Effects of treatment with clofibrate, bezafibrate, and ciprofibrate on the metabolism of cholesterol in rat liver microsomes

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Abstract The effects of treatment of rats with clofibrate, bezafibrate, and ciprofibrate on the hepatic metabolism of cholesterol were studied in rat liver microsomes. HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase activity, regulating cholesterol biosynthesis, was unaffected by clofibrate and ciprofibrate and slightly decreased (20%) by bezafibrate. Also cholesterol 7α-hydroxylase activity, governing bile acid biosynthesis, was unaffected by clofibrate and was reduced by 25-30% in the two other groups of rats. A major new finding was that all three fibric acid derivatives reduced ACAT (acyl-coenzyme A:cholesterol acyltransferase) activity, catalyzing the esterification of cholesterol, by 50-70%.

The hepatic content of free and esterified cholesterol was determined in the bezafibrate-treated rats. The concentration of microsomal cholesteryl ester was about 60% lower in the treated rats compared to the controls whereas the concentration of total cholesterol was unchanged. — Ståhberg, D., B. Angelin, and K. Einarsson. Effects of treatment with clofibrate, bezafibrate, and ciprofibrate on the metabolism of cholesterol in rat liver microsomes. J. Lipid Res. 1989. 30: 953-958.

Supplementary key words acyl-coenzyme A:cholesterol acyltransferase • cholesteryl ester • cholesterol 7α-hydroxylase • 3-hydroxy-3-methylglutaryl coenzyme A reductase

Clofibrate and its analogues bezafibrate and ciprofibrate are, like several other fibric acid derivatives, potent hypolipidemic agents. Their hypotriglyceridemic effect is due mainly to activation of lipoprotein lipase and to a lesser extent to a decreased production of triglycerides (1, 2). The cholesterol-lowering effect is less well understood.

The hepatic metabolism of cholesterol involves three key enzymes. HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase controls the synthesis of cholesterol (3) and cholesterol 7α-hydroxylase governs the catabolism of cholesterol to bile acids (4). The formation of cholesteryl esters, the storage form of cholesterol in the liver, is catalyzed by ACAT (acyl-coenzyme A:cholesterol acyltransferase) (5). Systematic studies on the effect of clofibrate or other fibric acid derivatives on the enzymes involved in hepatic metabolism of cholesterol are not available. Cohen et al. (6) have reported that clofibrate inhibits the HMG-CoA reductase activity in rat liver. In a previous study we did not find any influence of clofibrate on the 7α-hydroxylation of cholesterol in rat liver microsomes (7). As fibric acid derivatives are widely used as hypolipidemic drugs, we found it important to study their influence on the hepatic metabolism of cholesterol more thoroughly. In the present work, we have determined the activities of HMG-CoA reductase, cholesterol 7α-hydroxylase, and ACAT in liver microsomes from rats treated with clofibrate, bezafibrate, and ciprofibrate. The most important finding was that all three drugs inhibited the ACAT activity.

MATERIALS AND METHODS

Materials

Deuterium-labeled cholesterol was obtained from Larodan Fine Chemicals, Malmö, Sweden. Deuterium-labeled 7α-hydroxycholesterol was synthesized as described previously (8). [3-14C]HMG-CoA(sp act 19 mCi/mmol), [1-14C]oleoyl coenzyme A (sp act 57.8 mCi/mmol), and [1,2,6,7-3H]cholesteryl oleate (sp act 82.7 Ci/mmol) were obtained from New England Nuclear Corp., Boston, MA. DL-[2-3H]Mevalonic acid (sp act 125 mCi/mmol) was obtained from Radiochemical Center, Amersham, England. Unlabeled mevalonic acid lactone, cholesteryl olate, NADP, NADPH, EDTA, dithiothreitol, (DTT), glucose-6-phosphate, glucose-6-phosphate dehydrogenase. and human serum albumin were purchased from Sigma Chemical Co., St Louis, MO.

Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; DTT, dithiothreitol.
Experimental procedure

In the first series of experiments four groups of six male Sprague-Dawley rats, each weighing about 180 g, were used. They had free access to a crushed commercial pellet diet for 2 weeks. In three of the groups the food was supplemented (w/w) with 0.016% ciprofibrate (9), 0.1% bezafibrate (10), and 0.3% clofibrate (6, 7), respectively. In the second series of experiments only controls and bezafibrate-treated rats were used.

