A quantitative analysis of fine structure and drug metabolism in livers of clofibrate-treated young adult and retired breeder rats

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Abstract The effects of clofibrate on the fine structure and drug-metabolizing capacity of livers of normolipidemic young adult virgin (YA) and hypercholesterolemic retired breeder (RB) male rats were measured by morphometric and biochemical procedures. The oral administration of clofibrate for 7 days significantly increased liver weight and reduced the cholesterol concentrations in the serum and liver tissue in both groups of animals. The hepatic triglyceride (TG) concentration and the volume of cytoplasmic lipid droplets, presumably TG, as well as the serum TG concentration, increased only in the drug-treated RB rats. Clofibrate treatment resulted in significant increases in the volumes of the hepatocytes and their constituent mitochondria and microbodies and caused a proliferation of the smooth-surfaced endoplasmic reticulum. Although the magnitude of the hypocholesterolemic response was considerably greater in the RB animals, the morphological changes were much more marked in the YA group. However, the surface area of the rough-surfaced endoplasmic reticulum was reduced in the livers of the drug-treated RB rats. NADPH cytochrome *c* reductase specific activity was significantly increased in both the RB and YA animals, but the concentration of cytochrome P-450 (per mg microsomal protein) increased only in the YA rats. Neither the cytochrome b_5 concentration nor the rate of ethylmorphine *N*-demethylation was significantly affected by clofibrate administration. The results suggest that there is no positive correlation between the hypocholesterolemic response to clofibrate and the degree of subcellular changes in the hepatocytes and that this hypolipidemic drug elicits a minimal effect on the concentrations of the components of the hepatic microsomal drug-metabolizing system.

Supplementary key words quantitative electron microscopy · hepatic drug metabolism · hepatic fine structure · membrane proliferation · endoplasmic reticulum · microbodies · microsomal enzymes

Clofibrate (chlorophenoxyisobutyrate) is a widely used hypolipidemic agent which is reported to lower the serum lipid levels in man (1, 2) and experimental animals (3, 4; see 5 for a review). Although numerous qualitative morphologic and biochemical studies have examined the effects of clofibrate and related analogs on the liver (6–24), there have been few correlated structural and functional analyses. Kolde, Roessner, and Themann (25) quantitatively evaluated the hepatic morphological response to this drug and correlated these findings with changes in liver catalase activity. However, there has been no extensive study of the clofibrate-induced hepatic ultrastructural alterations, particularly changes in the endoplasmic reticulum membranes, in conjunction with an analysis of the drug-metabolizing system. The close association between the hepatic smooth-surfaced endoplasmic reticulum (SER) and (*1*) drug metabolism, (*2*) lipoprotein synthesis, and (*3*) cholesterol synthesis and catabolism suggests the need to evaluate the effects of clofibrate and other hypolipidemic agents on this and other intracellular membrane systems. Such studies are particularly important in order to define specific mechanisms of drug action and to observe potential hepatotoxicity and drug interactions.

The retired breeder rat, used as a hyperlipidemic model in the present study (26), is characterized by hypercholesterolemia, a high incidence of arteriosclerosis, and a shortened lifespan (27–30). Recent morphometric studies showed that centrolobular hepatocytes of RB rats contained less SER than similar cells in the livers of normolipidemic young adult virgin animals of the same strain and that they

Abbreviations: RB, retired breeder; YA, young adult; SER, smooth-surfaced endoplasmic reticulum; RER, rough-surfaced endoplasmic reticulum; TG, triglyceride.

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responded similarly to the potent cholesterol-lowering drug oxandrolone (31). In the present study, we measured the effects of clofibrate on several hepatic parameters, including fine structure, lipid concentrations, drug-metabolizing capacity, and serum lipid-lowering ability in both RB and YA rats.

METHODS

Animals

Three-month-old YA (200–250 g) and six-month-old RB (500 g) male Sprague-Dawley rats obtained from the Holtzman Company, Madison, WI, were employed throughout the study. The animals were housed in individual cages and provided tap water and a standard diet of laboratory chow ad libitum for at least one week before use. The rats were fasted for 18 hr prior to either collecting tail vein blood for serum lipid determinations or killing to evaluate hepatic fine structure, lipid concentrations, or drug-metabolizing enzymes.

To avoid the problems of reduced food intake and subsequent weight loss in animals administered clofibrate in the diet (32–34), the drug was administered by gavage. Clofibrate (Atromid-S, Ayerst Laboratories, New York, NY) at a dosage of 200 mg/kg body weight in corn oil or corn oil vehicle alone was administered twice daily for 7 consecutive days. This dosage is approximately 14 times the therapeutic level administered to humans. Separate experiments were conducted for the biochemical determinations and electron microscopic analyses.

Microscopy and morphometry procedures

Eight YA and eight RB rats, five drug-treated and three control animals per group, were used for morphometric analysis. The animals were anesthetized with sodium pentabarbital (IP) and their livers were perfused via the hepatic portal vein according to the method of Wisse (35) with 2.7% glutaraldehyde/0.8% paraformaldehyde in 0.02 M sodium bicarbonate buffer (pH 7.4; 770 *m*osmol). Following postfixation in 1% osmium tetroxide, the tissue was dehydrated and embedded in epoxy resin (36).

