Biochemical Pharmacology, Vol. 34, No. 19, pp. 3473–3482, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

INDUCTION OF FATTY ACID β-OXIDATION AND PEROXISOME PROLIFERATION IN THE LIVER OF RHESUS MONKEYS BY DL-040, A NEW HYPOLIPIDEMIC AGENT

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(*Received* 6 *November* 1984; *accepted* 15 *February* 1985)

Abstract—Many structurally unrelated hypolipidemic agents and certain phthalate-ester plasticizers induce hepatomegaly and proliferation of peroxisomes in liver parenchymal cells of rodents, but there is relatively limited evidence regarding the ability of such compounds to induce peroxisome proliferation in the livers of nonrodent species including man. The present study was designed to determine if DL-040 (4-(((1,3-benzodioxol)-5-yl)methyl)amino-benzoic acid), a newly developed hypolipidemic agent, induces peroxisome proliferation in the liver of adult rhesus monkeys. Feeding of DL-040 (300 mg/kg body wt for 1 week; and 400 mg/kg body wt for 10 weeks) caused a significant increase in peroxisome population as determined by ultrastructural and morphometric analyses. The DL-040-induced peroxisome proliferation was accompanied by increases in the levels of catalase, carnitine acetyltransferase and the peroxisomal fatty acid β-oxidation system. As expected, DL-040 caused a significant reduction of serum cholesterol and low density lipoprotein content. These data suggest that hepatic peroxisome proliferation is inducible in nonhuman primates at dose levels that exceed therapeutic levels.

The induction of peroxisome proliferation and of selected peroxisomal enzymes in liver by several hypolipidemic drugs and certain phthalate-ester plasticizers is a well-known phenomenon in rats and mice [1–7]. A marked increase in peroxisome number also occurs in the liver cells of hamsters treated with certain hypolipidemic compounds and to a lesser extent in hamsters treated with phthalate-ester plasticizers [8, 9]. However, there is very limited information about the extent to which the hypolipidemic drugs and plasticizers induce peroxisome proliferation in non-rodent species including man [10–14]. We have shown recently that ciprofibrate, 2-[4-(2,2-dichlorocyclopropyl)phenoxy]-2-methylpropanoic acid, a hypolipidemic drug [6, 15, 16], induces peroxisome proliferation and increases the activities of certain peroxisome associated enzymes in the livers of chickens, pigeons, cats, rhesus monkeys and cynomolgus monkeys [17]. Our studies with ciprofibrate suggested that hypolipidemic drug-induced hepatic peroxisome proliferation is not specific to rodents as generally envisaged [11–13, 18, 19], and that it can be induced in several non-rodent species [17]. We now report that DL-040 (4-(((1,3-benzodioxol)-5-yl)methyl)amino-benzoic acid; Fig. 1), a newly developed hypolipidemic agent, also induces peroxisome proliferation and certain peroxisomal enzymes in the liver of rhesus monkeys.

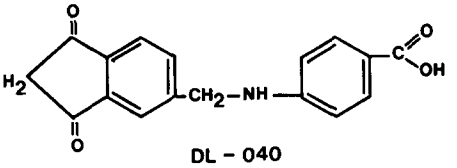

Fig. 1. Chemical structure of 4-(((1,3-benzodioxol)-5-yl)methyl)amino-benzoic acid (DL-040).

MATERIALS AND METHODS

Chemicals. The hypolipidemic compound DL-040 (purity > 99% as assessed by high performance liquid chromatography) was provided by Merrell-Dow Pharmaceuticals Inc., Indianapolis, IN. Crotonyl CoA, NAD, NADP, CoA, palmitoyl CoA and carnitine were obtained from the Sigma Chemical Co. St. Louis, MO. [1-^{14}C]Palmitoyl CoA was obtained from the Radiochemical Center, Amersham, Arlington Heights, IL. Cholesterol oxidase reagent was purchased from Isolab Inc., Akron, OH. Agarose films were purchased from Corning, Palo Alto, CA.

Animals and treatment. Adult male rhesus monkeys (*Macaca mulatta*) were a gift from the Searle Research and Development Division of G. D. Searle & Co., Skokie, IL. The animals were housed in individual cages with free access to water, were fed Purina monkey chow (Ralston Purina, St. Louis, MO) *ad lib.*, and received supplements of one-half

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an orange on alternate days. The monkeys were maintained on a 12-hr light/dark period in the primate facility of the Center for Experimental Animal Research of Northwestern University Medical School. Four monkeys were fed a daily dose of DL-040 (300 mg/kg body wt for 1 week, followed by 400 mg/kg body wt for 10 weeks) mixed freshly in ~2 g fruit jelly and spread on one slice of bread. Three monkeys served as controls. The monkeys ate the piece of bread and, in general, the spillage of jelly with drug was kept to a minimum. The dose of DL-040 used in this study represents a 2-fold increase over the dose of ciprofibrate used in a previous study [17]. Stability of DL-040 has been found to be 100.76% after 78 months. In the present studies, DL-040 was mixed in the jelly 5 min before feeding. The blood samples were collected in tubes containing EDTA, prior to treatment and after 7 and 11 weeks of treatment. DL-040-treated monkeys were killed under ketamine chloride-induced anesthesia at the end of 11 weeks.

