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Effects of Peroxisome Proliferators Gemfibrozil and Clofibrate on Syntheses of Dolichol and Cholesterol in Rat Liver

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The effects of two peroxisome proliferators, gemfibrozil and clofibrate, on syntheses of dolichol and cholesterol in rat liver were investigated. Gemfibrozil did not affect the overall content of dolichyl phosphate, but it changed the chain-length distribution of dolichyl phosphate, increasing the levels of species with shorter isoprene units. Gemfibrozil suppressed synthesis of dolichyl phosphate from [^{3}H]mevalonate and [^{3}H]farnesyl pyrophosphate in rat liver. In contrast, clofibrate increased the content of dolichol (free and acyl ester forms). It remarkably enhanced dolichol synthesis from mevalonate, but did not affect dolichol synthesis from farnesyl pyrophosphate. Gemfibrozil elevated cholesterol synthesis from [^{14}C]acetate, but did not affect the synthesis from mevalonate. Clofibrate suppressed cholesterol synthesis from acetate, but did not affect cholesterol synthesis from mevalonate. These results suggest that gemfibrozil suppresses synthesis of dolichyl phosphate by inhibiting, at the least, the pathway from farnesyl pyrophosphate to dolichyl phosphate. As a result, the chain-length pattern of dolichyl phosphate may show an increase in shorter isoprene units. Clofibrate may increase the content of dolichol by enhancing dolichol synthesis from mevalonate. Gemfibrozil may increase cholesterol synthesis by activating the pathway from acetate to mevalonate. Unlike gemfibrozil, clofibrate may decrease cholesterol synthesis by inhibiting the pathway from acetate to mevalonate.

Key words: cholesterol, clofibrate, dolichol, gemfibrozil, peroxisome.

Abbreviations: FPP, farnesyl pyrophosphate; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase; IPP, isopentenyl pyrophosphate.

Dolichols are a group of α-saturated polyprenol lipids that generally contain from 14 to 24 isoprene units. Dolichol derivatives found in eukaryotes include free alcohols, monophosphate esters and acyl esters of dolichol. Several studies have appeared in recent years that suggest a role of dolichyl phosphate and its pyrophosphate as carriers of saccharide residues in *N*-linked glycoprotein synthesis (*1–3*). However, little is known about the functions of free and acyl dolichols. They may play a role in the properties of model and plasma membranes, such as fluidity, stability, and permeability (*4–11*).

The biosynthetic pathways of cholesterol, dolichol, and ubiquinone are the same up to farnesyl pyrophosphate (FPP) (*12*, *13*), from where they diverge. Recent investigations have demonstrated various localizations of enzymes involved in the mevalonate pathway (*14*, *15*), which is partly associated with peroxisomes. The subsequent steps from isopentenyl pyrophosphate (IPP) to cholesterol or dolichol are also partly associated with peroxisomes.

Gemfibrozil and clofibrate are both known to be hypolipidemic drugs and also peroxisome proliferators (*16–23*). Therefore, their effects are almost the same as those related to peroxisomal enzymes, such as catalase, the fatty acyl-CoA oxidase and others, except for 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), the rate-limiting enzyme of cholesterol synthesis (*24–26*). Clofibric acid inhibits HMG-CoA reductase activity of rat liver and decreases cholesterol synthesis from acetate in the body and in cultured cells (*21*, *26–28*). In earlier studies, we observed that (i) gemfibrozil decreased the HMG-CoA reductase activity and inhibited cholesterol synthesis from acetate in cultured cells (*28*, *29*); (ii) unlike in culture cells, gemfibrozil unexpectedly increased the HMG-CoA reductase activity in the body and stimulated biosynthesis of cholesterol from acetyl-CoA derived from peroxisomal β-oxidation (*25*, *30*). In the case of dolichol synthesis, we reported that gemfibrozil suppresses dolichol synthesis from mevalonate in cultured cells, and clofibrate enhances its synthesis (*29*).

