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Triglyceride Accumulation by Peroxisome Proliferators in Rat Hepatocytes

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Peroxisome proliferators (PxPs) induce peroxisomal β-oxidation (Px-ox) in the liver of rodents and have a hypolipidemic function. To investigate hypolipidemic effect of PxPs, the relationship between TG fluctuation and Px-ox activity, as an indicator of the function of PxPs, was studied in primary cultured rat hepatocytes. Nafenopin (Nf) treatment of hepatocytes caused an increase in Px-ox activity in association with cellular TG accumulation in a time-dependent manner with a coefficient of $r=0.918$. This relationship between the activity and cellular TG were obtained using structurally diverse PxPs with a correlation coefficient of $r=0.747$. Treatment of the hypolipidemic drug, but non-PxP Pravastatin, decreased TG in the medium, but did not have the effects on cellular TG and Px-ox activity. The total amount of TG and diacylglycerol acyltransferase activity, the last enzyme in the TG *de novo* synthesis pathway, were not affected by Nf treatment. When hepatocytes were cultured with Brefeldin A, cellular TG was accumulated, the same as with Nf, however, Px-ox activity was not enhanced. Nf treatment markedly decreased the level of apolipoprotein B (apo B) in very low density lipoprotein (VLDL) fractions prepared from conditioned media and increased that of cellular apoB by Western blot analysis. Microsomal triglyceride transfer protein activity was not influenced by Nf. Together, with regards to TG lowering effect of PxPs, it is suggested that PxPs cause hepatocellular accumulation of TG without effects on TG biosynthesis and VLDL construction, and they might have inhibitory effect on VLDL secretion process.

Key words   peroxisome proliferator; peroxisomal β-oxidation; triglyceride; apolipoprotein B

Peroxisome proliferators (PxPs) are structurally diverse compounds, such as fibrates (hypolipidemic drugs), phthalate ester plasticizers, fatty acid analogues, cause induction of several proteins related to lipid metabolism, including peroxisomal β-oxidation (Px-ox),[1,2] accompanied by a hypolipidemic effect in rodents.[3] Since the peroxisome proliferator-activated receptors (PPARs), which are nuclear transcriptional receptors,[4] were found, they have been regarded as the wide lipid metabolism regulatory factors. Among the 3 subtypes of PPARs, α, β/δ and γ, PPARα is mainly involved in peroxisome proliferation, fatty acid β-oxidation, and plasma lipid metabolism, and is expressed predominantly in the livers and kidneys of rodents.[5] On hypolipidemic action, plasma triglyceride (TG) lowering effect of PxPs, which are PPARα ligands, are responses produced by PPARα-mediated transcriptional regulation.[6—8] However, in a contrasting line of studies, the TG lowering effect of PxPs was found to be exerted by a mechanism independent of PPARα.[9—11] Thus, the mechanism of the hypolipidemic effect of PxPs has not been clearly established. Further, the implications of the two different and simultaneous responses by PxPs, which develop in a distant and distinct tissues, and their mechanisms, including physiological aims, and if it were, which is the initiator of another response, have not been elucidated.

The plasma TG lowering effect of PxPs have been essentially ascribed to the induction of lipoprotein lipase (LPL) expression[12] and the inhibition of apolipoprotein C-III (apo C-III) gene transcription and activity.[13,14] The combined effects of PxPs toward LPL and apo C-III result in TG hydrolysis. On the other hand, fibrates induce fatty acid transporter protein in rat livers,[15] by which hepatic uptake of fatty acids is enhanced. A linear increasing correlation between Px-ox activity and cellular long chain fatty acid level by PxP was reported in rat cultured hepatocytes,[16] leading to hepatic TG accumulation when the TG synthesis process was completed, as well as a fibrate and a long chain fatty acid enhanced cellular TG level with a increase in Px-ox activity.[17] In *in vivo* experiments, PxPs including fatty acid analogues enhanced hepatic TG accumulation,[11,18,19] or in contrast, decrease it,[20—22] when the increase in Px-ox activity and the decrease in plasma TG were simultaneously observed. These discrepancy results could be due to the fact that physiological lipid metabolism is regulated by complicated, precise, and divers endogenous factors. Since the direct action of PxPs toward rat hepatocytes has been established,[23] we investigated the interrelationships among Px-ox activity, TG and apoprotein B (apo B) fluctuations in primary cultured rat hepatocytes in the present study, avoiding *in vivo* complex situations, especially hormonal modulations, organ–organ interrelation, moiety of blood components, physiological status and food intake.