Morphology and morphometry. Small pieces of liver were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 hr and post-fixed in 1% OsO_4 in 0.1 M *sym*-collidine buffer, pH 7.4, for 1 hr at 4° [17]. The cytochemical localization of peroxisomal catalase in liver tissue was carried out by incubating glutaraldehyde-fixed tissues in the alkaline 3,3′-diaminobenzidine medium [20] at 40°, the incubation temperature used for human liver by Roels and Goldfischer [21]. The morphometric analysis of peroxisome induction was carried out according to Weibel [22], using the point counting method as previously described [12].

Subcellular fractionation of liver. The livers were homogenized (10%, w/v) in ice-cold 0.25 M sucrose with a Potter–Elvehjem homogenizer. The homogenates were fractionated into nuclear, mitochondrial, light mitochondrial, microsomal and soluble fractions as suggested by Baudhuin *et al.* [23].

Enzyme assays. The activities of peroxisome-associated enzymes (catalase [17], carnitine acetyltransferase [24], urate oxidase [25] and heat-labile enoyl-CoA hydratase [26]) and peroxisomal palmitoyl CoA oxidation [27] were determined in homogenates and subcellular fractions as previously described [15]. Protein content was measured by the method of Lowry *et al.* [28].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The post-nuclear and large particle fractions were analyzed by SDS-PAGE according to the method outlined by Laemmli [29] to ascertain the increase in peroxisome proliferation associated M_r 80,000 polypeptide [30].

Determination of cholesterol and lipoproteins. The cholesterol content of plasma and plasma fractions was determined by the cholesterol oxidase reagent [31]. This reagent contained cholesterol ester hydrolase to hydrolyze cholesterol esters and catalase and dye for the assay of oxidized cholesterol.

The lipoproteins β, pre-β and α were estimated according to the procedure standardized by Hatch *et al.* [32] involving agarose gel electrophoresis. The electrophoresis of plasma samples was carried out using 0.05 M barbital buffer, pH 7.4. The gels were stained with Fat Red 7B in 50% methanol to stain lipoproteins. The electrophoretograms were analyzed by densitometric scans. The areas under the peak for β-, pre-β- and α-lipoproteins were converted to concentration in terms of cholesterol using conversion factors determined previously with several plasma samples of known lipoprotein concentrations which were calculated from plasma total cholesterol and triglyceride and the value of α-lipoprotein cholesterol concentration. The latter was determined independently in all samples after removing β- and pre-β-lipoproteins by affinity chromatography on heparin-agarose (Isolab Inc.). The conversion factors (peak area to lipoprotein cholesterol, for β:pre-β:α were 1:0.3:0.6 in good agreement with those found by Hatch *et al.* [32] for human plasma lipoproteins.

RESULTS

Effect of DL-040 on cholesterol and lipoprotein distribution. As shown in Table 1, the control values of cholesterol and different lipoproteins in the plasma of rhesus monkeys were found to be similar to those reported earlier [33]. The total cholesterol and lipoprotein levels decreased gradually on treatment with DL-040 (Fig. 2). Except for stain intensity, the lipoprotein patterns in terms of the position of the β-; pre-β-and α-lipoprotein bands remained similar for control and experimental animals. The reductions in the levels of various lipoproteins deter-

Table 1. Total cholesterol and lipoprotein profile of plasma in rhesus monkeys fed with DL-040 for 11 weeks*

	Cholesterol† (mg/dl)			
Treatment	Total	β	Pre-β	α
Control	148.7 ± 8.7	70.3 ± 3.8	10.3 ± 1.5	67.8 ± 6.2
DL-040	46 ± 4.3	20.8 ± 2.1	2.3 ± 0.31	22.8 ± 1.8

* A total of nine specimens obtained from pretreatment and control monkeys was used for control values. Four male rhesus monkeys were given DL-040 at daily oral doses of 300 mg/kg body weight for 1 week and 400 mg/kg body weight for 10 weeks in 2 g jelly and spread on one slice of bread.