Since the effect of gemfibrozil on HMG-CoA reductase activity in the body is different from that in cultured cells, its effect on dolichol synthesis in the body is also expected to be different from that in cultured cells. We have not yet studied the influence of gemfibrozil on cholesterol synthesis from acetate in whole body. Therefore, in this study we investigated the effects of gemfibrozil and clofibrate on syntheses of dolichol and cholesterol in the body by using various precursors in order to elucidate more precisely the regulation of lipid synthesis.

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MATERIALS AND METHODS

Materials—Gemfibrozil, dolichol C_{80}-C_{105} and dolichyl monophosphate C_{80}-C_{105} were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). [1-^{14}C]Acetic acid (2.06 GBq/mmol), [RS]-[5-^{3}H]mevalonolactone (2.22 TBq/mmol) and [1-^{3}H]FPP (2.22 TBq/mmol) were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). Liquifluor was purchased from New England Nuclear (Boston, MA, USA). All other reagents were of analytical grade from Wako Pure Chemicals (Osaka).

Animals—Male Wistar rats (200–250 g) were allowed free access to a standard chow, CE-2 (Japan Clea, Tokyo, Japan), and kept on a 12-h light-dark cycle. The treated rats were fed a chow containing 0.25% clofibrate (w/w) or 0.2% gemfibrozil (w/w) for 2 wk. The rats then fasted overnight. On the following day, a radiolabeled chemical, [1-^{14}C]acetic acid (400 kBq), [RS]-[5-^{3}H]mevalonolactone (200 kBq) or [1-^{3}H]FPP (100 kBq), was injected into a thigh vein of the rats under anesthesia. The rats were sacrificed and the livers were excised after perfusion with cold saline.

Extraction and Separation of Isoprenoid Lipids—The liver (1 g) was minced, homogenized in 5 ml of 60% KOH/methanol (1:1) and saponified at 100°C for 1 h. Lipid was extracted twice with 5 ml of diethyl ether. The ether extract was washed with 2 ml of 5% acetic acid (v/v), the ether was evaporated under nitrogen, and the residue was dissolved in 1 ml of chloroform/methanol (2:1). This solution was applied to an Accell QMA Sep-Pak equilibrated with chloroform/methanol (2:1). Dolichol (free and acyl ester type) and cholesterol were both eluted with 10 ml chloroform/methanol (2:1). Thereafter, dolichyl phosphate was eluted with 10 ml chloroform/methanol (2:1) containing 0.5 M ammonium acetate. The eluate containing dolichyl phosphate was evaporated to dryness, and the residue was dissolved in 300 μl of the following HPLC solvent. The eluate containing dolichol and cholesterol was evaporated, and the residue was dissolved in 1 ml of methanol. Dolichol and cholesterol were further separated on a C_{18} Sep-Pak equilibrated with methanol. Cholesterol was eluted with 10 ml of methanol, then dolichol was eluted with 10 ml of *n*-hexane. The respective lipid fractions were evaporated to dryness and the residues were dissolved in 300 μl of the following HPLC solvents.

Analysis of Isoprenoid Lipids by HPLC—Isoprenoid lipids were analyzed using a Shimadzu HPLC LA-10 at 40°C, with a Merk Lichrospher 100 RP-18 column (4 × 250 mm, 5 mm particles) (Darmstadt, Germany). For cholesterol and dolichyl phosphate, the HPLC elution solvents were methanol/2-propanol (1:1) and water/methanol/2-propanol/*n*-hexane (1:8:15:5), respectively. The flow rate was 1 ml/min. For dolichol, methanol/2-propanol/*n*-hexane (3:1:1) was used at a flow rate of 1.3 ml/min. Since dolichol, dolichyl phosphate and cholesterol all have absorbance at 210 nm, the fractions around the peak at 210 nm were collected, and the radioactivity of the pooled fractions was measured using an Aloka LSC 700 Scintillation Counter (Tokyo) with Liquifluor as a scintillator.