MATERIALS AND METHODS

Materials   Clofibrate and diethyl hexyl phthalate (DEHP) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.) and other peroxisome proliferators were kindly provided by Dr. Janardan K. Reddy, Northwestern University Medical School (Chicago, IL, U.S.A.). Pravastatin was kindly donated by Sankyo Seiyaku Co. (Tokyo, Japan). Antibodies were purchased from Chemicon International Co. (Temecula, CA, U.S.A.).

Cell Cultures and Treatments   Rat parenchymal hepatocytes were prepared from male Wistar rats (7 weeks-old, SLC Co. Shizuoka, Japan) fed a standard pellet diet for 1 week. The cells were obtained by collagenase perfusion and centrifugation at $50 \times g$, for 1 min, according to the method of Seglen.[24] Hepatocytes which have viability greater than 90% by trypan blue exclusion test were seeded on fibronectin coated dishes at a density of 2.0×10^6 cells/3 ml/60 mm-dish

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in DMEM with 5% calf serum, 10^{-6} M insulin and 10^{-7} M dexamethasone, and subcultured in a humidified atmosphere with 5% CO_2 for 20 h at 37 °C. The hepatocytes were then cultured in medium with 0.1 mM nafenopin (Nf) dissolved in dimethylsulfoxide (final concentration 0.2%) for indicated periods with the medium change every 24 h. At the end of the culture periods, the cells were rinsed with ice-cold PBS, scraped into adequate solutions, and sonicated for 20 s at 25 mW. The cell homogenates or the supernatants were subjected to enzyme assays, TG determination, and lipoprotein preparation.

Apolipoprotein B (apo B) Detection VLDL fractions were obtained by a density ultracentrifugation method from 2 ml of conditioned medium at the end of the culture periods. Briefly, the medium was mixed with 0.03% benzamidine and 5% Fat Red solution, then placed into tubes, and layered with a NaCl solution adjusted to a density of 1.006 g/ml and centrifuged at 50000 rpm for 20 h at 16 °C. The VLDL fractions were then concentrated 5-fold by molecular cut centrifugation (Kurabo, Japan) and subjected to SDS-PAGE on a 2—15% gradient gel (Multigel: Daiichi Pure Chemicals, Japan), after which Western blot analysis was performed using goat anti-human apolipoprotein B antibody as the first antibody, and rabbit anti-goat IgG HRP-labeled antibody as the second antibody. Cell homogenates containing 0.03% benzamidine were centrifuged to remove cell debris and the supernatants were incubated with protein A-Sepharose for 1 h at 4 °C, then centrifuged at 12000 rpm at 4 °C. The resultant supernatants were individually mixed with the first antibody for 2.5 h at 4 °C, then protein A-Sepharose was added, and they were shaken for 1 h and centrifuged. The thoroughly washed precipitates were subjected to SDS-PAGE as the same as VLDL-apoB.

Biological Determinations For peroxisomal β-oxidation activity, cell homogenates in 50 mM Tris–HCl buffer (pH 8.0) were subjected to enzyme determination according to ^{14}C-labeled acetyl CoA production from ^{14}C-palmitoyl CoA, using the method of Lazarow.[25] Microsome fractions, and enzyme sources were prepared for 1,2-diacylglycerol acyltransferase (DGAT) and microsomal triglyceride transfer protein (MTP) activities according to the report of Wetterau et al.[26] Cells from 5 dishes were homogenized in 50 mM Tris–HCl buffer containing 50 mM KCl, 5 mM EDTA and 0.03% benzamidine, then the homogenates were incubated with deoxycholate for 30 min at 4 °C and centrifuged at 103000×g for 60 min. The