Five tissue blocks were randomly selected from each animal, providing they contained at least one central vein (terminal hepatic venule) in cross-section as determined by light microscopy. The selected tissue blocks were retrimmed to within a ten-cell radius around the central vein. Evidence from a number of studies that have evaluated drugs, such as phenobarbital (37), and hepatotoxins, such as carbon tetrachloride (38), suggested that morphological alterations first occur in the centrolobular hepatocytes. Both Loud (39) and Jones et al. (40, 41) reported a heterogeneity in certain fine structural parameters across the hepatic lobule. Thus, the knowledge of the sublobular orientation of the sample electron micrographs used in the present study facilitated comparisons between the clofibrate-treated and control livers and provided a certain degree of uniformity. Photomicrographs of random thick sections at a final magnification of 1000× were used to determine the average hepatocyte volumes and the nuclear numerical densities according to the method of Loud (39).

Three electron micrographs per block were taken at each of two primary magnifications, 4600× and 15,200×, and photographically enlarged to 13,800× and 45,600×, respectively. The sampling and morphometry procedures employed were similar to those previously described (31, 40–42). The volume data are expressed in (*1*) geometrical units or volume densities (cm^3/cm^3 of intralobular tissue), and (*2*) biological units or volumes per average mononuclear hepatocyte (μm^3/cell). The surface areas of the intracellular membranes are expressed per volume of hepatocellular ground substance (surface density; M^2/cm^3) and per average cell (μM^2/cell). Since these data represent relative estimates, no attempt was made to correct for possible systematic errors introduced by section thickness (~600 Å), angle, or specific membrane configurations that may have resulted in either under- or overestimations of membrane surface areas. A total of 500 electron micrographs were analyzed and the data were subjected to statistical analysis (Student's *t*-test). All values of $P < 0.05$ were considered indicative of a significant difference between two estimates.

Biochemical procedures

Initial serum samples, i.e., prior to drug or vehicle administration, were collected from the tail vein, extracted in chloroform-methanol 2:1, and analyzed for cholesterol (43) and triglycerides using the Biochimica Test Combination for Triglycerides (Boehringer Mannheim Corporation, Mannheim, West Germany) (44).

At the termination of the experiments, the rats were killed by decapitation, serum samples were collected, and the livers were quickly removed. A portion of liver tissue was homogenized in saline (1:8 w/v) and extracted in chloroform–methanol. Post-drug or vehicle-treated serum and liver tissue