Statistical Analysis of the Data—Statistical analysis of the data was carried out using Student's t test. Significance was set at $p < 0.05$.

RESULTS

Effects of Gemfibrozil and Clofibrate on the Amount of Dolichol in the Liver—Liver weight of the control group was 7.2 ± 0.9 g/200 g body weight. Gemfibrozil and clofibrate both increased the liver weight (gemfibrozil: 11.5 ± 1.1 g/200 g body weight, clofibrate: 9.9 ± 1.2 g/200 g body weight), suggesting that they caused hepatomegaly. Figure 1 shows the effects of gemfibrozil and clofibrate on the hepatic amount of dolichol (free and acyl ester type), dolichyl phosphate and cholesterol. In control rats, 1 g of the liver contained about 35 μg of dolichol (free and acyl ester type), 20 μg of dolichyl phosphate and 2.3 mg of cholesterol, agreeing approximately with an earlier report (*31*). Gemfibrozil treatment did not change these levels, whereas clofibrate treatment increased the dolichol level to 130% of the control, decreased the cholesterol level to 80% of the control, but did not affect the amount of dolichyl phosphate.

Effects of Gemfibrozil and Clofibrate on the Chain Length of Dolichol—Liver of control rats contained dolichols with mainly 17–21 isoprene units: the highest content was of 18 isoprenoid units, and the next was of 19 units. Gemfibrozil treatment slightly increased the contents of 17 and 18 isoprene units, but it hardly affected those of 19, 20 and 21 isoprene units. In contrast, clofibrate increased contents of all isoprene units (Fig. 2). This increase by clofibrate may be the cause of the increase in the amount of dolichol (Fig. 1).

Figure 3 shows the effects of gemfibrozil and clofibrate on the chain length of dolichyl phosphate. Control rat liver contained approximately half the amount of dolichyl phosphate compared with dolichol as described in the legend of Fig. 1, and the distribution of chain lengths of dolichyl phosphate was somewhat different from that of dolichol (Figs. 2 and 3). In the liver of control rats, dolichol with 18 isoprene units was more abundant than that with 19 isoprene units (Fig. 2), but dolichyl phosphates

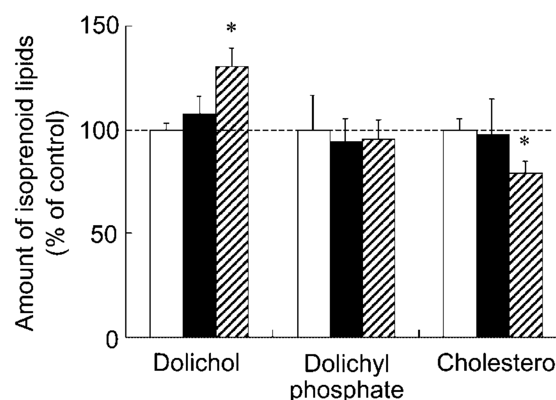

Fig. 1. **Influence of administration of gemfibrozil and clofibrate on amount of isoprenoid lipids.** Rats were maintained on either normal chow (open bars), or a chow containing 0.2% gemfibrozil (closed bars), or 0.25% clofibrate (shaded bars) for 2 wk. Isoprenoid lipids were extracted from the liver and separated using an HPLC system equipped with a UV detector. Data are means ± SD of 5–6 experiments. Asterisks indicate significant differences (*p < 0.01). One gram of normal liver contained 35.2 ± 1.1 μg, 19.7 ± 3.3 μg, and 2.25 ± 0.13 mg of dolichol (free and acyl ester type), dolichyl phosphate and cholesterol, respectively.

