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Changes in Isoprenoid Lipid Synthesis by Gemfibrozil and Clofibric Acid in Rat Hepatocytes

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ABSTRACT. We studied whether gemfibrozil and clofibric acid alter isoprenoid lipid synthesis in rat hepatocytes. After incubation of the cells with the agent for 74 hr, [^{14}C]acetate or [^{3}H]mevalonate was added, and the cells were further incubated for 4 hr. Gemfibrozil and clofibric acid increased ubiquinone synthesis from [^{14}C]acetate and [^{3}H]mevalonate. The effect of gemfibrozil was greater than that of clofibric acid. Also, gemfibrozil decreased dolichol synthesis from [^{14}C]acetate and [^{3}H]mevalonate. However, clofibric acid increased dolichol synthesis from [^{3}H]mevalonate. Gemfibrozil decreased cholesterol synthesis from [^{14}C]acetate and [^{3}H]mevalonate. Clofibric acid decreased cholesterol synthesis from [^{14}C]acetate, but did not affect synthesis from [^{3}H]mevalonate. These results suggest that both agents, at different rates, activate the synthetic pathway of ubiquinone, at least from mevalonate. Gemfibrozil may inhibit the synthetic pathway of dolichol, at least from mevalonate. Contrary to gemfibrozil, clofibric acid may activate the synthetic pathway of dolichol from mevalonate. Gemfibrozil may inhibit the synthetic pathway of cholesterol from mevalonate in addition to the pathway from acetate to mevalonate inhibited by both agents. BIOCHEM PHARMACOL **59**;10:1203–1210, 2000.

KEY WORDS. gemfibrozil; clofibric acid; peroxisome; ubiquinone; dolichol; cholesterol

Clofibrate, a fibric acid derivative, can cause the proliferation of peroxisomes upon activation of the peroxisome proliferator-activated receptor α both in the whole body and at the cell culture level, and is used as a standard peroxisomal proliferator. Gemfibrozil is also a fibric acid derivative. We demonstrated that after oral administration of gemfibrozil to rats, activity of some peroxisomal enzymes (catalase and the cyanide-insensitive fatty acyl-CoA oxidizing system) was increased [1]. We reported that at the cell culture level, gemfibrozil, like clofibrate, induces catalase and the peroxisomal fatty acyl-CoA oxidizing system [2]. In other words, gemfibrozil proliferates peroxisomes both in the whole body and at the cell culture level.

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 [3–9]. Clofibrate, at least, is thought to suppress cholesterol synthesis by inhibiting HMG-CoA reductase†, the rate-limiting enzyme of cholesterol synthesis in the whole body [10–13]. We reported that oral administration of gemfibrozil to rats unexpectedly increases syntheses of cholesterol and bile acid [1], and that it remarkably increases HMG-CoA reductases of peroxisomes and microsomes [1, 14]. We demonstrated that this increase in reductase activity is not caused by direct activation of the enzyme itself by gemfibrozil *in vitro*. Instead, gemfibrozil showed direct inhibition of the enzyme activity at high concentrations [14]. Therefore, we studied in a previous work, the effect of gemfibrozil on HMG-CoA reductase at the cell culture level. We found that, in contrast to whole body results, gemfibrozil suppresses cholesterol synthesis from [^{14}C]acetate through the inhibition of HMG-CoA reductase in a similar pattern to clofibric acid [2].

Cholesterol is synthesized in the mevalonate pathway, and ubiquinone and dolichol are formed in the same route up to farnesyl pyrophosphate in the mevalonate pathway. Recently, there have been many reports suggesting a relationship between peroxisomes and the synthesis of these isoprenoid lipids [15–21]. Krisans and colleagues reported that enzymes which participate in the pathway from mevalonate to farnesyl pyrophosphate are predominantly localized in peroxisomes [22–24]. Therefore, we expected that the synthesis of these isoprenoid lipids might be affected by gemfibrozil and clofibric acid at the cell culture level. Furthermore, similar effects of both agents on the synthesis were expected, because both inducing compounds are members of the same class of fibrates and have a similar pattern of activity as peroxisomal proliferator and HMG-CoA reductase inhibitor at the cell culture level. As a result, we found that both agents act differently on several parameters in the synthesis of ubiquinone, dolichol, and cholesterol from [^{14}C]acetate or [^{3}H]mevalonate.

