Effects of Clofibrate on some Microsomal Hydroxylations involved in the Formation and Metabolism of Bile Acids in Rat Liver

By BO ANGELIN, INGEMAR BJÖRKHEM and KURT EINARSSON

Department of Medicine, Serafimerlasarettet, and Department of Clinical Chemistry, Huddinge Sjukhus, Karolinska Institutet, Stockholm, Sweden

(Received 19 December 1975)

1. The liver microsomal metabolism of [4-¹⁴C]cholesterol, endogenous cholesterol, 7 α -hydroxy-4-[6 β -³H]cholesten-3-one, 5 β -[7 β -³H]cholestane-3 α ,7 α -diol and [³H]lithocholic acid was studied in control and clofibrate (ethyl *p*-chlorophenoxyisobutyrate)-treated rats. 2. The extent of 7 α -hydroxylation of exogenous [4-¹⁴C]cholesterol and endogenous cholesterol, the latter determined with a mass fragmentographic technique, was the same in the two groups of rats. The extent of 12 α -hydroxylation of 7 α -hydroxy-4-cholesten-3-one and 5 β -cholestane-3 α ,7 α -diol was increased by about 60 and 120% respectively by clofibrate treatment. The 26-hydroxylation of 5 β -cholestane-3 α ,7 α -diol was not significantly affected by clofibrate. The 6 β -hydroxylation of lithocholic acid was about 80% higher in the clofibrate-treated animals than in the controls. 3. The results are discussed in the context of present knowledge about the liver microsomal hydroxylating system and bile acid formation in patients with hypercholesterolaemia, treated with clofibrate.

Clofibrate (ethyl *p*-chlorophenoxyisobutyrate) is a widely used hypolipidaemic drug that lowers the concentrations of serum cholesterol and triglycerides in man and in the rat. Its cholesterol-lowering effect is as yet not fully understood. In a previous investigation evidence was given that the 7 α -hydroxylation of cholesterol, which is the rate-limiting step in bile acid formation, was not influenced by clofibrate treatment in rat liver (Einarsson *et al.*, 1974a). In contrast Cohen *et al.* (1974) showed that clofibrate-treated rats have a decreased faecal bile acid excretion compared with control rats. It was suggested by Cohen *et al.* (1974) that our results might be explained by the system for enzyme assay *in vitro* used for the 7 α -hydroxylase. With the type of assay used, only 7 α -hydroxylation of exogenous cholesterol is determined, and the possibility cannot be excluded that this conversion does not reflect changes in 7 α -hydroxylation of endogenous cholesterol.

To clarify further the influence of clofibrate on the formation of bile acids, the 7 α -hydroxylation of cholesterol was studied with a recently developed mass-fragmentographic technique by which the mass of 5-cholestene-3 β ,7 α -diol formed from endogenous cholesterol can be determined (Björkhem & Danielsson, 1974a). In addition the effects of clofibrate on some other hydroxylases important in the biosynthesis and metabolism of bile acids were studied.

Materials and Methods

Radioactive steroids

[4-¹⁴C]Cholesterol (specific radioactivity 58 μ Ci/ μ mol) was from The Radiochemical Centre,

Amersham, Bucks., U.K. Before use [4-¹⁴C]cholesterol was purified by chromatography on a column of Al₂O₃ (grade III; Woelm, Eschwege, Germany). 7 α -Hydroxy-4-[6 β -³H]cholesten-3-one (specific radioactivity 2.7 μ Ci/ μ mol) was prepared as described by Björkhem (1969) and 5 β -[7 β -³H]cholestane-3 α ,7 α -diol (specific radioactivity 6.5 μ Ci/ μ mol) was synthesized as described by Björkhem & Gustafsson (1973). [³H]Lithocholic acid (specific radioactivity 4 μ Ci/ μ mol) was from New England Nuclear Corp., Boston, MA, U.S.A. NADP⁺, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were from Sigma Chemical Co., St. Louis, MO, U.S.A. Clofibrate (Atromidin) was from Scanmeda, Göteborg, Sweden.