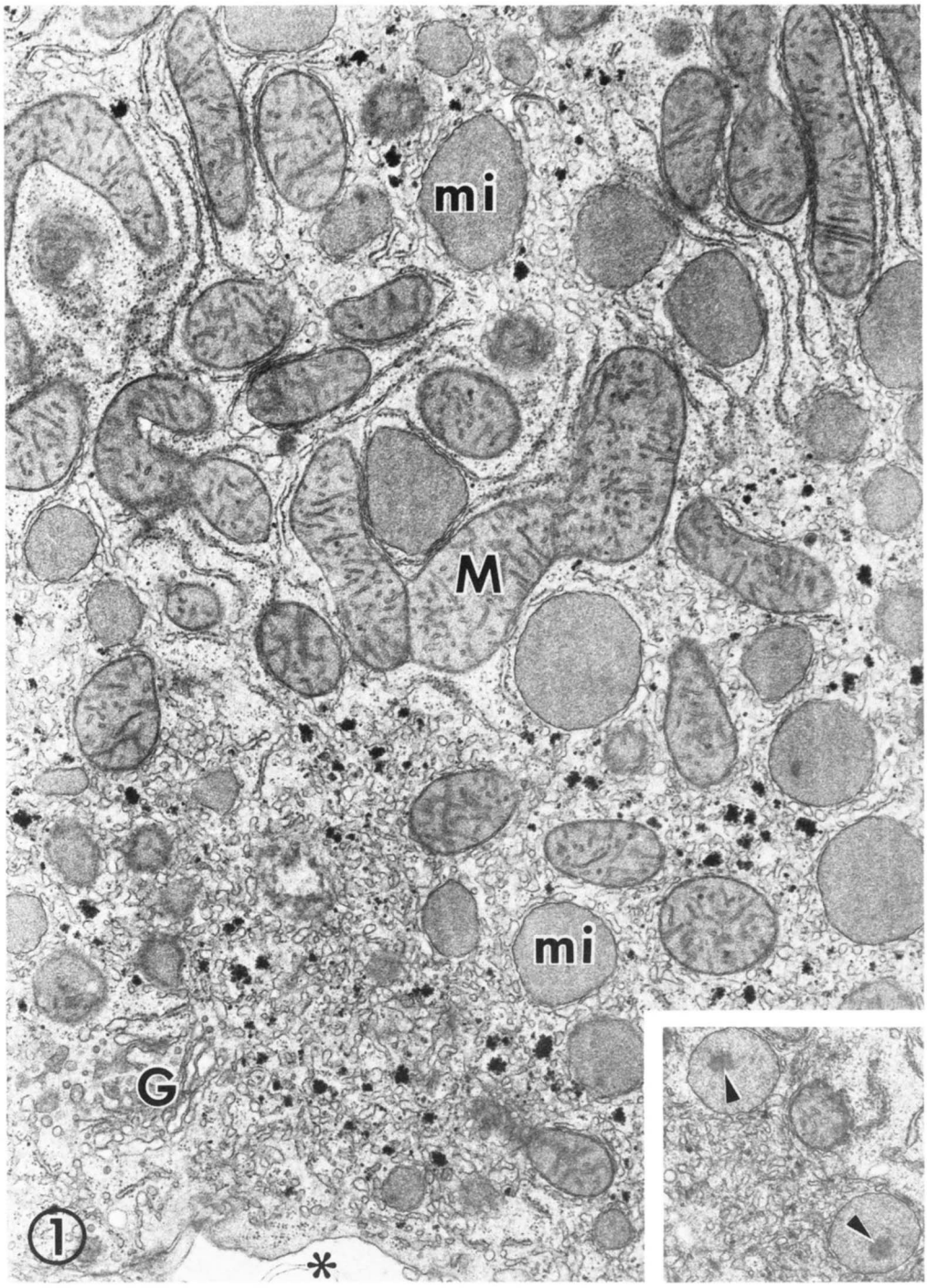

Fig. 1. Portion of a centrolobular hepatocyte from a clofibrate-treated virgin rat. The numerous microbodies (mi) lack a crystalline nucleoid. Otherwise, the fine structural appearance of these cells, including the mitochondria (M), is similar to that observed in animals receiving the corn oil vehicle alone. Inset: Typical appearance of hepatic microbodies in the control animals at the same magnification. Note the relatively small size of these organelles and the electron dense nucleoids in the matrices (arrows). G, Golgi; *, bile canaliculus. 17,300×.

samples were analyzed for cholesterol and TG concentrations as described above. Another portion of liver tissue was homogenized in ice-cold water (1:10 w/v) and the protein concentration was determined by the method of Lowry et al. (45).

The remaining liver tissue was minced, homog-

enized in 10 volumes of 0.25 M sucrose, and the microsomal fraction was prepared by differential centrifugation as described by Anthony (46). Microsomal protein content was measured by the method of Gornall, Bardawill, and David (47), using bovine serum albumin as standard. Cytochrome P-450 and b_5 concentrations were determined by the procedure described by Omura and Sato (48), using an Aminco-Chance spectrophotometer in the split-beam mode. NADPH cytochrome *c* reductase specific activity was measured by the method of Masters, Williams, and Kamin (49). The rate of hepatic microsomal *N*-demethylation was measured as previously described (50), using ethylmorphine as substrate (6.5 mM). Aliquots (1 ml) of the mixture were taken at 1-min intervals for 10 min and mixed with 1 ml of 10% (w/v) trichloroacetic acid. The reaction was linear with time under these conditions. The formaldehyde produced was determined according to Nash (51). All of the data were analyzed by Student's *t*-test for differences between the means.

RESULTS

Qualitative fine structural observations

Qualitatively, the fine structural appearance of the liver tissue from the clofibrate- and vehicle-treated YA and RB rats was unremarkable. The only exception was that the livers from the drug-treated animals exhibited a marked proliferation of microbodies (peroxisomes) in comparison to the control tissues (**Fig. 1**). The matrices of these drug-induced organelles appeared electron dense and the crystalline nucleoids were often absent. No other obvious changes in liver ultrastructure could be detected by qualitative examination.

Morphometric measurements

Morphometric analysis of the electron micrographs revealed several significant drug-induced alterations. The mean volume of the hepatocytes increased significantly, 78% and 50% in the YA and RB rats, respectively. However, the volume densities of the hepatocytes did not change appreciably, which is consistent with the marked hepatomegaly that occurred in both groups of clofibrate-treated animals (Tables 1, 2, and 4). The increases in average cell size are attributable to concomitant increases in the cytoplasmic compartments (YA, 84%; RB, 44%) and certain of the constituent organelles. Neither the volume densities nor the specific volumes of the hepatocyte nuclei in either group were affected by clofibrate administration. The nuclear

TABLE 1. Hepatic morphometric parameters in clofibrate-treated and control YA rats[a]

Component[b]	Control	*P*	Clofibrate-treated
Extrahepatocyte (V_v)	0.14 ± 0.02	NS	0.10 ± 0.01
Biliary space (V_v)	0.008 ± 0.001	NS	0.007 ± 0.001
Hepatocyte (V_v)	0.86 ± 0.02	NS	0.89 ± 0.02
(μm^3)	*5147 ± 323*	<0.005	*9143 ± 1113*
Nuclei (V_v)	0.08 ± 0.01	NS	0.06 ± 0.01
(μm^3)	*454 ± 73*	NS	*630 ± 83*
(N_v)[c]	170 ± 11	<0.001	102 ± 12
Cytoplasm (V_v)	0.78 ± 0.02	NS	0.84 ± 0.01
(μm^3)	*4693 ± 89*	<0.001	*8626 ± 134*
Mitochondria (V_v)	0.16 ± 0.01	<0.001	0.22 ± 0.01
(μm^3)	*967 ± 66*	<0.001	*2263 ± 103*
Microbodies (V_v)	0.011 ± 0.001	<0.001	0.068 ± 0.004
(μm^3)	*66 ± 6*	<0.001	*703 ± 41*
Dense bodies (V_v)	0.004 ± 0.001	NS	0.006 ± 0.001
(μm^3)	*26 ± 4*	<0.001	*63 ± 7*
Lipid droplets (V_v)	0.003 ± 0.001	NS	0.002 ± 0.001
(μm^3)	*18 ± 6*	NS	*21 ± 11*
RER (S_v)	3.85 ± 0.47	NS	3.25 ± 0.30
(μm^2)	*14010 ± 1726*	NS	*18223 ± 1665*
SER (S_v)	7.06 ± 0.33	<0.001	10.57 ± 0.48
(μm^2)	*25673 ± 1200*	<0.001	*59186 ± 2705*
Golgi (S_v)	0.33 ± 0.00	NS	0.52 ± 0.10
(μm^2)	*1200 ± 400*	NS	*2906 ± 549*
Golgi-rich area (V_v)	0.018 ± 0.003	NS	0.013 ± 0.001
(μm^3)	*105 ± 15*	NS	*149 ± 16*