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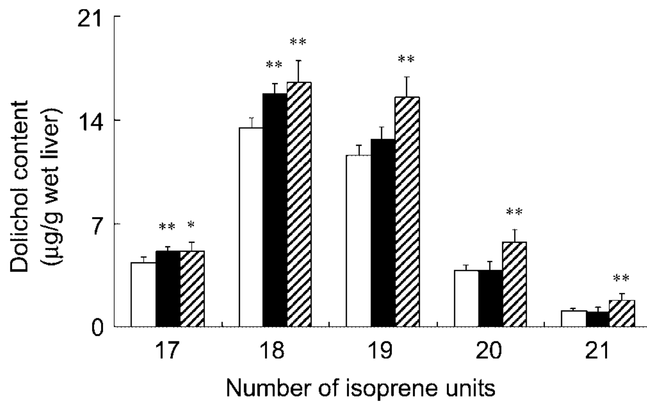

Fig. 2. **Effects of gemfibrozil and clofibrate on the chain length of dolichol.** Rats were maintained on either a normal chow (open bars), or a chow containing 0.2% gemfibrozil (closed bars) or 0.25% clofibrate (shaded bars) for 2 wk. Dolichols were isolated from the liver and separated according to their number of isoprene units by HPLC. Data are means ± SD of 5–6 experiments. Asterisks indicate significant differences (*$p < 0.05$; **$p < 0.01$).

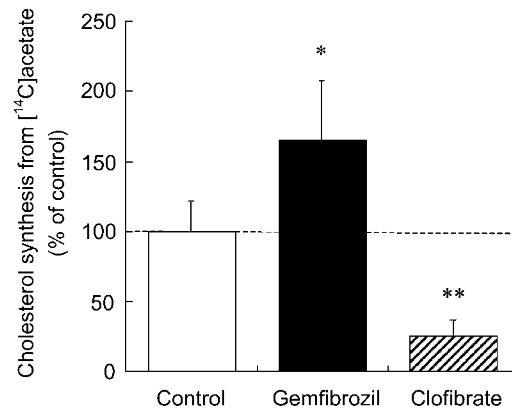

Fig. 4. **The effects of gemfibrozil and clofibrate on hepatic cholesterol synthesis from [^{14}C]acetate.** Rats were fed normal chow containing peroxisome proliferators for 2 wk. [^{14}C]Acetate was intravenously injected into the rats. After 3 h, cholesterol was extracted from the livers and analyzed as described in the text. Data are means ± SD of 5–6 experiments. Asterisks indicate significant differences (*$p < 0.05$; **$p < 0.01$). The radioactivity of normal liver was 423 ± 93 dpm/g wet liver of cholesterol.

with 18 and 19 isoprene units were present in the same quantity (Fig. 3). Gemfibrozil did not change the total amount of dolichyl phosphate (Fig. 1), but it changed the distribution of isoprene units: dolichyl phosphates with 17 and 18 isoprene units increased in content, while those with 19–21 units decreased. On the other hand, clofibrate did not affect these contents (Fig. 3).

Effects of Gemfibrozil and Clofibrate on Hepatic Cholesterol Synthesis from [1-^{14}C]Acetic Acid—Clofibric acid inhibits HMG-CoA reductase activity of rat liver in the whole body and in cultured cells (*21*, *26–28*). We reported that gemfibrozil decreases the HMG-CoA reductase activity in cultured cells but remarkably increases the activity in the whole body (*25*, *28–30*). Therefore, we studied cholesterol synthesis from [1-^{14}C]acetate after administration of gemfibrozil and clofibrate in a whole-body experiment. In control rats, incorporation of [^{14}C]acetate into cholesterol after 3 h was 423 ± 93 dpm/g wet liver. Gemfibrozil elevated cholesterol synthesis from [^{14}C]acetate to 170% of the control, but clofibrate suppressed the biosynthesis to only 25% that of the control (Fig. 4). Thereafter, we tried to determine dolichol synthesis from [^{14}C]acetate, but the recovery of the radioactivity in dolichol was too small to be detected. Therefore, we could not study the effect of the two agents on dolichol synthesis from [^{14}C]acetate.