† Values are mean ± S.E. All values for the DL-040 group are significantly different from controls ($P < 0.05$).

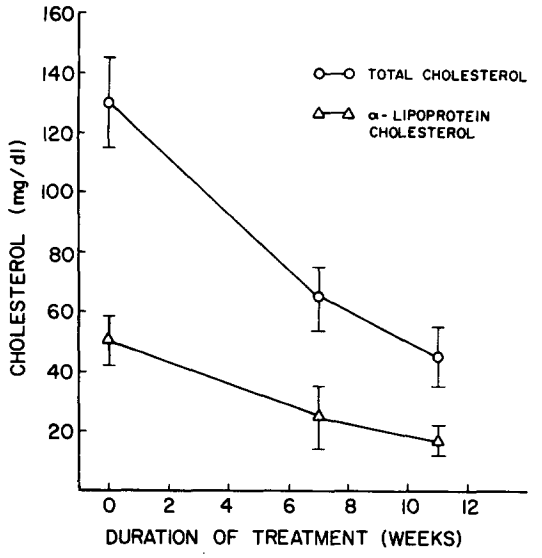

Fig. 2. Changes in plasma cholesterol (○—○) and α-lipoprotein cholesterol (△—△) content of rhesus monkeys given DL-040 over an 11-week period. Each point represents the mean plasma concentration of four monkeys (±S.E.).

mined electrophoretically were almost parallel, including that of α-lipoprotein which was quantitated independently by the heparin column method in animals treated with DL-040. The ratio of $\alpha/(\beta +$ pre-β) was, however, slightly higher (0.98) in the DL-040-treated monkeys as compared to controls (0.84).

Electron microscopic observations. As reported earlier [17, 34], peroxisomes in rhesus monkey hepatocytes appear as single membrane limited structures measuring about 0.2 to 0.8 μm in diameter. These organelles in this primate species lacked urate oxidase containing core or nucleoids (Fig. 3), although urate oxidase activity has been demonstrable in the liver [35]. Although the low levels of urate oxidation observed in rhesus monkey liver are attributable to uricase activity, the reasons for the absence of uricase core in rhesus monkey hepatic peroxisomes are not clear. However, it has been claimed that uricase activity of old world monkeys is quite unstable [35]. Peroxisomes were few in number in normal rhesus monkey liver (Fig. 3). At the end of 11 weeks of treatment with DL-040, there was a substantial increase in peroxisome number in the hepatocytes of these animals (Fig. 4). These peroxisomes showed intense electron dense reaction product when incubated in alkaline 3,3′-diaminobenzidine medium at 40° for the cytochemical localization of catalase (Fig. 5). Table 2 shows the morphometric data on liver peroxisomes and mitochondria. Peroxisome volume density increased 5-fold in rhesus monkeys treated with DL-040, but the mitochondrial population did not change significantly when compared to controls.

Peroxisomal enzymes. The liver homogenates of DL-040-treated monkeys showed significantly higher activities of peroxisomal catalase, uricase and cyanide-insensitive peroxisomal fatty acid β-oxidation (Table 3). Heat-labile enoyl-CoA hydratase and carnitine acetyltransferase activities were almost nondetectable in normal rhesus monkey livers, but showed marked increases in the livers of monkeys treated with DL-040 (Table 3).

The subcellular distribution of the peroxisomal enzymes in normal and DL-040-treated monkeys is depicted in Fig. 6. On treatment with DL-040, the peroxisomal enzyme activities increased in heavy and light mitochondrial fractions (Fig. 6).

Peroxisome proliferation associated 80,000 molecular weight polypeptide. Peroxisome proliferation induced by hypolipidemic drugs is accompanied by an increase in the amount of 80,000 molecular weight protein in large particle, post-nuclear, and microsomal fractions of liver [6, 17, 30]. SDS-PAGE of the post-nuclear pellets prepared from livers of DL-040-treated rhesus monkeys showed a substantial increase in the content of 80,000 molecular weight peroxisome proliferation associated protein, whereas this protein was almost undetectable in the post-nuclear pellets prepared from the livers of normal rhesus monkeys (Fig. 7).

DISCUSSION

Our earlier studies with ciprofibrate have demonstrated unequivocally that hepatic peroxisome proliferation can be induced in cats, chickens, pigeons

Table 2. Morphometric analysis of DL-040-induced peroxisome proliferation in livers of rhesus monkeys*

	Volume density (% of cytoplasmic volume)	
Treatment	Mitochondria	Peroxisomes
Control	19.4 ± 1.7	1.9 ± 0.44
DL-040	20.9 ± 2.3	10.3 ± 0.88†

* Thirty electron micrographs of randomly selected areas of liver cell cytoplasm from each group (three animals per group, ten micrographs per animal) were subjected to morphometric measurement as described by Weibel [22]. Points overlying cytoplasm, mitochondria and peroxisomes were determined to obtain the volume density of mitochondria and peroxisomes. The values are expressed as percentage of cytoplasmic volume. DL-040 was administered for 11 weeks. Values are mean ± S.E.

