Effects of WY-14643 on Peroxisomal Enzyme Activity and Hormone Secretion in Immortalized Human Trophoblast Cells

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In our previous report, clofibric acid increased both the enzyme activities of peroxisomes (catalase and fatty acyl-CoA oxidase) and the secretion of progesterone in immortalized human extravillous trophoblast cells (TCL-1) (F. Hashimoto *et al.*, *Biochem. Pharm.*, 68, 313 (2004)). WY-14643 is reported to be stronger inducer of peroxisomes in rodents than clofibric acid. Therefore, the effects of WY-14643 on the activities of peroxisomal enzymes and hormone secretion in TCL-1 were studied. After incubation for 3 d with WY-14643, WY-14643 (≧0.15 mM) suppressed the rate of increase in DNA and protein. The specific activities of catalase were increased by 0.1 mM WY-14643. The specific activities of fatty acyl-CoA oxidase were hardly changed by WY-14643. The concentration of progesterone in the medium was increased by 0.1 mM WY-14643, but human chorionic gonadotropin was decreased by 0.2 mM WY-14643. After a discontinuous Nycodenz-density gradient centrifugation of the light mitochondrial fraction of the cells, catalase activity was distributed in lower density fractions than cytochrome-*c* oxidase (a mitochondria marker enzyme) activity, but the distribution was not changed by WY-14643. These results suggest that WY-14643 inhibits the proliferation of trophoblast cells. The density of peroxisomes in human trophoblast cells is lower than that of mitochondria, and it is not affected by WY-14643. WY-14643 may increase the progesterone secretion. Effects of WY-14643 on metabolism of human trophoblast cells are different from those of clofibric acid.

Key words WY-14643; clofibric acid; peroxisome; trophoblast; progesterone

There are three kinds of trophoblasts in the placenta: cytotrophoblasts, syncytiotrophoblasts, and extravillous trophoblasts. Each of the three plays an important role in the development and maintenance of gestation. In human trophoblasts, peroxisomes are known to be present in cytotrophoblast cells[1,2] and are detected later during gestation in syncytiotrophoblast cells.[2] Extravillous trophoblast cells are difficult to obtain; therefore, the peroxisomes in these cells have not been elucidated.

We detected catalase and fatty acyl-CoA oxidase activity (marker enzymes of peroxisomes) in the homogenate of extravillous trophoblast (TCL-1) cells.[3] Using electron microscopy and immunoelectron microscopy, peroxisomes were detected in the cells. The size and number of peroxisomes in the trophoblast cells were smaller than those in the rat liver. Namely, microperoxisomes, which have a density smaller than those of rat hepatic peroxisomes, exist in human extravillous trophoblast cells.[4]

Clofibric acid and gemfibrozil (fibric acid derivative) are not only a hypolipidemic drug, but also a peroxisome proliferator-activated receptor (PPAR)α agonist in rodents.[5—7] However, it is unclear whether PPARα agonists affect peroxisomal enzyme activity in human liver cells. In all organs except the liver, the effects of PPARα agonists on peroxisomes are rarely reported in rats,[8] and even less so in humans.[9] We reported that gemfibrozil activates the peroxisomal fatty acid β-oxidation system of the rat liver.[10] Furthermore, we reported that the enzymes that compose the peroxisomal fatty acid β-oxidation system are activated by gemfibrozil.[11]

Previously, we reported that clofibric acid and gemfibrozil increase the catalase activity of human extravillous trophoblast cells.[3] In the preceding paper, we clarified that clofibric acid induces the proliferation of trophoblast cell microperoxisomes.[4]

Rat liver cells are useful for experiment of peroxisomes, but they do not secrete hormone. However, trophoblast cells secrete both steroid hormone and protein hormone. We reported that progesterone secretion from TCL-1 is stimulated by clofibric acid.[3] Steroid hormone is synthesized from cholesterol. Peroxisomes take part in cholesterol synthesis.[12] Peroxisomal sterol carrier protein 2 may participate in the transport of steroid such as cholesterol.[13—16] Therefore, we speculate that peroxisomes in trophoblast cells participate in synthesis and/or transport of steroid hormone. In order to study these points, we need a large number of purified normal peroxisomes of TCL-1.