Animals and preparation of homogenates

Adult male rats of the Sprague–Dawley strain weighing approx. 200 g were used. They were divided into two groups of six animals and were fed on crushed rat diet pellets (R3; Astra-Ewos AB, Södertälje, Sweden) for 1 month *ad lib*. For one of the groups the food was supplemented with 0.3% clofibrate. The drug was dissolved in aq. 95% (v/v) ethanol, which was added to the crushed pellets and left to evaporate at room temperature (20°C). At this dose the drug is reported to have a maximum effect on the plasma cholesterol concentration without producing toxic manifestations (Thorp & Waring, 1962).

After overnight starvation, the rats were killed with a blow on the neck. The livers were excised, immediately chilled on ice, blotted, weighed and minced into small pieces. A 20% (w/v) homogenate was prepared in 0.3M-sucrose containing 75 mM-nicotinamide,

2mM-EDTA and 20mM-2-mercaptoethanol, by using a Potter-Elvehjem homogenizer equipped with a loosely fitting pestle. The homogenate was centrifuged at 20000g for 15 min. The microsomal fraction was isolated by centrifuging the 20000g supernatant for 1 h at 100000g, and washed once in the homogenizing medium by resuspension and re-centrifugation at 100000g for 30 min. A part of the microsomal fraction, intended for assay of cholesterol 7 α -hydroxylase activity, was suspended in 0.1M-potassium phosphate buffer, pH 7.0, containing 28mM-nicotinamide. The remaining part of the microsomal fraction was suspended in a modified Bucher medium (Bergström & Gloor, 1955). The protein concentrations of the microsomal fractions were determined by the method of Lowry *et al.* (1951) with albumin as standard.

Incubations with [4-¹⁴C]cholesterol and endogenous cholesterol

To a mixture of 5 ml of liver microsomal fraction and an NADPH-generating system [consisting of: NADP⁺, 3 μ mol; glucose 6-phosphate, 10 μ mol; glucose 6-phosphate dehydrogenase (EC 1.1.1.49), 5 units; MgCl₂, 3 μ mol] was added 10 μ g of [4-¹⁴C]-cholesterol, dissolved in a suspension of 0.1 ml of above mentioned buffer containing 3 mg of Tween 80 (Björkhem & Danielsson, 1975). The incubation was for 15 min at 37°C and was terminated by the addition of 20 vol. of chloroform/methanol (2:1, v/v). Just before addition of chloroform/methanol to the incubation mixture, ³H-labelled 5-cholestene-3 β ,7 α -diol (3 μ g) was added as internal standard. The chloroform phase was analysed by t.l.c. (Björkhem & Danielsson, 1975). Conversion of the trace amount of added [4-¹⁴C]cholesterol into 5-cholestene-3 β ,7 α -diol was determined by radioscanning of the t.l.c. plate. The mass of 5-cholestene-3 β ,7 α -diol formed from endogenous cholesterol was determined by means of combined gas-liquid chromatography-mass

spectrometry of the appropriate chromatographic fraction by using an LKB instrument equipped with a 1.5% SE-30 column (Björkhem & Danielsson, 1974a, 1975).

Incubations with 7 α -hydroxy-4-[6 β -³H]cholesten-3-one

To 4 ml of liver microsomal fraction, supplemented with an NADPH-generating system, 7 α -hydroxy-4-[6 β -³H]cholesten-3-one (50 μ g) was added. The incubation was for 12 min at 37°C and was terminated as described above. Further analysis was performed as reported previously (Björkhem & Einarsson, 1970) except that the conversion of the labelled substrate was determined by scanning the chromatoplate with a thin-layer radioscanner.

Incubations with 5 β -[7 β -³H]cholestane-3 α ,7 α -diol

To a mixture of 3 ml of liver microsomal fraction and an NADPH-generating system, 5 β -[7 β -³H]-cholestane-3 α ,7 α -diol (250 μ g) was added. The incubation was for 20 min and was terminated as described above. Further analysis was performed as described by Björkhem & Gustafsson (1973).

Incubations with [³H]lithocholic acid

To 2 ml of liver microsomal fraction and an NADPH-generating system, [³H]lithocholic acid (50 μ g) was added. The incubation was for 20 min and was terminated with an equal volume of aq. 95% (v/v) ethanol. Further analysis was performed as described by Einarsson & Gustafsson (1974).

Statistical analysis

Student's *t* test was used.