† $P < 0.05$, as determined by a two-tailed t-test.

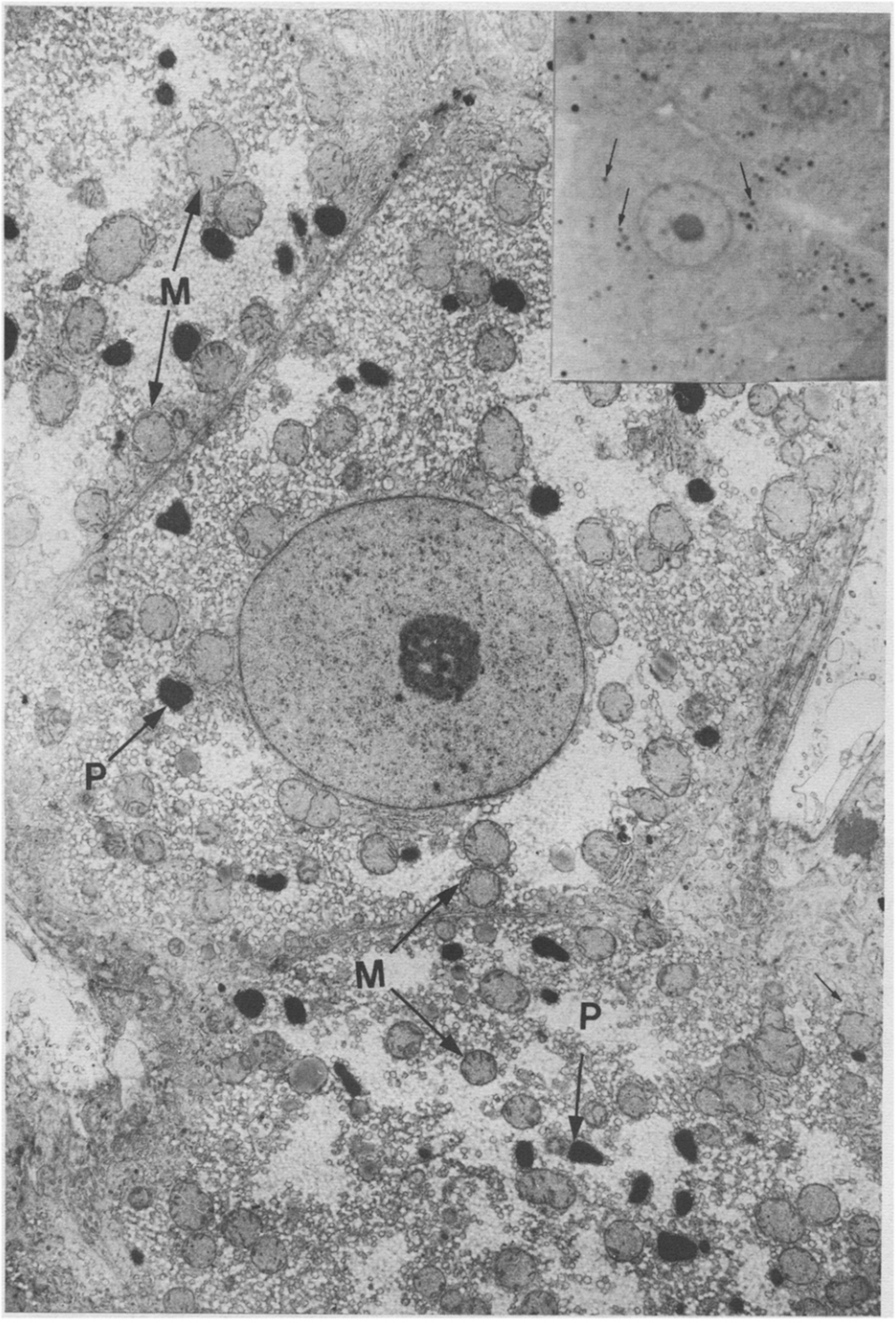

Fig. 3. Electron micrograph showing portions of liver cells from a male rhesus monkey (control). Peroxisomes (P) appear as electron dense, single membrane limited organelles. Mitochondria (M). Stained with uranyl acetate lead citrate. Magnification: ×5400. Inset: thick section (0.5 μm thick) of Epon-embedded normal liver which was incubated in the alkaline, 3,3′-diaminobenzidine HCl medium prior to embedding. Peroxisomes (arrows) appear as black dots in this picture. Magnification: ×1100.