[a] All values are expressed as the mean ± SEM.
[b] V_v is volume density or relative volume; S_v is surface density or relative surface area. Italicized values represent the volumes or surface areas per average mononuclear hepatocyte.
[c] NV is nuclear numerical density or the number of hepatocyte nuclei per unit volume of hepatocytes.

numerical densities, i.e., the number of hepatocytes per volume of liver tissue, in the YA and RB drug-treated animals are approximately 40% less than these values in their respective controls (**Tables 1** and **2**). Thus, an increase in the volume of the individual hepatocytes accompanies the clofibrate-induced hepatomegaly in both groups of animals.

Although there were no obvious alterations in the fine structural appearance of the mitochondria after clofibrate treatment, the specific volumes of this organelle were significantly larger in the YA (134%) and RB (58%) animals. The mitochondrial volume density increased significantly in the clofibrate-treated YA rats (38%), suggesting a higher concentration of this organelle. The smaller increase in this parameter in the RB animals probably reflects the increase in hepatocyte volume rather than a higher concentration of mitochondria (Tables 1 and 2).

Clofibrate administration caused a marked proliferation of hepatic microbodies in both the YA and RB animals (**Fig. 2**). The increase in the specific volume was more marked in the YA rats (~11×) in comparison to the RB animals (~6×). The significant increases in the volume densities of this organelle, 518% and 292% in the YA and RB

TABLE 2. Hepatic morphometric parameters in clofibrate-treated and control RB rats[a]

Component[b]	Control	P	Clofibrate-treated
Extrahepatocyte (V_V)	0.13 ± 0.02	NS	0.12 ± 0.02
Biliary space (V_V)	0.009 ± 0.002	NS	0.007 ± 0.001
Hepatocyte (V_V)	0.87 ± 0.02	NS	0.88 ± 0.01
(μm^3)	*5542 ± 691*	<0.005	*8302 ± 1113*
Nuclei (V_V)	0.07 ± 0.02	NS	0.08 ± 0.02
(μm^3)	*424 ± 93*	NS	*738 ± 137*
(N_V)[c]	168 ± 21	<0.01	106 ± 6
Cytoplasm (V_V)	0.81 ± 0.02	NS	0.80 ± 0.02
(μm^3)	*5259 ± 157*	<0.001	*7575 ± 136*
Mitochondria (V_V)	0.19 ± 0.01	NS	0.20 ± 0.01
(μm^3)	*1196 ± 75*	<0.001	*1891 ± 69*
Microbodies (V_V)	0.013 ± 0.001	<0.001	0.051 ± 0.003
(μm^3)	*82 ± 6*	<0.001	*482 ± 19*
Dense bodies (V_V)	0.006 ± 0.002	NS	0.005 ± 0.001
(μm^3)	*38 ± 78*	NS	*47 ± 9*
Lipid droplets (V_V)	0.003 ± 0.002	<0.05	0.014 ± 0.004
(μm^3)	*18 ± 10*	<0.02	*129 ± 35*
RER (S_V)	4.12 ± 0.36	<0.01	2.67 ± 0.33
(μm^2)	*15667 ± 1356*	NS	*14546 ± 1859*
SER (S_V)	07.00 ± 0.34	<0.001	9.00 ± 0.33
(μm^2)	*26670 ± 1294*	<0.001	*46190 ± 1695*
Golgi (S_V)	0.29 ± 0.09	NS	0.35 ± 0.08
(μm^2)	*1661 ± 398*	NS	*1801 ± 414*
Golgi-rich area (V_V)	0.031 ± 0.014	NS	0.012 ± 0.001
(μm^3)	*211 ± 102*	NS	*110 ± 12*

[a] All values expressed as the mean ± SEM.
[b] V_V is volume density or relative volume; S_V is surface density or relative surface area. Italicized values represent the volumes or surface areas per average mononuclear hepatocyte.
[c] NV is nuclear numerical density or the number of hepatocyte nuclei per unit volume of hepatocytes.

rats, respectively, suggest a higher concentration of microbodies, i.e., per volume of liver tissue, following clofibrate treatment.

The volume densities of the dense bodies (lysosomes) increased in both drug-treated groups, but the variabilities among the individual samples precluded any significant differences. Similarly, the increases in the specific volumes of this organelle reflect the increases in hepatocyte volumes, particularly in the YA animals (Tables 1 and 2).