The rats were killed by decapitation after a 12-h fast. Blood samples were collected for determination of triglyceride and cholesterol values (Boehringer Mannheim enzymatic tests). The livers were excised, weighed, and cut into two or, in some cases, three pieces which were then put into cold buffer solutions.

Preparation of liver microsomes

One piece of the liver was minced and homogenized with a loose-fitting Teflon pestle in nine volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose, 10 mM DTT, 10 mM EDTA, and 50 mM NaCl. In some cases another piece of liver was prepared in the same way, but the 50 mM NaCl buffer was replaced by a 50 mM NaF-containing buffer and used for assay of the HMG-CoA reductase. The homogenate was centrifuged at 20,000 g for 15 min. The supernatant solution was centrifuged at 100,000 g for 60 min. The contents of protein in the microsomal fraction and the liver homogenate were determined by the method of Lowry et al. (11).

Another piece of the liver was homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose and 1 mM EDTA. The homogenate was centrifuged at 20,000 g for 15 min. The supernatant solution was centrifuged at 100,000 g for 60 min. The pellet obtained was resuspended in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and used for assay of the ACAT activity.

The contents of protein in the microsomal fraction and the liver homogenate were determined by the method of Lowry et al. (11).

Assay of HMG-CoA reductase activity

The HMG-CoA reductase activity was assayed essentially as described recently (12). In this assay, the microsomal fraction, 40 μl, is preincubated for 15 min at 37°C in a total volume containing 0.1 M phosphate buffer, pH 7.4, 10 mM imidazole buffer, pH 7.4, 5 mM DTT, 10 mM EDTA, 3 mM NADP, 12 mM glucose-6-phosphate, and one unit of glucose-6-phosphate dehydrogenase. The reaction is then initiated with the addition of 90 nmol (0.5 μCi) of [3-14C]HMG-CoA. The incubation is run for 15 min and stopped by the addition of 6 M HCl. Tritium-labeled mevalonic acid (0.01 μCi) is added, as an internal standard, together with 3 mg of unlabeled mevalonic acid lactone to the incubation mixture, which is then further lactonized, subjected to thin-layer chromatography, and analyzed for radioactivity.

Assay of cholesterol 7α-hydroxylase activity

The standard assay system consisted of 1.0 ml of the microsomal fraction, 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM NADPH. The enzyme assay was carried out according to the method of Björkhjem and Kallner (8) where the product 7α-hydroxy-cholesterol is measured by isotope dilution-mass spectrometry after addition of deuterium-labeled 7α-hydroxy-cholesterol as an internal standard.

Assay of ACAT activity

The standard assay system contained 0.1 ml of the microsomal preparation and 1 mg of fatty acid-free bovine serum albumin in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA to give a final volume of 1.0 ml. The mixture was preincubated for 5 min at 37°C. The reaction was initiated by the addition of 25 nmol (1.45 μCi) of [1-14C]oleoyl coenzyme A. After 4 min the assay was stopped by the addition of 10 ml chloroform-methanol 2:1 (v/v). Tritium-labeled cholesteryl oleate (0.01 μCi) was added as internal standard to estimate recovery followed by 1 ml 0.9% (w/v) NaCl. The chloroform phase was collected and evaporated to dryness under N2. The residue was resuspended in chloroform-methanol 2:1 and subjected to thin-layer chromatography together with unlabeled cholesteryl oleate as marker. The chromatogram was developed in hexane-ethyl acetate 95:5 (v/v). The cholesteryl oleate zone was visualized with iodine vapor and scraped off into a counting vial. A Packard liquid scintillation spectrometer, Model 3003, was used for determining the radioactivity using Aquasol as scintillator liquid. The rate of formation of cholesteryl oleate was linear with time up to at least 10 min. An incubation time of 4 min was therefore chosen to assure optimal assay conditions. The rate of formation of cholesteryl oleate was proportional to increasing amounts of microsomal protein up to at least 0.9 mg per assay. The dependence of ACAT activity on oleoyl CoA concentration was clearly demonstrated. At the concentration of endogenous cholesterol in the microsomes, saturation of the enzyme was obtained at an oleoyl coenzyme A concentration of 25 μM.
In some experiments the assay mixture was preincubated in the presence of exogenous cholesterol, dissolved in 600 mg of Triton X-100. A preincubation time of 30 min was optimal. The addition of cholesterol increased the enzyme activity about threefold. Saturation of the enzyme was obtained at the addition of 50 nmol of cholesterol.