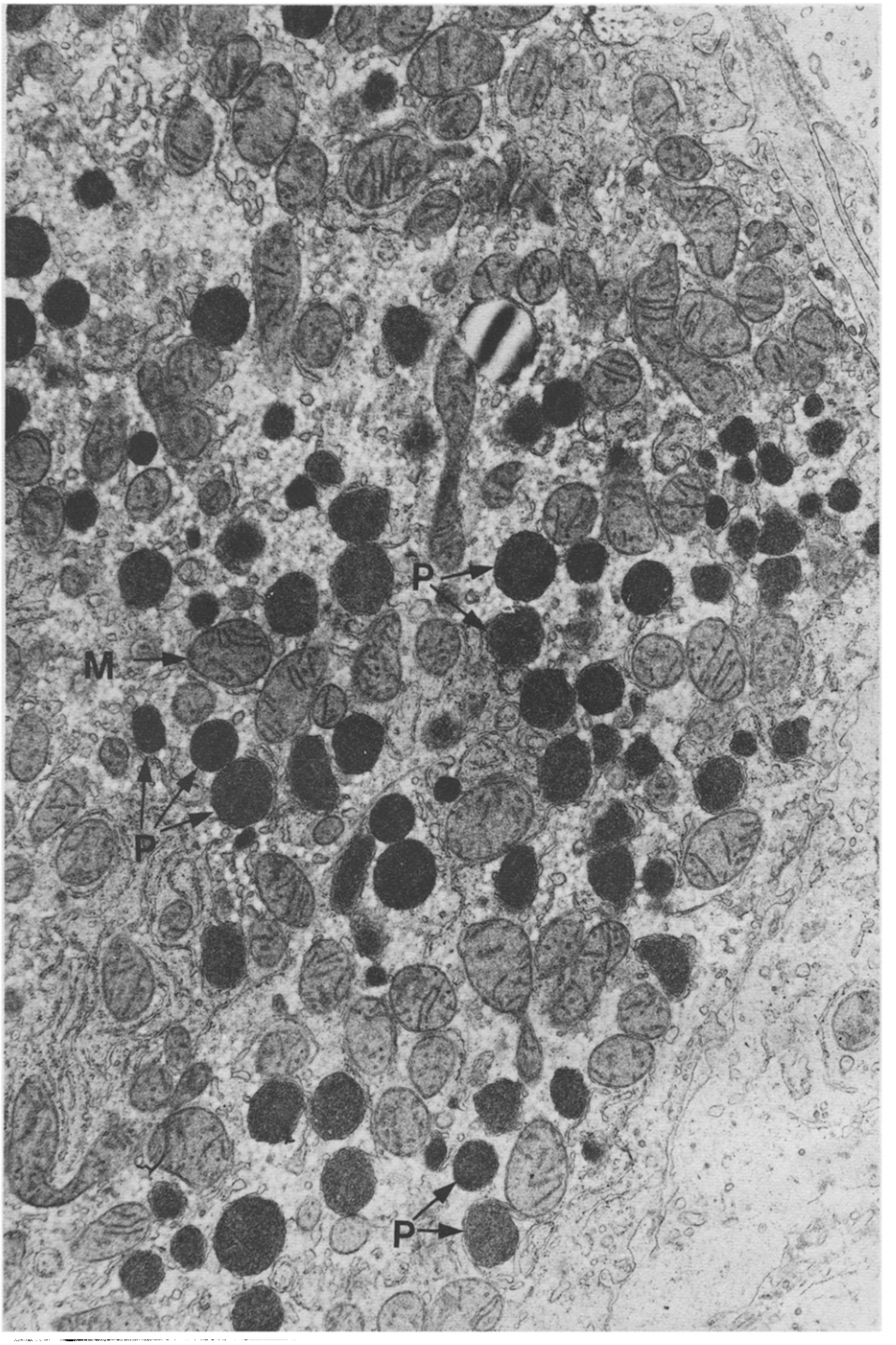

Fig. 4. Electron micrograph representing a portion of a liver cell from a rhesus monkey fed DL-040 for 11 weeks. Peroxisomes (P) are increased in number. Mitochondria (M). Stained with lead citrate-uranyl acetate. Magnification: ×9000.

