PPARα Agonists Clofibrate and Gemfibrozil Inhibit Cell Growth, Down-Regulate hCG and Up-Regulate Progesterone Secretions in Immortalized Human Trophoblast Cells

Fumie Hashimoto[a,*], Yoshinobu Oguchi[a], Mieko Morita[a], Kikumi Matsuoka[b], Satoru Takeda[b], Masayuki Kimura[c], Hidenori Hayashi[a]

[a]*Department of Pathological Biochemistry, Faculty of Pharmaceutical Sciences, Josai University, Keyakidai, Sakado, Saitama 350-0295, Japan*

[b]*Department of Obstetrics and Gynecology,* [c]*Department of Pharmacy, Saitama Medical Center, Saitama Medical School, Kawagoe, Saitama 350-8550, Japan*

*Corresponding author: Tel. : 0492-71-7678 ; Fax : 0492-71-7984

E-mail address: hasimoto@josai.ac.jp (Fumie Hashimoto)

Abbreviations: FBS, fetal bovine serum; hCG, human chorionic gonadotropin; PPARα, peroxisome proliferator-activated receptor α

Abstract.

We studied effects of PPARα agonists clofibric acid and gemfibrozil on cell growth and functions of immortalized human extravillous trophoblast cells. Levels of DNA and protein gradually increased during incubation for 4 days. Gemfibrozil (> 0.25 mM) and clofibric acid (2.5 mM) suppressed the rate of increase in DNA and protein. Specific activities of fatty acyl-CoA oxidase and catalase were increased to about 1.2 ~ 2.0 times the control value by 0.05 mM gemfibrozil and 1.0 and 2.5 mM clofibric acid after incubation for 3 days. Acid phosphatase activity showed a small increase in response to both agents, but esterase activity changed little. The secretion of progesterone from the cells into the medium was increased by 0.25 mM gemfibrozil and 1.0 and 2.5 mM clofibric acid after incubation for 3 days, but that of human chorionic gonadotropin (hCG) was decreased by 0.35 mM gemfibrozil and 2.5 mM clofibric acid. The specific activity of lactate dehydrogenase in the cells was hardly changed at all after incubation for 3 days.

These results suggest that gemfibrozil and clofibric acid inhibit the proliferation of trophoblast cells. Cell metabolism is probably affected by both agents. The two agents may down-regulate hCG and up-regulate progesterone secretions.

Key words: gemfibrozil; clofibric acid; peroxisome; PPARα; trophoblast; progesterone.

1. Introduction

Although reports about the peroxisomes of rat liver are abundant [1-16], much less is known about those of other rat organs [17, 18]. Moreover, there are few reports about peroxisomes of human liver [2, 3, 5, 6, 8-10, 14, 19], and very few about those of other human organs [20-22]. In regard to the effect of PPARα agonist on peroxisomal enzymes and proliferation, there are many reports about rat liver [1-16]. In human liver cells, some reports revealed an increase in peroxisomal enzyme activity caused by PPARα agonist [5, 6, 10, 12, 19], while others found no effect [2, 3, 8, 9, 14]. In all organs except the liver, the effects of PPARα agonist on peroxisomes are little reported in rats [17], and even less so in humans [21].

Fibric acid derivatives are not only PPARα agonists in rodents, but also hypolipidemic agents. Clofibrate, at least, is thought to suppress the synthesis of cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), the rate-limiting enzyme of cholesterol synthesis in the whole body [23, 24] and cell cultures [13]. We reported that gemfibrozil suppresses the production of cholesterol through the inhibition of HMG-CoA reductase in cell culture [13, 15], but unexpectedly increases it via a remarkable stimulation of peroxisomal and microsomal HMG-CoA reductase after oral administration to rats [7, 11]. Furthermore, we reported that the two agents affect differently the synthesis of isoprenoid lipids such as ubiquinone, dolichol and cholesterol in the whole body and cultured cells of rats [13, 15, 25].

There are three kinds of trophoblasts in the placenta: cytotrophoblasts,

syncytiotrophoblasts and extravillous trophoblasts. Each of the three plays important roles in development and the maintenance of gestation. In human trophoblasts, peroxisomes are known to be present in cytotrophoblast cells [20, 22], and are detected later during gestation in syncytiotrophoblast cells [22]. Almost all cells which produce hormones secrete either steroid hormone or protein hormone, but the trophoblast cells secrete both. Steroid hormone is synthesized from cholesterol. Peroxisomes take part in cholesterol synthesis [26]. Sterol carrier protein 2 may participate in steroid hormone synthesis [27-29], and is reported to be primarily localized in peroxisomes [30-32]. However, less is known about the effect of PPARα agonist on hormone synthesis (secretion) [21, 33, 34]. Therefore, we studied the effects of gemfibrozil and clofibric acid on the secretion of progesterone (steroid hormone) and human chorionic gonadotropin (hCG) (protein hormone) from extravillous trophoblast cells, in addition to cell growth and metabolism. The characteristics of immortalized cells are not similar to those of cancer cells, but are rather similar to those of normal cells. Thus, we used an immortalized trophoblast cell-line (TCL-1) [35] for the present study.

2. Materials and methods

2.1. Materials

The gemfibrozil used in this study was a gift from Warner-Lambert. Clofibric acid, palmitoyl-CoA, leuco-2,7-dichlorofluorescin diacetate (Leuco-DCF), *o*-nitrophenylacetate, NAD, Triton X-100, and fetal calf serum were obtained from Sigma. RPMI 1640 medium was purchased from Nissui Chemicals. The progesterone EIA kit used was from the Cayman Company. The assay kit for hCG was obtained from Wako Pure Chemicals. All other reagents were of analytical grade and were purchased from Wako Pure Chemicals.

2.2. Culture of trophoblast cells and drug treatment

The trophoblast cell line-1 (TCL-1) was obtained by immortalizing third-trimester human trophoblast with the large-T antigen from the SV40 virus, as described in detail elsewhere [35]. The cells in RPMI 1640 medium (12 mL) containing 2 mM glutamine, 0.2% gentamycin sulfate, and 10% fetal bovine serum (FBS) were plated in plastic plates (100 mm diameter) at 10^5 cells/mL and then cultured at 37°. The medium was changed 24 hr after plating. Drug treatment was initiated 24 hr after plating by changing the medium to a drug-containing one, and thereafter the medium was changed every 24 hr. Gemfibrozil or clofibric acid solubilized in ethanol was added. The final concentration of ethanol was 0.2% (v/v).

Ethanol was also added in the medium of control cells. In the last 24 hr of the culture the medium was collected for the assay of hormone concentrations and lactate dehydrogenase activity. 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 cell homogenates were then prepared with a Potter-Elvehjem type Teflon homogenizer. These homogenates were used for the assay of enzyme activity and protein content.