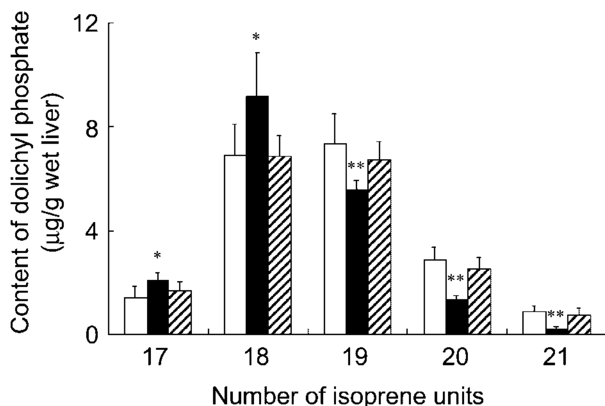

Fig. 3. **Effects of gemfibrozil and clofibrate on the chain length of dolichyl phosphate.** Rats were fed normal chow (open bars), or a chow containing 0.2% gemfibrozil (closed bars) or 0.25% clofibrate (shaded bars) for 2 wk. Dolichyl phosphates were isolated from the liver, and individual isoprene species were separated by HPLC. Data are means ± SD of 5–6 experiments. Asterisks indicate significant differences (*$p < 0.05$; **$p < 0.01$).

Effects of Gemfibrozil and Clofibrate on Dolichol Biosynthesis from [5-^{3}H]Mevalonate—To elucidate the effects of gemfibrozil and clofibrate on dolichol synthesis in steps subsequent to the HMG-CoA reductase reaction, [5-^{3}H]mevalonate was used as a precursor of dolichol. One hour after the administration of [^{3}H]mevalonate into control rats, incorporation of radioactivity into cholesterol was approximately 20,000 dpm/g of wet liver, while that into dolichyl phosphate was approximately 490 dpm/g of liver (*ca*. one thirtieth that of cholesterol). A plateau of incorporation into cholesterol and dolichyl phosphate was maintained from 1 h to 6 h after the administration of mevalonate. The radioactivity incorporated into dolichol was only 80 dpm/g of liver at 1 h, about 130 dpm at 3 h, and 160 dpm at 6 h (data not shown). Figure 5 shows the results of rats treated with gemfibrozil and clofibrate. The results are shown as incorporation 3 h after the administration of [^{3}H]mevalonate relative to that of the control. Clofibrate increased both dolichol synthesis (280%) and dolichyl phosphate synthesis (170%) from [^{3}H]mevalonate. Conversely, gemfibrozil suppressed biosynthesis of dolichyl phosphate synthesis (*ca*. 45% of the control) from [^{3}H]mevalonate. Neither agent affected cholesterol synthesis from [^{3}H]mevalonate.

Effects of Gemfibrozil and Clofibrate on Dolichol Synthesis from [1-^{3}H]FPP—As shown in Figs. 1–5, our results indicate that both gemfibrozil and clofibrate affect dolichol synthesis at some point after the formation of mevalonate. Therefore, we investigated their effects at a branch-point step using [1-^{3}H]FPP. The result is shown

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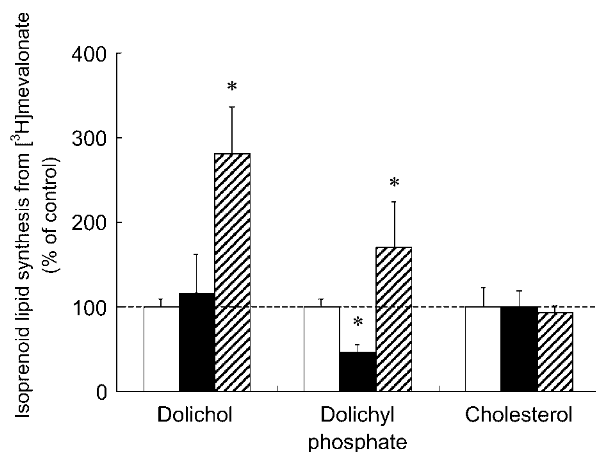

Fig. 5. **The effects of gemfibrozil and clofibrate on isoprenoid lipid synthesis from [^{3}H]mevalonate.** Rats were fed normal chow (open bars), or a chow containing 0.2% gemfibrozil (closed bars) or 0.25% clofibrate (shaded bars) for 2 wk. Three hours after intravenous injection of [^{3}H]mevalonate, isoprenoid lipids were extracted from the liver and analyzed as described in the text. Results are means ± SD of 5–6 experiments. Asterisks indicate significant differences (*p < 0.01). The radioactivity of normal liver was 167 ± 15, 427 ± 39 and 20,000 ± 4,700 dpm/g wet liver of dolichol, dolichyl phosphate and cholesterol, respectively.