**Determination of liver cholesterol**

The concentrations of total cholesterol in liver homogenates and microsomal fractions were determined by isotope dilution-mass spectrometry after addition of deuterium-labeled cholesterol as an internal standard as described previously (13). Free cholesterol was determined by the same method but the hydrolysis step was omitted. The concentration of esterified cholesterol was calculated as the difference between the total and free cholesterol in the same sample.

**Statistical analysis**

Data are presented as means ± SEM (standard error of the mean). The significance of differences was evaluated by Student's t-test.

**RESULTS**

Weight gain during the 2 weeks of treatment was similar in the four groups of animals (Table 1). Compared to the controls, the liver weight was about 50% higher in the rats treated with clofibrate and about 90% higher in those given ciprofibrate or bezafibrate (Table 1). The protein contents in the microsomal fraction and the liver homogenate were about the same in the treated groups and in the controls. The serum cholesterol level was 15–30% lower in the treated groups, whereas the triglyceride concentration was not significantly changed (Table 1).

In the control animals, the HMG-CoA reductase activity averaged $196 \pm 35$ pmol·min$^{-1}·mg$ protein$^{-1}$. Corresponding values in the treated animals expressed as % of the controls are shown in Table 2. Treatment with clofibrate and ciprofibrate did not significantly affect the HMG-CoA reductase activity. Bezafibrate treatment decreased the enzyme activity by about 20%.

The hepatic HMG-CoA reductase exists in both active and inactive forms (14, 15). The inactive form of the enzyme may be converted into the active one by dephosphorylation during the preparation of microsomes. This activation is inhibited in NaF-containing buffers. In the present study the state of activation, calculated as the ratio between the activity of NaF-prepared microsomes and that of NaCl-prepared microsomes, averaged 22 ± 5% in the controls. The state of activation was higher in both the clofibrate-treated rats (42 ± 5%) and the bezafibrate-treated animals (52 ± 9%).

The cholesterol 7α-hydroxylase activity was $108 \pm 8$ pmol·min$^{-1}·mg$ protein$^{-1}$ in the control rats. Clofibrate did not affect the enzyme activity (Table 2). Treatment with bezafibrate and ciprofibrate reduced the cholesterol 7α-hydroxylase activity by 25 to 30%.

In the control rats, the ACAT activity averaged $300 \pm 37$ pmol·min$^{-1}·mg$ protein$^{-1}$. Treatment with the fibrin acid derivatives reduced the enzyme activity by 50 to 70%, bezafibrate being the most potent agent (Table 2). The enzyme activity was reduced also when related to the total liver weight.

When the enzyme activities were related to the total liver weight, no decrease of the HMG-CoA reductase activity was obtained in the bezafibrate-treated rats and no reduction of the cholesterol 7α-hydroxylase activity was seen in the rats treated with bezafibrate and ciprofibrate. The ACAT activity, however, was reduced in the treated animals also when related to the total liver weight.

More detailed studies in bezafibrate-treated and control rats revealed that preincubation with added exogenous cholesterol raised the ACAT activity about

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Clofibrate</th>
<th>Bezafibrate</th>
<th>Ciprofibrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain, g</td>
<td>108 ± 9</td>
<td>113 ± 10</td>
<td>106 ± 8</td>
<td>95 ± 8</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>10.3 ± 0.4</td>
<td>15.9 ± 1.1***</td>
<td>19.2 ± 0.9***</td>
<td>19.8 ± 1.8***</td>
</tr>
<tr>
<td>Protein content in liver homogenate, mg/ml</td>
<td>20.8 ± 1.5</td>
<td>24.4 ± 1.7</td>
<td>24.5 ± 1.5</td>
<td>24.4 ± 1.0</td>
</tr>
<tr>
<td>Protein content in microsomal fraction, mg/ml</td>
<td>2.0 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Serum cholesterol, mmol/l</td>
<td>2.3 ± 0.1</td>
<td>1.8 ± 0.1**</td>
<td>2.0 ± 0.1*</td>
<td>1.6 ± 0.1***</td>
</tr>
<tr>
<td>Serum triglyceride, mmol/l</td>
<td>1.2 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
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</table>

Data represent means ± SEM of six animals. Values significantly different from controls: *, $P < 0.02$; **, $P < 0.01$; ***, $P < 0.001$. 

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threefold in both groups (Fig. 1). The inhibiting effect of bezafibrate on ACAT activity was about the same in microsomes preincubated in the presence and absence of exogenous cholesterol. The concentration of microsomal cholesterol ester was reduced by about 60% in the bezafibrate-treated rats (Table 3). The content of total cholesterol was not significantly changed.