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† *Abbreviation:* HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

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MATERIALS AND METHODS

Materials

The gemfibrozil used in this study was a gift from Warner-Lambert. [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. Clofibrate, cholesterol, HMG-CoA, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, mevalonolactone, palmitoyl-CoA, NAD, and 25-hydroxycholesterol were obtained from Sigma. All other reagents were of analytical grade and were purchased from Wako Pure Chemicals.

Animals

Male Wistar rats weighing about 200–250 g and obtained from Saitama Laboratory Animals were used. They had free access to standard laboratory chow, CE-2 (Nihon Clea Inc.) 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 based on the trypan blue exclusion test were used for experiments. The cells in L-15 medium (2 mL) containing 28 mM HEPES, 1 μM dexamethasone, 1 μM insulin, 100 U/mL of penicillin G-K, 75 U/mL of streptomycin sulfate, 100 μg/mL of 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°. The medium was changed 4 hr after plating. Drug treatment was initiated 24 hr after plating by changing the medium to a drug-containing medium, and thereafter the medium was changed every 24 hr. 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 indicated times. 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), and then cell homogenates were prepared by sonication. These homogenates were used for assay of enzyme activity and protein content [2, 25].

Assay Methods

The activity of the peroxisomal fatty acyl-CoA oxidizing system was determined by the method described by Lazarow and de Duve [26] with slight modifications [27]. 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 described by Keller *et al.* using [3-^{14}C] HMG-CoA and [5-^{3}H]mevalonolactone as the substrate and the internal standard, respectively [28]. 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 the standard [29].

Metabolic Labeling of Isoprenoid Lipid

Cells (6×10^6) were preincubated in 12 mL of L-15 medium containing hormones, antibiotics, and fetal bovine serum, as stated above, with or without gemfibrozil or clofibric acid in collagen-coated plastic plates (100-mm diameter) for 2 or 74 hr at 37°. In the 74-hr preincubation, the medium was changed every 24 hr. After the preincubation, 888 kBq (24 μCi) of [1-^{14}C]acetate or 222 kBq (6 μCi) of [5-^{3}H]mevalonolactone was added, followed by incubation in the same medium for 4 hr [30].

Isolation of Isoprenoid Lipid

Following the incubation, the medium was removed, and the attached cells were washed with PBS. In the case of cholesterol and dolichol, the cells were harvested twice with 1.5 mL of methanol. Before the final vortexing, 6 mL of chloroform was added to the sample. The samples were sonicated for 1 min. As carriers, 20 μg of unlabeled cholesterol and 25 μg of dolichol (mixture of C_{80}–C_{105}) were added to the sonicated sample. The chloroform extracts were reduced to dryness under N_2. Mild alkaline hydrolysis was achieved by refluxing the mixture for 1 hr at 80°. The non-saponifiable lipids were isolated from the saponification mixture by extraction with petroleum ether. The non-saponifiable lipids were purified on a C18 reversed-phase Sep-Pak mini-column (Waters) previously equilibrated with methanol. Cholesterol was eluted with 10 mL of ethanol, and dolichol was subsequently eluted with 10 mL of hexane. The solvent was removed from each eluate under vacuum. The residual material was redissolved in a known volume of methanol before HPLC analysis [30].

In the case of ubiquinone, the washed cells were harvested twice with 3 mL of methanol. Before the final vortexing, 3.5 mL of petroleum ether was added to the samples. The samples were sonicated for 1 min. In each case, 10 μg of unlabeled ubiquinone-9 was added as a carrier. The petroleum ether extracts were reduced to dryness under N_2. The lipids were purified on a Sep-Pak Silica mini-column (Waters) previously equilibrated with chloroform. Ubiquinone was eluted with 20 mL of chloroform. The solvent was removed from the eluate under vacuum. The residual material was redissolved in a known volume of isopropanol before HPLC analysis [31, 32].