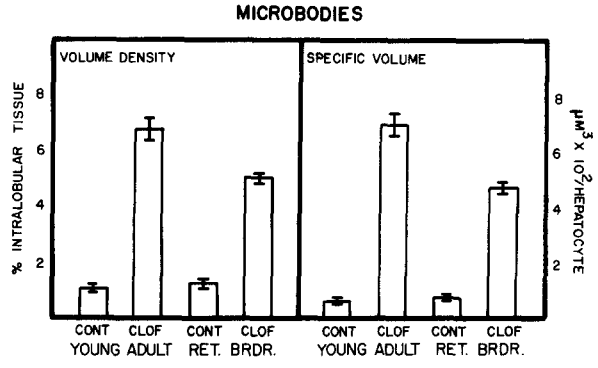

Fig. 2. Histogram demonstrating clofibrate-induced microbody proliferation. The left panel represents the data expressed as the relative volume or the percent of intralobular liver tissue occupied by microbodies. The right panel is expressed as the volume of microbodies per average liver cell. All values are means ± SEM.

Morphometric analysis revealed significant increases in both the volume density (~5×) and specific volume (~7×) of the cytoplasmic lipid droplets in the drug-treated RB rats. These data correlated well with the biochemical analysis showing a significant increase in the hepatic TG concentration (see Table 4). However, clofibrate did not affect the volume of cytoplasmic lipid droplets in the YA animals.

Changes in the amount of SER were of particular interest since a number of drugs cause an adaptive hepatic response that is manifested morphologically by a hypertrophy of this membrane system (see 52 and 53 for reviews). The mean surface densities of the SER, i.e., membrane surface area per volume of hepatocyte ground substance, increased significantly in the YA (50%) and the RB (29%) animals after clofibrate administration (Tables 1 and 2). This drug-induced hypertrophy was even more spectacular when the data were expressed as specific surface areas, resulting in increases of 130% and 73% in the YA and RB rats, respectively.

The surface density of the rough-surfaced endoplasmic reticulum (RER) decreased 16% in the clofibrate-treated YA rats, although each individual hepatocyte contained approximately 30% more membrane than these cells in animals receiving corn oil vehicle alone. These data suggest that there is no net loss of RER membrane in the livers of the YA animals. On the other hand, the significant reduction in RER surface density (35%) and the small decrease in the specific surface area of the RER (7%) in the clofibrate-treated RB rats suggests that this drug may cause a net loss of RER in the liver of this hyperlipidemic animal model. Regardless of the changes in the amount of RER per liver, clofibrate administration causes a significant reduction in the concentration of this membrane system in the RB animals (Tables 1 and 2).

The specific surface areas of the total endoplasmic reticulum were significantly greater in the hepatocytes of both the YA (95%) and RB (43%) animals following clofibrate treatment (**Fig. 3**). This overall increase in membrane surface area was more marked in the YA rats in comparison to the RB animals since the latter group exhibited a larger reduction in RER surface density or concentration and a smaller increase in SER specific surface area (Tables 1 and 2, Fig. 3).

The surface densities and the specific surface areas of the Golgi membranes increased in both groups of animals following clofibrate treatment,

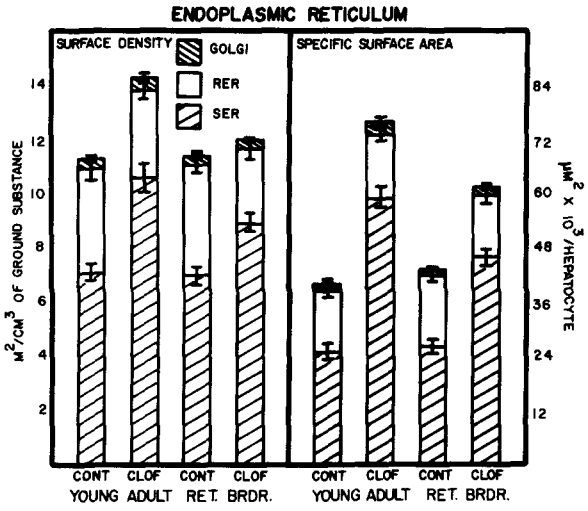

Fig. 3. Histogram demonstrating the effect of clofibrate on the surface area of the endoplasmic reticulum membranes. The left panel represents the relative surface areas, i.e., per unit volume of hepatocyte ground substance, whereas the right panel is expressed as surface area per average hepatocyte. Note that the virgin animals exhibit a greater response and that the SER is more markedly affected by clofibrate. All values are means ± SEM.

although neither change was significant (Tables 1 and 2). Since the small number of Golgi membrane profiles observed in the random high magnification electron micrographs resulted in a low confidence level of sampling, the volumes of the Golgi-rich areas were estimated on the low magnification micrographs according to the method described by Jones et al. (40, 41). The relative volume of Golgi-rich area decreased by 28% with clofibrate treatment in the YA rats, whereas each individual hepatocyte contained 30% more Golgi-rich area. These differences were not significant (Table 1).

In summary, clofibrate administration to normolipidemic YA and hyperlipidemic RB rats causes (*1*) a significant increase in the volume of individual hepatocytes, (*2*) greater volume densities and/or specific volumes of mitochondria and microbodies, (*3*) an increased concentration of hepatic SER, and (*4*) a greater volume of cytoplasmic lipid droplets in the RB rats. In addition, there appears to be a negative correlation between clofibrate treatment and the concentration of hepatic RER, particularly in the RB animals.