The homogenate was also used for preliminary experiment of cell fractionation. The homogenate of control cells was centrifuged at 500 x g for 1 min at 4°, and unbroken cells were then removed. The resulting supernatant was centrifuged at 10,5000 x g for 1 hr at 4°. Activity of catalase and fatty acyl-CoA oxidase in the particulate and supernatant fractions obtained was assayed.

2.3. Assay of enzymes and protein

Fatty acyl-CoA oxidase and catalase are marker enzymes of peroxisomes. Fatty acyl-CoA oxidase activity was determined by measuring the H_2O_2-dependent oxidation of leuco-2,7-dichlorofluorescin diacetate (Leuco-DCF) at 502 nm catalyzed by exogenous horseradish peroxidase [12, 36]. This spectrophotometric assay has been demonstrated to be mUch more sensitive than previous methods of measuring peroxisomal fatty acid oxidation [37], due to the large absorption coefficient of the dye DCF and the favorable stoichiometry of the peroxidation reaction. Catalase activity was measured according to the method of Leighton et al. [38] with slight

modification [39]. Esterase is a marker enzyme of microsomes. Its activity was determined using *o*-nitrophenyl acetate as a substrate according to Beaufay et al. [40]. The acid phosphatase activity was determined as a marker enzyme of lysosomes, using the method described previously [39].

Cytotoxity was evaluated by measuring the activity of lactate dehydrogenase in the cells and medium and by viewing morphological changes with an inverted microscope. Lactate dehydrogenase activity was measured according to Glascott et al. by monitoring the oxidation of NADH at 340 nm in the presence of pyruvate [41].

Protein levels were determined by the Lowry method, using BSA as a standard [42].

2.4. Assay of hormone concentration

A progesterone EIA kit (Cayman Company) was used to quantitate progesterone in the culture medium. Human chorionic gonadotropin was quantitated using a solid phase immUnoassay kit purchased from Wako Pure Chemicals.

3. Results

3.1. TCL-1 cells possess functional peroxisomes

Functional peroxisomes in TCL-1 cells were demonstrated by content in active peroxisomal marker enzymes (fatty acyl-CoA oxidase and catalase) and their recovery (about 80%) in the particulate fraction. After incubation of TCL-1 cells for 3 days, the activity of both enzymes was clearly detectable in the cells, and increased dependent on the concentration of cell protein. Activity levels of the fatty acyl-CoA oxidase and catalase of the trophoblast cells was 0.265 mU/mg protein and 1.35 U/mg protein, respectively.

3.2. Clofibric acid and gemfibrozil inhibit cell growth rates in a dose-dependent manner

First, we studied the effect of PPARα agonists, clofibric acid and gemfibrozil, on the proliferation of the cells. The immortalized cells were cultured with or without the agents for 4 days. The cells were harvested at the time indicated in Fig. 1, and the DNA and protein content was then determined. Figure 1 A (1) shows the time courses of the changes in DNA content at various concentrations of the agents. The DNA content of control cells gradually increased during incubation for 4 days, indicating normal growth of the cells. Gemfibrozil at not less than 0.25 mM

suppressed the rate of increase in DNA content as compared to the control. Clofibrate at not less than 2.5 mM clearly lowered the rate. However, even at the highest concentrations of both agents, the DNA content on day 4 was higher than that on the first day.

Figure 1 A (2) illustrates the effect of the concentrations of the agents on DNA content 3 or 4 days after incubation. All values are relative to the control. The content after treatment with 0.25 and 0.35 mM gemfibrozil for 3 days was about 71.1 and 57.3 % of the control, respectively. On the other hand, that after treatment with 2.5 mM clofibric acid for 3 days was approximately 47.2% of the control. After incubation for 4 days, the DNA content after treatment with 0.25 and 0.35 mM gemfibrozil was significantly decreased to about 61.7 and 52.1% of the control, respectively. The content after treatment with 2.5 mM clofibric acid was about 42.3% of the control.

Figure 1 B (1) shows the time courses of the changes in protein content at various concentrations of the agents. The protein content of control cells increased in a time-dependent manner during 4 days. As it did the DNA content, gemfibrozil at not less than 0.25 mM decreased the rate of increase in protein content as compared to the control. Clofibrate at not less than 2.5 mM clearly decreased the rate.

Figure 1 B (2) illustrates the concentration dependency of gemfibrozil and clofibric acid on protein content after incubation for 3 or 4 days. All values are relative to the control. The content after treatment with 0.25 and 0.35 mM gemfibrozil for 3 days was about 70.1 and 66.4 % of the control value, respectively. On the other hand, that after treatment with 2.5 mM gemfibrozil for 3 days was about

51.2% of the control. The protein content after treatment with 0.25 and 0.35 mM gemfibrozil for 4 days was significantly decreased to about 68.2 and 57.4%, respectively. The content after treatment with 2.5 mM clofibric acid was approximately 48.5% of the control.

Fig. 1

Protein content was decreased at the same concentrations of the agents as DNA content. These results suggest that gemfibrozil and clofibric acid beyond particular concentrations suppress the proliferation of immortalized human trophoblast cells. Matsuo and Strauss reported that numbers of JEG-3 choriocarcinoma cells which are derived from syncytiotrophoblasts were 52% of control values, after 3 days in culture in the presence of 3 mM clofibric acid. The diminished cell number was not due to syncytium formation or cell death, as there was no evidence of abnormal cell morphology or an increase in cell detachment. Moreover, the effects were reversible, as cell growth returned to normal after removal of the drug [21]. In the present study, we used clofibric acid at concentrations lower than 3 mM, and could detect neither abnormal cell morphology nor cell death under an inverted microscope. Therefore, clofibric acid may suppress the proliferation of immortalized TCL-1 cells as it does JEG-3 choliocarcinoma cells.

