Comparison of the Effects of Gemfibrozil and Clofibric Acid on Peroxisomal Enzymes and Cholesterol Synthesis of Rat Hepatocytes

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We studied whether the peroxisomal proliferation, induction of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) and activation of cholesterol synthesis by gemfibrozil shown in whole body (Hashimoto F., Ishikawa T., Hamada S. and Hayashi H., *Biochemical. Pharm.*, 49, 1213—1221 (1995)) is also detected at a culture cell level, and we made a comparative analysis of the effects of clofibric acid. Gemfibrozil at 0.25 mM increased the activity of some peroxisomal enzymes (catalase and the cyanide-insensitive fatty acyl-CoA oxidizing system) after incubation for 72 h. However, contrary to whole body experiments, gemfibrozil decreased the activity of HMG-CoA reductase and cholesterol synthesis from [^{14}C]acetate. At 1 mM, gemfibrozil decreased not only the activity of HMG-CoA reductase and cholesterol synthesis, but also the protein content of the cells and peroxisomal enzyme activity, indicating nonspecific inhibition at this concentration. Clofibric acid (0.25 and 1 mM) increased the activity of peroxisomal enzymes, but decreased the activity of HMG-CoA reductase and cholesterol synthesis. With respect to the direct effect on HMG-CoA reductase in the cell homogenate, gemfibrozil at 0.25 mM did not affect the activity, but it clearly inhibited the activity at 2 mM and above. Clofibric acid at 2 mM hardly affected the activity, but it clearly decreased the activity at 5 mM and over. That is, gemfibrozil directly inhibited the activity more strongly than clofibric acid. The direct inhibition of the enzyme itself required higher concentrations of both agents than did inhibition at the culture cell level.

These results suggest that the cytotoxicity of gemfibrozil is greater than that of clofibric acid, and that gemfibrozil, as well as clofibric acid, can induce peroxisomal enzymes in the culture cell level. In contrast to whole body results, gemfibrozil may suppress cholesterol synthesis from [^{14}C]acetate through the inhibition of HMG-CoA reductase at the culture cell level. The decreases in the reductase activity caused by gemfibrozil and clofibric acid at the culture cell level may not be caused by the direct inhibition of the enzyme.

Key words gemfibrozil; clofibric acid; peroxisome; HMG-CoA reductase; cholesterol; hepatocyte

Clofibrate, a fibric acid derivative, can cause the proliferation of peroxisomes both in whole body and at the culture cell level, and is used as a standard peroxisomal proliferator. Gemfibrozil is also a fibric acid derivative. We demonstrated that after the oral administration of gemfibrozil to rats, the activity of some peroxisomal enzymes (catalase and the cyanide-insensitive fatty acyl-CoA oxidizing system) was increased.[1] However, Kocarek and Feller reported that at the culture cell level, unlike clofibrate, gemfibrozil did not induce the peroxisomal enzyme fatty acyl-CoA oxidase.[2]

Fibric acid derivatives are not only peroxisomal proliferators, but also hypolipidemic agents. The mechanism by which clofibrate and gemfibrozil act as hypolipidemic agents has not been thoroughly investigated. Clofibrate is considered, at least, to suppress cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), the rate-limiting enzyme of cholesterol synthesis in whole body.[3—9] We reported that the oral administration of gemfibrozil to rats increases the syntheses of cholesterol and bile acid from acetyl-CoA derived from peroxisomes, and that it remarkably increases the HMG-CoA reductases of microsomes and peroxisomes.[1] We demonstrated that this increase in the reductase activity is not caused by the direct activation of the enzyme itself by gemfibrozil in such intracellular particles. Gemfibrozil rather showed direct inhibition of the activity of the particles at a high concentration.[10]

In this experiment, we studied whether the peroxisomal proliferation, induction of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) and activation of cholesterol synthesis by gemfibrozil shown in whole body[1] is also detected at a culture cell level. Both gemfibrozil and clofibrate are derivatives of fibric acid, but they showed different effects as stated above, and so we also made a comparative analysis of the effects of clofibric acid at the culture cell level. We found that gemfibrozil, as well as clofibric acid, can induce peroxisomal enzymes at the culture cell level, but in contrast to whole body results, gemfibrozil may suppress the cholesterol synthesis from [^{14}C]acetate through the inhibition of HMG-CoA reductase.