Serum lipid concentrations, body weight, and liver weight

Clofibrate exerted a significant hypocholesterolemic effect on the serum of both the YA and RB rats, 32% and 38%, respectively (**Table 3**). The serum TG concentrations were increased in both groups after clofibrate treatment, although the difference was significant only in the RB animals (30%).

The administration of clofibrate by gavage did not affect the body weights of either the YA or RB animals. However, the liver weights, expressed as a percentage of the final body weights, were significantly greater in both groups of drug-treated rats, 58% and 61% in the YA and RB animals, respectively (**Table 4**).

Hepatic lipid and protein concentrations

Hepatic protein concentrations were unaffected by clofibrate in either group of animals. Liver cholesterol concentrations were significantly reduced in the drug-treated YA (29%) and RB (27%) rats (Table 4). Although the concentrations of the hepatic TG were elevated in both groups of animals following clofibrate administration, the increase was significant only in the RB rats (70%).

Hepatic drug metabolism

The effects of clofibrate on hepatic microsomal drug-metabolizing enzymes and on the drug-metabolizing capacity are summarized in **Table 5.** Liver microsomal cytochrome P-450 concentrations, expressed as nmol/mg microsomal protein, were increased in both the YA and RB rats, 20% and 33%, respectively. Due to the variability among individual sample values, only the YA rats showed a significant elevation in this parameter in comparison to animals receiving the corn oil vehicle alone. Clofibrate

TABLE 3. Pre- and post-treatment serum lipid values in male YA and RB rats treated for 7 days with clofibrate or corn oil vehicle[a]

Group	Serum Cholesterol (mg%)			Serum Triglyceride (mg%)		
	T_0	P^b	T + 7d	T_0	P^b	T + 7d
RB/Control (10)[c]	103 ± 5	NS	98 ± 5	71 ± 5	NS	74 ± 9
RB/Clofibrate (16)	102 ± 8	<0.001	63 ± 6	79 ± 8	<0.05	103 ± 7
YA/Control (11)	89 ± 3	NS	88 ± 3	60 ± 5	NS	50 ± 4
YA/Clofibrate (17)	87 ± 3	<0.001	61 ± 3	59 ± 4	NS	76 ± 6

[a] All values expressed as the mean ± SEM.
[b] Difference between initial and post-treatment determinations.
[c] Parentheses denote the number of animals.

TABLE 4. Body weight, liver weight, liver protein, and liver lipids in male YA and RB rats treated for 7 days with clofibrate or corn oil vehicle[a]

	Retired Breeder			Young Adult		
	Control	P^b	Clofibrate	Control	P^b	Clofibrate
Body weight (g)	496 ± 9 (19)[c]	NS	487 ± 12 (18)	301 ± 7 (17)	NS	289 ± 8 (17)
Liver weight (g)	12.4 ± 0.4 (12)	<0.001	19.9 ± 0.8 (13)	8.8 ± 0.1 (11)	<0.001	14.2 ± 0.5 (10)
Liver weight (g/100 g body wt)	2.5 ± 0.1 (12)	<0.001	4.1 ± 0.1 (13)	3.0 ± 0.1 (11)	<0.001	5.2 ± 0.1 (10)
Liver protein (mg/g)	187 ± 9 (10)	NS	179 ± 3 (7)	192 ± 5 (6)	NS	203 ± 4 (9)
Liver triglycerides (mg/g)	10 ± 0.8 (10)	<0.001	17 ± 1.4 (11)	9.9 ± 0.6 (7)	NS	13.1 ± 1.3 (9)
Liver cholesterol (mg/g)	0.43 ± 0.02 (8)	<0.001	0.31 ± 0.04 (9)	0.40 ± 0.01 (7)	<0.001	0.29 ± 0.01 (9)

[a] All values expressed as the mean ± SEM.
[b] Difference between vehicle- and drug-treated animals.
[c] Parentheses denote the number of animals.

also caused a small reduction in the microsomal cytochrome b_5 concentrations in both groups of rats, although neither change was statistically significant.

The most noticeable changes occurred in the specific activity of hepatic microsomal NADPH cytochrome *c* reductase. The administration of clofibrate resulted in enhanced levels of activity in the YA rats (37%) and, more significantly, in the RB animals (106%). Clofibrate did not affect the hepatic capacity to metabolize ethylmorphine when the data were expressed per mg microsomal protein (Table 5). Although the hepatic microsomes from drug-treated animals exhibited a slightly enhanced ability to produce formaldehyde by the *N*-demethylation of ethylmorphine, the increases were not significant in either the YA (17%) or the RB (25%) rats. However, in view of the significant increases in liver size and in the concentrations of hepatic SER, the drug-metabolizing capacities of the whole livers of clofibrate-treated rats appear markedly increased (Tables 1, 2, 4, and 5).