3.3. Clofibric acid and gemfibrozil affect marker enzyme activities from several subcellular compartments

The ability of the fibrate compounds to influence cell metabolism has been assessed in TCL-1 cellls by studying drug impact on markers from subcellular compartments including peroxisomes (fatty acyl-CoA oxidase and catalase), lysosomes (acid phosphatase) and microsomes (esterase). Figure 2 gives an overview of drug-induced changes in marker enzyme activities. Significant drug-induced changes in fatty acyl-CoA oxidase included at the third incubation day a 1.53 increase by 0.05 mM gemfibrozil, 1.93 and 1.96 stimulation by 1.0 and 2.5 mM clofibric acid respectively. At the incubation 4, the peroxisomal oxidase was significantly increased by 0.25 mM clofibric acid. Significant drug-induced changes in catalase included at the third incubation day a 1.23 increase by 0.05 mM gemfibrozil, 1.30 and 1.34 stimulation by 0.25 and 1.0 mM clofibric acid respectively. The activity of acid phosphatase tended to increase by both agents, but that of esterase hardly changed,

Fig. 2

3.4. Clofibric acid and gemfibrozil up-regulate progesterone and down-regulate hCG secreted by TCL-1 cells

The ability of gemfibrozil and clofibric acid to affect hormone secretion has been assessed in TCL-1 cellls by studying drug impact on progesterone and hCG. Significant drug-induced changes in progesterone secretion included at the third incubation day a 1.48 increase by 0.25 mM gemfibrozil, 1.75 and 3.04 stimulation by 1.0 and 2.5 mM clofibric acid respectively. At the incubation 4, significant drug-induced changes in progesterone secretion included 1.62 and 2.4 stimulation by 1.0 and 2.5 mM clofibric acid respectively (Fig. 3 (1)). Significant drug-induced changes in hCG secretion included at the third incubation day 0.745 and 0.505 decrease by 0.35 mM gemfibrozil and 2.5 mM clofibric acid, respectively. At the incubation 4, significant drug-induced changes in hCG secretion included 0.762 and 0.681 decrease by 0.25 and 0.35 mM gemfibrozil, 0.641 and 0.435 inhibition by 1.0 and 2.5 mM clofibric acid, respectively (Fig. 3 (2)).

As illustrated in Figure 3 (2), increasing doses of drugs gradually depress TCL-1 cell hCG secretion with, at the third incubation day, significant decreases in hormone concentration amounting to 25.5% and 49.5% upon 0.35 mM gemfibrozil and 2.5 mM clofibrate, respectively. At incubation day 4, increasing doses of drugs depress hCG secretion with, significant decreases in hormone concentration amounting to 23.8% and 31.9% upon 0.25 and 0.35 mM gemfibrozil, 35.9% and 56.5% upon 1.0 mM and 2.5 mM clofibric acid, respectively.

Fig. 3

3.5. Clofibric acid and gemfibrozil depress TCL-1 cell lactate dehydrogenase and do not exhibit cytotoxicity

We biochemically studied the cytotoxity of gemfibrozil and clofibric acid in trophoblast cells by measuring the activity of a marker enzyme, lactate dehydrogenase, in the cells and medium. As illustrated in Figure 4 (1), increasing doses of drugs gradually depress TCL-1 cell lactate dehydrogenase with, at incubation day 4, significant decreases in enzyme activity amounting to 31.3% and 46.6% upon 0.35 mM gemfibrozil and 2.5 mM clofibrate, respectively. At drug dose and incubation day tested, the compounds did not exhibit cytotoxic lysis as attested by the absence of significant increases induced in cell media lactate dehydrogenase (Figure 4 (2)).

Fig. 4

4. Discussion

Since human extravillous trophoblast cells are difficult to obtain, there are fewer reports about these cells than villous trophoblast cells. Osumi et al. reported that rat hepatoma cells become larger by treatment with ciprofibrate [4]. In the present study, at the higher concentrations of clofibric acid and gemfibrozil the relative amount of protein was suppressed at almost the same rate as the relative amount of DNA (Fig. 1). Therefore, differing from rat hepatoma cells, TCL-1 cells treated with the PPARα agonists might not become larger than control cells.

In our previous report, the protein content of rat hepatic cells was not decreased by the treatment with 0.25 mM gemfibrozil for 3 days [13]. However, the present data clearly showed that 0.25 mM gemfibrozil suppresses the proliferation of the cells (Fig. 1). These results suggest that trophoblast cells are more susceptible to gemfibrozil than rat hepatic cells.

Clofibrate is reported to induce apoptosis in cultured human hepatocytes [12]. Goll et al. reported that apoptosis was easily induced by peroxisome proliferators in confluent cells of human hepatic carcinoma cells, HepG2 cells, but not in growing cells [14, 16]. Canute et al. reported that apoptosis induced by clofibrate in HepG2 cells is not connected to the activation of PPARα [43]. Matsuo and Strauss reported that clofibric acid suppresses proliferation of JEG-3 choliocarcinoma cells by

stimulating p53 (tumor suppressor) expression through PPAR [21]. It is known that p53 protein which induces apoptosis also blocks cell cycle progression in the G1 phase. Gemfibrozil and clofibric acid probably block cell cycle progression of TCL-1 cells by stimulating p53 expression, and suppress the proliferation. However, further study is needed for clarification of this point.

This is the first report which proposes the existence of peroxisomes in human extravillous trophoblast cells. Fatty acyl-CoA oxidase and catalase activities were increased by the treatment with gemfibrozil and clofibric acid (Fig. 2). In human liver cells, some reports indicate an increase in peroxisomal enzyme activity by peroxisomal proiliferators [5, 6, 10, 12, 19], but others do not [2, 3, 8, 9, 14]. Our data support the former. However, because of the restricted extent of induction by gemfibrozil and clofibric acid, the absolute activation of PPARα is yet unclear. In the other reports, in order to study the effects of PPARα agonist on peroxisomal enzymes in human cells, clofibric acid and/or ciprofibrate were mostly used [2, 3, 5, 6, 8-10, 12, 14, 19], and so the effect of gemfibrozil was first studied this time.

It is reported that apoptosis was easily induced by peroxisome proliferators in confluent cells of HepG2 cells [16]. In the present study, after incubation for 4 days the cell population was nearly confluent. Therefore, some changes possibly occurred in the cells on day 4, and so a significant increase of peroxisome marker enzyme activities was rarely detected.

The activity of acid phosphatase tended to increase by gemfibrozil and clofibric acid (Fig. 2 (3)), but that of esterase hardly changed (Fig. 2 (4)), suggesting that the agents somewhat affect lysosomes, but not microsomes. The increase in peroxisome enzyme activities and no change in microsome enzyme activitiy suggest that the increase in the specific activity of peroxisome enzymes was not caused by the decrease in protein content.

Matsuo and Strauss reported that the decrease in progesterone secretion by 3 mM clofibric acid depends on reduced cell growth of JEG-3 cells [21]. In the present study, 1 mM clofibric acid did not reduce the cell growth (Fig. 1), but increased progesterone secretion (Fig. 3 (1)). These results indicate that even at higher concentrations of the agent, the increase in progesterone secretion is caused by the agents. It was also reported that levels of mRNA encoding the key steroidogenic enzyme, cytochrome P450scc, were modestly increased by 3 mM clofibric acid in JEG-3 cells [21], indicating that these findings are compatible with our result of the increase in progesterone secretion.