The structure of WY-14643 (a derivative of [2-pyrimidinylthio]acetic acid) is different from that of fibrate derivatives such as gemfibrozil and clofibric acid. WY-14643 is not only a hypolipidemic drug, but is also a PPARα agonist.[17—19] WY-14643 is reported to be stronger inducer of peroxisomes in rodents than clofibric acid.[20,21] Therefore, we expected that WY-14643 would proliferate peroxisomes more strongly than clofibric acid. In the present study, we studied the effects of WY-14643 on peroxisomal enzyme activity and hormone secretion in immortalized human trophoblast cells.

MATERIALS AND METHODS

Materials The WY-14643 was purchased from Tokyo Kasei (Japan). Gemfibrozil was a gift from Warner-Lambert (U.S.A.). Clofibric acid, palmitoyl-CoA, leuco-2,7-dichlorofluorescin diacetate (Leuco-DCF), *o*-nitrophenylacetate, nicotinamide adenine dinucleotide (NAD), Triton X-100, and fetal calf serum were obtained from Sigma (U.S.A.). RPMI 1640 medium was purchased from Nissui Chemicals (Japan). The progesterone EIA kit used was from the Cayman Company (U.S.A.). The assay kit for human chorionic go-

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nadotroin (hCG) was obtained from Wako Pure Chemicals (Japan). All other reagents were of an analytical grade and were purchased from Wako Pure Chemicals (Japan).

Culture of Trophoblast Cells and Drug Treatment The trophoblast cell-line (TCL-1) was obtained by immortalizing third-trimester human trophoblast cells with the large-T antigen from the SV40 virus, as described in detail elsewhere.[22] Cells were cultured at 37 °C, and drug treatment was initiated 24 h after plating as described in the previous paper.[3] WY-14643 in ethanol was added. The final concentration of ethanol was 0.2% (v/v). In the last 24 h of the culture the medium was collected for the hormone concentration and lactate dehydrogenase activity assays. The attached cells were washed and suspended in 0.25 M sucrose containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM Tris–HCl (pH 7.4), and cell homogenates were then prepared.[3] These homogenates were used for the enzyme activity and protein content assays.

Cell Fractionation and Nycodenz Density Gradient Centrifugation The cells were homogenized in 0.25 M sucrose containing 5 mM HEPES (pH 7.4), 1 mM EDTA, and 0.1% ethanol (SVEH) using a Teflon homogenizer with a tight Teflon pestle of the Potter–Elvehjem type.[4] The light mitochondrial fraction prepared from the homogenate was subjected to Nycodenz density gradient centrifugation. A discontinuous Nycodenz density gradient (9.6 ml) was prepared in centrifuge tubes as follows: 1.12, 1.125, 1.13, 1.135, 1.14, 1.145, 1.15, and 1.16. Next, 1.8 ml of the light mitochondrial fraction was layered on top of the gradient. The tubes were centrifuged at 33000 rpm at 4 °C for 5 h in a Hitachi model SCP70H ultracentrifuge with a Hitachi RPS40T-948 rotor (Hitachi, Tokyo, Japan). After centrifugation, the tubes were divided into 10 fractions of 1.2 ml each from the top of the gradient with an ISCO gradient fractionater (U.S.A.). Each fraction was appropriately diluted with SVEH and centrifuged at $23500\times\boldsymbol{g}$ for 30 min at 4 °C. The respective precipitates were suspended in 0.25 M sucrose, and the enzyme activity of each fraction was determined.

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)[23,24] Catalase activity was measured according to the method of Leighton *et al.*[25] with slight modifications.[26] Cytochrome-*c* (Cyt-*c*) oxidase is a marker enzyme of mitochondria. Its activity was estimated by the method described by Wharton and Tzagoloff[27] with a slight modification.[28] The acid phosphatase activity was determined as a marker enzyme of lysosomes using a previously described method.[26]

Cytotoxicity was evaluated by measuring the activity of lactate dehydrogenase[29] in the cells and medium and by viewing morphological changes with an inverted microscope. Protein levels were determined by the Lowry method, using BSA as a standard.[30]

Hormone Concentration Assay A progesterone EIA kit (Cayman Company (U.S.A.)) 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 (Japan).