HPLC

Analysis of cholesterol, dolichol, and ubiquinone was achieved by separate analysis of each Sep-Pak eluate on

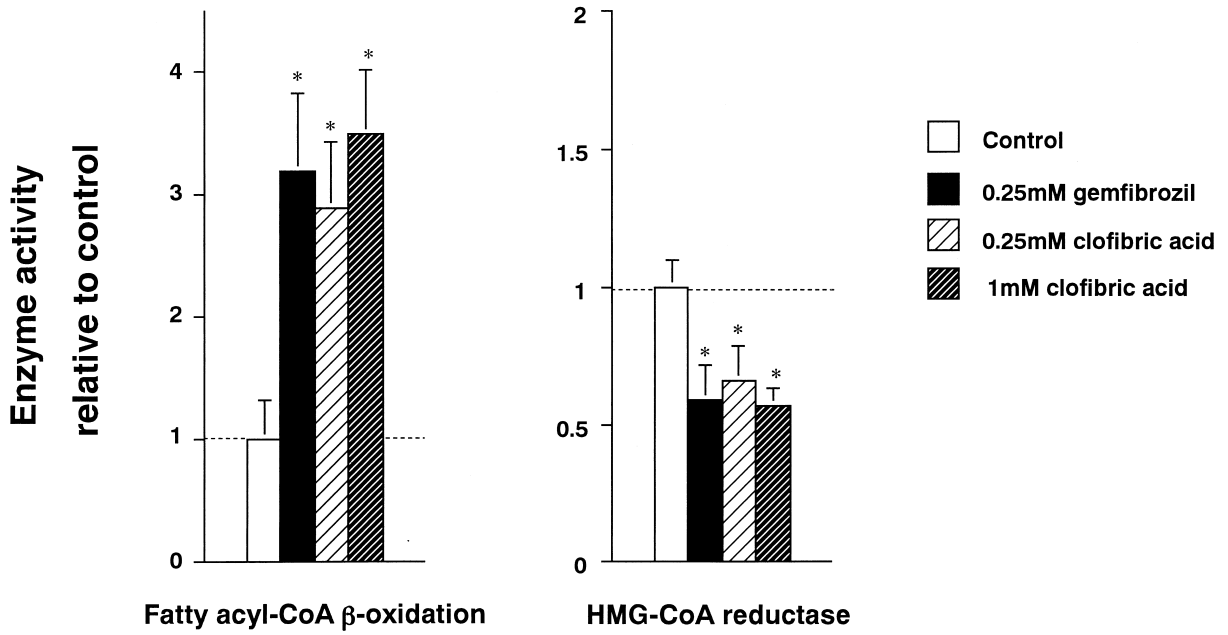

FIG. 1. Changes in activity of the peroxisomal fatty acyl-CoA β-oxidation system and the HMG-CoA reductase of hepatocytes treated with gemfibrozil or clofibric acid. Drug treatment was initiated 24 hr after plating (10^6 cells/plate) by changing the culture medium to a medium containing 0.25 mM gemfibrozil, or 0.25 or 1 mM clofibric acid, and the medium was then changed every 24 hr during the incubation period. The cells were harvested after incubation for 78 hr, and cell homogenates were prepared by sonication and used for assay of β-oxidation and HMG-CoA reductase. β-Oxidation activity (U/mg protein) and HMG-CoA reductase activity (pmol/min/mg protein) were expressed as the value relative to the control. Data are mean values ± SD of 5 experiments. * indicates significant difference ($P < 0.005$). The activity of β-oxidation and HMG-CoA reductase of control cells was 0.90 ± 0.29 U/mg protein and 19.0 ± 2.5 pmol/min/mg protein, respectively.

straight phase HPLC. A Shimazu Series LA-10 HPLC apparatus (Shimazu) was used with a reversed-phase 5-μm LiChrospher 100 RP-18 column (4 × 250 mm) (Merck). The mobile phase of methanol, isopropanol/methanol/hexane (5:2:1), and methanol/isopropanol (5:1) was used for analysis of cholesterol, dolichol, and ubiquinone, respectively. The mobile phase was pumped at a constant rate of 1 mL/min at 40°. The ultraviolet absorbance of the column eluate was monitored with an SPD-10A Shimazu UV spectrophotometric detector at 210 nm. In the case of ubiquinone, the absorbance at 275 nm, a specific absorbance for ubiquinone, was also monitored. Retention times of cholesterol, dolichol, and ubiquinone were 13.3, 6.8, and 23.5 min, respectively. Peak fractions corresponding to cholesterol, dolichol, and ubiquinone were collected and concentrated. Radioactivity was measured using an Aloka-LSC 700 scintillation counter, with Liquifluor (New England Nuclear) as a scintillator.