DISCUSSION

In the present study, clofibrate treatment did not significantly affect the hepatic HMG-CoA reductase activity in rat. In contrast to our results, Cohen et al. (6) have reported that administration of clofibrate to rats inhibited hepatic HMG-CoA reductase activity by about 50% and decreased the total cholesterol synthesis by about 25%. Whether the discrepancy between our results and those of Cohen et al. is due to different experimental conditions or to the difference in species of rats is difficult to assess. In accordance with our results, Turley and Dietschy (16) found normal hepatic cholesterol synthesis in clofibrate-treated rats. It may also be mentioned that clofibrate-treated hyperlipidemic patients have normal levels of hepatic HMG-CoA reductase activity (15).

Also, ciprofibrate had no effect on hepatic HMG-CoA reductase activity in the rat. Bezafibrate slightly decreased the HMG-CoA reductase activity level, but when correlated to the total liver weight this decrease was eliminated. The hypocholesterolemic action of fibrate preparations, which was confirmed in the present study, is thus not explained by a suppression of hepatic cholesterol synthesis.

In accordance with a previous study from our laboratory (7), clofibrate treatment did not affect cholesterol 7α-hydroxylase activity. On the other hand, bezafibrate as well as ciprofibrate treatment inhibited cholesterol 7α-hydroxylase activity by 25 to 30%, an effect that was compensated for by the increase in liver weight, however. Again, in contrast to our results, Cohen et al. (6) have reported a 25% reduction of the fecal excretion of bile acids in rats during treatment with clofibrate. Previous studies in humans have shown that clofibrate treatment does not generally affect bile acid synthesis in patients with hypercholesterolemia (2). Treatment of hypertriglyceridermic patients with clofibrate or ciprofibrate may, however, normalize the overproduction of bile acids often seen in these patients (2). The difference between the in vivo measurement of bile acid production reported by Cohen et al. (6) and the present in vitro assay of the cholesterol 7α-hydroxylase activity might be the result of a decreased availability of substrate, namely cholesterol, for the enzyme, or may, as discussed for the HMG-CoA reductase activity, be ascribed to difference in the species of rats used.

A major new finding of the present study was the pronounced inhibition of the ACAT activity in clofibrate-, bezafibrate-, as well as ciprofibrate-treated rats. In accordance with these results, the hepatic microsomal content of esterified cholesterol was decreased in the bezafibrate-treated animals. Previously Turley and Dietschy (16) have reported a reduced level of cholesteryl ester in the liver of clofibrate-treated rats. The mechanism of the inhibitory effect of fibrates on the ACAT activity can only be spe-
ulated on. It has earlier been claimed that ACAT is not a true rate-determining enzyme, but instead reflects the availability of unesterified cholesterol in the vicinity of the enzyme (5, 17, 18). However, the microsomal pool of unesterified cholesterol was not decreased in the microsomal preparation from the bezafibrate-treated rats compared with microsomes prepared from untreated animals. It may be argued that the pool of cholesterol accessible to the ACAT enzyme is only a fraction of the total pool of free cholesterol in microsomes, and that a decrease in the size of a small pool near the enzyme thus may occur without significant changes in the total concentration of free cholesterol in the microsomes. Such a mechanism cannot be completely excluded, even if it would seem less likely since preincubation of the microsomal fraction with unlabelled cholesterol increased the enzyme activity to the same extent in both bezafibrate-treated and untreated rats. A more likely explanation is that fibrates affect the formation of cholesteryl esters by directly inhibiting ACAT or by decreasing the amount of enzyme protein. To distinguish between these two possibilities we have to await experiments performed with purified enzyme preparations.

It is of interest to consider the possible clinical implications of the fibrate-induced reduction in hepatic ACAT activity. Treatment of hyperlipidemic patients with clofibrate, as well as with bezafibrate and ciprofibrate, causes an increase in cholesterol saturation of bile mainly because of an increased biliary output of cholesterol (2). The mechanism of this hypersecretion of biliary cholesterol remains to be studied. Whether such a mechanism actually operates in human treatment situations remains to be studied. The skillful technical assistance of Ms. Gunvor Alvelius, Ms. Lisbet Benthin, and Ms. Ingela Svensson is gratefully acknowledged. This study was supported by grants from the Swedish Medical Research Council (project 03X-04793) and Karolinska Institutet.

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