RESULTS

The Effects of WY-14643 on the Proliferation of Immortalized Human Chorionic Cells We reported the effects of gemfibrozil and clofibric acid on the DNA and protein contents of TCL-1 cells.[3] In the present experiment, the immortalized cells were cultured with or without WY-14643 for 3 d, and their DNA and protein contents were then determined. The DNA and protein contents of the control cells gradually increased during the incubation (data not shown), indicating the normal growth of the cells. The DNA and protein contents of control cells at 3 d were 1.109 ± 0.286 mg/plate and 6.04 ± 1.58 mg/plate, respectively. The DNA content after treatment with 0.15 and 0.2 mM WY-14643 for 3 d was about 60 and 40% of the control, respectively. The protein content after treatment with 0.15 and 0.2 mM WY-14643 for 3 d was about 60 and 40% of the control, respectively (data not shown).

Protein content was decreased at the same concentrations of the agent as DNA content. These results suggest that WY-14643 beyond particular concentrations suppresses the proliferation of immortalized human trophoblast cells. In the present study, we could detect neither abnormal cell morphology nor cell death using an inverted microscope. Therefore, WY-14643 suppresses the proliferation of immortalized TCL-1 cells.

We biochemically studied the cytotoxicity of WY-14643, in trophoblast cells by measuring the activity of a marker enzyme, lactate dehydrogenase, in the cells and medium. The lactate dehydrogenase activity of the control cells at 3 d was 3.22 ± 1.35 u/mg protein. The specific activity of the lactate dehydrogenase of the cells was hardly changed after incubation with WY-14643 for 3 d (data not shown). We studied the effects of WY-14643 on the percentage of total activity of lactate dehydrogenase in the cells and in the medium. The activity levels in the cells were maintained at 96% even at the highest concentration of WY-14643 (data not shown).

The Effects of WY-14643 on the Activity of Peroxisomal Enzymes and the Hormone Secretion of Immortalized Human Chorionic Cells We reported the effects of gemfibrozil and clofibric acid on the peroxisomal enzyme activity of TCL-1 cells.[3] To elucidate whether WY-14643 activates peroxisomal enzymes, we measured the activity levels of catalase and fatty acyl-CoA oxidase after incubation with or without the agent. Figures 1(1) and (2) illustrate the concentration dependence of the catalase and fatty acyl-CoA oxidase activity, respectively 3 d after the incubation. Values are relative to the control. WY-14643 at 0.05 and 0.1 mM significantly increased catalase activity to about 1.2 and 1.4 times that of the control at 3 d of incubation, respectively (Fig. 1(1)). WY-14643 did not show a clear increase in fatty acyl-CoA oxidase activity after incubation for 3 d (Fig. 1(2)).

We reported the effects of gemfibrozil and clofibric acid on the hormone secretion of TCL-1 cells.[3] In the present study, the cells were incubated in culture medium with or without WY-14643. After incubation for 3 d, the concentrations of progesterone and hCG secreted into the medium for the last 24 h were measured. Figures 1(3) and (4) show the concentration dependence of progesterone and hCG secretion, respectively. All values are relative to the control. WY-14643 at 0.1 mM significantly increased the secretion of prog-

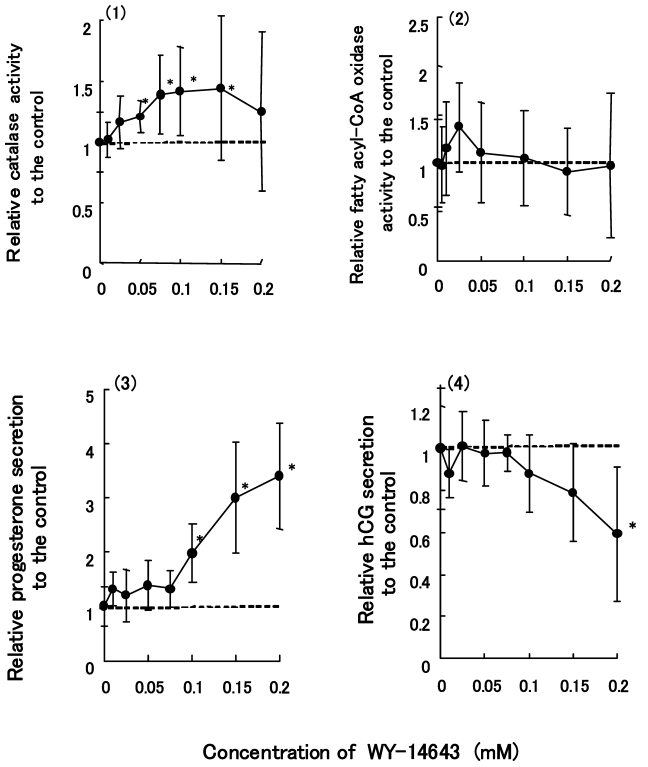

Fig. 1. The Effects of WY-14643 on Activities of Catalase and Fatty Acyl-CoA Oxidase of Trophoblast Cells, and the Levels of Progesterone and Human Chorionic Gonadotropin Secreted from Trophoblast Cells

