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Stimulation of Bile Acid Biosynthesis by Clofibrate

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The hypocholesterolemic effect of clofibrate (ethyl *p*-chlorophenoxyisobutyrate) was investigated in rats under conditions of severe hypercholesterolemia produced by administering relatively large amounts of cholesterol. The rats were fed a normal chow (control rats) or a chow containing 0.25% clofibrate (treated rats) for 14 d, then Triton WR-1339 was administered intraperitoneally (100 mg/100 g body weight). Four days after this administration, the blood cholesterol level of the control rats was markedly higher at 9335 mg/dl serum, while the level of the treated rats was about 30% lower than that of the control. The bile acid level in pooled 24-h bile was proportionally higher (about 1.3-fold) in the treated rats than that in the control. In the initial bile (within 4 h after bile duct cannulation), the bile acid level in the treated bile was 2.4 times higher than in the control bile.

[4-^{14}C]Cholesterol and 100 mg non-labeled cholesterol were injected intraperitoneally into the control and clofibratetreated rats, and the radioactivities excreted into the bile were determined. The early bile (within 8 h) of the treated rats contained 5.6-fold higher radioactivity than the control bile, and the radioactivities were largely recovered in the bile acid fraction; scarcely any radioactivity was found in the cholesterol fraction.

From these results, we conclude that one of the mechanisms by which the hypocholesterolemic effect of clofibrate is mediated is stimulation of the degradation of cholesterol, that is, stimulation of the biosynthesis of bile acids.

Keywords——clofibrate; CPIB; hyperlipidemia; hypercholesterolemia; bile acid

Introduction

Clofibrate (ethyl *p*-chlorophenoxyisobutyrate) is extensively used in the treatment of hyperlipidemias.[1-3] However, its mechanism of action is not clearly understood. Studies on the hypocholesterolemic effect of clofibrate suggest that the drug lowers plasma cholesterol level by inhibition of cholesterol synthesis.[4,5] The inhibition site has been reported to be the pathway from acetate to mevalonic acid,[5] or that from mevalonic acid to isopentenylpyrophosphate.[4]

On the other hand, Mitchell *et al.*[6,7] investigated the effect of clofibrate on the degradation of cholesterol, but observed no clear effect and concluded that the drug does not stimulate the degradation of cholesterol. The degradation products of cholesterol are bile acids; after hydroxylation of the steroid rings of cholesterol, the 27-methyl group of the side chain is subject to β-oxidation. Cholesterol gives rise to either 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) or 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA), depending on the extent of the hydroxylation. The side chains of THCA and DHCA then undergo β-oxidation by analogy with fatty acids, and THCA and DHCA are transformed to cholic acid and chenodeoxycholic acid, respectively.

Recently, it was reported that clofibrate enhances fatty acid β-oxidation in both mitochondria[11] and peroxisomes[12-14] of hepatocytes. Further, the conclusion of Mitchell *et al.*[6,7] regarding the hypocholesterolemic effect of clofibrate seemed questionable because they used only moderately hypercholesterolemic patients as experimental subjects. Therefore, it seemed desirable to reinvestigate whether or not clofibrate does participate in the degradation of cholesterol.

The present report shows that clofibrate does affect the degradation of cholesterol, that is, the synthesis of bile acids, in severely hypercholesterolemic rats.

Experimental

Materials——Clofibrate was kindly provided by Sumitomo Kagaku Co., (Japan). Triton WR-1339 (oxyethylated tertiary octylphenol–polymethylene polymer) was obtained from Ruger Chemicals (U.S.A.). Cholic acid and chenodeoxycholic acid were purchased from Sigma Chemicals (U.S.A.). [4-^{14}C]Cholesterol (50 mCi/mmol) was obtained from RCC Amersham (England). All other reagents were of analytical grade from Wako Pure Chemicals (Japan).

Treatments of the Rats——Male Wistar rats (*ca.* 200 g) were fed *ad libitum* on chow pellets (Oriental MF) (Japan) containing 0.25% (w/w) clofibrate for 2 weeks. Control rats were similarly fed on the normal chow (MF alone). Then both groups of rats were injected with Triton WR-1339 or [^{14}C]cholesterol.