RESULTS

Changes in Activity of the Peroxisomal Fatty Acyl-CoA β-Oxidation System and HMG-CoA Reductase of Hepatocytes Treated with Gemfibrozil or Clofibric Acid

The cells were incubated in the culture medium containing 0.25 mM gemfibrozil, or 0.25 or 1 mM clofibric acid. After incubations for 78 hr, the activity of the peroxisomal fatty acyl-CoA β-oxidation system and HMG-CoA reductase was determined. We did not use gemfibrozil at 1 mM because of the damage to hepatocytes that the agent causes at this concentration [2]. Gemfibrozil at 0.25 mM increased β-oxidation activity to 3.2 times that of the control. Clofibric acid at 0.25 and 1 mM increased the activity to 2.9 and 3.5 times the control, respectively. After incubation with 0.25 mM gemfibrozil, HMG-CoA reductase activity was decreased to 59.0% of the control. Clofibric acid at 0.25 and 1 mM decreased the reductase activity to 66.0% and 57.1%, respectively (Fig. 1). After incubation for 6 hr, neither gemfibrozil nor clofibric acid affected the activity of β-oxidation and HMG-CoA reductase (data not shown). These results indicate that gemfibrozil and clofibric acid proliferate peroxisomes and inhibit the activity of HMG-CoA reductase in rat hepatocytes after incubation for 78 hr.

Ubiquinone Synthesis from [^{14}C]Acetate and [^{3}H]Mevalonate after Incubation with Gemfibrozil and Clofibric Acid

Figure 2a shows the effects of gemfibrozil and clofibric acid on ubiquinone synthesis from [^{14}C]acetate. Cells were preincubated in the presence of gemfibrozil or clofibric acid for 74 hr, and further incubated with [^{14}C]acetate for 4 hr. After incubation (78 hr: 74 plus 4 hr) with 0.25 mM gemfibrozil, ubiquinone synthesis was increased to about

