and K^+ content and LDH leakage

Liver slices incubated with 5 mM paracetamol for 2 hr and then transferred to paracetamol free medium for 4 hr showed leakage of LDH at the end of the

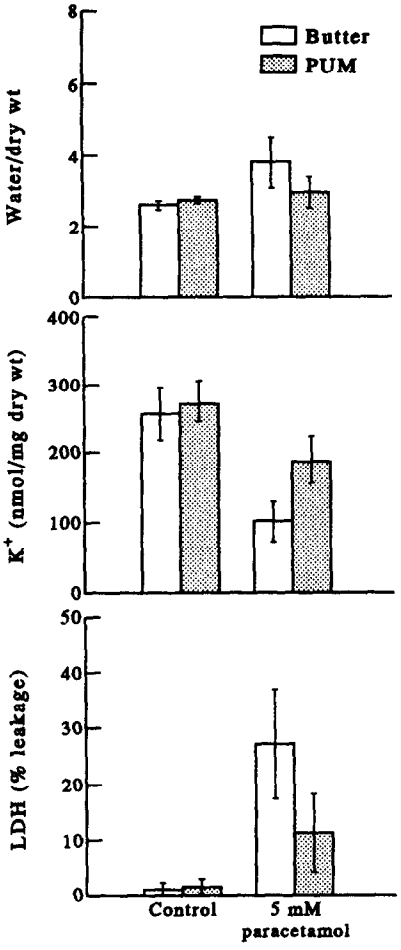

Fig. 1. Effect of dietary fat on paracetamol toxicity in liver slices, after 2 hr exposure to 5 mM paracetamol. Slice K^+ and water content, and leakage of LDH from slice into the medium were measured at 6 hr as described in Materials and Methods. Results represent mean values ± SD of at least four experiments performed in triplicate assays.

second incubation period. This was accompanied by loss of K^+ and an increase in water content. These *in vitro* toxic effects were significantly less ($P < 0.05$) in liver slices from rats fed a polyunsaturated fat source compared to slices from rats fed butter-containing diets (Fig. 1).

In contrast, slices incubated without paracetamol showed minimal leakage of enzyme and maintained a high potassium content.

Covalent binding of radiolabelled paracetamol

In liver slices incubated for 2 hr with 10 mM paracetamol containing 1 μCi [^{14}C]paracetamol, covalent binding was significantly higher ($P < 0.01$) in slices from butter-fed rats (2.61 ± 0.01 nmol [^{14}C]paracetamol/mg protein) compared to those from polyunsaturated fat-fed rats (1.81 ± 0.145 nmol [^{14}C]paracetamol/mg protein).

Fatty acid content of liver microsomes

The fatty acid composition of the liver microsomes (Fig. 2) reflected the nature of the dietary fat source. This was particularly significant with linoleic and

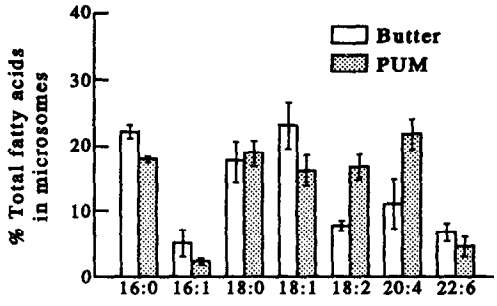

Fig. 2. Fatty acid content of liver microsomes from rats fed, for 7–10 days, semi-purified diets containing 10% fat as either butter or PUM.

arachiodonic acid. In liver microsomes from rats fed a polyunsaturated fat source, levels of linoleic and arachidonic acid were twice those found in liver microsomes from butter-fed rats.