Triton WR-1339 was administered intraperitoneally at a dose of 100 mg per 100 g body weight. The rats were fed on the chows for 2 or 4 d, then bile-duct cannulation was carried out to obtain bile.

In the case of [4-^{14}C]cholesterol treatment, 0.5 μCi of [^{14}C]cholesterol was mixed with 100 mg of non-labeled cholesterol in 1 ml of 2% Tween 80, and the mixture was sonicated in a Branson Sonifier (Model 200, U.S.A.) at 200 mA, 100 V for 10 min, then intraperitoneally administered to the clofibrate-treated and control rats immediately after the bile-duct cannulation.

After these treatments, the rats were kept on the same chows and physiological saline in Bollman cages to collect bile.

Fractionation of Bile——A 2 ml bile sample was extracted twice with 20 ml of petroleum ether (bp 60—80°C). The bile was separated from the petroleum ether layer, and further extracted twice with 20 ml of ethanol. After centrifugation, the ethanol layer was separated from the precipitate (bile pigments, mutin and others). The petroleum ether and ethanol extracts were taken to dryness under nitrogen and each residue was dissolved in 1 ml of ethanol. The former extract was used as cholesterol fraction and the latter extract, as bile acids fraction (Table III).

In order to hydrolyze the bile acid conjugates, 10 ml of 10% (w/w) KOH–50% (v/v) ethanol solution was added to the ethanol extract, and the mixture was autoclaved at 100—115°C for 15 h. The hydrolysate was then acidified to pH 4 with 1N HCl and extracted three times with equal volumes of ethyl acetate. The ethyl acetate extract was appropriately concentrated under nitrogen and an aliquot was subjected to thin-layer chromatography. For thin-layer chromatography, Silicagel G (Merck, U.S.A.) was used, and the bile acids were separated with an ethyl acetate–isooctane–acetic acid system (25:5:0.2). Standard mixtures of cholic acid and chenodeoxycholic acid were run in each case to facilitate identification. After the development, the line of the standard mixture as sprayed with 20% H_2SO_4. The areas corresponding to the positions of the standards thus located were scraped from the plate into centrifuge tubes and the remaining areas were also collected to isolate other bile acids. Each bile acid was extracted twice from the silica gel with a certain volume of ethanol. The quantities of bile acids were determined according to the method of Eastwood *et al.*[15]

Determination of Radioactivity——After the administration of [^{14}C]cholesterol, the radioactivities in bile and fractionated samples were determined with an Aloka scintillation counter (Model LSC-700, Japan) in scintillation fluid consisting of PPO (4 g) and POPOP (0.04 g) in 1 liter of toluene and 500 ml of Triton X-100.

Assays of Lipids——Cholesterol was assayed by the *o*-phtalaldehyde method[16] and triglyceride was assayed by the method of Sardesai and Manning.[17] Bile acids were determined by the method of Eastwood *et al.*[15]

Results

Effect of Clofibrate on Serum Lipid Content in severely Hypercholesterolemic Rats

Table I shows the effect of clofibrate on the serum lipid content of the rats 4 d after administration of Triton WR-1339. Triglyceride and cholesterol levels of normal rats were 100.2±10.1 and 90.7±8.5 mg/dl, respectively. Therefore, Triton WR-1339 enhanced the triglyceride level 93-fold and the cholesterol level 27-fold, in accord with the results of Hayashi *et al.*[18] Even under such severe hyperlipidemia, clofibrate lowered the serum lipid levels. Cholesterol fell to 60% of the control after the treatment, and triglyceride, to 72%.