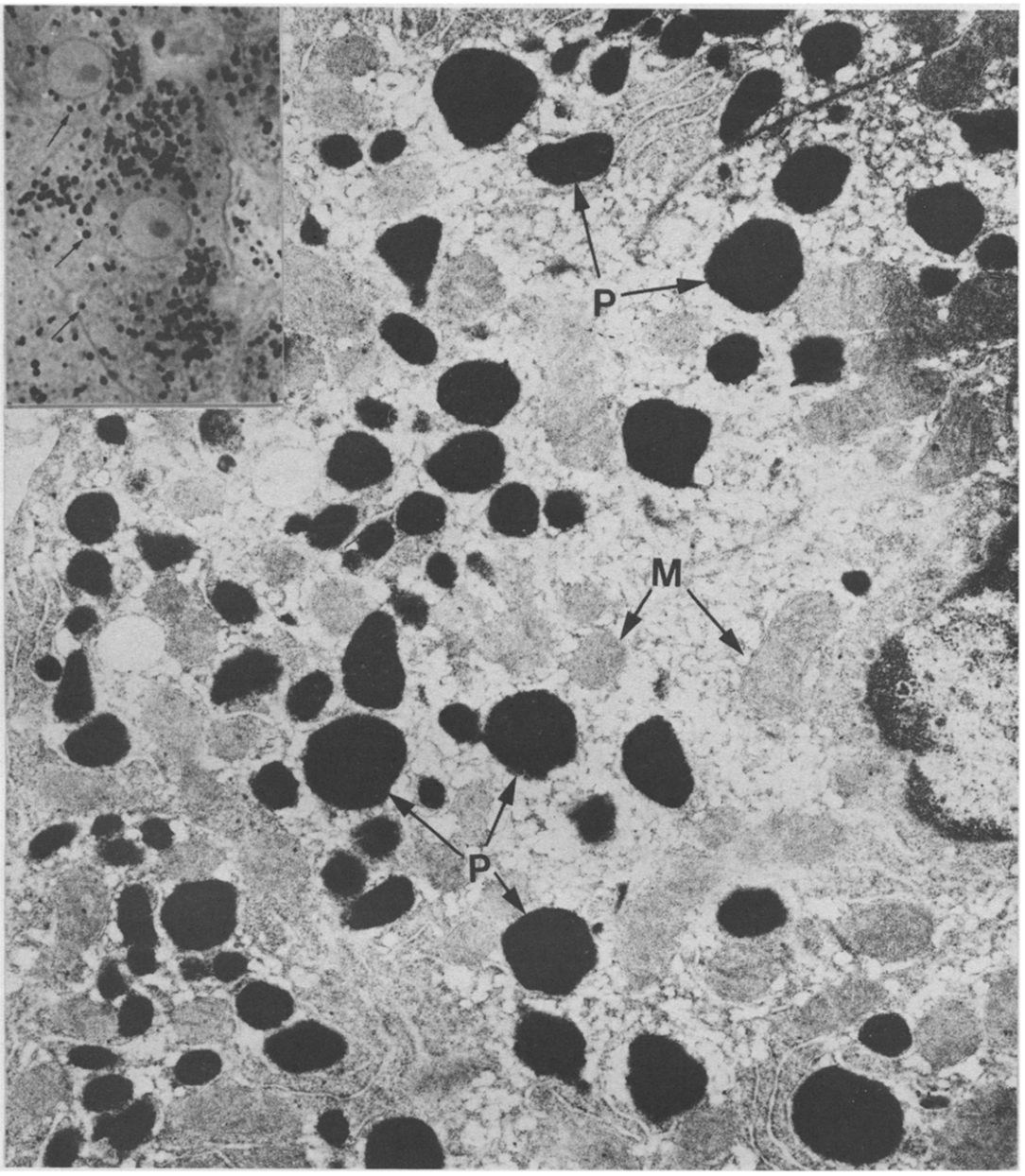

Fig. 5. Cytochemical localization of peroxisomal catalase in liver cell of a rhesus monkey treated with DL-040 for 11 weeks. Glutaraldehyde fixed. Liver pieces were incubated in 3,3′-diaminobenzidine medium for cytochemical localization of catalase. Peroxisomes (P) appear darkly stained due to the electron dense reaction product. Mitochondria (M); magnification: ×14,000. Inset: the ultraphot III light microscopic appearance of catalase specific reaction product in peroxisomes (arrows) in liver parenchymal cells of a DL-040-treated monkey. Magnification: ×1100.