Results

Liver weight and microsomal protein

The liver weights calculated as g/100g body wt., were 20–25% higher in the clofibrate-treated rats

Table 1. Effects of clofibrate treatment on some steroid hydroxylases in the microsomal fraction of rat liver homogenates

The values listed are the means $\pm$ S.E.M. of experiments with six rats. N.S., No significance ($P > 0.05$).

Substrate	Reaction	Hydroxylase activity (pmol/15 min per mg of protein)		Significance of differences
		Control rats	Clofibrate- treated rats	
Cholesterol	7 α -Hydroxylation of labelled cholesterol	11.6 $\pm$ 1.8	11.5 $\pm$ 1.6	N.S.
	7 α -Hydroxylation of endogenous cholesterol	133 $\pm$ 14	141 $\pm$ 17	N.S.
(nmol/20 min per mg of protein)				
7 α -Hydroxy-4-cholesten-3-one	12 α -Hydroxylation	6.0 $\pm$ 1.0	9.8 $\pm$ 1.3	$P < 0.05$
5 β -Cholestane-3 α ,7 α -diol	12 α -Hydroxylation	3.9 $\pm$ 0.5	8.5 $\pm$ 0.8	$P < 0.001$
	26-Hydroxylation	5.3 $\pm$ 0.8	7.9 $\pm$ 1.2	N.S.
Lithocholic acid	6 β -Hydroxylation	3.7 $\pm$ 0.2	6.6 $\pm$ 0.3	$P < 0.001$

(5.5 ± 0.2 g; mean $\pm$ S.E.M.) compared with the control rats (4.5 ± 0.1 g) ($P < 0.005$). The protein concentration of the microsomal fraction was not significantly increased during clofibrate treatment: 2.0 ± 0.2 mg/ml in the treated animals against 1.5 ± 0.1 mg/ml in the control animals.

Incubations

Table 1 summarizes the results. The extent of 7α -hydroxylation of the trace amount of added [4^{14}C]cholesterol was the same in the two groups of rats. Also the amount of 5β -cholestene- $3\beta,7\alpha$ -diol formed from endogenous cholesterol was similar in the two groups of animals.

The principal metabolite formed from 7α -hydroxy-4-cholesten-3-one in the microsomal fraction is $7\alpha,12\alpha$ -dihydroxy-4-cholesten-3-one (Einarsson, 1968; Björkhem & Einarsson, 1970). The extent of 12α -hydroxylation was increased by about 60% in the clofibrate-treated rats compared with the control rats.

The two main metabolites formed from 5β -cholestane- $3\alpha,7\alpha$ -diol in the microsomal fraction of rat liver are 5β -cholestane- $3\alpha,7\alpha,12\alpha$ -triol and 5β -cholestane- $3\alpha,7\alpha,26$ -triol (Björkhem & Gustafsson, 1973). The extent of 12α -hydroxylation was more than twice as great in clofibrate-treated rats as in control animals. Also the 26 -hydroxylase activity tended to increase on clofibrate treatment, but no significant difference was obtained.

Lithocholic acid has previously been shown to be mainly 6β -hydroxylated in the microsomal fraction (Einarsson & Johansson, 1969). The extent of 6β -hydroxylation was increased by about 80% by clofibrate treatment.

Discussion

The main bile acids formed from cholesterol in rat liver as well as in human liver are cholic acid ($3\alpha,7\alpha,12\alpha$ -trihydroxy- 5β -cholanoic acid) and chenodeoxycholic acid ($3\alpha,7\alpha$ -dihydroxy- 5β -cholanoic acid) (for a review see Danielsson, 1973). During intestinal passage both cholic acid and chenodeoxycholic acid are partly 7α -dehydroxylated to yield deoxycholic acid ($3\alpha,12\alpha$ -dihydroxy- 5β -cholanoic acid) and lithocholic acid (3α -hydroxy- 5β -cholanoic acid) respectively. In rat liver reabsorbed deoxycholic acid is rehydroxylated in the 12α -position and lithocholic acid is 6β -hydroxylated. Chenodeoxycholic acid is also further hydroxylated in the 6β -position. The 6β -hydroxylase active on chenodeoxycholic acid has similar properties to the lithocholic acid 6β -hydroxylase. Human liver has a very low ability to hydroxylate bile acids (Björkhem *et al.*, 1973a).