Effect on Biliary Bile Acids

Table II shows the effect of clofibrate on biliary bile acids in rats with severe hypercholesterolemia. The bile used in this experiment was pooled 24-h bile obtained after 4

TABLE I. Effect of Clofibrate on Blood Lipid in severely Hyperlipidemic Rats

	Triglyceride (mg/dl serum)	Cholesterol (mg/dl serum)
Control	9335±808	2428±247
Clofibrate	6743±799[a)]	1448± 73[a)]

Triton WR-1339 was injected into the control and clofibrate-treated rats. After 4 d, serum lipid levels were determined.
Values represent the means±S. D. of 8 animals.
a) Statistically significant decrease ($p<0.01$).

TABLE II. Effect of Clofibrate on Biliary Bile Acids in severely Hyperlipidemic Rats

	Bile acids (as cholate) (mg/ml bile)
Control	25.6±4.2
Clofibrate	35.6±5.6[a)]

Rats were given clofibrate for 2 weeks, and then were injected with Triton WR-1339 (100 mg/100 g b. w.). Four days after the administration, bile-duct cannulation was carried out, and bill was collected for 24 h. Values are the means±S. D. of 4 experiments.
a) Statistically significant increase ($p<0.01$).

d of Triton WR-1339 administration. Bile acid content in the bile was 1.39-fold higher in the clofibrate group than in the control. The results suggest that one of the mechanisms of the hypocholesterolemic effect of clofibrate may be stimulation of the degradation of cholesterol, that is, synthesis of bile acids.

The bile acids in bile listed in Table II were investigated (data not shown). Chenodeoxycholic acid predominated (about 60% of the total bile acids) over cholic acid (*ca.* 30%) in both control and clofibrate-treated rats.

Effect of Clofibrate on Changes in Excretion of Biliary Bile Acids in severely Hypercholesterolemic Rats

Since a slight effect of clofibrate on the degradation of cholesterol could be observed using the pooled 24 h bile (Table II), we sought to confirm by a more detailed experiment that clofibrate enhances the degradation of cholesterol. Figure 1 shows the time course of biliary bile acids 4 d after the administration of Triton WR-1339. The bile acid content was highest in the initial bile (up to 4 h) after the cannulation in both groups. The bile acids content was approximately 2.4 times higher in the initial bile (0 to 4 h) of the treated rats, while it was 1.8 times higher in the bile from 4 to 8 h after cannulation, as compared with the control. Though it is not shown in the figure, the biliary excretion rates remained almost constant during the experiment. This result also suggests that clofibrate acts on the degradation of cholesterol, besides inhibiting its synthesis.

Effect of Clofibrate on Biosynthesis of Bile Acids

No clear-cut results relating to the biosynthesis of bile acids after administration of clofibrate were obtained previously.[6,7] However, the results in Table II and Fig. 1 suggest that clofibrate does stimulate the biosynthesis of bile acids. In order to confirm this idea, a mixture of ^{14}C-labeled cholesterol and a large amount (100 mg) of non-labeled cholesterol in 1 ml of 2%. Tween suspension was intraperitoneally injected into control rats and rats that had received clofibrate for 14 d (treated rats). The biliary radioisotope excretions are shown in Fig. 2. High radioactivities were found in the bile immediately after the administration. The highest radioactivities were found in the initial bile of the control rats (up to 4 h), and in 4—7 h bile of the treated rats. Although the bile of control rats after 8 h contained little radioactivity, that of the treated rats slightly increased again after 24 h. This experiment was continued for 48 h, but the radioactivity recovered in the total bile accounted for no more than 7% of the injected amount even with the treated rats. The remaining radioactivity was probably excreted by some other route and/or remained in the bodies.

Distribution of the Radioactivity in the Early Bile

It can be seen from Fig. 2 that the early bile (up to 8 h after administration of the radioactive cholesterol) is most affected by clofibrate. Table III shows the distribution of the radioactivity in the early bile. The excretion rate of bile was almost unaffected by the