After incubation with various concentrations of WY-14643 for 3 d, the cells were harvested and the medium was collected. The catalase (1) and fatty acyl-CoA oxidase (2) activity of the cells was determined as described in the text. The specific activities (U/mg protein) were expressed as values relative to the control (0 mM) at each concentration of the agents. Data are shown as mean values±S.D. of 7—9 experiments. * Indicates a significant difference ($*p<0.05$). The catalase and fatty acyl-CoA oxidase activity of the control cells at 3 d were 1.717±0.424 U/mg protein and 0.285±0.129 U/mg protein, respectively. The progesterone (3) and human chorionic gonadotropin (4) concentrations in the medium were determined as described in the text. The contents of progesterone and human chorionic gonadotropin per cell protein (pg/mg protein/24 h) were expressed as values relative to the control (0 mM) at each concentration of the agents. Data are shown as mean values±S.D. of 6—9 experiments. * Indicates a significant difference ($*p<0.05$). The progesterone and human chorionic gonadotropin contents secreted from the control cells at 3 d were 98.1±36.5 pg/mg protein/24 h, and 13.19±3.76 mIU/mg protein/24 h, respectively.

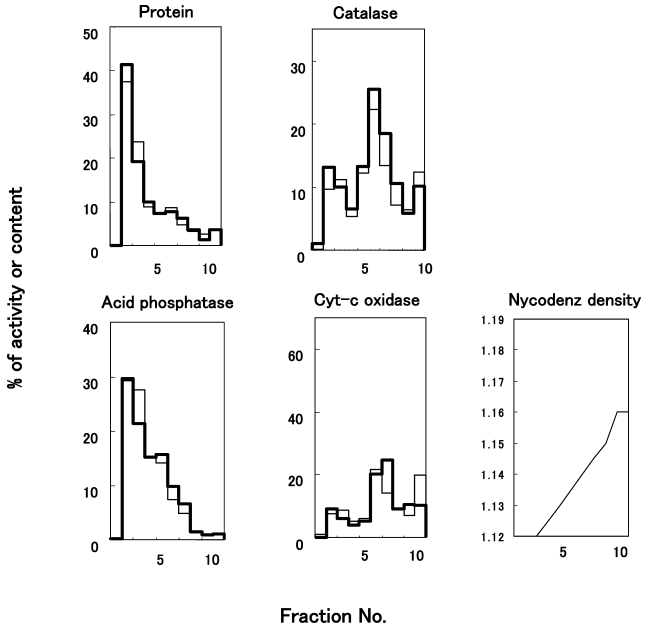

Fig. 2. The Distribution Patterns of Enzyme Activity and Protein Content after Nycodenz Density Gradient Centrifugation of the Light Mitochondrial Fraction of TCL-1 Cells

TCL-1 cells were treated with (—) or without (—) WY-14643 for 3 d. The light mitochondrial fraction was prepared from TCL-1 cell homogenate and subjected to Nycodenz density gradient centrifugation. The ordinates represent the enzyme activity or protein content of the fractions relative to the total activity of the L fractions. The abscissa represents fraction numbers from the top of the tube.

esterone from the cells into the medium to about 2 times the control value after incubation for 3 d, but it did not affect the secretion at concentrations under 0.075 mM (Fig. 1(3)). WY-14643 at 0.2 mM significantly decreased the secretion of hCG from the cells into the medium to about 60% of the control value after incubation for 3 d, but it did not affect the secretion at concentrations under 0.15 mM (Fig. 1(4)). WY-14643 at the concentrations used did not directly affect the determination of progesterone and hCG using the assay kit (data not shown).