Secretion of hCG was decreased by gemfibrozil and clofibric acid (Fig. 3 (2)). Matsuo and Strauss reported that 3 mM clofibric acid suppresses the mRNA expression of hCG in JEG-3 cells [21]. The decrease of hCG secretion in TCL-1 cells may be caused by the same mechanism as in JEG-3 cells. Maruo et al. reported that progesterone selectively suppresses the production and secretion of hCG

by normal placenta [44]. The intra-regulatory relationship between steroid and protein hormones in the placenta may be also related to the decrease of hCG secretion.

The percent activity of lactate dehydrogenase in the cells was large and remained at about 95% even at the highest concentrations of gemfibrozil and clofibric acid (Fig. 4), indicating that the increase in progesterone secretion from the cells treated with the agents does not depend on cell damage.

Diseases of peroxisome function including adrenoleukodystrophy and neonatal adrenoleukodystrophy, are associated with deficits in steroidogenesis [45]. In addition, it is reported that many data pointed out the important role of peroxisomes in steroid biosynthesis [46]. We considered a possible mechanistic link between peroxisomes and progesterone synthesis as follows. Peroxisomes participate in fatty acid β-oxidation and mevalonate pathway [47]. Acetyl-CoA derived from β-oxidation may be used for cholesterol synthesis in peroxisomes. Cytochrome $P450_{scc}$ is localized in mitochondria and catalyzes the pathway from cholesterol to pregnenolone. Progesterone may be synthesized from pregnenolone in mitochondria/microsomes [48, 49]. Peroxisomal sterol carrier protein 2 may participate in the transport of steroid such as cholesterol [27-29]. If somewhere on these pathways is stimulated by PPARα agonist, progesterone biosynthesis/secretion is possibly increased.

In conclusion, PPARα agonists gemfibrozil and clofibric acid inhibit the proliferation of trophoblast cells. Cell metabolism is probably affected by both agents. Gemfibrozil and clofibric acid may enhance the secretion of progesterone from the cells, but suppress that of hCG. Gemfibrozil at lower concentrations than clofibric acid affects these cellular functions. Therefore, immortalized human trophoblast cells (TCL-1) seem to be useful for investigating changes in the functions of human cells caused by PPARα agonists.

Figure legends

Fig. 1. Changes in DNA and protein content of human trophoblast cells treated with gemfibrozil and clofibric acid. Drug treatment was initiated 24 hr after plating (12 x 10^5 cells/plate) by changing the culture medium to one containing gemfibro**z**il or clofibric acid, and the medium was then changed every 24 hr. (1) Time course of change in the DNA and protein content. The cells were harvested at the times indicated. The DNA and protein content of cells was determined as described in the text. Data are mean values of 5 experiments. (2) Changes in DNA and protein content relative to the control after 3 (●) or 4 (○) days of incubation. The DNA and protein content (mg/plate) was expressed as a value relative to the control (0 mM). Data are the mean ± SD of 5 experiments. * indicates significant difference (*, $p<0.05$).

Fig. 2. Effects of gemfibrozil and clofibric acid on enzyme activity of trophoblast cells after 3 or 4 days of incubation. After incubation with various concentrations of gemfibrozil and clofibric acid for 3 (●) or 4 (○) days, the cells were harvested. The fatty acyl-CoA oxidase (1), catalase (2), acid phosphatase (3) and esterase (4) activity of the cells was measured as described in the text. The specific activity (u or mU/mg protein) was expressed relative to the control (0 mM) at each concentration of the agents. Data are the mean ± SD of 5-7 experiments. * indicates significant difference (*, $p<0.05$). The fatty acyl-CoA oxidase activity of control cells at 3 and 4 days was 0.265 ± 0.138 and 0.282 ± 0.117 mU/mg protein, respectively. The catalase activity of control cells at 3 and 4 days was 1.35 ± 0.35 and 1.79 ± 0.44 U/mg protein, respectively. The acid phosphatase activity of control cells at 3 and 4 days was 16.9 ± 3.5 and 16.7 ± 2.9 mU/mg protein, respectively. The esterase activity of control cells at 3 and 4 days was 71.7 ± 12.8 and 105.3 ± 13.0 U/mg protein, respectively.

Fig. 3. Influence of gemfibrozil and clofibric acid on levels of progesterone and hCG secreted from trophoblast cells. After incubation with various concentrations of gemfibrozil and clofibric acid for 3 (●) or 4 (○) days, the medium was collected. The progesterone (1) and hCG (2) concentration was measured as described in the text. The amounts of progesterone and hCG per protein (pg/mg protein/24 h) were expressed as values relative to the control (0 mM) at each concentration of the agents. Data are the mean ± SD of 5 experiments. * indicates significant difference (*, $p<0.05$). The concentration of progesterone secreted from the control cells at 3 and 4 days was 85.7 ± 36.3 and 88.3 ± 32.5 pg/mg protein/24 h, respectively. The concentration of hCG secreted from control cells at 3 and 4 days was 16.7 ± 3.8 and 27.5 ± 5.1 mIU/mg protein/24 h, respectively.

Fig. 4. Cytotoxic effects of gemfibrozil and clofibric acid on trophoblast cells after 3 or 4 days of incubation. After incubation with various concentrations of gemfibrozil and clofibric acid for 3 (●) or 4 (○) days, the medium and cells were harvested. The lactate dehydrogenase activity of the cells and medium was measured as described in the text. (1) The specific activity (U/mg protein) was expressed relative to the control (0 mM) at each concentration of the agents. Data are the mean ± SD of 6 or 7 experiments. * indicates significant difference (*, $p<0.05$). The lactate dehydrogenase activity of control cells at 3 and 4 days was 3.46 ± 1.45 and 4.63 ± 0.97 U/mg protein, respectively. (2) Effects of gemfibrozil and clofibric acid on the percentage of total activity of lactate dehydrogenase in the trophoblast cells and in the medium. Opened bars show the percentage of enzyme activity in the cells, and closed bars show that in the medium.

References

[1] Butterworth BE, Smith-Oliver T, Earle L, Loury DJ, White RD, Doolittle DJ, Working PK, Cattley RC, Jirtle R, Michalopoulos G, Strom S. Use of primary cultures of human hepatocytes in toxicology studies. Cancer Res 1989;49:1075-1084.

[2] Bichet N, Cahard D, Fabre G, Remandet B, Gouy D, Cano J-P. Toxicological studies on a benzofuran derivative. III. Comparison of peroxisome proliferation in rat and human hepatocytes in primary culture. Toxcol Appl Pharmacol 1990;106:509-517.

[3] Blaauboer BJ, van Holsteijn CWM, Bleumink R, Mennes WC, van Pert FNAM, Yap SH, van Pelt JF, van Iersel AAJ. The effect of beclobric acid and clofibric acid on peroxisomal β-oxidation and peroxisome proliferation in primary cultures of rat, monkey and human hepatocytes. Biochem Pharmacol 1990;40:521-528.