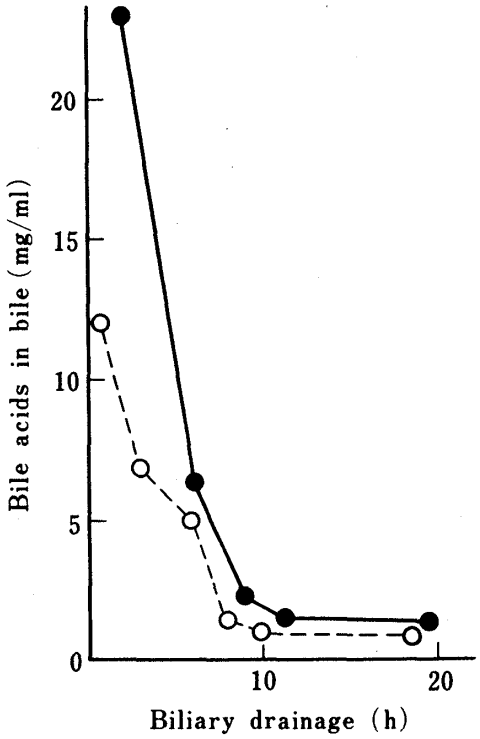

Fig. 1. Changes in Amount of Biliary le Acids in Rats treated with Clofibrate after Administration of Triton WR-1339

Triton WR-1339 was injected into the control and clofibrate-treated rats. After 4 d, bile-duct cannulation of rats was carried out for the collection of bile acids.
●—●, clofibrate-treated rats; O----O, control rats. Data are means of 3 animals.

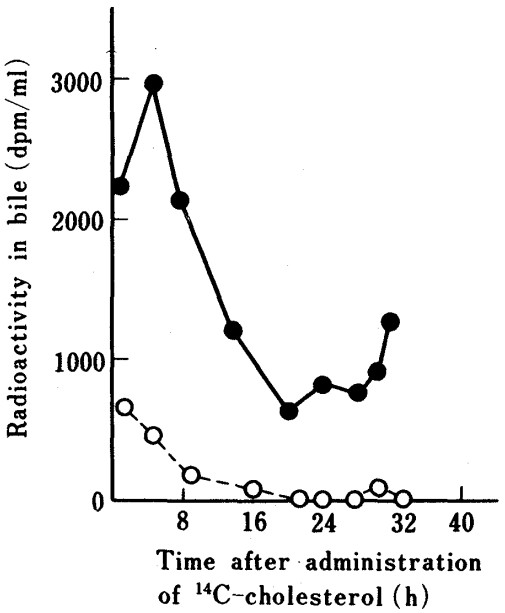

Fig. 2. Changes of Biliary Radioactivities in Clofibrate-treated Rats after Administration of ^{14}C-Cholesterol

[^{14}C]-Cholesterol was injected into the control and clofibrate-treated rats immediately after bile-duct cannulation.
●—●, clofibrate-treated rats; O----O, control rats. Data are means of 3 animals.

TABLE III. Comparison of Radioactivities in Early Bile of Control and Clofibrate-treated Rats after Administration of ^{14}C-Cholesterol

		Excretion rate			
	Collecting time (h)	Bile (ml/h)	Total (dpm/h)	Cholesterol (dpm/h)	Bile acids (dpm/h)
Control	8	0.75	243	0	339
Clofibrate	7	0.64	1358	25.7	1352

Two weeks after the administration of clofibrate, rats were subjected to bile-duct cannulation, and immediately administered 100 mg of cholesterol containing 0.5 μCi ^{14}C-labeled compound in 1 ml of Tween 80. The bile was collected and fractionated as described in "Experimental". See also the legend to Fig. 2.

administration of clofibrate. Total radioactivity in the bile was increased 5.6-fold by clofibrate and the bulk of the activity was found in the bile acids, with only a little in cholesterol. This result also shows that clofibrate stimulates the degradation of cholesterol, *i.e.*, the biosynthesis of bile acids.