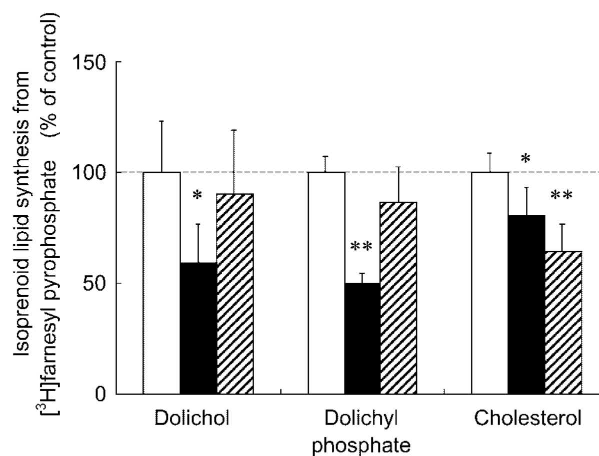

Fig. 6. **Effects of gemfibrozil and clofibrate on isoprenoid lipid synthesis from [^{3}H]FPP.** Rats were fed normal chow (open bars), or a chow containing 0.2% gemfibrozil (closed bars) or 0.25% clofibrate (shaded bars) for 2 wk. Three hours after intravenous injection of [^{3}H]FPP, isoprenoid lipids were extracted from the liver and analyzed as described in the text. Data are means ± SD of 5–6 experiments. Asterisks indicate significant differences (*p < 0.05; **p < 0.01). The radioactivity of normal liver was 84.8 ± 19.5, 84.6 ± 19.5, and 15,800 ± 1,400 dpm/g wet liver of dolichol, dolichyl phosphate and cholesterol, respectively.

in Fig. 6. Gemfibrozil suppressed biosyntheses of all isoprenoid lipids in this experiment. Dolichol was decreased to 60%, dolichyl phosphate to 50% and cholesterol to 80% of the control. Clofibrate suppressed cholesterol biosynthesis, but it did not affect biosyntheses of dolichol and dolichyl phosphate from [^{3}H]FPP.

DISCUSSION

We investigated the effects of gemfibrozil and clofibrate, two well-known antilipidemic agents and peroxisome proliferators, on the syntheses of isoprenoid lipids in rat liver. Gemfibrozil hardly affected the overall content of dolichol (free and acyl ester type) (Fig. 1), but it slightly increased the levels of dolichols with shorter isoprene units (Fig. 2). It similarly modified the chain-length pattern of dolichyl phosphate without changing the total amount (Figs. 1 and 3). On the other hand, clofibrate increased the total content of dolichol (Fig. 1) and the contents of dolichol species with different numbers of isoprene units (Fig. 2). However, it affected neither the amount nor the chain length pattern of dolichyl phosphate (Figs. 1 and 3). These results suggest that gemfibrozil and clofibrate have different effects on individual isoprene units of dolichol and dolichyl phosphate, even though both drugs are fibrate derivatives. Dolichol increases fluidity of the membrane (*5*). Therefore, the increase of dolichol content induced by clofibrate may increase the fluidity of the membrane and decrease its stability. Palamarczyk *et al*. reported that the chain length of polyprenyl derivatives is involved in the specificity of glycosyl transferase for polyprenyl derivatives (*32*). Therefore, the change of chain-length pattern of dolichyl phosphate induced by gemfibrozil may cause an alteration in the biosynthesis of glycoprotein.