MATERIALS AND METHODS

Materials Gemfibrozil was a kind gift from Warner-Lambert (U.S.A.). [3-^{14}C]HMG-CoA (2.1 GBq/mmol, 57.6 mCi/mmol), [5-^{3}H]mevalonolactone (1221 GBq/mmol, 33.0 Ci/mmol), [1-^{14}C]acetate (2.035 GBq/mmol, 55.0 mCi/mmol) and Aquazol 2 were purchased from New England Nuclear (U.S.A). Clofibrate, cholesterol, HMG-CoA, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, mevalonolactone, palmitoyl-CoA, NAD and 25-hydroxycholesterol were obtained from Sigma (U.S.A.). All other reagents were of analytical grade from Wako Pure Chemicals (Japan).

Animals Male Wistar rats weighing about 200—250 g, obtained from Saitama Laboratory Animals (Japan), were used. They had free access to standard laboratory chow, CE-2 (Nihon Clea Inc., Japan) and water.

Preparation of Primary Cultured Hepatocytes and Drug Treatment Hepatocytes were isolated by the collagenase perfusion method. Isolated rat hepatocytes showing more than 85% cell viability in terms of the trypan blue exclusion test were used for the experiments. The cells in L-15 medium (2 ml) containing 28 mM Hepes, 1 μM dexametha-

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zone, 1 μM insulin, 100 U/ml penicillin G-K, 75 U/ml streptomycin sulfate, 100 μg/ml kanamycin sulfate and 5% fetal bovine serum (FBS) were plated in collagen-coated plastic plates (35 mm diameter) at 10^6 cells/plate and then cultured at 37 °C. Unless otherwise indicated, normal FBS was used for the culture. When low-density lipoprotein (LDL)-deficient FBS was used, it was prepared according to the method of Goldstein *et al.*[12] The medium was changed 4 h after plating. Drug treatment was initiated 24 h after plating by changing the medium to a drug-containing medium, and thereafter the medium was changed every 24 h. Gemfibrozil or clofibric acid solubilized in ethanol was added to the above medium. The final concentration of ethanol was 0.2% (v/v). Medium was removed by aspiration at the times indicated in the Figures. The attached cells were washed with phosphate-buffered saline and suspended in 0.25 M sucrose containing 1 mM EDTA and 10 mM Tris–HCl (pH 7.4), then the cell homogenates were prepared by sonication. These homogenates were used for assaying the enzyme activities and protein content.

Assay Methods Catalase activity was determined according to the method of Leighton *et al.*[13] with slight modification.[14] The activity of the peroxisomal fatty acyl-CoA oxidizing system was determined by the method of Lazarow and de Duve[15] with slight modification.[16] One unit of activity was defined as the amount of enzyme that reduced 1 nmol NAD per min.

The activity of HMG-CoA reductase was determined by the method of Keller *et al.* using [3-^{14}C] HMG-CoA and [5-^{3}H]mevalonolactone as the substrate and internal standard, respectively.[17] The enzyme sample was diluted in 50 mM potassium phosphate buffer, pH 7.4, containing 30 mM EDTA, 200 mM NaCl and 10 mM dithiothreitol. Approximately 50 μg of protein was used.

Protein was determined by the Lowry method using bovine serum albumin as a standard.[18]

Metabolic Labelling of Cholesterol Cells (2×10^6) were preincubated in 4 ml of L-15 medium containing hormones, antibiotics and FBS as stated above with or without gemfibrozil or clofibric acid in collagen-coated plastic plates (60 mm diameter) for 2, 50 or 74 h at 37 °C. In the case of the preincubation for 50 or 74 h, the medium was changed every 24 h. After the preincubation, 2 μCi of [1-^{14}C]acetate was added, followed by incubation in the same medium for 4 h.

Isolation of Cholesterol Following the incubation, the medium was removed. The attached cells were washed with phosphate-buffered saline and extracted twice with 1 ml of methanol. To the samples, 4 ml of $CHCl_3$ was added before final vortexing. The samples were sonicated for 1 min. In each case 20 μg of unlabelled cholesterol was added as a carrier. The $CHCl_3$ extracts were reduced to dryness under N_2. Mild alkaline hydrolysis was achieved by refluxing the mixture for 1 h at 80 °C. The non-saponifiable lipids were isolated from the saponification mixture by extraction with petroleum ether. The non-saponifiable lipids were purified on a C_{18} reverse phase Sep-Pak mini-column (Waters, U.S.A.) previously equilibrated with methanol. Cholesterol was eluted with 10 ml of ethanol. The solvent was removed from the ethanol eluate under a vacuum. The residual material was redissolved in a known volume of methanol before HPLC analysis.