Enzyme Activity and Protein Content after Nycodenz Density Gradient Centrifugation We reported the peroxisomes of gemfibrozil-treated rats were concentrated in fractions of higher density compared with control rats.[11] Furthermore, we reported effects of clofibric acid on the distribution patterns of enzyme activity of TCL-1 cells in a continuous Nycodenz density gradient centrifugation (1.12—1.18 g/ml).[4] In the present experiment, for good separation of the peroxisomes from other organella we performed a discontinuous Nycodenz density gradient centrifugation (1.12—1.16 g/ml) of the light mitochondrial fraction of the cells treated with WY-14643. We compared the result of WY-14643 with that of gemfibrozil and clofibric acid.

The highest peak of catalase activity was found in the density fraction of about 1.14 g/ml (fraction No. 6), and moderate levels of catalase activity also appeared in the density fraction around 1.14 g/ml. WY-14643 did not affect the density of the peroxisomes (Fig. 2). Gemfibrozil and clofibric acid did not also affect the density (data not shown).

Acid phosphatase (lysosome marker enzyme) activity (fraction No. 2, around 1.125 g/ml) was separated from the peroxisomal fractions (fraction No. 5—7), but a low activity of Cyt-*c* oxidase (mitochondria marker enzyme) was found in the peroxisomal fractions (Fig. 2). Therefore, we performed Nycodenz gradient centrifugation of the heavy mitochondrial fraction. Cyt-*c* oxidase activity was detected in heavier fractions (fractions No. 9 and 10, around 1.16 g/ml) than that of catalase (data not shown).

DISCUSSION

WY-14643 is a stronger PPARα agonist in rodents than gemfibrozil and clofibric acid.[20,21] Therefore, we expected it to have strong effects on peroxisomes in TCL-1 cells. In the present study, we studied the effects of WY-14643 on peroxisomal enzyme activity (catalase and fatty acyl-CoA oxidase) and hormone secretion in TCL-1 cells. The data of WY-14643 was compared with that of gemfibrozil and clofibric acid.[3]

Incubation for 3 d with one of the drugs, WY-14643 ($\geqq$0.15 mM) (data not shown), gemfibrozil ($\geqq$0.2 mM), and clofibric acid ($\geqq$2.5 mM)[3] suppressed the rate of increase in DNA and protein. These results suggest that trophoblast cells

are more susceptible to WY-14643 and gemfibrozil than clofibric acid. Matsuo and Strauss[9] reported that the proliferation of JEG-3 choriocarcinoma cells, which are derived from syncytiotrophoblasts, was decreased to about 50 and 52% after 3 d in culture in the presence of 0.25 mM WY-14643 and 3 mM clofibric acid, respectively. In the present study, proliferation (DNA and protein contents) was decreased to about 40 and 45% after 3 d in culture in the presence of 0.2 mM WY-14643 (Fig. 1) and 2.5 mM clofibric acid,[3] respectively. These results suggest that trophoblast cells are somewhat more susceptible to WY-14643 and clofibric acid than choriocarcinoma cells.

In human cells, there are reports that rodent peroxisome proliferators do not affect peroxisomal enzyme activity. Goll *et al.*[31] reported that fatty acyl-CoA oxidase activity in human hepatic carcinoma cells, HepG2 cells, was not increased by 0.25 mM clofibric acid. Hwang *et al.*[18] reported that the activity of the fatty acyl-CoA oxidation system in human hepatic cells was not increased by 0.1 mM WY-14643, but was increased in rat hepatocytes. On the other hand, Perrone *et al.*[23] reported that fatty acyl-CoA oxidase activity in human hepatic cells was significantly increased to about 2.4 times the control value by 1.0 mM clofibric acid. Furthermore, Chance *et al*.[32] reported that fatty acyl-CoA oxidase and catalase activity in HepG2 cells was significantly increased by 0.5 mM clofibric acid. In the present study, we used human trophoblast cells. Catalase activity was increased by WY-14643 (Fig. 1(1)), gemfibrozil, and clofibric acid[3] after incubation for 3 d. Furthermore, WY-14643 and gemfibrozil increased catalase activity at lower concentrations than clofibric acid. Therefore, we support the positive effect of rodent peroxisome proliferators on human cells. Chance *et al*.[32] reported that catalase activity in HepG2 cells was increased to about 1.4 times higher than the control value by 1.0 mM clofibric acid. In our results, the activity in trophoblast cells was increased to about 1.34 times the control value by 1.0 mM clofibric acid,[3] indicating that the sensitivity of catalase in trophoblast cells to clofibric acid is the same as that of human hepatocytes.