and two species of sub-human primates [17]. In agreement with our studies on ciprofibrate, the newly developed hypolipidemic compound DL-040 also induced peroxisome proliferation in the liver of sub-human primate *M. mulatta*. In these animals fed DL-040, the cyanide-insensitive peroxisomal fatty acid β-oxidation enzyme system increased approximately 7-fold. The hepatic activities of other peroxisome-associated enzymes such as catalase, carnitine acetyltransferase and enoyl-CoA hydratase also increased significantly. Consistent with these changes was the increase in the content of peroxisome proliferation associated 80,000 molecular weight polypeptide in the post-nuclear fractions of liver of rhesus monkeys treated with DL-040. These results unequivocally suggest that hypolipidemic drug-induced hepatic peroxisome proliferation is not a rodent specific response and that it can be induced in other species if tested appropriately. However, the extent of induction of hepatic peroxisome proliferation and peroxisomal enzyme induction including the content of PPA-80 in this primate species was

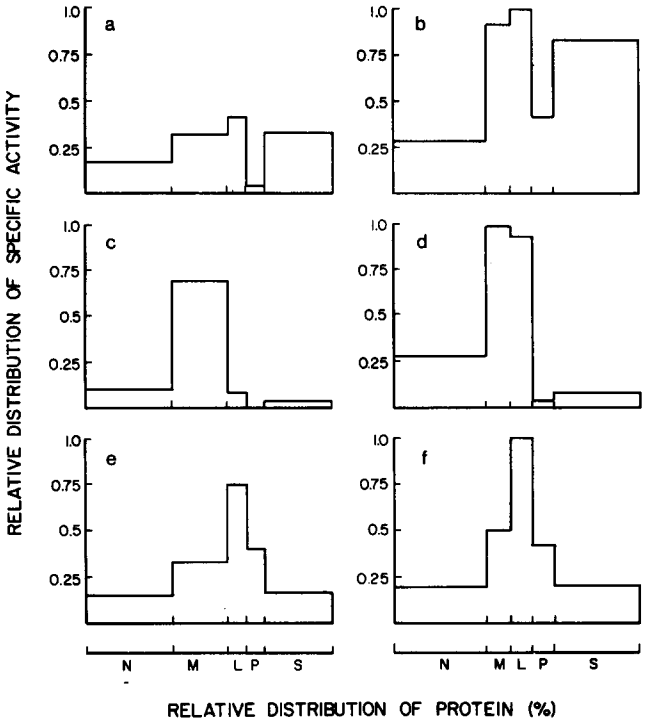

Fig. 6. Subcellular distribution of enzymes catalase (a, b), palmitoyl-CoA oxidation enzyme system (c, d) and uricase (e, f) in liver subcelluar fractions of control (a, c, e) and DL-040 fed (b, d, f) rhesus monkeys. The homogenates were fractionated according to the method outlined by Baudhuin *et al.* [23]. The ordinates represent the distribution of specific activities in subcellular fractions in normal liver, relative to the highest activities in normal and DL-040-treated rhesus monkey livers. The abscissas indicate the relative distribution of protein content of nuclear (N), mitochondria (M), light mitochondria (L), microsomal (P) and supernatant (S) fractions.

considerably lower than that inducible in the rat liver. It is also important to note that the magnitude of peroxisome proliferative response may depend on a variety of factors including the potency of the agent and the dose administered [10, 12, 14].

Attempts are currently being made to elucidate the mechanism(s) regulating the induction of peroxisomal enzymes by peroxisome proliferators [36–38]. The presence of a nafenopin-binding protein in the liver and kidney cortex suggests that peroxisome proliferators may act by a receptor-mediated pathway [36]. Tissue levels of nafenopin binding protein in the liver and kidney correlate well with the ability of these tissues to respond to peroxisome proliferation and to the induction of certain peroxisomal enzymes [36]. The tissue specificity of peroxisome proliferative response is further illustrated by our recent studies on the induction of peroxisome proliferation in hepatocytes transplanted at extrahepatic locations in syngeneic hosts or heterotransplanted into athymic nude mice [37, 38]. Further studies are required to determine whether different species share a common mechanism responsible for the peroxisomal enzyme expression.

The induction of peroxisome proliferation in rats and mice by a variety of hypolipidemic compounds has been associated with statistically increased incidences of hepatocellular carcinomas [12, 39]. DL-040 also induced peroxisome proliferation and liver tumors in rats and mice (unpublished observations). However, the carcinogenic peroxisome proliferators do not exert mutagenicity or chromosomal damage [40–43] in the Ames *Salmonella*/microsome test system [44] or bind covalently to DNA [45–47]. Although the events governing peroxisome proliferation in different species, and those culminating in the development of hepatocellular carcinomas in rats and mice, remain to be fully elucidated [12], it appears that the induction of peroxisome proliferation *vis-à-vis* peroxisomal fatty acid β-oxidation provides a potential means for the cell to generate excessive amounts of H_2O_2 and possibly other oxygen species that ultimately could interact with and damage cellular macromolecules [39, 48, 49]. Damage to DNA by this endogenous peroxisome proliferation mediated pathway might contribute to the development of hepatocellular carcinomas in rats and mice exposed to these non-mutagenic chemicals [12, 40, 49, 50]. In this context, it is pertinent to note that peroxisome proliferators also induce hepatic microsomal ω-oxidation enzyme system [51] and cytosolic epoxide hydratase [52] which may also further contribute to the lipid peroxidation related endogenous initiation process. Additional studies are needed to determine if the peroxidative damage leads to DNA damage *in vivo*.

