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EFFECT OF PHENOBARBITAL ON LIPID PEROXIDATION IN THE LIVER*

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Abstract — It has been established that the administration of phenobarbital to rats produces an accumulation of triglyceride in the liver. In the present study, it has been demonstrated that phenobarbital increases hepatic lipid peroxidation as reflected by the observed diene conjugation absorption of microsomal lipids as well as the increase in malonic dialdehyde formation. Both the fatty infiltration and the lipoperoxidation were found to be inhibited by an antioxidant, *N, N'*-diphenyl-*p*-phenylenediamine. These data suggest that the peroxidative process may play a role in the phenobarbital-induced fatty liver.

Phenobarbital (PB), as well as many other commonly administered drugs, has the ability to induce the drug-metabolizing microsomal enzyme systems of the liver. In addition, PB also has a profound influence on other facets of hepatic metabolism [1–4]. It was recently demonstrated in our laboratory that PB administration produced an accumulation of triglyceride in the liver [5, 6]; however, the mechanism responsible for this effect is still unknown.

Hepatic microsomal lipid peroxidation *in vivo* has been proposed as a mechanism responsible for the development of a fatty liver which can progress to cell necrosis [7, 8]. One of the most extensively studied model systems of lipid peroxidation has been the hepatotoxicity produced by carbon tetrachloride [7–9]. The peroxidation of subcellular lipids can occur by both an enzymatic and non enzymatic process [10–14]. The enzymatic system has an absolute requirement for the NADPH-dependent flavoprotein, NADPH cytochrome *c* reductase [11, 15–17]. This same flavoprotein is also involved in the oxidative metabolism of drugs [4, 15, 18]. Thus, there exists a close relationship between hepatic microsomal lipid peroxidation and the mixed function oxidase system. Since the induction of the components of the mixed function oxidase system including NADPH cytochrome *c* reductase by PB has been well established [1, 19, 20], the possibility exists that PB could also induce peroxidation. In addition, PB has been shown to increase the activity of microsomal NADPH oxidase [1, 21]. This could result in enhanced H_2O_2 production which could also enhance the peroxidative process. The purpose of this study was to determine if PB increased lipid peroxidation in the liver and to determine if peroxidation was involved in the formation of the fatty liver.

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EXPERIMENTAL

Male, Sprague–Dawley rats (250 g) were maintained on a synthetic diet as previously described [6]. This diet contained 35% fat, 24% protein and 2·1% choline dihydrogen citrate. The animals receiving phenobarbital (PB) were injected intraperitoneally with 50 mg/kg body weight every 12 hr for 5 days. PB suspended in mineral oil was injected in some animals, while others received injections of PB dissolved in saline. Control rats received equivalent amounts of mineral oil or saline. *N,N'*-diphenyl-*p*-phenylenediamine (DPPD) was suspended in mineral oil as a vehicle and administered by intraperitoneal injection at a dose of 60 mg/100 g body weight at intervals of 72 and 24 hr prior to phenobarbital injections and 24 hr before sacrifice. The rats in the various groups were group fed and were given water *ad lib*. The animals were killed by decapitation; their livers were removed immediately and weighed. One portion of liver was kept frozen at −50° for those analyses not requiring fresh tissue.

Triglycerides were determined according to Kessler and Lederer [22]. Malonic dialdehyde (MDA) was determined using the thiobarbituric acid method of Ghoshal and Recknagel [23] as modified by Scheig and Klatskin [24]. MDA production was measured in the 9000 *g* supernatant after a 30-min incubation period at 37°. Proteins in the 9000 *g* supernatant were determined according to Lowry *et al.* [25]. Conjugated dienes were determined in the extracted microsomal lipids as described by Srinivasan and Recknagel [26]. The recovered lipids were dissolved in 3 ml cyclohexane and read against a cyclohexane blank in a Beckman model 25 Spectrophotometer in cuvettes with a 1-cm path length. The samples were scanned over a range of 360 to 220 nm. Lipid phosphorus was determined according to Bartlett [27]. The results of diene conjugation were expressed as optical density readings from 232 to 234 nm and adjusted to a uniform base (per mg phospholipid/ml of cyclohexane).