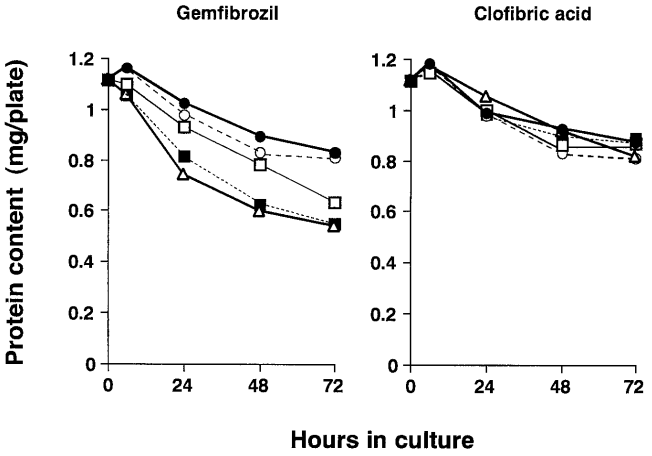

Fig. 1. Time Course of Protein Content of Rat Hepatocytes Treated with Gemfibrozil or Clofibric Acid

Drug treatment was initiated 24 h after the plating (10^6 cells/plate) by changing the culture medium to the medium containing gemfibrozil or clofibric acid (○, 0; ●, 0.25; □, 0.5; ■, 0.75; △, 1 mM), and the medium was then changed every 24 h. The cells were harvested at the indicated times, and cell homogenates were prepared by sonication and used for assay of protein content. Data are mean values of 5 experiments.

HPLC Analysis of cholesterol was achieved by separate analysis of the methanol Sep-Pak eluate on straight phase HPLC. A Shimadzu Series LA-10 HPLC apparatus (Shimadzu, Japan) was used with a 5 μm LiChrospher 100 RP-18 column (4×250 mm) (Merck, Germany). A mobile phase of methanol was pumped at a constant rate of 1 ml/min at 40 °C. The ultraviolet absorbance of the column eluate was monitored with an SPD-10A Shimadzu UV spectrophotometric detector at 210 nm. Radioactivity was monitored by a radio HPLC/LS analyzer (Ramona/LS System (Easy), Germany).

RESULTS

Effect of Gemfibrozil and Clofibric Acid on Protein Content of Rat Hepatocytes In order to study the cytotoxicity of gemfibrozil and clofibric acid, primary rat hepatocytes were cultured with or without these agents. The cells were harvested at the time indicated in Fig. 1, and the protein content was then determined. Figure 1 shows the time courses of protein content at the various concentrations of the agents. Figure 2 illustrates the effect of the concentrations of the agents on relative protein content 72 h after incubation. As shown in Fig. 1, the protein content of all cells gradually decreased in a time- dependent manner. As both Figs. 1 and 2 indicate, gemfibrozil decreased the protein content and clearly decreased the relative content at a concentration of not less than 0.5 mM after incubation for 72 h. The content after treatment with 1 mM gemfibrozil was 66.2% of the control. On the other hand, clofibric acid at all concentrations tested hardly affected the content.

Cells treated with 1 mM gemfibrozil for 72 h were more strongly stained with trypan blue than those treated with 1 mM clofibric acid (data not shown). This indicates that the cytotoxicity of gemfibrozil is greater than that of clofibric acid.

Influence of Gemfibrozil and Clofibric Acid on Activity of Peroxisomal Marker Enzymes Whether gemfibrozil induces peroxisomal proliferation at the cellular level was un-

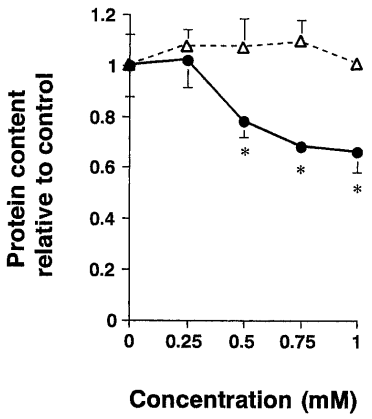

Fig. 2. Changes in Protein Content of Hepatocytes Relative to the Control after 72 h of Incubation

After incubation with various concentrations of gemfibrozil (●) or clofibric acid (△) for 72 h, the cells were harvested. Cell homogenates were prepared by sonication, and used for the assay of protein. The protein content (mg/plate) was expressed as the value relative to the control (0 mM). Data are mean values±S.D. of 5 experiments. * indicate significant differences (*, $p<0.01$).