We reported that gemfibrozil inhibited the synthesis of dolichol from mevalonate and the activity of HMG-CoA reductase in cultured cells (*29*). However, in the present study, gemfibrozil did not affect dolichol synthesis from mevalonate in the whole body (Fig. 5), but it decreased dolichol synthesis from FPP (Fig. 6). It also decreased synthesis of dolichyl phosphate from mevalonate and FPP (Figs. 5 and 6). These results suggest that gemfibrozil suppresses synthesis of dolichyl phosphate by inhibiting, at the least, the conversion of FPP to dolichyl phosphate (Fig. 6). As a result, the chain-length distribution of dolichyl phosphate may be changed to one with shorter isoprenoid lipids (Fig. 3). Gemfibrozil may suppress *cis*-prenyltransferase, which mediates the sequential *cis*-addition of IPP units, commencing with the addition of IPP to all-*trans*-FPP in the body.

Clofibrate remarkably increased dolichol synthesis from mevalonate in the whole body (Fig. 5). This finding is consistent with the earlier report that clofibrate increased synthesis of dolichol from mevalonate in cultured cells (*29*). However, clofibrate did not affect dolichol synthesis from FPP (Fig. 6). Consequently, clofibrate seems to stimulate the steps between mevalonate and FPP in the synthesis of dolichol. These results support the report that the FPP synthase activity of peroxisomes of rat liver was elevated severalfold and that of cytosol only moderately upon treatment with chow including 5% clofibrate for 7 d (*33*). It is reasonable to consider that the stimulation by clofibrate of the pathway from mevalonate to FPP in dolichol synthesis induced the increase of dolichol content (Fig. 1) and enhanced the levels of all isoprene units (Fig. 2). The effect of clofibrate on dolichyl phosphate synthesis from mevalonate was similar to that on dolichol synthesis (Fig. 5), but clofibrate did not increase the content of dolichyl phosphate (Fig. 1). This

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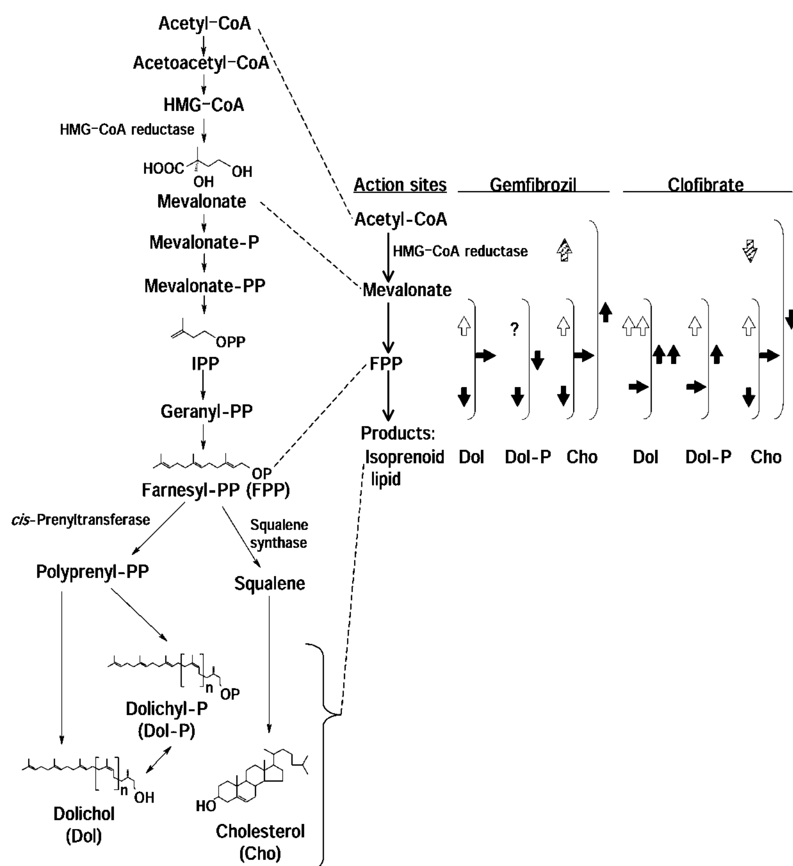