supernatants were dialyzed against 15 mM Tris–HCl buffer with 40 mM NaCl, 1 mM EDTA and 0.03% benzamidine (15/40 buffer), and the resulting dialysates were used as enzyme solutions. DGAT activity was determined by measuring the counts of incorporated ^{14}C-palmitoyl CoA into the TG fraction extracted with a heptane–isopropanol–H_2SO_4 mixture, using the method of Hosaka et al.[27] MTP activity was determined by measuring the amount of radiolabeled TG transferred from donor small unilamellar vesicles (SUVs) to acceptor SUV. To prepare the SUVs, egg phosphatidyl choline, ^{14}C-radiolabeled TG, cardiolipin for the donor, and egg phosphatidyl choline, unlabeled TG for the acceptor, were dissolved in chloroform, then evacuated and sonicated in 15/40 buffer for 60 min at 50 °C. According to the method of Wetterau et al.,[28] a typical transfer reaction mixture containing donor, acceptor vesicles, bovine serum albumin and enzyme solution in 1 ml of reaction mixture was reacted for 1 h at 37 °C. The reaction was terminated by the addition of DEAE-sepharose CL-6B. The mixture was then agitated for 5 min in an ice-bath and centrifuged, and the counts in 0.5 ml of each supernatant were determined. The activity is expressed as radioactive counts per mg of protein. For TG determination, enzymatic analysis was employed using a commercial kits (Wako Pure Chemical Ind., Japan), with some modifications. Protein was measured using a DC protein assay (BIO-RAD) with bovine serum albumin as the standard.

RESULTS

Relationship between Px-ox Activity and TG The correlation between Px-ox activity and TG in cultured rat hepatocytes was examined. Addition of Nf, a PxPs, to the culture medium increased either enzyme activity or cellular TG time-dependently, whereas TG in the medium was decreased in the same manner (Fig. 1). The relationship between the activity and cellular TG was summarized with a correlation coefficient of $r=0.918$ (Fig. 3A), and a linear negative correlation between the activity and TG in medium with a coefficient of $r=-0.817$ was observed (data not shown). As shown in Fig. 2, not only Nf but also other structurally diverse PxPs, clofibrate, DEHP and Wy 14,643, produced the same relationship between the activity and cellular TG, which was summarized with a coefficient of $r=0.747$ (Fig. 3B). Although a enhancement of hepatocellular TG level by bezafi-
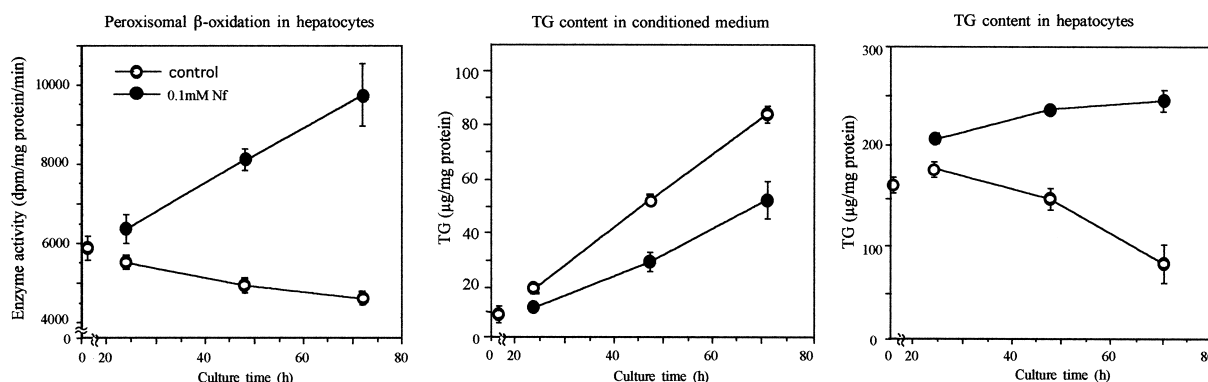

Fig. 1. Effect of Nf on Peroxisomal β-Oxidation and TG in Rat Cultured Hepatocytes

Rat hepatocytes were cultured with 0.1 mM Nf for the indicated periods. Values represent the mean±S.D. of 3 to 4 dishes.
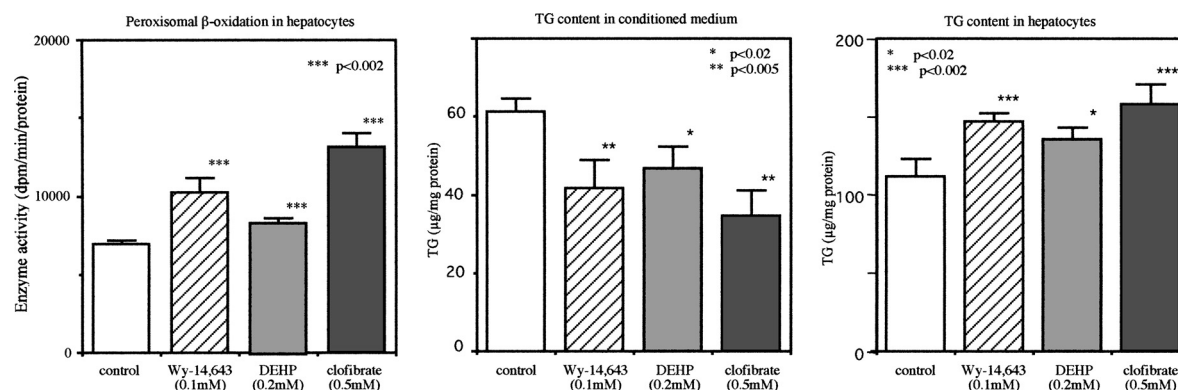
Fig. 2.   Effects of Various Peroxisome Proliferators on Peroxisomal β-Oxidation and TG