DISCUSSION

The present study indicates that *in vitro* paracetamol hepatotoxicity is significantly decreased in rats fed a polyunsaturated fat source (10% vegetable margarine) in the diet, compared to rats fed an equal quantity of saturated fat (10% butter). This protective effect was manifested in the liver slices as a lower water content, decreased LDH leakage and decreased potassium loss. Liver slices from margarine-fed rats also had significantly less covalent binding of radioactivity after exposure to radiolabelled paracetamol. Although the mechanism of this protective effect is unclear, there are several possible explanations.

Liver toxicity caused by paracetamol is due to the formation of a toxic metabolite by the cytochrome P450 system, a system known to be susceptible to dietary manipulation. Several workers [4, 7, 8, 20] have shown increased P450 levels and MFO activity in animals fed diets containing PUFA compared to those given saturated fat, suggesting a PUFA requirement for optimal activity of the MFO system. Specifically, linoleic acid as well as the highly unsaturated, long chain fatty acids found in fish oils (eicosopentaenoic acid and docosohexanoic acid) have all been shown to be important in this respect [14, 20]. In the present study, P450 levels were marginally higher in rats fed the PUFA (margarine) diet as compared to those fed the butter diet, although these differences were not statistically significant. With higher levels of P450 one might expect increased activation of paracetamol and thus greater toxicity in the PUFA-fed rats. In the present study, the reverse effect was seen, with greater paracetamol toxicity in rats fed a saturated fat diet. However, the method used to measure P450 in the present study (spectrophotometrically as the reduced carbon monoxide complex) does not distinguish specific P450 isoenzymes which may selectively activate paracetamol.

When considering the effects of dietary manipulation on drug metabolism and toxicity, it is important to examine the effects on specific P450 isoenzymes. In a recent study [21] investigating the effect of dietary lipids on both constitutive and inducible

levels of specific cytochrome P450 isoenzymes, it was shown that, compared to a fat-free diet, corn oil diet doubled the constitutive level of cytochrome P450IIE. This isoenzyme has been implicated in paracetamol activation to *N*-acetyl-*p*-benzoquinoneimine, and it would thus be expected that induction of this specific isoenzyme by polyunsaturated fat would enhance paracetamol toxicity. However, it has been shown that both constitutive and inducible levels of glutathione-*S*-transferase are also increased in rats fed PUFA-containing diets, suggesting that the balance between activating and inactivating pathways of metabolism may be altered by dietary manipulation.

In contrast, other workers [22] have shown that in rats fed a fat-free or 10% corn oil diet, levels of P450 isozymes were similar. These workers propose that rather than altering P450 levels *per se*, dietary fat may alter the activity of P450 by altering its intramembrane positioning. Hammer and Wills [23] have shown that the fatty acid composition of the cell membrane is highly susceptible to dietary manipulation, and observed changes in the fatty acid composition of the endoplasmic reticulum after only 1–2 days on an experimental diet. Essential fatty acids, when incorporated into membrane phospholipids, can alter membrane fluidity which in turn can influence the conformation, mobility and function of membrane-bound proteins, including enzymes.

In the present study we have shown marked differences in the microsomal membrane fatty acid composition of the livers from rats fed the two diets. Rats fed polyunsaturated fat diets had a significantly higher microsomal membrane content of linoleic and arachidonic acid. These differences in membrane composition from animals fed the two diets may cause alterations in enzyme activity and subsequent differences in drug metabolism and toxicity in the two groups of animals. Recently, Speck and Lauterburg [14] have suggested a membrane effect to explain the protective effect of fish oil against paracetamol toxicity in the mouse *in vivo*. These workers propose that the major protective effect of fish oil appears to be due to an increased clearance of paracetamol by stimulation of the glucuronidation pathway, possibly caused by fluidization of microsomal membranes by fish oil. It would be of interest to examine the effect of the diets used in the present study on the *in vivo* metabolism of paracetamol.

Alterations in dietary fat have been shown to affect both membrane fatty acid composition and drug toxicity, both of which may have far reaching consequences in man. These observations warrant further investigation, particularly with the current trend towards an increased polyunsaturated fat consumption in the human diet.

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