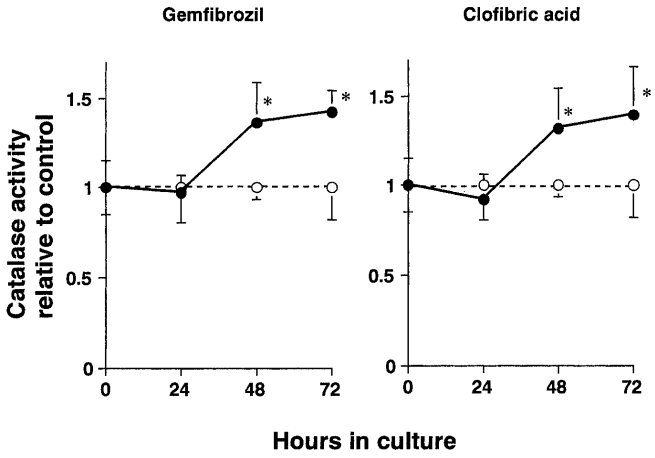

Fig. 3. Increase in Catalase Activity of Hepatocytes Treated with Gemfibrozil or Clofibric Acid

The cells were incubated with gemfibrozil or clofibric acid (○, 0; ●, 0.25 mM). At the indicated times the cells were harvested, and homogenates were prepared by sonication and used for the assay of catalase activity. The catalase activities (U/mg protein) were expressed as values relative to the control at each time. Data are mean values±S.D. of 5 experiments. * indicate significant differences (*, $p<0.05$). Catalase activities of control cells at 0, 24, 48 and 72 h were 101±15, 88.5±16.9, 76.0±5.0 and 74.0±13.0 U/mg protein, respectively.

known, so we studied activity of catalase and the cyanide-insensitive fatty acyl-CoA oxidizing system after incubation with or without the agent. Figure 3 shows the time course of catalase activity. The activity was enhanced after 48 h of incubation with 0.25 mM gemfibrozil or clofibric acid, and both activities were approximately 1.4 times the control at 72 h. The results with clofibric acid are in agreement with the findings of Ozasa *et al.*[19]

As shown in Fig. 4, the activity of the cyanide-insensitive fatty acyl-CoA oxidizing system was hardly changed after 24 h incubation with 0.25 mM gemfibrozil and clofibric acid, then gradually increased to reach 3.1 and 2.9 times the control, respectively, after 72 h. The β-oxidation system activity increased to 3.5 times the control after incubation with 1 mM clofibric acid for 72 h (Fig.4), but no activity was detected after incubation with 1 mM gemfibrozil as expected from the data of Figs. 1 and 2 (data not shown). Gemfibrozil, as well

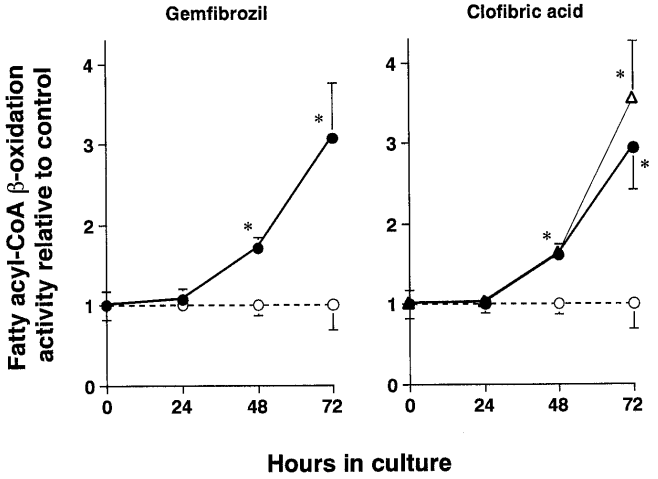

Fig. 4. Changes in Activity of Peroxisomal Fatty Acyl-CoA β-Oxidation System

Cells were incubated with gemfibrozil or clofibric acid (○, 0; ●, 0.25; △, 1 mM). At the indicated times the cells were harvested, then the homogenates were prepared by sonication and used for the assay of β-oxidation activity. The β-oxidation activities (U/mg protein) were expressed as values relative to the control at each time. Data are mean values±S.D. of 5 experiments. * indicate significant differences (*, $p<0.005$). The β-oxidation activities of control cells at 0, 24, 48 and 72 h were 1.96±0.33, 1.69±0.10, 1.29±0.15 and 0.821±0.263 U/mg protein, respectively.