Rat hepatocytes were cultured for 48 h. Values represent the mean±S.D. of 3 to 4 dishes. *p* values are significant difference from control.
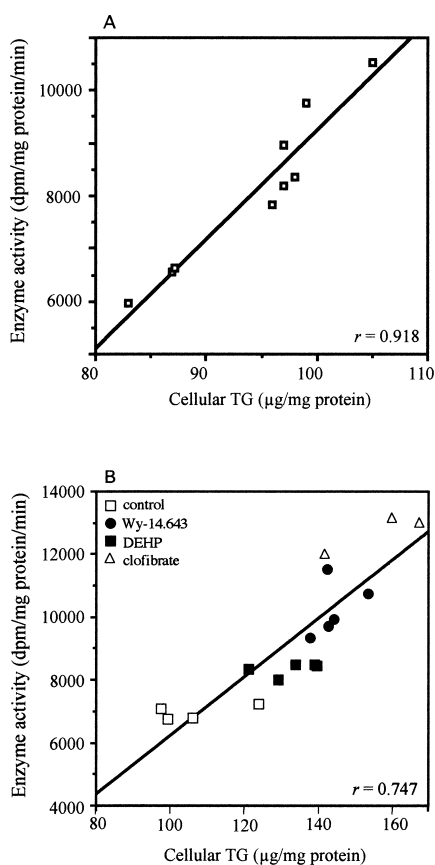

Fig. 3.   Correlations between Peroxisomal β-Oxidation and TG in Hepatocytes

(A) Each point is derived from the corresponding culture dishes in Fig. 1. (B) Each point is derived from the corresponding culture dishes in Fig. 2.

brate treatment in rat cultured hepatocytes was shown in a previous report,[17] an obvious relationship between Px-ox activity and TG fluctuation was shown in the present study. Total TG amount, hepatic TG+TG in medium, in each treatment was not significantly different as compared to total TG in the control cells (data not shown). Figure 4 shows the results when hepatocytes were treated with the non-PxP pravastatin. TG level in medium was decreased, whereas cellular TG and Px-ox activity were not affected. Therefore, it is suggested that hepatic TG accumulation associated with the increase in Px-ox activity is specific action of PxPs. In order

to investigate the relationship between TG accumulation and Px-ox activity, hepatocytes were treated with Brefeldin A, a blocker of Golgi apparatus function.[29] Figure 5 shows that Brefeldin A markedly increases cellular TG content, however, Px-ox activity was not enhanced.

Effect of Nf on TG Synthesis and VLDL Assembly in Hepatocytes   To confirm whether PxPs increase TG synthesis activity in hepatocytes, the effect of Nf on the activity of DGAT, the last enzyme in TG *de novo* synthesis, was investigated. Microsome fractions prepared from cell homogenates were used as the enzyme source, and an increase in the activity that was dependent on the amount of protein was confirmed. The results presented in Fig. 6 show that 0.1 mm Nf did not have an effect on enzyme activity. Also, in regards to TG secretion from hepatocytes, microsomal triglyceride transfer protein (MTP)[30] activity, which is required for VLDL construction, was examined. As shown in Fig. 7, enzyme activity after 24 and 48 h of culture was not significantly influenced by Nf.