[4] Osumi T, Yokota S, Hashimoto T. Proliferation of peroxisomes and induction of peroxisomal β-oxidation enzymes in rat hepatoma H4IIEC3 by ciprofibrte. J Biochem 1990;108:614-621.

[5] Brocard C, E-Souni M, Ramirez LC, Latruffe N, Bournot P. Stimulation of peroxisomal palmitoyl-CoA oxidase activity by ciprofibrate in hepatic cell lines:

Comparative studies in Fao, MH_1C_1 and HepG2 cells. Biol Cell 1993;77:37-41.

[6] Scotto C, Keller JM, Schohn H, Dauca M. Comparative effects of clofibrate on peroxisomal enzymes of human (Hep EBNA2) and rat (FaO) hepatoma cell lines. Eur J Cell Biol 1995;66:375-381.

[7] Hashimoto F, Ishikawa T, Hamada S, Hayashi H. Effect of gemfibrozil on lipid biosynthesis from acetyl-CoA derived from peroxisomal β-oxidation. Biochem Pharmacol 1995;49:1213-1221.

[8] Richert L, Price S, Chesne C, Maita K, Calmichael N. Comparison of the induction of hepatic peroxisome proliferation by the herbicide oxadiazon in vivo in rats, mice, and dogs and in vitro in rat and human hepatocytes. Toxicol Appl Pharmacol 1996;141:35-43.

[9] Elcombe CR, Bell DR, Elias E, Hasmall SC, Plant NJ. Peroxisome proliferators: Specific differences in response of primary hepatocyte cultures. Ann N Y Acad Sci 1996;804:628-635.

[10] Duclos S, Bride J, Ramirez LC, Bournot P. Peroxisome proliferation and β-oxidation in Fao and MH_1C_1 rat hepatoma cells, HepG2 human hepatoblastoma cells and cultured human hepatocytes: effect of ciprofibrate. Eur. J Cell Biol 1997;72:314-323.

[11] Hashimoto F, Hamada S and Hayashi H. Effect of gemfibrozil on centrifugal behavior of rat peroxisomes and activities of peroxisomal enzymes involved in lipid

metabolism. Biol Pharm Bull 1997;20:315-321

[12] Perrone CE, Shao L, Williams GM. Effect of rodent hepatocarcinogenic peroxisome proliferators on fatty acyl-CoA oxidase, DNA synthesis, and apoptosis in cultured human and rat hepatocytes. Toxicol Appl Pharmacol 1998;150:277-286.

[13] Hashimoto F, Taira S, Hayashi H. Comparison of the effects of gemfibrozil and clofibric acid on peroxisomal enzymes and cholesterol synthesis of rat hepatocytes. Biol Pharm Bull 1998;21:1142-1147.

[14] Goll V, Alexandre E, Viollon-Abadie C, Nicod L, Jaeck D, Richert L. Comparison of the effects of various peroxisome proliferators on peroxisomal enzyme activities, DNA synthesis, and apoptosis in rat and human hepatocyte cultures. Toxicol Appl Pharmacol 1999;160:21-32

[15] Hashimoto F, Taira S, Hayashi H. Changes in isoprenoid lipid synthesis by gemfibrozil and clofibric acid in rat hepatocytes. Biochem Pharmacol 2000;59:1203-1210

[16] Goll V, Viollon-Abadie C, Nicod L, Richert L. Peroxisome proliferators induce apoptosis and decrease DNA synthesis in hepatoma cell lines. Hum Exp Toxicol 2000;19:193-202

[17] Nemali MR, Usuda N, Reddy MK, Oyasu K, Hashimoto T, Osumi T, Rao MS, Reddy JK. Comparison of constitutive and inducible levels of expression of peroxisomal β-oxidation and catalase genes in liver and extrahepatic tissues of rat.

Canc Res 1988;48:5316-5324.

[18] Mendis-Handagaya SMLC, Watkins PA, Gelber SJ, Scallen TJ, Zirkin BR, Ewing LL. Luteinizing hormone causes rapid and transient changes in rat leydig cell peroxisome volume and intraperoxisomal sterol carrier protein-2 content. Endocrinology 1990;127:2947-2954.

[19] Chance DS, Wu S-M, McIntosh MK. Inverse relationship between peroxisomal and mitochondrial β-oxidation in HepG2 cells treated with dehydroepiandrosterone and clofibric acid. P Soc Exp Biol Med 1995;208:378-384

[20] King BF. Morphologic and cytochemical identification of microperoxisomes in human and non-human primate placental trophoblast. Histchemistry 1982;74:115-121

[21] Matsuo H, Strauss JF III. Peroxisome proliferators and retinoids affect JEG-3 choliocarcinoma cell function. Endocrinology 1994;135:1135-1144.

[22] Watson AL, Skepper JN, Jauniaux E, Burton GJ. Changes in concentration, localization and activity of catalase within the human placenta during early gestation. Placenta 1998;19:27-34.

[23] Cohen BI, Raicht RF, Shefer S, Mosbach H. Effects of clofibrate on sterol metabolism in the rat. Biochim Biophys Acta 1974;369:79-85.

[24] Hayashi H, Takahata S. Role of peroxisomal fatty acyl-CoA β-oxidation in phospholipids biosynthesis. Arch Biochem Biophys 1991;284:326-331.

[25] Shiota Y, Ikeda M, Hashimoto F, Hayashi H. Effects of peroxisome proliferators gemfibrozil and clofibrate on syntheses of dolichol and cholesterol in rat liver. J Biochem 2003;134:197-202.

[26] Kovacs WJ, Olivier LM, Krisans SK. Central role of peroxisomes in isoprenoid biosynthesis. Prog Lipid Res 2002;41:369-391.

[27] Vahouny GV, Canderbhan R, Noland BJ, Irwin D, Deniss P, Lambeth JD, Scallen TJ. Sterol carrier protein$_2$ Identification of adrenal sterol carrier protein$_2$ and site of action for mitochondrial cholesterol utilization. J Biol Chem 1983;258:11731-11737.

[28] Vahouny GV, Dennis P, Chanderbhan R, FiskU/mg, Noland BJ, Scallen TJ. Sterol carrier protein$_2$ (SCP$_2$)-mediated transfer of cholesterol to mitochondrial inner membranes. Biochem Biophys Res CommUn 1984;122:509-515.

[29] Pfeifer SM, Furth EE, Ohba T, Chang YJ,Rennert H, Sakuragi N, Billheimer JT, Straus III JF. Sterol carrier protein 2: A role in steroid hormone synthesis? 1993;47:167-172.

[30] Tsuneoka M, Yamamoto A, Fujiki Y, Tashiro Y. Nonspecific lipid transfer protein (sterol carrier protein-2) is located in rat liver peroxisomes, J Biochem 1988;104:560-564.