NADPH cytochrome *c* reductase activity was determined by measuring the reduction of cyto-

Table 1. Effect of PB and DPPD on body weight, liver weight and microsomal enzymes*

Treatment	Body weight (g)	Liver weight (g)	Cytochrome *c* reductase†	Cytochrome P-450‡
Saline	290 ± 21 (8)	10·72 ± 0·58 (8)	19·15 ± 3·61 (4)	290 ± 52 (4)
Saline + PB	287 ± 27 (7)	15·01 ± 2·37§ (7)	26·50 ± 2·57§ (6)	544 ± 162 (5)
Mineral oil	277 ± 33 (10)	10·70 ± 1·19 (10)	17·33 ± 4·66 (6)	247 ± 53 (6)
Mineral oil + PB	301 ± 14 (8)	15·73 ± 1·04§ (8)	31·80 ± 7·50 (6)	937 ± 232‖ (6)
DPPD	233 ± 36 (9)	10·53 ± 1·89 (9)	17·80 ± 2·75 (6)	354 ± 61 (6)
DPPD + PB	238 ± 36 (7)	14·04 ± 1·81§ (7)	27·40 ± 5·20 (6)	928 ± 130 (6)

* Values are expressed as means ± S. D. Numbers of animals is shown in parentheses.
† Activity is expressed as μmoles cytochrome *c* reduced min/total liver/100 g body wt ± S. D.
‡ Content is expressed as nmoles/total liver/100 g body wt ± S. D.
§ Compared to the respective control, significance at $P < 0{\cdot}025$.
‖ Compared to the respective control, significant at $P < 0{\cdot}001$.

chrome *c* at 550 nm as described by Williams and Kamin [28]. The cytochrome P-450 content in liver was measured according to Schoene *et al.* [29] using an extinction coefficient of 91 cm^{-1} mM^{-1}.

RESULTS

In accordance with many previous studies [1, 2, 19, 20, 30], PB administration increased liver weight, NADPH cytochrome *c* reductase activity and the content of cytochrome P-450 (Table 1). It is noteworthy that the antioxidant, DPPD, did not interfere with these inductive effects of PB in the liver; however, the DPPD-treated rats did evidence a decrease in body weight.

Estimation of diene conjugation of microsomal lipids has been used by many investigators to determine the extent of lipid peroxidation *in vivo* [26, 31–34]. As can be seen in Table 2, administration of PB caused a marked increase in diene conjugation absorption over that observed in both the saline and mineral oil controls. Administration of DPPD to the controls did not alter the u.v. absorption of the microsomal lipids; however, DPPD was effective in suppressing the increased lipid peroxidation resulting from PB treatment. To further demonstrate the above results, Fig. 1 shows the ultraviolet spectra for microsomal lipids and the difference spectrum between the mineral oil control and PB-treated animals. These spectra represent an average sample containing 2·5 mg phospholipid/3 ml of cyclohexane. The increase in the diene conjugation peak of the microsomal lipids of the PB-treated rats ranged from 232 to 235 nm throughout the experiment. The ability of DPPD to suppress the PB-induced increase in diene conjugation is clearly demonstrated in Fig. 2. The u.v. difference spectrum of the microsomal lipids between the DPPD- and DPPD plus PB-treated animals does not exhibit a peak. The peak seen at 300 nm in Fig. 2 is due to DPPD absorption.

The effects of PB and DPPD on hepatic triglyceride levels are depicted in Table 2. Because of the changes in liver weight and body weight due to the experimental conditions, hepatic triglyceride content is expressed using three different standards of reference. PB administration caused a 3- to 5-fold increase in triglycerides as compared to both the saline and mineral oil controls. These results compare favorably with those previously reported by this laboratory [5, 6]. Administration of DPPD in a suspension of mineral oil did not significantly modify hepatic triglycerides; however, administration of DPPD to rats which received PB resulted in a 60–70 per cent inhibition of triglyceride accumulation. This partial