as clofibric acid, seems to be able to induce peroxisomal enzymes. The increase in the β-oxidation caused by clofibric acid at the culture cell level is in agreement with the findings of Watanabe *et al.*[20] The induction of catalase and the β-oxidation system by gemfibrozil at the culture cell level supports our previous results in whole body.[1,10]

Changes in HMG-CoA Reductase Activity Caused by Gemfibrozil and Clofibric Acid In previous reports, we demonstrated a remarkable increase in HMG-CoA reductase activity by whole body treatment with gemfibrozil.[1,10] Therefore, we tested the effect of gemfibrozil and clofibric acid on the enzyme activity at the culture cell level. Figure 5 shows the time course of the HMG-CoA reductase activity of rat hepatocytes. Neither gemfibrozil nor clofibric acid at 0.25 mM affected the activity after 6 h of incubation. However, they decreased the activity after 24 h, with the gemfibrozil and clofibric acid causing levels of 59.5 and 66.5% of the control, respectively, after incubation for 72 h. Gemfibrozil at 1 mM drastically inhibited the reductase activity as expected from the results of Figs. 1 and 2. Clofibric acid inhibited the activity at 1 mM more strongly than at 0.25 mM, but the differences between 1 mM and 0.25 mM were not significant.

Figure 6 illustrates the dependency of HMG-CoA reductase activity on concentrations of gemfibrozil and clofibric acid after incubation for 72 h. Clofibric acid at 0.1 mM reduced the activity to approximately 75.8% of the control, and the reductase activity was gradually decreased by clofibric acid in a dose-dependent manner. On the other hand, gemfibrozil at 0.1 mM reduced the activity to 79.9% of the control, and the activity was sharply decreased in a dose-dependent manner. At concentrations of 0.5 mM and above, the effect expected from the data of Figs.1 and 2 was detected.

The activity of HMG-CoA reductase was barely detectable after 72 h of incubation with 1 mM gemfibrozil (Figs. 5 and 6). Mevalonate metabolites (non-sterol products) are reported to regulate HMG-CoA reductase and to be necessary for liv-

ing cells.[21] Therefore, we speculated that 1 mM gemfibrozil primarily inhibited HMG-CoA reductase, resulting in the decrease in such metabolites. This decrease might in turn cause nonspecific damage to hepatocytes, resulting in decreases in protein content and β-oxidation activity. Hence, we added mevalonate (0.2, 0.4, 0.6 or 0.8 mM) to the culture medium together with 1 mM gemfibrozil, then incubated the mixture for 72 h. However, the reductase activity was not restored (data not shown). Consequently, the inhibition of HMG-CoA reductase by 1 mM gemfibrozil seems to be not primary, but nonspecific.

We reported that when gemfibrozil was administered to rats in the whole body, HMG-CoA reductase was markedly increased.[1,10] Therefore, a stimulating effect of gemfibrozil on HMG-CoA reductase was also expected at the culture cell level. However, an increase in reductase activity was not detected; the activity was, instead, suppressed (Figs. 5 and 6). Since LDL-cholesterol, contained in the FBS added to the culture medium, has been reported to suppress HMG-CoA reductase activity,[22] we considered it possible that the overall activity of the enzyme was apparently decreased through feedback regulation by cholesterol even though the activity should have been induced by gemfibrozil. Therefore, we tried to use LDL-deficient FBS instead of normal FBS in the experiment. However, we could not detect any increase in the HMG-CoA reductase activity by gemfibrozil (data not shown).

We added hormones to the culture medium to stimulate cell attachment to the dishes. To our knowledge, the effect of dexamethazone on HMG-CoA reductase is unknown. Insulin is reported to stimulate HMG-CoA reductase activity,[23] hence the reductase activity in the control cells has probably already been sufficiently stimulated by insulin contained in the culture medium. Thus, we speculated that the reductase activity sufficient for the cells prevented any further increase in the reductase by gemfibrozil. Therefore, we tried to use a hormone-free medium for culture, resulting in the low activity of HMG-CoA reductase in control cells after incubation for 72 h. However, the reductase activity was not induced by gemfibrozil (data not shown).