[31] Keller GA, Scallen TJ, Clarke D, Maher PA, Krisans SK, Singer SJ. Subcellular localization of sterol carrier protein-2 in rat hepatocytes: Its primary

localization to peroxisomes. J Cell Biol. 1989;108:1353-1361.

[32] van Amerongen A, van Noort M, van Beckhoven JR, Rommerts FF, Orly J, Wirtz KWA. The subcellular distribution of the nonspecific lipid transfer protein (sterol carrier protein 2) in rat liver and adrenal gland. Biochim Biophy. Acta 1989;1001:243-248.

[33] Liu RCM, Hahn C, Hurtt ME. The direct effect of hepatic peroxisome proliferators on rat leydig cell function in vitro. Fund Appl Toxcol 1996;30:102-108.

[34] Gazouli M, Yao Z-X, Boujrad N, Corton JC, Culty M, Papadopoulos V. Effect of peroxisome proliferators on leydig cell peripheral-type benzodiazepine receptor gene expression, hormone-stimulated cholesterol transport, and steroidogenesis: Role of the peroxisome proliferator-activator receptor α. Endocrinology 2002;143:2571-2583.

[35] Lewis MP, Clements M, Takeda S, Kirby PL, Seki H, Lonsdale LB, Sullivan MHF, Elder MG, White JO. Partial characterization of an immortalized human trophoblast cell-line, TCL-1, which possesses a CSF-1 autocrineloop. Placenta 1996;17:137-146.

[36] Small GM, Burdett K, Connock MJ. A sensitive spectrophotometric assay for peroxisomal acyl-CoA oxidase. Biochem J 1985;227:205-210

[37] Osumi T, Hashimoto T. Acyl-CoA oxidase of rat liver: A new enzyme for

fatty acid oxidation. Biochem Biophys Res Commun 1978;83:479-485.

[38] Leighton F, Poole B, Beaufay H, Baudhuim P, Coffey L W, Fowler S, de Duve C. The large scale-separation of peroxisomes, mitochondria and lysosomes from the livers of rats injected with TritonWR-1339. J Cell Biol 1968; 37: 482-513.

[39] Hayashi H, Suga T. Some characteristics of peroxisomes in the swlime mold, *Dictyostelium discoideum*. J Biochem 1978;84:513-520.

[40] Beaufay H., Amar-Costesec A, Feytmans E, Thines-Sempoux D, Wibo M, Berthet T. Analytical study of microsomes and isolated subcellular membranes from rat lover. J Cell Biol 1974;61:188-200.

[41] Glascott Jr PA, Gilfor E, Farber JL. Effects of vitamin E on the killing of cultured hepatocytes by *tert*-butyl hydroperoxide. Mol Pharmacol 1992;41:1155-1162.

[42] Lowry OH, Rosebrongh NJ, Farr A L, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-275.

[43] Canute RA, Muzio G, Bonelli G, Maggiora M, Autelli R, Barbiero G, Costelli P, Brossa O, Baccino FM. Peroxisome proliferators induce apoptosis in hepatoma cells. Canc Detec Prev 1998;22:357-366

[44] Maruo T, Matsuo HJG, Ohtani T, Hoshina M, Mochizuki M. Differential modulation of chorionic gonadotrolpin (CG) subunit messenger ribonucleic acid levels and CG secretion by progesterone in normal placenta and choriocarcinoma

cultured in vitro. Endocrinology 1986;119:855-864.

[45] Suzuki Y, Yamaguchi S, Orii T, Tsuneoka M, Tashiro Y. Nonspecific lipid transfer protein (sterol carrier protein 2) defective in patients with deficient peroxisomes. Cell Struct Funct 1991;15:301-308.

[46] Magalhaes MM, Magalhaes MC. Peroxisomes in adrenal steroidogenesis. Microsc Res Tech 1997;36:493-502.

[47] Aboushadi N, Engfelt WH, Paton VG, Krisans SK. Role of peroxisomes in isoprenoid biosynthesis. Hystechem Cytochem 1999;47:1127-1132.

[48] Cherradi N, Defaye G, Chambaz EM. Characterization of the 3β-hydroxysteroid dehydrogenase activity associated with bovine adrenocortical mitochondria. Endocrinology 1994;134:1358-1364.

[49] Sauer LA, Chapman JG, Dauchy RT. Topology of the 3β-hydroxy-5-en-steroid dehydrogenase/Δ^5-Δ^4-isomerase in adrenal cortex mitochondraia and microsomes□. Endocrinology 1994;134:751-759.