Fig. 7. **Proposed action sites of gemfibrozil and clofibrate on the mevalonate pathway at whole-body level.** Closed and open arrows indicate conclusions and speculations based on the present study, respectively. Shaded arrows indicate conclusions based on our previous study (*25*, *26*). Upward and downward arrows indicate activation and inhibition, respectively. Dol, Dol-P, Cho, P, and PP indicate dolichol, dolichyl phosphate, cholesterol, monophosphate and pyrophosphate, respectively.

may be because clofibrate activates the synthesis of dolichyl phosphate more weakly than that of dolichol (Fig. 5).

Gemfibrozil increased cholesterol synthesis from acetate in the whole body (Fig. 4), but it did not affect the synthesis from mevalonate (Fig. 5). In consequence, gemfibrozil may stimulate the biosynthesic pathway of cholesterol in the step from acetyl-CoA to mevalonate. This finding corresponds with the report that gemfibrozil enhanced HMG-CoA reductase activity, the rate-limiting enzyme of cholesterol synthesis in the body (*25*). It may be due to the very large cholesterol pool of the liver that the gemfibrozil-induced increase of cholesterol synthesis did not affect content of cholesterol (Figs. 1 and 4). In contrast, the result of Fig. 4 differs from our earlier report that gemfibrozil inhibited cholesterol biosynthesis from acetate in primary cultured rat hepatocytes (*29*). From these observations, gemfibrozil seems to have opposite effects on cholesterol biosynthesis in the whole body and in cultured hepatocytes.

Clofibrate suppressed cholesterol synthesis from acetate (Fig. 4), but it did not affect the synthesis from mevalonate (Fig. 5). Consequently, clofibrate may suppress the biosynthesis of cholesterol by inhibiting the synthetic pathway from acetyl-CoA to mevalonate in the whole body. This finding corresponds with the report that clofibrate inhibited the activity of HMG-CoA reductase in the body and in cultured cells (*26*, *28*, *30*). From the present results and others (*26–30*), clofibrate seems to have similar effects on cholesterol biosynthesis in the whole body and in cultured cells.

Figure 7 indicates the proposed action sites of gemfibrozil and clofibrate on the mevalonate pathway in the body. The steps from mevalonate to FPP in the syntheses of isoprenoid lipids tend to be enhanced in animals treated with gemfibrozil and clofibrate. Aboushadi and Krisans reported that the mevalonate pathway from mevalonate to FPP is localized in peroxisomes (*34*). Since gemfibrozil and clofibrate are peroxisome proliferators, it is likely that the proliferation of peroxisomes is related to this enhancement. Unlike clofibrate, however, gemfibrozil inhibited the syntheses of dolichol and dolichyl phosphate from FPP differing from clofibrate. Consequently, the sites affected by gemfibrozil in the mevalonate pathway may be different from those affected by clofibrate. The effects of gemfibrozil and clofibrate on the pathway from mevalonate to FPP are still hypothetical, however, and require further study. It is interest that gemfibrozil and clofibrate had different effects on the syntheses of dolichol, dolichyl phosphate and cholesterol, although both are included in the fibrate class of cholesterol-lowering drugs. This is the first report concerning the effects of gemfibrozil and clofibrate on biosyntheses of dolichol and dolichyl phosphate at the whole-body level. We clarified that gemfibrozil suppresses dolichyl phosphate synthesis at least from FPP, and clofibrate elevates dolichol synthesis by accelerating the pathway from mevalonate to FPP.

The liver plays an important part in the regulation of plasma lipid concentration. Since hypolipidemic agents are administered orally, in situ study of the whole body is very important, in order to elucidate the regulation

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mechanism of isoprenoid synthesis by gemfibrozil and clofibrate in rat liver.

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