Influence of Gemfibrozil and Clofibric Acid on Cholesterol Synthesis Gemfibrozil and clofibric acid decreased the activity of the rate-limiting enzyme of cholesterol synthesis (Figs. 5 and 6). In order to confirm this, we examined the effects of these agents on cholesterol synthesis from [^{14}C]acetate (Table 1). A typical inhibitor of cholesterol synthesis,

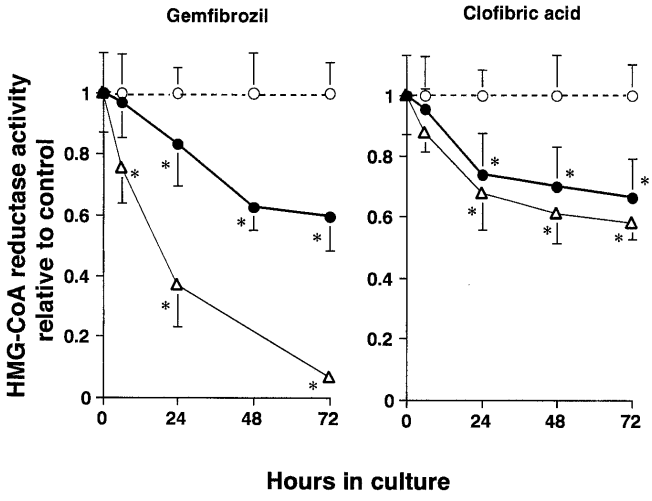

Fig. 5. Time Course of HMG-CoA Reductase Activity of Hepatocytes Treated with Gemfibrozil or Clofibric Acid

The cells were incubated with gemfibrozil or clofibric acid (○, 0; ●, 0.25; △, 1 mM). At the indicated times the cells were harvested, then the homogenates were prepared by sonication and used for the assay of HMG-CoA reductase activity. The activities (pmol/min/mg protein) were expressed as values relative to the control at each time. Data are mean values±S.D. of 5 experiments. * indicate significant differences (*, $p<0.05$). The HMG-CoA reductase activities of control cells at 0, 24, 48 and 72 h were 50.3±6.5, 37.8±3.2, 28.8±3.7 and 15.0±1.5 pmol/min/mg protein, respectively.

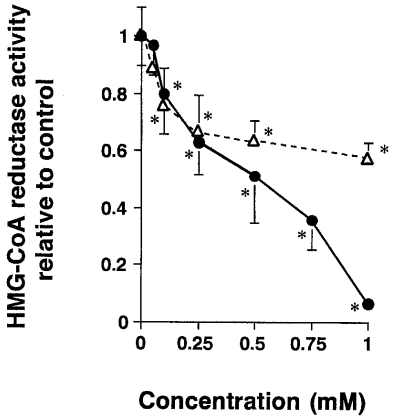

Fig. 6. Influence of Gemfibrozil and Clofibric Acid on HMG-CoA Reductase of Hepatocytes after 72 h of Incubation

After incubation with various concentrations of gemfibrozil (●) or clofibric acid (△) for 72 h, the cells were harvested, and homogenates of the cells were prepared by sonication and used for the assay of HMG-CoA reductase. The activities (pmol/min/mg protein) were expressed as values relative to the control (0 mM). Data are mean values±S.D. of 5 experiments. * indicate significant differences (*, $p<0.05$).

Table 1. Effect of Gemfibrozil and Clofibric Acid on Cholesterol Synthesis from [^{14}C]Acetate

Agent	Incorporation of [^{14}C]acetate into cholesterol (dpm×10^{-3}/plate)		
	Preincubation time 2 h	50 h	74 h
Control	7.39±1.07 (1.0)	4.34±0.70 (1.0)	3.11±0.57 (1.0)
2 μg/ml 25-hydroxycholesterol	5.03±0.84 (0.68)**	—	—
0.25 mM gemfibrozil	7.90±1.40 (1.07)	3.16±0.76 (0.73)*	2.00±0.35 (0.64)**
0.25 mM clofibric acid	7.90±1.41 (1.07)	3.23±0.56 (0.74)*	1.96±0.39 (0.63)**
1 mM clofibric acid	7.81±0.71 (1.06)	2.77±0.37 (0.64)**	1.36±0.41 (0.44)**

Cells (2×10^6) were preincubated in 4 ml of the culture medium with or without agent for 2, 50 or 74 h at 37 °C. The medium was changed every 24 h. After preincubation, 2 μCi [1-^{14}C]acetate was added, and cells were incubated for 4 h. Cholesterol was extracted from the cells as described in the text. Radioactivity in cholesterol was determined using radio-HPLC. Data are mean values±S.D. of five experiments. ** and * represent significant differences (**, $p<0.01$; *, $p<0.05$).