Table 2. Effect of PB and DPPD on hepatic lipid peroxidation and triglycerides*

Treatment	Diene conjugation absorption†	Triglycerides (mg/g)	Triglycerides (mg total liver)	Triglycerides (mg total liver/100 g body wt)
Saline	0·314 ± 0·039 (5)	10·97 ± 0·91	118 ± 11	40·8 ± 4·5
Saline + PB	0·449 ± 0·055‡ (6)	36·16 ± 7·94‡	473 ± 74‡	197·2 ± 88·0‡
Mineral oil	0·295 ± 0·070 (6)	15·19 ± 4·75	162 ± 50	59·3 ± 18·8
Mineral oil + PB	0·430 ± 0·100§ (6)	36·11 ± 7·31	557 ± 114‡	188·2 ± 37·9‡
DPPD	0·285 ± 0·064 (6)	10·71 ± 6·14	113 ± 67	47·6 ± 26·0
DPPD + PB	0·284 ± 0·065‖ (6)	18·39 ± 3·52¶,**	260 ± 68‡,¶	109·1 ± 28·2‡,¶

* Values are expressed as means ± S. D. Number of animals is shown in parentheses.
† Units of absorption are O.D./mg of phospholipid/ml.
‡ Compared to the respective control, significant at $P < 0{\cdot}001$.
§ Compared to the respective control, significant at $P < 0{\cdot}025$.
‖ Not significant.
¶ Compared to the saline + PB and mineral oil + PB groups, significant at $P < 0{\cdot}001$.
** Compared to the respective control, significant at $P < 0{\cdot}01$.

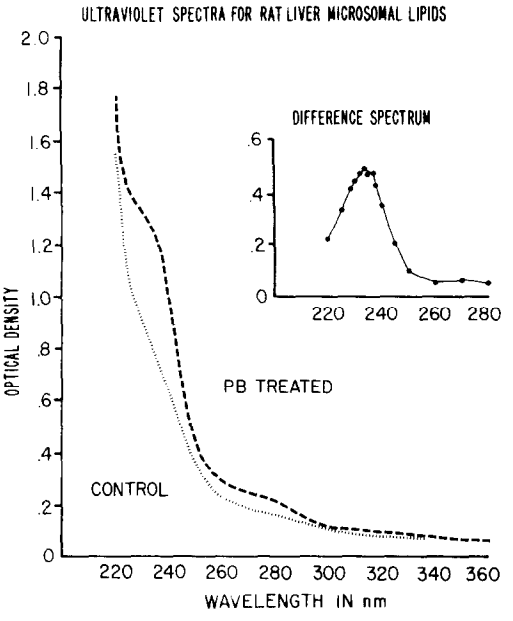

Fig. 1. PB-induced microsomal lipid peroxidation *in vivo*. The difference spectrum is also illustrated.

protection afforded by DPPD against the PB-induced fatty liver is similar to that observed for a CCl_4 fatty liver [35, 36].

Another index of assessing the effects of PB in lipid peroxidation was also studied. The generation of MDA, as measured by the thiobarbituric acid (TBA) method, has been used by many investigators as a measurement of the lipid peroxidation potential *in vitro* [7, 8, 23, 31]. The specificity of the TBA method for determining lipid peroxidation, however, has drawn recent criticism [37, 38]. Nevertheless, since many previous studies used MDA formation as an index of peroxidation, the effects of PB on MDA production by the liver were also studied. PB treatment

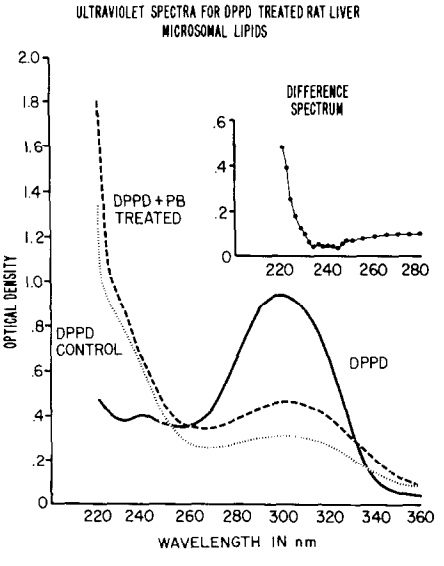

Fig. 2. Inhibition of PB-induced microsomal lipid peroxidation *in vivo* after DPPD pretreatment. DPPD spectrum is shown for comparison.

caused a 5-fold increase in MDA production (μg/mg of protein) as compared to the controls (0·594 ± 0·130 vs 0·106 ± 0·062). Thus, PB also increased MDA formation which reflects a stimulation of lipid peroxidation.