Effect of Nf on apo B   Since apo B is a pivotal protein during construction of VLDL particles, of which TG is a constituent and is secreted from hepatocytes to the vascular stream as particles, we examined the effect of Nf on the protein. Hepatocytes were cultured with Nf for various periods, and apo B masses in hepatocytes and media were developed by immunoblotting. The VLDL-apo B100 profiles of Nf-treated media were evidently slighter than the control after 48 and 72 h of culture, and cellular apo B100 amounts following Nf treatment were larger than in the control culture. In contrast to apo B100, cellular and medium apo B48 profiles were not affected by Nf. These results reflected the results of TG fluctuations, as shown in Figs. 1 and 2, and was very similar to the report of Linden *et al.*[31]

DISCUSSION

From the viewpoint that PPARα is expressed in hepatocytes[4] and is an indispensable transcription factor involved in primarily lipid metabolism, the effects of the exogenous ligands of PPARα, PxPs, on TG fluctuation were investigated.

The relationship between TG fluctuation and peroxisomal induction, including the peroxisome proliferation, the induction of several proteins, and increase in enzyme activities by
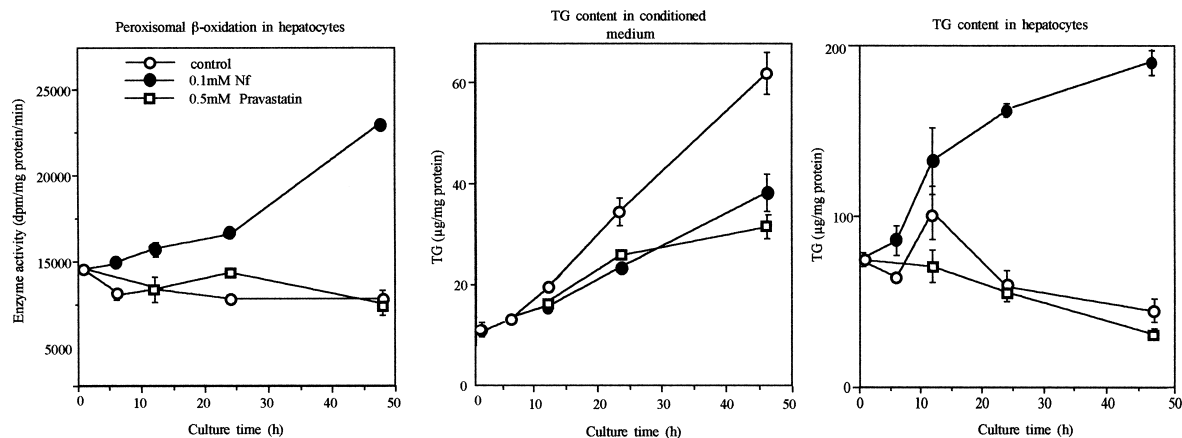
Fig. 4. Effect of Pravastatin on Peroxisomal β-Oxidation and TG
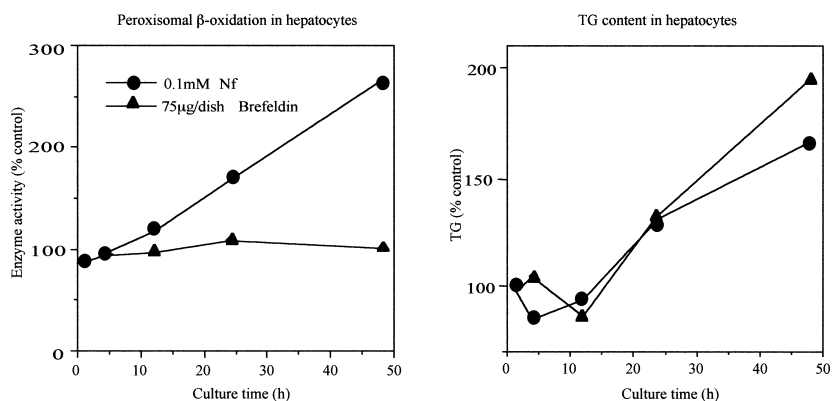
Values represent the mean±S.D. of 3 to 4 dishes.

Fig. 5. Effect of Brefeldin A on Peroxisomal β-Oxidation and TG

Hepatocyes were cultured with 3 to 4 dishes for each treatment. Data are expressed as % of average of control values at 0 time, which are 6055±282 dpm/mg protein/min and 48.5±4.1 mg/mg protein for Px-ox and TG, respectively.
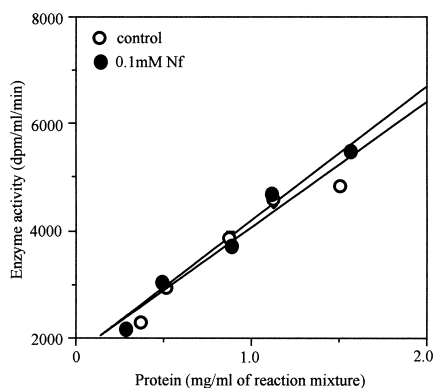

Fig. 6. Diacylglycerol Acyltransferase Activity

Rat hepatocytes were cultured for 48 h. The indicated amounts of microsomal protein were subjected to an enzyme reaction.