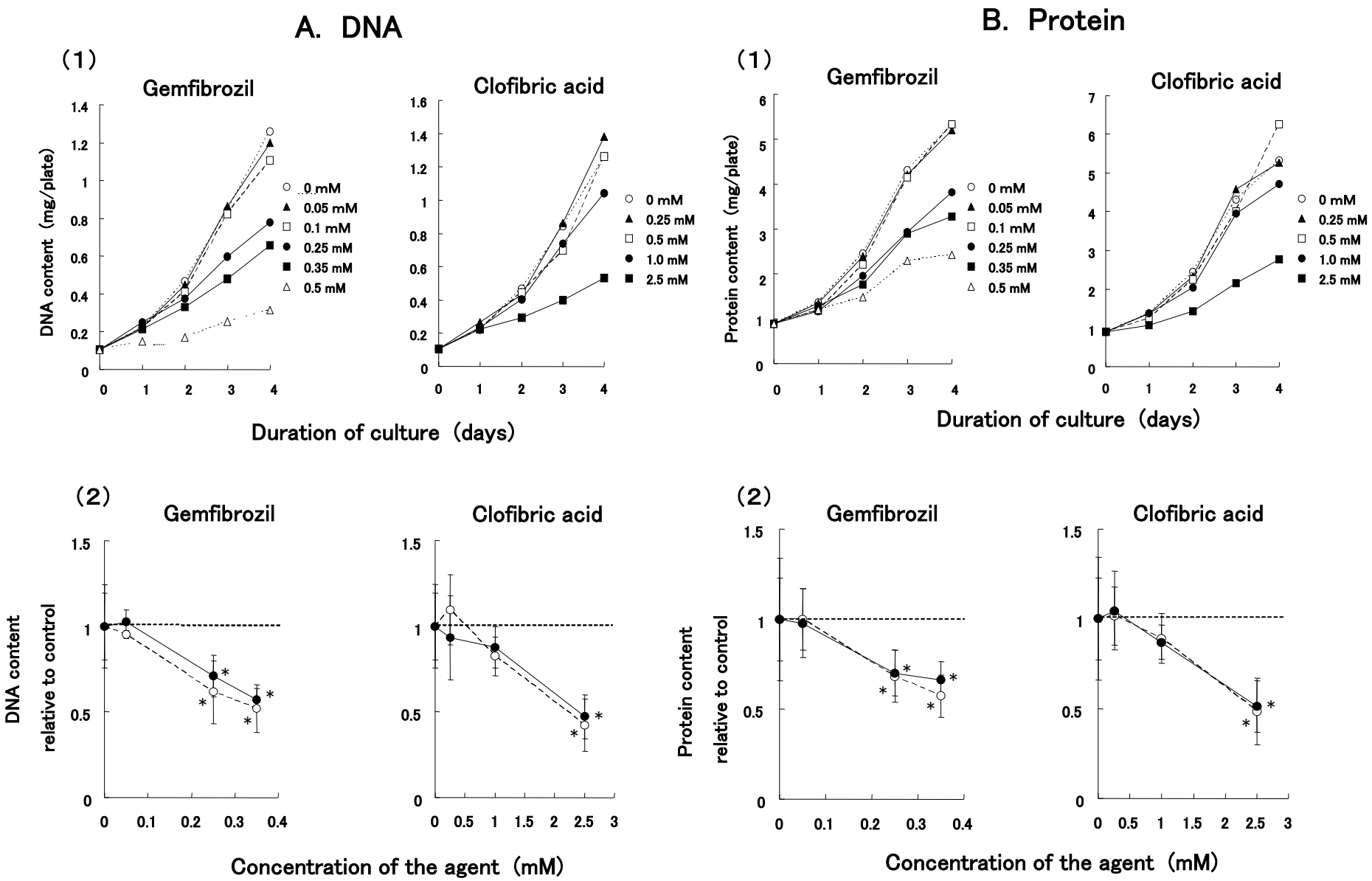

Fig.1 F. Hashimoto et al.

Fig. 2 → 4th page

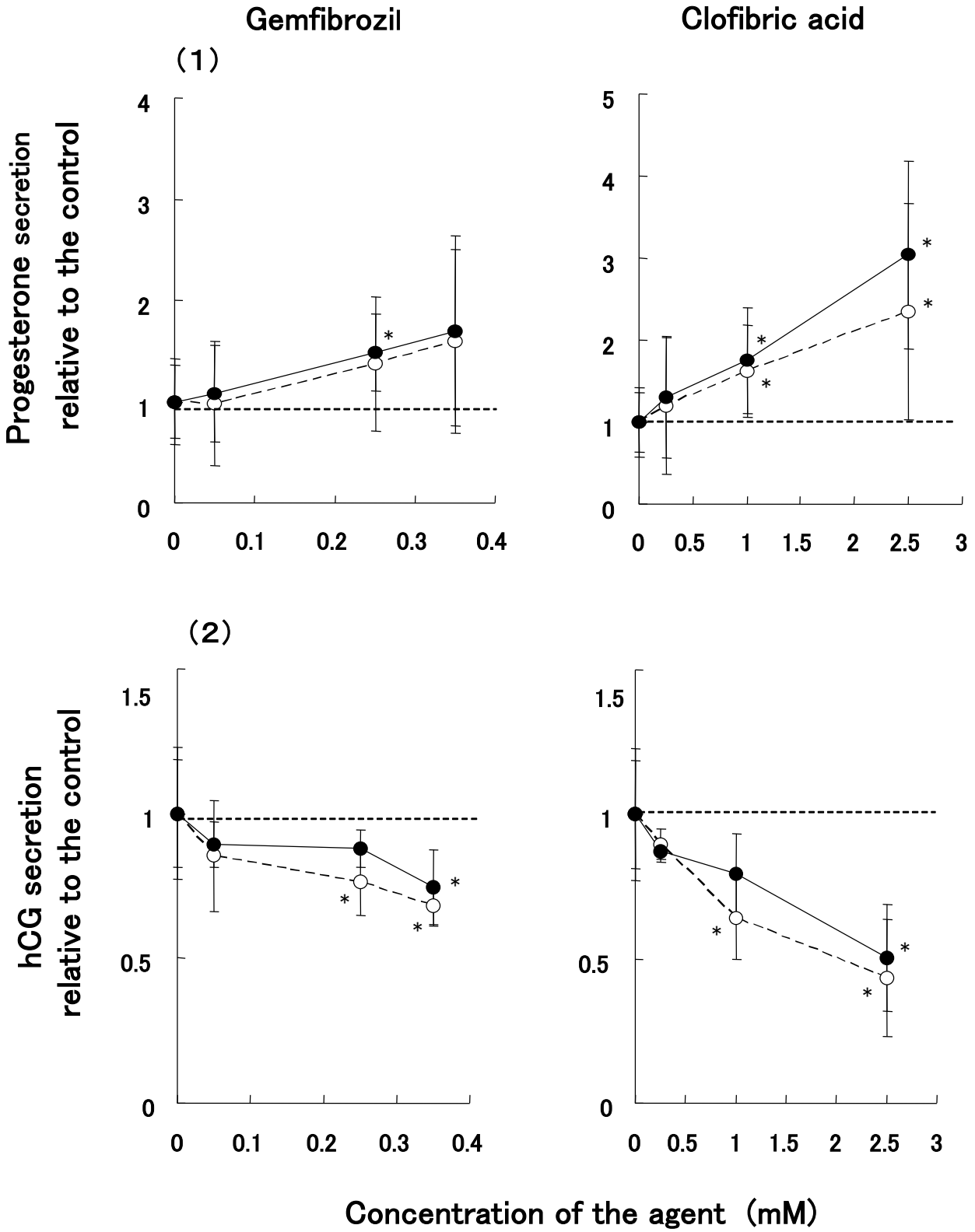

Fig. 3 F. Hashimoto et al.