The first and rate-limiting step in the degradation of cholesterol into bile acids is 7α -hydroxylation (for a review see Danielsson, 1973). Subsequent oxidation yields 7α -hydroxy-4-cholesten-3-one. This compound

is considered to be a key intermediate and might be further 12α -hydroxylated in cholic acid biosynthesis. If not 12α -hydroxylated, 7α -hydroxy-4-cholesten-3-one might be converted into 5β -cholestane- $3\alpha,7\alpha$ -diol in the soluble fraction of the cell. The latter compound might be either 12α -hydroxylated or 26 -hydroxylated. With 26 -hydroxylation, chenodeoxycholic acid will be the final metabolite, since introduction of a 26 -hydroxyl group seems to prevent subsequent 12α -hydroxylation. At least in the rat, the ratio between cholic acid and chenodeoxycholic acid formed appears to be determined by the relative activity of the microsomal 12α - and 26 -hydroxylase (Björkhem & Danielsson, 1974b; Cohen *et al.*, 1975). The main pathway for bile acid synthesis in human liver seems to be analogous to that in rat (Björkhem *et al.*, 1968, 1975). The possibility has been discussed, however, that side-chain oxidation starts with a 25 -hydroxylation instead of a 26 -hydroxylation in biosynthesis of bile acids in the human liver (Setoguchi *et al.*, 1974).

Earlier works have shown that clofibrate increases liver weight (Best & Duncan, 1964), induces proliferation of smooth-surfaced endoplasmic reticulum in rat liver cells (Hess *et al.*, 1965) and increases the content of cytochrome *P*-450 in rat liver microsomal fraction (Salvador *et al.*, 1970; Lewis *et al.*, 1974). Clofibrate influences hepatic drug metabolism in rats (Lewis *et al.*, 1974) and alters the metabolism of several steroid hormones, e.g. testosterone (Salvador *et al.*, 1970), androstenedione (Einarsson *et al.*, 1974a), progesterone and oestradiol (Einarsson *et al.*, 1973a). Thus clofibrate seems to belong to the group of drugs, among them phenobarbital, that stimulates the activity of a variety of hepatic microsomal hydroxylase activities towards several drugs, fatty acids, steroids etc. Previous investigations have given evidence that all hydroxylases studied in the present work are dependent on cytochrome *P*-450 (cf. Björkhem & Danielsson, 1974b). However, only the 6β -hydroxylase is stimulated by phenobarbital treatment and the other hydroxylases seem to be more specific (cf. Björkhem & Danielsson, 1974b). In the present experiments the 6β -hydroxylation of lithocholic acid was found to be stimulated by clofibrate treatment. Surprisingly the activity of the 12α -hydroxylase was also increased by clofibrate. 7α -Hydroxylation of cholesterol and 26 -hydroxylation of 5β -cholestane- $3\alpha,7\alpha$ -diol were not significantly increased by clofibrate treatment. It has been shown previously that 12α -hydroxylation is specifically increased by starvation (Johansson, 1970) and by hypothyroidism (Mitropoulos *et al.*, 1968). In the starved state, however, the activity of the cholesterol 7α -hydroxylase is decreased, which was not the case after clofibrate treatment. In the hypothyroid state, 26 -hydroxylase activity is decreased (Björkhem *et al.*, 1973b), which was also not observed after clofibrate treatment. It has been shown that the cytochrome

P-450 fraction is responsible for the stimulatory effect of starvation on 12 α -hydroxylase activity (Bernhardsson *et al.*, 1973). It is also possible that clofibrate induces the specific type of cytochrome P-450 activity involved in 12 α -hydroxylation.

The finding that 7 α -hydroxylation of cholesterol was uninfluenced by clofibrate treatment is in accordance with an earlier study (Einarsson *et al.*, 1974a). Cohen *et al.* (1974) reported that clofibrate-treated rats have about 25% lower faecal excretion of bile acids compared with controls. Whether the relatively small discrepancy between our results *in vitro* and those of Cohen *et al.* (1974) *in vivo* depend on the different experimental conditions or on differences in the species of rats is difficult to assess.

The finding that the 12 α -hydroxylase, in contrast with the 7 α -hydroxylase and the 26-hydroxylase, was stimulated by clofibrate treatment may have some bearing on recent observations in patients with hyperlipoproteinaemia type II (hypercholesterolaemia). These patients have a low ratio between cholic acid and chenodeoxycholic acid synthesis, depending on a subnormal formation of cholic acid (Einarsson *et al.*, 1974b). During treatment with clofibrate the combined synthesis of cholic acid and chenodeoxycholic acid was unchanged, but the ratio between cholic acid and chenodeoxycholic acid increased in five out of six patients (Einarsson *et al.*, 1973b).