PxPs, has not been fully elucidated, though an increase in Px-ox activity and a reduction in hepatic TG level,[9,22] or contrarily, the activity and hepatic TG accumulation[11,18,19] *in vivo* have been reported. Two mechanisms of Px induction[32] have been proposed: 1) the receptor mediated theory, termed PPARs by Isseman *et al.*,[4] which does not rule out the possibility that PxPs interact indirectly with the receptor, and 2) substrate overload, termed lipid pertubation, which is caused

by a high cellular level of long acyl CoA[20] and PxP metabolites, the corresponding CoA thioesters.[33] As for the latter, when peroxisomes are induced by exogenous PxPs, not by fatty acid analogues, which are destined to be physiologically metabolized, cellular TG status were studied in present studies.

Of the 2 major types of hypolipidemic drugs, statins and fibrates, it is widely acceptable for lowering plasma lipids in clinical pharmacology that statins, which are inhibitor for HMG CoA reductase a key enzyme in the steps of cholesterol synthesis, are applied to lower plasma cholesterol, while fibrates are aimed to lower TG. Additionally, statins and fibrates have a simultaneous potency to decrease TG and cholesterol, respectively. However, simvastatin administration to rats decreased TG concentration, due to increase in LPL mRNA and activity, accompanied by decrease in the mRNA as well as plasma apo C-III protein,[34] as well as PxPs.[12—14] Moreover, a recent study showed that lipid modulating functions of statins are through mechanism involving PPARα.[35,36] There are several studies that hepatic peroxisomal inductions and the TG lowering effect by fibrates are not directly related.[8—10] According to a report by Foxworthy *et al.*,[19] the sequence of events in the liver after administration of PxPs to rats, including transient hepatic TG accumulation in the early stage, followed by an increase in Px-ox, implies
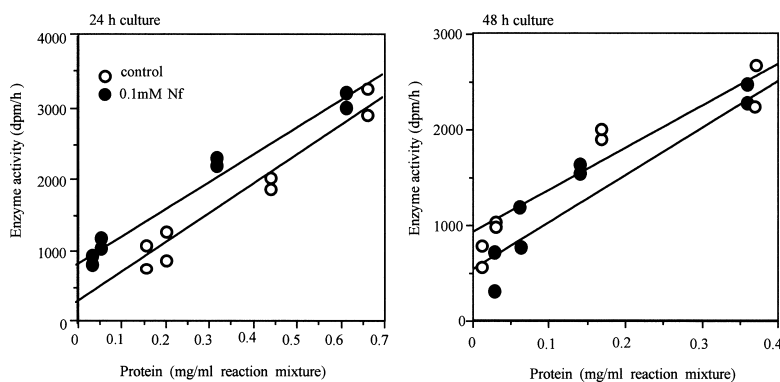
Fig. 7. TG Transfer Activity of Microsomal Triglyceride Transfer Protein

Rat hepatocytes were cultured for 24 and 48 h. The indicated amounts of microsomal protein were subjected to an enzyme reaction.
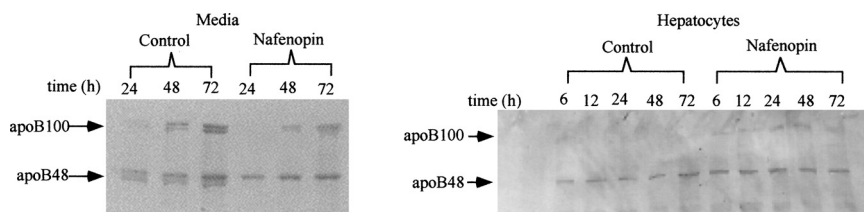

Fig. 8. Effect of Nf on Apo B Levels in Hepatocytes and Media

Cells were treated with Nf for the indicated periods. Media were collected from 2 dishes and combined. The detections of apo B in the cells and medium are described in Materials and Methods.

that hepatic TG accumulation is responsible for the induction of Px-ox. However, hepatic lipid accumulation by chlorpromazine administration resulted in no indication of Px-ox activity in rats,[37] in accordance with the results of Brefeldin A treatment (Fig. 5). Therefore, it is suggested that hepatic TG accumulation has no responsibility for increasing Px-ox activity and lowering plasma TG both *in vivo* and *in vitro*.