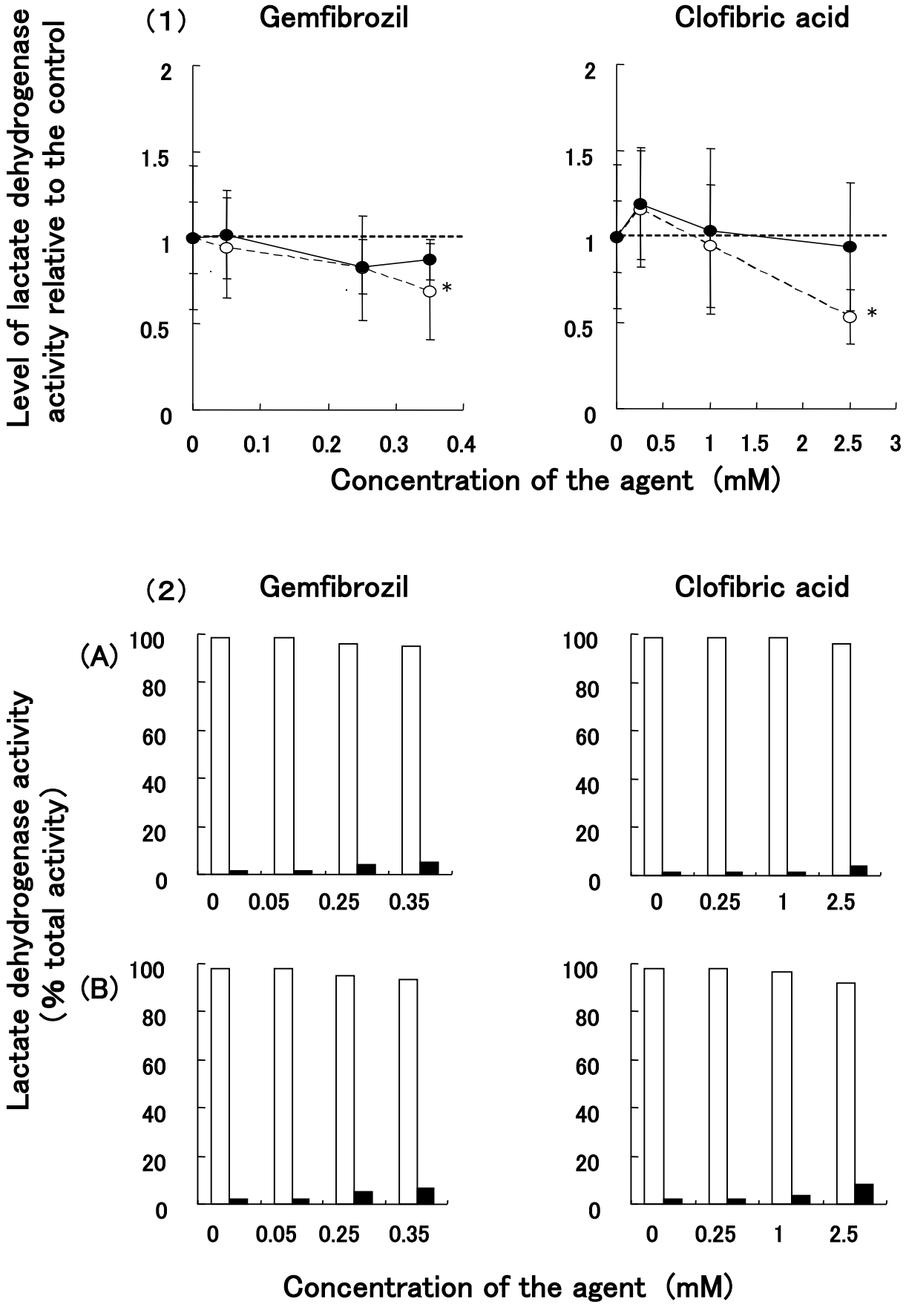

Fig. 4 F. Hashimoto at al.

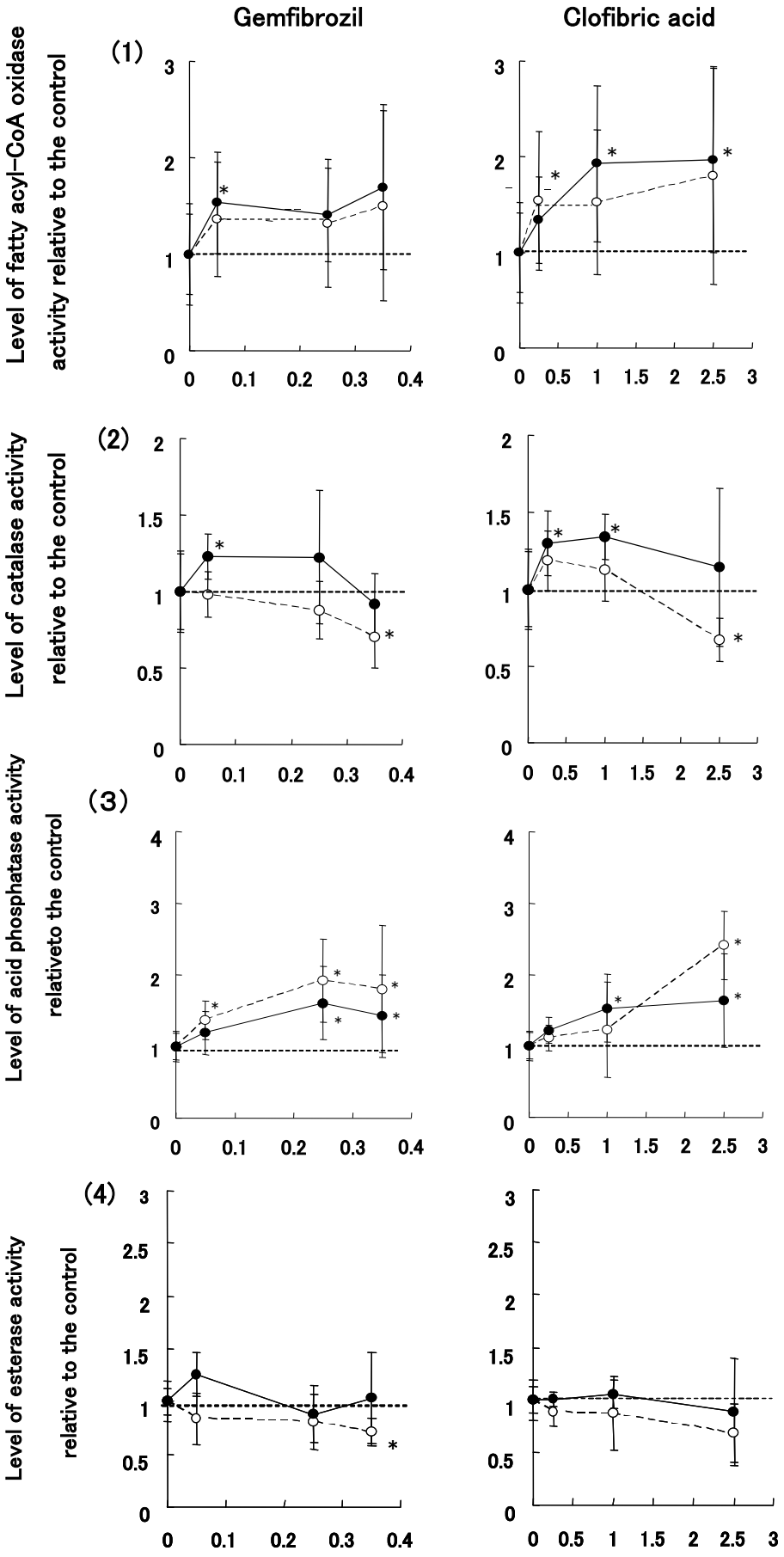

Fig. 2 F. Hashimoto et al.