Effect of Clofibrate on the Excretion Rate of Bile Acids after Administration of Triton WR-1339

It is known that after the administration of Triton WR-1339 very-low-density lipoprotein (VLDL) appears in the serum,[18,19] and that VLDL is altered to low-density lipoprotein (LDL).[19-21] Figure 3 shows the excretion rate of bile acids in initial bile (up to 4 h). The excretion rate of bile acids of clofibrate-treated rats before the administration of Triton WR-

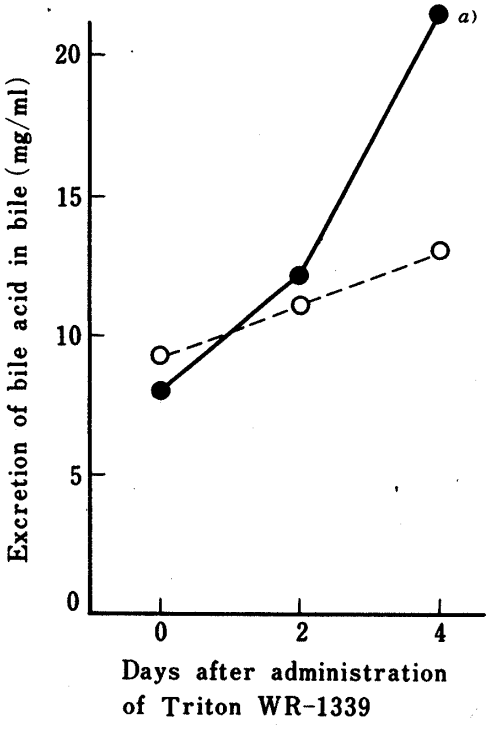

Fig. 3. Changes in Bile Acid Excretion in Early Bile after Triton WR-1339 Administration

Triton WR-1339 was injected into the control and clofibrate-treated rats. Bile-duct cannulation was carried out on uninjected rats (day 0) and on Triton-injected rats at days 2 and 4 after the injection for the collection of initial bile (0—4 h after cannulation).
●—●, clofibrate-treated rats; O----O, control rats. Each circle is the mean of 3 animals.
a) Statistically significant difference ($p<0.01$).

1339 was slightly lower than that of control rats, but at 2 d after the administration, the excretion rate of clofibrate-treated rats became higher than that of the control rats, and further increased sharply at 4 d. However, the results at day 0 and day 2 for control and treated rats were not significantly different statistically. These results indicate that cholesterol in LDL is more affected by clofibrate, and is more likely to be taken up into the liver for the biosynthesis of bile acids than that in VLDL.

Discussion

Since Thorp and Waring[22] reported in 1962 that clofibrate decreases serum cholesterol in experimental animals, many investigators have tried to elucidate the mechanism of the hypocholesterolemic effect. Though clofibrate was reported to inhibit the biosynthesis of cholesterol, the apparent sites of action were different in different experiments.[4,5] Therefore, even the inhibition of cholesterol biosynthesis, which is a generally accepted action of clofibrate, is not yet established in a satisfactory manner.

In the present experiments, it has become apparent that clofibrate stimulates the degradation of cholesterol as well as inhibiting its biosynthesis. The degradation was mainly dependent on the biosynthesis of bile acids. The reason why we were able to obtain such clear-cut data is considered to be our use of severely hypercholesterolemic rats and further, the administration of a large amount of cholesterol in this study as compared with previous experiments.[6,7] Even if clofibrate had enhanced the capacity for degradation in the previous experiments, the extent of the hypercholesterolemia might have been too low for the stimulation of bile acid biosynthesis to be observable because the amount of the substrate was insufficient.

It was reported that the bile of normal rats contains more cholic acid than chenodeoxycholic acid,[23] while that of rats administered Triton WR-1339 contains more of the latter than the former acid, suggesting that Triton may inhibit cholesterol 12α-hydroxylase activity, and consequently depress the formation of cholic acid.

When the ease of conversion from cholesterol in lipoprotein to bile acids was compared for VLDL and LDL, cholesterol in LDL was more readily used as a substrate for bile acid biosynthesis. The main product of degradation of cholesterol is bile acids, and the pathway of bile acids biosynthesis involves fatty acid β-oxidation, which is markedly enhanced by administration of clofibrate. The β-oxidation system is present in both peroxisomes and mitochondria, but the properties of the system in the two particles are different, and it is not known yet where the β-oxidation for bile acid synthesis occurs. This point will be investigated in a future study.

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