The mechanism of the plasma TG lowering effect by fibrates has been ascribed to decrease in apo C-III expression[13,14] and induce LPL gene expression in rat liver.[12] Although LPL is functional on the surface of endothelial cells in blood vessels, the enzyme is synthesized in the parenchymal cells in adipose tissues and muscle, hepatic Kupffer cells and macrophages in the lung and spleen,[38] though its synthetic potency and activity in the liver is very low in adults.[12,39] Since fibrates have a direct effect on intracellular events in cultured hepatocytes, it is generally agreed that TG fluctuation and Px induction by PxPs are not mediated by LPL or hormones, and that the lower level of induction in isolated cells is due to a lack of several endogenous components. Even if the TG lowering effects of PxPs *in vivo* are due to an increase of hepatic uptake of TG or enhancement of LPL activity, the relationship between TG and Px-ox remains controversial, because animal strain, age, food intake, and circadian rhythm must also be taken into consideration.

Hepatic TG accumulation by Nf could be due to 3 phenomena in the liver, 1) enhancement of TG synthesis, 2) inhibition of TG secretion, and 3) decrease in TG degradation. Since long chain fatty acids are accumulated in rat hepatocytes following treatment with PxPs,[16] hepatic DGAT activity that catalyzes the final reaction in TG synthesis was examined. A previous study showed that fibrates raised overt (cytosol-facing) DGAT activity and inhibited latent (endoplasmic reticulum lumen-facing) DGAT activity,[40] thus even

if the apparent activity was not affected by Nf treatment using microsomal fractions, as the enzyme source, we considered that the results on DGAT (Fig. 6) might be acceptable as a rough estimation. In previous *in vivo* studies, however, a linear correlation between DGAT activity and hepatic TG content was observed.[18] Apo B-containing VLDL is the responsible particle for TG secretion from the liver. Further, during intrahepatic VLDL construction, assembly of apo B with TG to form the particles requires the function of MTP.[41—43] MTP locates in the lumen of the ER of hepatocytes, then transports TG from the ER membrane, where lipid molecules are synthesized, to within the lumen of the ER.[44] In a recent study, Wy 14,643 increased the mRNA expression of MTP protein, and enhanced the activity.[45] This report, however, implies that TG secretion from hepatocytes might be stimulated as the results of enhancement of VLDL assembly by Wy 14,643, in spite of its hypolipidemic action. As shown in Fig. 8, the fluctuation of apo B100 exactly reflected the decrease in TG in the medium and increase in cellular TG. Thus, cellular TG amounts and apo B protein in hepatocytes might be provided to secrete or to form VLDL particles.

The involvement of hepatocellular triglyceride hydrolase (hepatic triglyceride lipase HTGL) in cellular TG level remains to be studied, though the enzyme function has not been well established. With regard to TG hydrolysis in the liver, the PPAR agonist, clofibrate and fatty acid enriched-diets did not have effects on hepatic TG hydrolysis in mice.[46] In the present study, HTGL activity using conditioned media from heparin-treated cells was not different between Nf treated and control cells (data not shown).

In summary, PxPs induce hepatic TG accumulation associated with increase in Px-ox activity with a linear correlation in cultured hepatocytes. Based on our results, we concluded

the following as reasons for the hepatocellular TG accumulation. Fatty acid transporter up-regulation by PxPs[15] increases the influx of fatty acids to hepatocyes.[16] Though TG is normally synthesized utilizing the transported fatty acids, TG is accumulated in hepatocytes due to the inhibition of VLDL secretion by PxPs, which is consistent with the report of Schoonjans et al.,[6] leading to a lowering TG in the medium. Together with the results of recent studies on the regulation of membrane efflux transporters in the ABC family by PxPs, such as ABCA1 in intestines by Wy 14,643[47] and multidrug resistance-associated proteins in the liver by clofibrate,[48] it is hypothesized that hepatocellular membrane molecules or structures related to VLDL secretion might be modified by PxPs.

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