DISCUSSION

The lipid peroxidation concept of liver injury has been proposed as a mechanism responsible for the hepatotoxicity of a variety of chemical agents such as CCl_4, hydrazine, ethanol and phosphorus [7, 8, 39–41]. Increased lipid peroxidation results in the destruction of phospholipid-containing intracellular membranes which can lead to triglyceride accumulation and cellular necrosis. The results of this study indicate that PB also enhances hepatic lipid peroxidation. This is based on the findings that PB increased the diene conjugation absorption of microsomal lipids which has been shown by other investigators [26, 31–34] to be an estimation of the extent of lipid peroxidation *in vivo*. In addition, MDA production (measurement of lipid peroxidation potential *in vitro*) [7, 8, 23, 31] was increased 5-fold in the liver of the PB-treated rats. This compares with a 2-fold increase observed by Fukuzawa and Uchiyama [42] in mouse liver and a 2-fold increase observed by Skutches and Smith [43] in rat liver. However, in neither of the above instances were diene conjugation studies conducted.

PB administration, in addition to inducing lipid peroxidation, also caused an accumulation of triglyceride in the liver. This finding confirms our previous studies [5, 6] which showed that PB induces a fatty liver in rats. The hepatotoxic effects of PB were also recently confirmed by Saito *et al.* [44] who reported a similar increase of triglyceride levels due to PB treatment. Administration of a lipid-soluble antioxidant, DPPD, was effective in reducing the accumulation of neutral fat in the liver caused by PB (Table 2). The antioxidant properties of DPPD have been well documented [8, 35, 36, 41, 45]. This compound is a strong free radical scavenger and is an effective antioxidant both *in vitro* and *in vivo*. Administration of DPPD has been shown to modify the hepatic lesions produced by CCl_4 [36, 41, 46], ethanol [47] and hydrazine [39]. Evidence has been reported demonstrating that the hepatic injury produced by these agents, especially in the case of CCl_4, proceeds via involvement of the peroxidation of subcellular lipids [7, 8, 40, 41]. In this study, DPPD was effective in preventing the increase in lipid peroxidation as well as reducing the triglyceride accumulation resulting from PB treatment (Table 2). This suggests that the peroxidative process may play a role in the PB-induced fatty changes in the liver.

Nothing in the present data indicates how PB functions to generate free radicals which stimulate lipid peroxidation. PB is a known inducer of NADPH cytochrome *c* reductase which is the enzyme required for the NADPH-dependent enzymatic lipid-peroxidizating system [1, 15–17] Thus, as is indicated by the results in Table 1, PB may increase hepatic lipid peroxidation by virtue of its ability to induce the enzymatic peroxidizing system. Recent studies, however, by Levin *et al.* [48] showed that PB treatment did

not appear to alter enzymatic lipid peroxidation which was determined by measuring MDA formation by isolated liver microsomes that were incubated with an NADPH-generating system. In view of this, it is possible that the pro-oxidant activity of PB may reside in its ability to alter other factors involved in the total lipid peroxidation process of the hepatocyte. These other factors could include: (1) the level of unsaturated fatty acids which serve as substrates for peroxidation [49], (2) the levels of cofactors which are required in the enzymatic (NADPH) and nonenzymatic (ascorbic acid) processes of lipoperoxidation [10, 12], and (3) the level of water and lipid-soluble antioxidants. All these factors are involved and interrelated in the total process of lipid peroxidation. The effect of PB on these factors is currently being investigated in our laboratory.

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