If the peroxisome proliferation is responsible for the initiation of hepatocarcinogenesis, it may be

Table 3. Alteration in liver weight and liver peroxisomal enzymes in rhesus monkeys treated with DL-040, a hypolipidemic compound, for 11 weeks*

Treatment	Liver weight (g/100 g body wt)	Catalase (units/mg protein)	Carnitine acetyl-transferase (units/mg protein)	Enoyl-CoA hydratase (units/mg protein)	[^{14}C]Palmitoyl CoA oxidation (μmoles/min/g liver)	Urate oxidase (units/g liver)
Control (3)	1.66 ± 0.17	94 ± 1.0	Less than 1	0.05 ± 0.02	0.14 ± 0.03	0.49 ± 0.1
DL-040 (4)	2.16 ± 0.25	177 ± 29†	18.9 ± 7.0†	0.32 ± 0.07†	1.01 ± 0.37†	0.92 ± 0.05

* Numbers of animals are given in parentheses. Enzyme activities were determined on liver homogenates as described in Materials and Methods. Values represent mean ± S.E.

† Value is significantly different from control ($P < 0.05$). It should be noted that control animals were killed after 7 weeks, whereas the DL-040 animals were killed at 11 weeks. Rhesus monkeys were fed with DL-040 in fruit jelly 300 mg/kg body weight for 1 week followed by 400 mg/kg body weight for 10 weeks.

Fig. 7. SDS-polyacrylamide gel electrophoretic profile of post-nuclear fractions from livers of control (lane A) and DL-040 (lane B) fed rhesus monkeys. Lane C represents molecular weight standards. The arrow indicates the peroxisome proliferation associated 80,000 molecular weight protein, which is increased in DL-040-treated liver.

possible to predict the risk of hepato-carcinogenesis of a particular hypolipidemic drug, in part, by assessing the extent of peroxisome proliferative response and enzymatic imbalances within this organelle (i.e. increase in catalase relatively less than the increases in H_2O_2 generating fatty acid β-oxidation leading to excessive H_2O_2 fluxes within the cell in a given species). As pointed out elsewhere [12, 17], the observed differences in hepatic peroxisome proliferative response in different species may be attributable to quantitative or qualitative differences in the cytosolic receptors and to the bioavailability of the chemical.

Finally, at the dose of DL-040 used in this study on normolipidemic monkeys, there was a drastic reduction in plasma cholesterol from a control value of 149 mg/dl to about 46 mg/dl after 11 weeks of treatment (Table 1). The different plasma lipoproteins (β, pre-β and α) decreased in a parallel manner; a specific effect on any particular lipoprotein could not be established although, as mentioned earlier, the ratio $\alpha/(\beta + \text{pre-}\beta)$ seemed to be

increased slightly. The decrease in α-lipoprotein on DL-040 appears to be a specific feature in this species. It may be mentioned that the β-, pre-β- and α-lipoproteins determined by the electrophoretic method have been shown to correlate highly with low, very low and high density lipoproteins (LDL, VLDL and HDL), respectively, as determined by ultracentrifugation [33]. Furthermore, the α-lipoprotein determined by the heparin complexing method has been shown to correlate highly (r = 0.93) with HDL [53], and the ratio $\alpha/(\beta + \text{pre-}\beta)$ cholesterols has been reported to show a greater discrimination between atherosclerotic and control humans.

In summary, the results of the present study in the rhesus monkeys with DL-040 as well as those obtained with ciprofibrate [17] and gemfibrozil [14] suggest that induction of peroxisome proliferation in primates and other non-rodent species requires relatively high dose levels of the drug that apparently exceed the therapeutic dose levels required to lower serum lipids. Although available data on peroxisome proliferation in liver biopsies from patients receiving either clofibrate [11] or gemfibrozil [19] are equivocal, additional studies are needed to document the effects of newly identified hypolipidemic drugs, such as ciprofibrate, which are several orders of magnitude more effective than clofibrate in lowering serum lipids [16].

Acknowledgements—This study was supported by Grant GM 23750 from the National Institutes of Health. We thank Ms. Lowella Rivero for her excellent secretarial assistance.

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