Fatty acyl-CoA oxidase activity was significantly increased to about 1.93 times higher than the control value by 1.0 mM clofibric acid,[3] but it was hardly changed by WY-14643 (Fig. 1(2)). Watanabe *et al.*[21] reported that fatty acyl-CoA oxidase activity in rat hepatic cells was increased to about 3.5 times higher than the control value by 0.09 mM WY-14643 and 0.25 mM clofibric acid. These results suggest that the sensitivity of the fatty acyl-CoA oxidase of human trophoblast cells to clofibric acid is lower than that of rat hepatocytes.

In many reports related to peroxisome proliferators, the increase in the number of peroxisomes caused by the proliferators is accompanied by an increase in catalase activity.[33,34] In the preceding paper, using morphological and biochemical methods, we clarified that clofibric acid induces the proliferation of microperoxisomes in human trophoblast cells.[4] We tried to purify the TCL-1 peroxisomes by a discontinuous Nycodenz density gradient centrifugation, but a small number of mitochondria contaminated the peroxisomal fractions (Fig. 2). Therefore, from the data of partially purified peroxisomes, we could not conclude the possibility that the peroxisome proliferation is induced by WY-14643.

The secretion of progesterone was significantly increased to about 2.0 and 1.8 times higher than the control value by incubation with 0.1 mM WY-14643 (Fig. 1(3)) and 1.0 mM clofibric acid,[3] respectively. With the exception that the agent concentration affects proliferation, gemfibrozil hardly affect progesterone secretion.[3] Matsuo and Strauss[9] reported that the levels of mRNA encoding of the key steroidogenic enzyme, cytochrome P450scc, were modestly increased by 3 mM clofibric acid in JEG-3 cells, indicating that these findings are compatible with our results concerning the increase in progesterone secretion.

There are many reports that peroxisomes play important roles in steroid biosynthesis.[35,36] We considered a possible mechanistic link between peroxisomes and progesterone synthesis as follows. Peroxisomes participate in fatty acid β-oxidation and the mevalonate pathway.[36,37] Acetyl-CoA derived from β-oxidation may be used for cholesterol synthesis in peroxisomes. Prognenolone is synthesized from cholesterol by cytochrome P450scc and is used for progesterone synthesis. Peroxisomal sterol carrier protein 2 may participate in the transport of steroids such as cholesterol.[13—16,38—40]

We reported the peroxisomes of gemfibrozil-treated rats were concentrated in fractions of higher density (around 1.20 g/ml) compared with control rats (around 1.19 g/ml).[11] However, in the present study, the density of TCL-1 peroxisomes (around 1.14 g/ml) was not changed by WY-14643 (Fig. 2), gemfibrozil and clofibric acid (data not shown), indicating that the density of TCL-1 peroxisomes are not susceptible to the drugs.

In rats, the density of peroxisomes (around 1.19 g/ml) is higher than that of mitochondria (around 1.16 g/ml).[11] In the present experiment, the density of lysosomes of TCL-1 (around 1.125 g/ml) (Fig. 2) was lower than that of rat liver (around 1.155 g/ml).[11] We expected that the density of mitochondria of TCL-1 would also be lower than that of rat liver (around 1.16 g/ml), resulting in the higher density of peroxisomes of TCL-1 than that of mitochondria of TCL-1. However, after Nycodenz gradient centrifugation of the heavy mitochondrial fraction of TCL-1, the distribution patterns of Cyt-*c* oxidase and catalase activity indicated that the density of peroxisomes (around 1.14 g/ml) is lower than that of mitochondria (around 1.16 g/ml) (Fig. 2). During the centrifugation, the mitochondria may have moved to the higher density fractions through the peroxisomal fraction. Therefore, a small number of mitochondria may have contaminated the peroxisomal fractions. Usually, we can obtain purified peroxisomes from rat liver by a discontinuous Nycodenz density gradient centrifugation. In order to obtain completely purified peroxisomes from trophoblast cells, further purification methods are needed.

In conclusion, WY-14643 inhibits the proliferation of trophoblast cells at lower concentrations than clofibric acid. WY-14643 may increase the progesterone secretion. Effects of WY-14643 on metabolism of human trophoblast cells are different from those of clofibric acid. In human trophoblast cells, WY-14643 may not be stronger inducer of peroxisomes than clofibric acid.

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