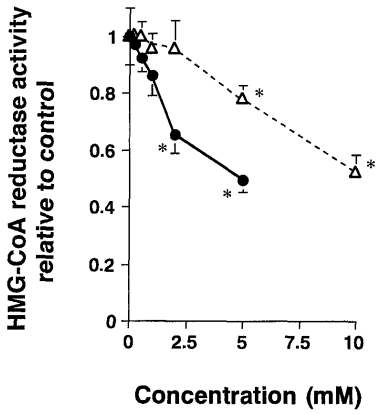

Fig. 7. Direct Effect of Gemfibrozil and Clofibric Acid on the Activity of HMG-CoA Reductase of Rat Hepatocyte Homogenate

Cells were harvested 24 h after the plating. The cell homogenate was prepared by sonication, and HMG-CoA reductase activity was assayed under various concentrations of gemfibrozil (●) or clofibric acid (△). Gemfibrozil or clofibric acid solubilized in ethanol was used. The final concentration of ethanol was 1.3% (v/v). The activities (pmol/min/mg protein) were expressed as values relative to the control (0 mM). Data are mean values±S.D. of the 3 experiments. * indicate significant differences (*, $p<0.05$). Activity of HMG-CoA reductase in the control cell was 41.7±4.1 pmol/min/mg protein.

25-hydroxycholesterol, inhibited the synthesis after a short preincubation (2 h) and after further incubation (4 h) with [^{14}C]acetate. Cholesterol synthesis was nearly unchanged after the short preincubation with 0.25 mM gemfibrozil, but decreased after a long preincubation (50 or 74 h), resulting in 64% of the control value after 74 h. The induction of activity by gemfibrozil after long preincubation, expected on the basis of the whole body results,[1,7] was not detected. Clofibric acid (0.25 mM) barely changed the activity after short preincubation, but decreased it after long preincubation (50 and 74 h). The activity was 63% of the control level after 74 h. Clofibric acid inhibited the activity more strongly at 1 mM than at 0.25 mM.

Direct Effects of Gemfibrozil and Clofibric Acid on HMG-CoA Reductase of Rat Hepatocyte Homogenates Figure 7 shows the direct effects of gemfibrozil and clofibric acid on HMG-CoA reductase of rat hepatocyte homogenates. Both gemfibrozil and clofibric acid inhibited the reductase activity. Gemfibrozil inhibited it more strongly than clofibric acid, and the activity was decreased to half the control level at 5 mM gemfibrozil. Clofibric acid decreased the activity to half the control level at 10 mM. From these results, gemfibrozil and clofibric acid seem to directly inhibit the enzyme activity of cell homogenates, but higher concentrations of both agents are needed for direct inhibition compared to that needed at the cellular level.

DISCUSSION

We studied whether the peroxisomal proliferation, the induction of HMG-CoA reductase and the activation of cholesterol synthesis by gemfibrozil shown in whole body[1] are also detected at the culture cell level, and compared the effects of gemfibrozil with those of clofibric acid.

The induction of fatty acid oxidase by clofibrate at the culture cell level has been reported by many laboratories.[2,19,24,25] Kocarek and Feller reported that gemfibrozil, unlike clofibrate, did not induce peroxisomes based on the study of fatty acyl-CoA oxidase at the culture cell level.[2] However, the present study indicates that gemfibrozil, as well as clofibric acid, can induce peroxisomes at the culture cell level. Since we reported that similarly to clofibric acid, gemfibrozil induces peroxisomes in whole body,[1] the induction by gemfibrozil in whole body may also apply at the culture cell level. Fibrates are known to activate peroxisome proliferator-activated receptor alpha (PPARα).[26—29] Gemfibrozil and clofibric acid may induce peroxisomes at the cellular level by the activation of PPARα, but we have not confirmed this yet.

We reported that the oral administration of gemfibrozil to rats increases the synthesis of cholesterol from acetyl-CoA derived from peroxisomes by using [1-^{14}C]lignocerate, and that it increases HMG-CoA reductases in microsomes more strongly than that in peroxisomes.[1] These results suggest that total cholesterol synthesis from the acetyl-CoA pool in whole cells is also increased by elevated microsomal reductase. Therefore, we used [^{14}C]acetate as the precursor for cholesterol synthesis in these experiments.

After a short incubation of the cells, gemfibrozil (0.25 mM) and clofibric acid (0.25 and 1 mM) hardly affected the activity of HMG-CoA reductase and cholesterol synthesis from [^{14}C]acetate, but the activities were suppressed after long incubation (Fig. 5 and Table 1). Concerning the metabolism of gemfibrozil, there have been reports that 45% of incorporated gemfibrozil is excreted unchanged in urine, and the rest is composed of four metabolites.[30,31] Clofibrate is known to be excreted in the urine mainly as the glucuronide of clofibric acid.[11] It is unclear whether such metabolite(s) of the agent and/or the agent itself exhibit(s) the inhibitory effect on the reductase.