This work was supported by grants from Karolinska Institutets Forskningsfonder.

References

- Bergström, S. & Gloor, U. (1955) *Acta Chem. Scand.* **9**, 34–38
- Bernhardsson, C., Björkhem, I., Danielsson, H. & Wikvall, K. (1973) *Biochem. Biophys. Res. Commun.* **54**, 1030–1038
- Best, M. M. & Duncan, C. H. (1964) *J. Lab. Clin. Med.* **64**, 634–642
- Björkhem, I. (1969) *Eur. J. Biochem.* **8**, 337–344
- Björkhem, I. & Danielsson, H. (1974a) *Anal. Biochem.* **59**, 508–516
- Björkhem, I. & Danielsson, H. (1974b) *Mol. Cell. Biochem.* **4**, 79–95
- Björkhem, I. & Danielsson, H. (1975) *Eur. J. Biochem.* **53**, 63–70
- Björkhem, I. & Einarsson, K. (1970) *Eur. J. Biochem.* **13**, 174–179
- Björkhem, I. & Gustafsson, J. (1973) *Eur. J. Biochem.* **36**, 201–212
- Björkhem, I., Danielsson, H., Einarsson, K. & Johansson, G. (1968) *J. Clin. Invest.* **47**, 1573–1582
- Björkhem, I., Einarsson, K. & Hellers, G. (1973a) *Eur. J. Clin. Invest.* **3**, 459–465
- Björkhem, I., Gustafsson, J. & Danielsson, H. (1973b) *FEBS Lett.* **31**, 20–22
- Björkhem, I., Gustafsson, J., Johansson, G. & Persson, B. (1975) *J. Clin. Invest.* **55**, 478–486
- Cohen, B. I., Raicht, R. F., Shefer, S. & Mosbach, E. H. (1974) *Biochim. Biophys. Acta* **369**, 79–85
- Cohen, B. I., Raicht, R. F., Nicolau, G. & Mosbach, E. H. (1975) *Lipids* **10**, 168–174
- Danielsson, H. (1973) in *The Bile Acids: Chemistry, Physiology and Metabolism*. (Nair, P. P. & Kritchevsky, D., eds.), vol. 2, pp. 1–32, 305–306, Plenum Press, New York
- Einarsson, K. (1968) *Eur. J. Biochem.* **5**, 101–108
- Einarsson, K. & Gustafsson, J.-Å. (1974) *Biochem. Pharmacol.* **23**, 9–12
- Einarsson, K. & Johansson, G. (1969) *FEBS Lett.* **4**, 177–180
- Einarsson, K., Gustafsson, J.-Å. & Hellström, K. (1973a) *Biochem. J.* **136**, 623–628
- Einarsson, K., Hellström, K. & Kallner, M. (1973b) *Eur. J. Clin. Invest.* **3**, 345–351
- Einarsson, K., Gustafsson, J.-Å. & Hellström, K. (1974a) *Biochem. Pharmacol.* **23**, 13–19
- Einarsson, K., Hellström, K., & Kallner, M. (1974b) *J. Clin. Invest.* **54**, 1301–1311
- Hess, P., Staubli, W. & Riess, W. (1965) *Nature (London)* **208**, 856–858
- Johansson, G. (1970) *Eur. J. Biochem.* **17**, 292–295
- Lewis, N. J., Witiak, D. T., & Feller, D. R. (1974) *Proc. Soc. Exp. Biol. Med.* **145**, 281–285
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mitropoulos, K. A., Suzuki, M., Myant, N. B. & Danielsson, H. (1968) *FEBS Lett.* **1**, 13–15
- Salvador, R. A., Haber, S., Atkins, C., Gommi, B. W. & Welch, R. M. (1970) *Life Sci.* **9**, 397–407
- Setoguchi, T., Salen, G., Tint, G. S. & Mosbach, E. H. (1974) *J. Clin. Invest.* **53**, 1393–1401
- Thorp, J. M. & Waring, W. S. (1962) *Nature (London)* **194**, 948–949