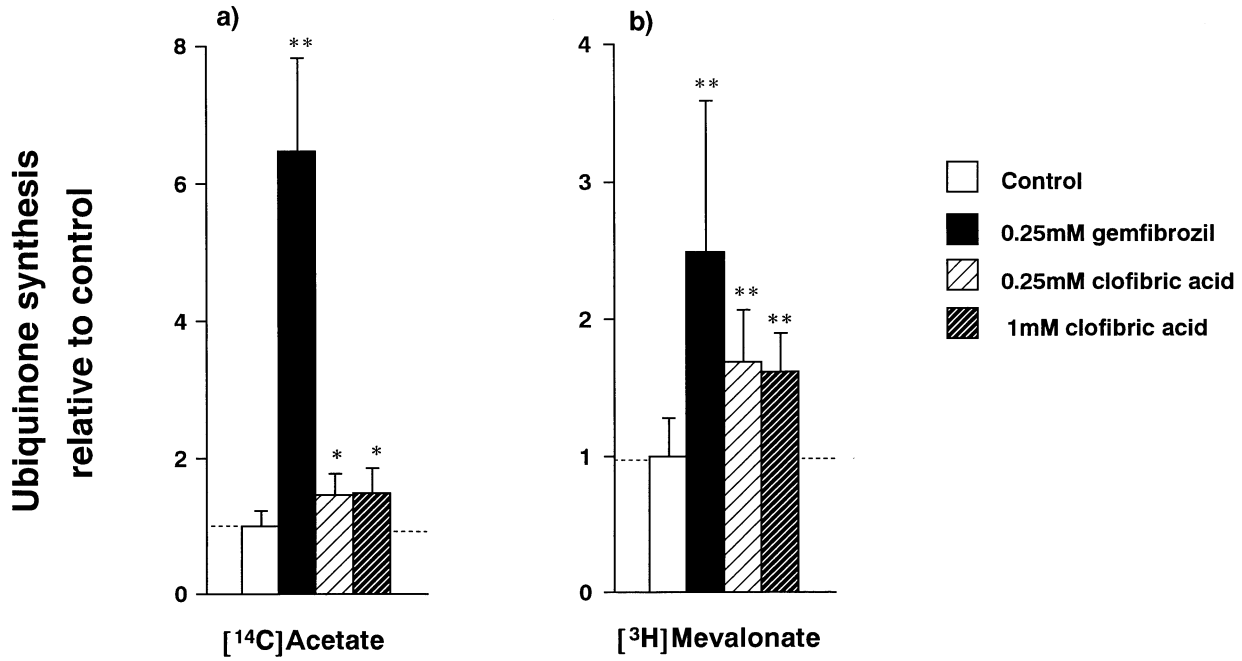

FIG. 2. Ubiquinone synthesis from [^{14}C]acetate and [^{3}H]mevalonate in cells treated with gemfibrozil or clofibric acid. Cells (6×10^6) were preincubated in 12 mL of the culture medium with or without agent for 74 hr at 37°. The medium was changed every 24 h. After preincubation, 24 μCi [1-^{14}C]acetate (a) or 6 μCi [5-^{3}H]mevalonate (b) was added, and cells were incubated for 4 hr. Ubiquinone was extracted from the cells as described in the text. Radioactivity in the ubiquinone was determined. Data are mean values ± SD of 6 experiments. * and ** represent significant differences (* $P < 0.05$; ** $P < 0.01$). The radioactivity of ubiquinone synthesized from [^{14}C]acetate and [^{3}H]mevalonate of control cells was 72.1 ± 16.9 and 230 ± 64 dpm/10^6 cells, respectively.

6.5 times the control. After incubation with 0.25 and 1 mM clofibric acid, ubiquinone synthesis was increased to 1.46 and 1.47 times that of the control, respectively. These results indicate that both agents activate ubiquinone synthesis from acetate.

In the experiment represented in Fig. 2b, [^{3}H]mevalonate instead of [^{14}C]acetate was used as a precursor. Mevalonate is formed from HMG-CoA by HMG-CoA reductase. In other words, isoprenoid lipid synthesis from [^{3}H]mevalonate is a reaction independent of HMG-CoA reductase. After incubation with 0.25 mM gemfibrozil, ubiquinone synthesis was increased to about 2.5 times that of the control. On the other hand, 0.25 and 1 mM clofibric acid increased the synthesis to 1.69 and 1.61 times that of the control, respectively. From these results, both agents would seem to activate the synthetic pathway of ubiquinone, at least from mevalonate. The effect of gemfibrozil was much greater than that of clofibric acid.

Aberg *et al.* reported that the ubiquinone content of rat liver was increased by long-term treatment with clofibrate in the whole body, suggesting a relationship between ubiquinone synthesis and peroxisomes [15]. Neither gemfibrozil nor clofibric acid caused peroxisomal proliferation after short incubation (6 hr), as stated above. If the increase in ubiquinone synthesis after a long incubation with gemfibrozil and clofibric acid depends on peroxisomal proliferation, neither agent would then be able to affect ubiquinone synthesis after a short incubation. Therefore, we studied the effect of both agents in a longer incubation. Cells were preincubated in the presence of the agents for 2 hr and further incubated with [^{14}C]acetate or [^{3}H]mevalonate for 4 hr. Unexpectedly, 0.25 mM gemfibrozil increased ubiquinone synthesis from [^{14}C]acetate to about 5.7 the control, and clofibric acid at 0.25 and 1 mM increased the synthesis to 1.23 and 1.37 times that of the control, respectively. Gemfibrozil at 0.25 mM increased ubiquinone synthesis from [^{3}H]mevalonate to about 2.6 times that of the control, and a small increase (1.35 times the control) was detected by 1 mM clofibric acid (data not shown). These results indicate that the increase by both agents is not necessarily dependent on peroxisomal proliferation.