With respect to cholesterol synthesis, Haughom and Spydevold reported that when rat hepatocytes were incubated with 1 mM clofibrate for 90 min, cholesterol synthesis from [^{14}C]acetate was inhibited.[6] Henry *et al.* reported that after the preincubation (4 h) of human leukemic T-lymphocyte cells (Molt-4) with 0.25 mM gemfibrozil or clofibrate, and further incubation (6 h) with [^{14}C]acetate or [^{3}H]mevalonate, the cholesterol synthesis from [^{14}C]acetate or [^{3}H]mevalonate was unchanged.[32] Our results with short time experiments are similar to the findings of Henry *et al.* If they performed long incubation, the cholesterol synthesis activity could be suppressed by fibrates. Hemingway *et al.* reported that after the incubation of rat hepatocytes with gemfibrozil for 1 h, cholesterol synthesis from $^{3}H_2O$ was decreased.[33] However, they did not take into consideration cytotoxicity by gemfibrozil (1 mM) and did not examine HMG-CoA reductase activity.

This is the first report which demonstrates the inhibition of HMG-CoA reductase by gemfibrozil at the culture cell level. Stange *et al.* reported the effects of gemfibrozil on the reductase of human mononuclear cells.[34] They found that gemfibrozil at 30 μg/ml (0.12 mM), which is considered to be equivalent to peak plasma levels during treatment with the standard dose, did not inhibit the enzyme after incubation for 24 h. As shown in Fig. 6, the reductase activity was reduced even by 0.1 mM gemfibrozil after incubation for 72 h. If the incubation had been carried out for a longer time, they might have found inhibition. From the results of Figs. 5—7, the decrease in HMG-CoA reductase activity by gemfibrozil shown at the culture cell level may not be caused by direct inhibition

of the reductase itself.

Compactin, a competitive inhibitor of HMG-CoA reductase, is reported to induce the reductase not only in whole body, but also at the culture cell level.[35, 36] The mechanism for this is considered to be as follows. Hepatic levels of cholesterol should first be reduced because of the inhibition of HMG-CoA reductase following the administration of compactin. Hence, in order to compensate for the reduced levels of hepatic cholesterol, the rate of hepatic sterol synthesis, *i.e.* HMG-CoA reductase activity, might be elevated. Gemfibrozil increased the reductase by whole body treatment,[1,10] similarly to compactin. However, contrary to the whole body results, gemfibrozil suppressed the enzyme activity at the culture cell level (Figs. 5 and 6). That is, the mechanisms of action of gemfibrozil on HMG-CoA reductase at the culture cell level seem to be different from those of compactin.

Compactin directly inhibits HMG-CoA reductase itself, so the enzyme activity in the cells was almost completely reduced, resulting in the drastic decrease in the cellular cholesterol level.[36] However, gemfibrozil at 0.25 mM did not directly affect the HMG-CoA reductase of the homogenate (Fig. 7). At the culture cell level, gemfibrozil at 0.25 mM decreased the enzyme activity, but about 60% of the activity still remained (Figs. 5 and 6). That is, the remaining HMG-CoA reductase can synthesize cellular cholesterol (Table 1), so the induction of HMG-CoA reductase may not be needed. This may be the reason for the different effects on enzyme induction at the culture cell level between compactin and gemfibrozil.

In any case, even though the experimental conditions were varied at the culture cell level, the induction of HMG-CoA reductase by gemfibrozil was not detected. Thus, the activation effect of HMG-CoA reductase in whole body may be caused by a complicated mechanism which cannot be explained only by the direct effects of the agent on the cells. In whole body, some mechanism by which gemfibrozil reduces the levels of hepatic cholesterol might be present, and consequently HMG-CoA reductase may be induced. This mechanism will be the subject of further study.

From these results, it is suggested that the cytotoxicity of gemfibrozil is greater than that of clofibric acid, but gemfibroizil, as well as clofibric acid, can induce peroxisomal enzymes at the culture cell level. In contrast to the whole body experiments, gemfibrozil may suppress cholesterol synthesis from [^{14}C]acetate through the inhibition of HMG-CoA reductase at the culture cell level. The decrease in the reductase activity caused by gemfibrozil and clofibric acid may not be caused by the direct inhibition of the reductase.

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