DISCUSSION

A number of investigators demonstrated that clofibrate causes hepatomegaly in the rat (4, 7, 54). In the present study, clofibrate caused significant increases in the liver weights of both YA and RB rats. These results are in close agreement with those of Kolde, Roessner, and Themann (25) who reported a 68% increase in liver weight in rats treated

TABLE 5. Cytochrome P-450, cytochrome b_5, NADPH-cytochrome *c* reductase and ethylmorphine *N*-demethylation in male YA and RB rats treated for 7 days with clofibrate or corn oil vehicle[a]

	Retired Breeder			Young Adult		
	Control	P^b	Clofibrate	Control	P^b	Clofibrate
Cytochrome P-450 (nmol/mg microsomal protein)	1.32 ± 0.11 (8)[c]	NS	1.76 ± 0.24 (8)	1.13 ± 0.05 (11)	<0.01	1.57 ± 0.07 (10)
Cytochrome b_5 (nmol/mg microsomal protein)	0.60 ± 0.01 (8)	NS	0.51 ± 0.06 (8)	0.53 ± 0.03 (11)	NS	0.48 ± 0.02 (10)
NADPH-cytochrome *c* reductase (nmol × mg^{-1} protein × min^{-1})	63 ± 9 (8)	<0.01	130 ± 14 (8)	54 ± 5 (11)	<0.01	74 ± 4 (10)
Ethylmorphine *N*-demethylation (nmol × mg^{-1} protein × min^{-1})	2.02 ± 0.02	NS	2.5 ± 0.2	2.3 ± 0.2	NS	2.7 ± 0.2

[a] All values expressed as mean ± SEM.
[b] Difference between vehicle- and drug-treated animals.
[c] Parentheses denote the number of animals.

with clofibrate for 8 days (0.5% in the diet). Kaneko et al. (18) and Dalton et al. (55) attributed clofibrate-induced hepatomegaly to a proliferation of hepatocyte intracellular membranes, particularly the SER. The increase in liver size observed in the present study is reflected in increases in the volumes of individual hepatocytes which, in turn, are due to concomitantly larger volumes of mitochondria and microbodies and increases in the surface areas of the SER. A recent morphometric analysis of clofibrate-induced changes in rat liver fine structure also revealed significant increases in average hepatocyte size, as well as in the volume or surface density of the hepatic microbodies and SER (25).

A comparison of the morphometric parameters between the YA and RB animals (Tables 1 and 2) shows that the magnitudes of most of the clofibrate-induced changes are greater in the former experimental group. Interestingly, the data from the clofibrate-treated YA rats correlate well with those of Kolde et al. (25) who employed animals of nearly similar size, age, and physiological status.

Since the average hepatocellular volumes were significantly increased, the hepatocyte nuclear numerical densities were significantly reduced, and the hepatocyte volume densities were unchanged in both the YA and the RB clofibrate-treated animals, the observed hepatomegaly resulted from an increase in individual cell size rather than from an increase in cell number.

Although numerous studies showed that clofibrate has a marked effect on hepatic fine structure, the extent of these alterations is variable. These differences may result from the different drug dosages and modes of administration employed in the various studies. Such factors as modified food intake (34) and weight gain (32, 34), which occur when clofibrate is incorporated into the diet, may influence the amount of drug ingested. However, the results of the present study, in which each experimental animal received a known amount of drug, suggest that physiological status is an important factor to consider in an evaluation of the hepatic response to clofibrate.

Lee, Kim, and Lee (14) reported changes in the ultrastructural appearance of liver cell mitochondria in swine fed clofibrate. Our qualitative evaluation of hepatocyte fine structure did not reveal any noticeable differences in mitochondrial morphology between the clofibrate-treated and control YA or RB animals. However, several studies reported that clofibrate increased the number of liver cell mitochondria and elevated the total mitochondrial protein content (13, 15). The present morphometric data provide the first quantitative morphological evidence indicating an increase in both the volume density and the specific volume of hepatocyte mitochondria in clofibrate-treated animals. In view of the conflicting reports concerning the effect(s) of clofibrate on hepatic mitochondrial oxidative phosphorylation (56–58), the relationship between the increased concentration of this organelle in the YA rats and the hypolipidemic action of this drug is unclear. The inability of clofibrate to significantly affect the volume density of liver cell mitochondria in RB animals suggests that this particular hepatic response may not be universal and that physiological status may be an influencing factor.

The administration of several hypolipidemic drugs, particularly clofibrate, results in a marked proliferation of hepatic microbodies and a concomitant increase in liver catalase activity (7–9, 12, 59–61). Most of the microbodies in the livers of both YA and RB clofibrate-treated rats lacked the typical crystalline nucleoid, thus confirming earlier reports (6, 25). Since this organelle substructure is generally assumed to represent urate oxidase, the absence of this crystalloid correlates well with the previous report of decreased levels of this microbody enzyme following clofibrate administration (60).

Several investigators suggested a positive relationship between hepatic microbody proliferation and the mechanisms of action of certain lipid-lowering compounds (61, 62). However, Reddy et al. (63, 64) suggested that hepatic catalase is not related to the hypolipidemic effect of clofibrate. Moreover, in our studies clofibrate resulted in a greater proliferation of microbodies in the YA rats in comparison with the RB animals, and the hypocholesterolemic response was more marked in the RB rats. This further suggests that hepatic microbodies and/or their constituent enzymes may not be related to the lipid-lowering effect of this drug. The fact that clofibrate elicits a hypolipidemic response in female rats in the absence of hepatic microbody proliferation also supports this contention (8). Recent reports suggesting that microbody proteins other than those indigenous to this organelle may respond to clofibrate demonstrate that additional studies are required to elucidate the relationship between hepatic microbodies and certain serum lipid-lowering drugs (17, 25, 64).