Dolichol Synthesis from [^{14}C]Acetate and [^{3}H]Mevalonate after Incubation with Gemfibrozil and Clofibric Acid

After incubation with 0.25 mM gemfibrozil for 78 hr, dolichol synthesis from [^{14}C]acetate was decreased to approximately 70% of the control. However, no significant change in dolichol synthesis caused by 0.25 and 1 mM clofibric acid was detected after incubation (Fig. 3a). Gemfibrozil decreased dolichol synthesis from [^{3}H]mevalonate to approximately 54% of the control. Gemfibrozil may inhibit the synthetic pathway of dolichol, at least from mevalonate. However, dolichol synthesis from [^{3}H]mevalonate was increased to 2.1 and 2.7 times the control by 0.25 and 1 mM clofibric acid, respectively (Fig. 3b). These results indicate that the effects on dolichol synthesis of

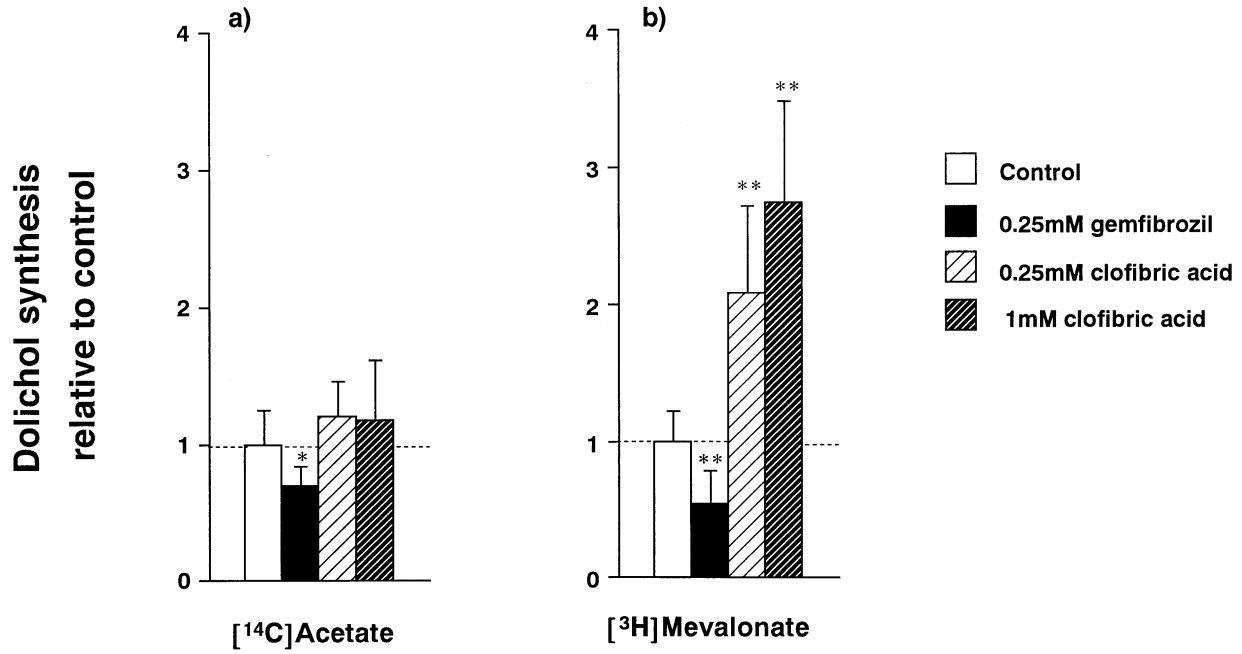

FIG. 3. Dolichol synthesis from [^{14}C]acetate and [^{3}H]mevalonate in cells treated with gemfibrozil or clofibric acid. Cells (6×10^6) were preincubated in the culture medium with or without agent for 74 hr. After preincubation, [^{14}C]acetate (a) or [^{3}H]mevalonate (b) was added, and the cells were further incubated for 4 hr. Dolichol was extracted from the cells, and radioactivity in the dolichol was determined. Data are mean values ± SD of 6 experiments. * and ** represent significant differences (* $P < 0.05$; ** $P < 0.01$). The radioactivity of dolichol synthesized from [^{14}C]acetate and [^{3}H]mevalonate of control cells was 114 ± 29 and 302 ± 65 dpm/10^6 cells, respectively.

gemfibrozil and clofibric acid are different, in fact, almost opposite. After incubation for 6 hr, 0.25 mM gemfibrozil decreased dolichol synthesis from [^{14}C]acetate and [^{3}H]mevalonate to approximately 70% of the control (data not shown). These data confirm the decrease in dolichol synthesis by gemfibrozil. No significant change in dolichol synthesis caused by 0.25 and 1 mM clofibric acid was detected after short incubation (data not shown).

Cholesterol Synthesis from [^{14}C]Acetate and [^{3}H]Mevalonate after Incubation with Gemfibrozil and Clofibric Acid

After incubation for 78 hr, cholesterol synthesis from [^{14}C]acetate was clearly decreased by both agents, resulting in approximately 54% of the control level caused by 0.25 mM gemfibrozil. The synthesis was decreased to 65% and 47% of the control by 0.25 and 1 mM clofibric acid, respectively (Fig. 4a). This decrease in synthesis after incubation with gemfibrozil and clofibric acid may have been caused by the inhibition of HMG-CoA reductase by the agents (Fig. 1).

Enzymes that participate in the pathway from mevalonate to farnesyl pyrophosphate have been recognized to be localized in cytosol. However, recently, Krisans and colleagues reported that these enzymes are predominantly localized in peroxisomes as stated above [22–24]. Therefore, we expected that cholesterol synthesis from [^{3}H]mevalonate might be increased by gemfibrozil and clofibric acid. However, cholesterol synthesis from [^{3}H]mevalonate was decreased approximately 56% by 0.25 mM gemfibrozil. No effect of 0.25 or 1 mM clofibric acid on cholesterol synthesis was detected (Fig. 4b). These results indicate that neither gemfibrozil nor clofibric acid activates cholesterol synthesis from [^{3}H]mevalonate unexpectedly, and gemfibrozil rather decreases the synthesis. After incubation for 6 hr, cholesterol synthesis from [^{14}C]acetate was only slightly affected by both agents. Cholesterol synthesis from [^{3}H]mevalonate was decreased approximately 64% by 0.25 mM gemfibrozil, but no effect of clofibric acid was detected (data not shown). These results confirm the decrease in cholesterol synthesis from [^{3}H]mevalonate by gemfibrozil at the cell culture level.

DISCUSSION

We studied whether gemfibrozil and clofibric acid alter isoprenoid lipid synthesis in rat hepatocytes. Considering the data from ubiquinone synthesis (Fig. 2) together with those from HMG-CoA reductase (Fig. 1), the increase in ubiquinone synthesis does not seem to depend on HMG-CoA reductase. In other words, HMG-CoA reductase may not be a rate-limiting enzyme of ubiquinone synthesis. There are several reports suggesting that ubiquinone synthesis is independent of HMG-CoA reductase [33–35]. Our data support these reports.