Although clofibrate is reported to have a hypotriglyceridemic effect in man (1, 2), its effect on this parameter in the rat is variable (33, 65). Several studies reported that clofibrate elicits a TG-lowering effect, perhaps by activating extrahepatic lipoprotein lipase, increasing the hepatic turnover rate of TG,

or decreasing hepatic TG synthesis (66–69). In the present study, clofibrate elevated the serum TG concentrations in both YA and RB rats, although the increase was significant only in the latter group. Furthermore, clofibrate caused significant increases in the concentration of hepatic TG and in the volume of the cytoplasmic lipid droplets. The present biochemical data are not compatible with previous reports of lower serum TG levels in clofibrate-treated virgin rats.

Kolde et al. (25) reported a disorganization of the hepatic RER and dissociation of the membrane-bound ribosomes and a significant reduction in the surface density of the RER in young virgin rats treated with clofibrate. Although we did not observe any marked changes in the appearance of this membrane system, our morphometric data reveal a similar decrease in the concentration of the RER in both the YA and RB rats. However, the difference is significant only in the drug-treated RB animals. To our knowledge, similar quantitative changes in the hepatic RER following clofibrate treatment have not been reported. The facts that we observed no change and that several studies reported significant increases in rat liver protein concentrations following clofibrate administration (18, 20, 25) suggest that there is no positive correlation between the loss of hepatic RER and a general reduction in protein synthesizing capacity.

Previous reports suggested that clofibrate caused a proliferation of the hepatic SER and enhanced microsomal drug-metabolizing activity in rats (6, 18, 21). The increases in hepatic cytochrome P-450 in the YA animals and in NADPH cytochrome *c* reductase activities in both groups of rats are consistent with these earlier studies. The absence of a statistically significant rise in the cytochrome P-450 concentrations in the RB rats correlates well with the smaller clofibrate-induced SER response observed in these animals. It should be noted that a specific change in hepatic NADPH cytochrome *c* reductase without a concomitant change in cytochrome P-450 and unrelated to SER proliferation has been observed following the administration of thyroid hormone or its analog (70). Lewis, Witiak, and Feller (20) found a 30% increase in the concentration of rat liver cytochrome P-450 and in the rate of ethylmorphine *N*-demethylation following clofibrate administration. These changes are of the same order of magnitude as those reported here (Table 5). Although Kaneko et al. (18) reported marked increases in these parameters, their dosage of clofibrate (1000 mg/kg body weight) was considerably higher than that employed in the present study (200 mg/kg body weight).

Recently, Gustafsson, Einarsson, and Gustafsson (71) suggested that elevated concentrations of cholesterol in the liver or in the microsomes may serve to induce hepatic microsomal hydroxylase activity either by affecting the catalytic activity of the hydroxylating enzymes or by inducing the synthesis of a specific cytochrome P-450 species. If in fact hepatic cholesterol is an important mediator of clofibrate-induced microsomal enzyme activities, the decrease in total liver cholesterol concentration (Table 4) may contribute to the minimal effects of clofibrate on the concentrations or activities of the constituents of the hepatic drug metabolizing system (Table 5).

Results from two studies in humans suggested that clofibrate increased the biliary excretion of endogenous cholesterol (72, 73). In addition, Cohen et al. (74) reported a significant reduction in hepatic cholesterol synthesis following clofibrate administration to rats. Clofibrate is reported either to have no effect (24) or to decrease (21) the activity of hepatic cholesterol 7-α-hydroxylase and to reduce biliary excretion of cholesterol in rats (74). However, this has not been studied in the hypercholesterolemic RB animal.

The SER is the primary intracellular site of both the rate-limiting enzymes of hepatic cholesterol synthesis (HMG Co-A reductase) and bile acid synthesis (cholesterol 7-α-hydroxylase). The marked proliferation of the SER coupled with the significant reductions in both serum and liver cholesterol concentrations suggest that this membrane system may be involved in the hypocholesterolemic effect of clofibrate.

Alternatively, the proliferation of hepatic SER may represent a nonspecific pharmacological response to clofibrate rather than a hepatic adaptation designed to permit enhanced cholesterol excretion. The facts that clofibrate routinely lowers serum TG levels in humans but not in rats and enhances the biliary excretion of cholesterol in humans while reportedly having the opposite effect in rats suggest that the mechanism of action of this compound may differ in these two species.

The present data confirm several previously reported clofibrate-induced changes in the liver, including marked hepatomegaly, a proliferation of the microbodies and mitochondria, and SER hypertrophy. The correlation of quantitative electron microscopy and biochemical techniques, coupled with the use of two different animal models, i.e.,

the YA and RB rats, resulted in several new observations: (*1*) clofibrate elicits a different set of serum and hepatic responses depending on the physiological status of the animal, (*2*) there is no positive correlation between the hypocholesterolemic effect and the extent of hepatic microbody proliferation, (*3*) clofibrate-induced SER proliferation occurs in the absence of marked increases in the overall rate of hepatic drug metabolism or in the concentrations of certain microsomal constituents, and (*4*) there is an apparent direct relationship between the degree of serum hypocholesterolemic response and the specific activity of microsomal NADPH cytochrome *c* reductase. In view of the reported defects in mitochondrial β-oxidation in the livers of clofibrate-treated animals (57, 58) and the absence of any increase in the hepatocellular concentration of this organelle in the RB rats, we suspect that depressed free fatty acid oxidation may be an important etiological factor leading to elevated serum and liver TG concentrations.

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