From the data from HMG-CoA reductase and dolichol

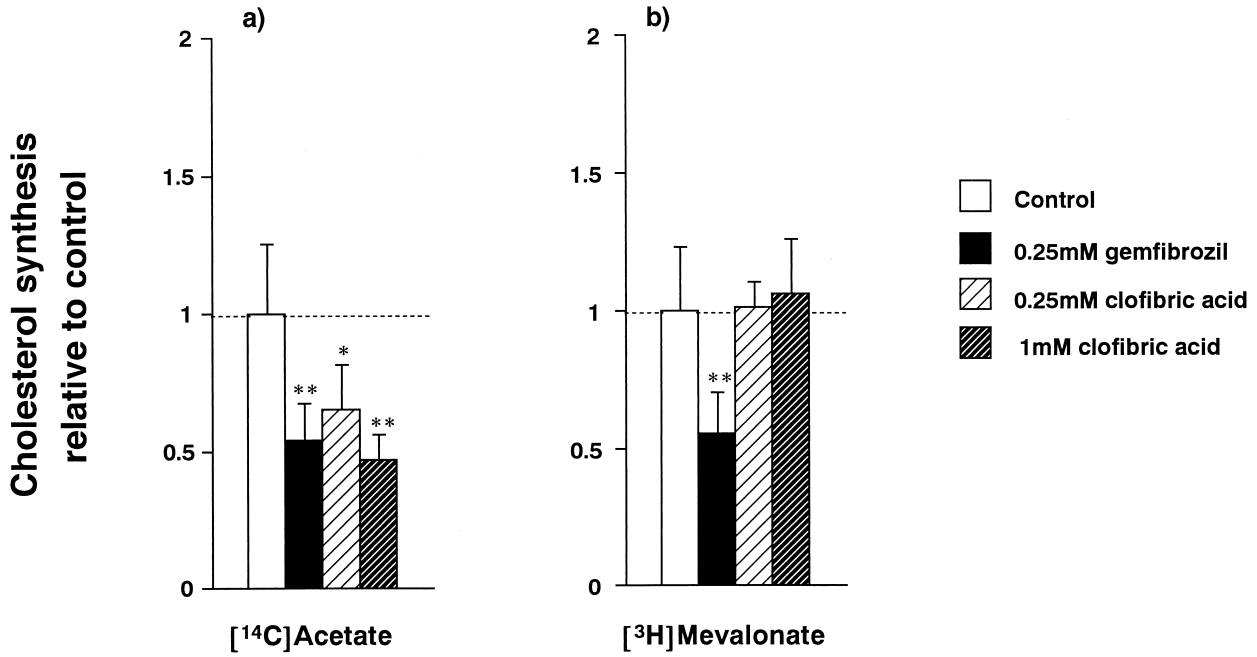

FIG. 4. Cholesterol synthesis from [^{14}C]acetate and [^{3}H]mevalonate in cells treated with gemfibrozil or clofibric acid. After preincubation of cells (6×10^6) in the culture medium with or without agent for 74 hr, the cells were incubated with [^{14}C]acetate (a) or [^{3}H]mevalonate (b) for 4 hr. Cholesterol was extracted from the cells, and radioactivity in the cholesterol was determined. Data are mean values ± SD of 6 experiments. * and ** represent significant differences (* $P < 0.02$; ** $P < 0.005$). The radioactivity of control cells at 78 hr was 3.82 ± 0.96 and 21.2 ± 5.0 dpm $\times 10^{-3}/10^6$ cells, respectively.

synthesis after short incubation, gemfibrozil seems to decrease dolichol synthesis independently of HMG-CoA reductase. Our data support several reports in which dolichol synthesis was found to be independent of HMG-CoA reductase [34–38]. Peroxisomal proliferation by clofibric acid after long incubation (Fig. 1) and the increase in dolichol synthesis from [^{3}H]mevalonate (Fig. 3b) suggest the participation of peroxisomes in dolichol synthesis. There are many reports that peroxisomes as well as microsomes participate in dolichol synthesis [16–21]. The present results support these reports. However, we do not exclude the possibility that the increase in dolichol synthesis was due to the effect of clofibric acid on other intracellular particulate(s), such as microsomes.

In the present study (Fig. 4), we could not detect any increase in cholesterol synthesis by gemfibrozil in the whole body [1, 14]. The increase seems to be caused by a complicated mechanism probably independent of the present effects of gemfibrozil.

From these results, it was clear that gemfibrozil and clofibric acid alter synthesis of not only cholesterol but also ubiquinone and dolichol in rat hepatocytes. The affected sites of gemfibrozil in the mevalonate pathway may be different from those of clofibric acid, as shown in Table 1. As a result, the effects of the agents on the synthesis of cholesterol, ubiquinone, and dolichol, which are formed in the same route up to farnesyl pyrophosphate in the mevalonate pathway, may be different. Gemfibrozil and clofibrate are used as hypolipidemic drugs in order to decrease blood levels of triglyceride and cholesterol. The present study suggests that these drugs act differently on the synthesis of ubiquinone, dolichol, and cholesterol in the body, resulting in different blood levels of these isoprenoid lipids. Recently, increasing attention has been paid to the

TABLE 1. Proposed action sites of gemfibrozil and clofibric acid on the mevalonate pathway

	Gemfibrozil			Clofibric acid		
Action sites:						
Acetyl-CoA						
↓	⬘⬘?	⬘?	⬙	⬘?	◊?	⬙
Mevalonate						
↓	⬙⬙	⬙	⬙	⬙	⬙⬙	◗
Products:						
Isoprenoid lipid	Ubiquinone	Dolichol	Cholesterol	Ubiquinone	Dolichol	Cholesterol

⬙, activation; ⬙, inhibition.

function of ubiquinone as antioxidant [39]. The large increase in ubiquinone synthesis by gemfibrozil detected in the present study seems to be useful for investigation of the function. Dolichol and cholesterol, as well as ubiquinone, play important roles in the body. Therefore, study of the regulation mechanism of the synthesis of these isoprenoid lipids by gemfibrozil and clofibric acid is very important.

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