Pretranslational regulation of cytochrome P4504A1 by free fatty acids in primary cultures of rat hepatocytes

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Abstract The effect of different fatty acids on cytochrome P4504A1 mRNA levels was studied in primary cultures of rat hepatocytes, using a solution hybridization assay. All fatty acids tested induced P4504A1 mRNA levels in a dose- and time-dependent manner. Most potent were docosahexaenoic acid (22:6) and arachidonic acid (20:4), both of which gave a 6-fold increase in mRNA levels at 300 μM, followed by linolenic acid (18:3) and lauric acid (12:0). The effect of three different sulfur-substituted fatty acids was investigated. Tetradecylthioacetic acid, which is blocked for β-oxidation by sulfur substitution of the β-carbon, induced P4504A1 mRNA levels 8-fold at 300 μM concentration, whereas tetradecylthiopropionic acid, which can undergo one round of β-oxidation, only gave a 2-fold increase at the same concentration. The most pronounced effect was seen with 3,14-dithiahexadecanedioic acid, a dicarboxylic acid with both β-carbons blocked for β-oxidation, which gave a 31-fold induction of mRNA levels at 300 μM. In time-course studies the effect of docosahexaenoic acid, 3,14-dithiahexadecanedioic acid and the potent peroxisomal proliferator Wy 14,643 on P4504A1 mRNA levels was already detectable after 2 h and maximal after 48 h of treatment, which was reflected in increased levels of P4504A1 protein. Taken together, these results show that endogenous fatty acids, such as docosahexaenoic acid and arachidonic acid, act as pretranslational regulators of P4504A1 when added to primary cultures of rat hepatocytes. Blocking their metabolism (β-oxidation) leads to significant enhancement of their activity.—**Tollet, P., M. Strömstedt, L. Frøyland, R. K. Berge, and J-Å. Gustafsson.** Pretranslational regulation of cytochrome P4504A1 by free fatty acids in primary cultures of rat hepatocytes. *J. Lipid Res.* 1994. **35:** 248–254.

Supplementary key words β-oxidation • sulfur-substituted fatty acids • docosahexaenoic acid • arachidonic acid • peroxisome proliferators

The cytochrome P450 superfamily consists of genes encoding multisubstrate monooxygenases (1). These enzymes are important in the metabolism of endogenous and exogenous hydrophobic compounds (2–4). Members of the CYP4A subfamily encode enzymes involved in the metabolism of fatty acids that catalyze the ω- and (ω-1)-hydroxylations of these substrates, including lauric, palmitic, and arachidonic acid, as well as the ω-hydroxylation of prostaglandins E_1 and $F_{2\alpha}$ (5–8). Cytochrome P4504A1 is expressed at a low level in rat liver and is induced by peroxisomal proliferators (9, 10) at the transcriptional level (6).

Peroxisomal proliferators are a structurally diverse group of chemicals that include hypolipidemic drugs, herbicides, and plasticizers (11–13). The administration of these compounds causes a transient lipid accumulation in the liver which is followed by a dramatic increase in fatty acid metabolism, including microsomal ω-hydroxylation (14–16) and peroxisomal β-oxidation (17, 18), accompanied by hepatomegaly (19) and an increase in the number of peroxisomes (20, 21). Similar effects on peroxisomal β-oxidation and peroxisomal proliferation have been observed after feeding rats a high-fat diet (22, 23). A common feature of some peroxisomal proliferators is inability to undergo mitochondrial β-oxidation, which is especially true for long-chain fatty acids (24) and fatty acids blocked for β-oxidation by sulfur substitution (25) or perfluorination (26). Feeding a high-fat diet may have the same effect by overloading the β-oxidation capacity. Although the molecular mechanism by which peroxisome proliferators act is not well understood, several studies indicate that these effects might be mediated by a member of the nuclear receptor supergene family of ligand-activated transcription factors, the peroxisomal proliferator activated receptor (PPAR) (27–29).

The PPAR has recently been shown to act as a transcription factor activated by peroxisome proliferating agents (30, 31), as well as fatty acids (32) in transactivating systems, and mediates peroxisome proliferator responsiveness of the promoter of the acyl coenzyme A oxi-

Abbreviations: PPAR, peroxisomal proliferator activated receptor; TNA, total nucleic acids; ME, tetradecylthioacetic acid; MP, tetradecylthiopropionic acid; TD, 3,14-dithiahexadecanedioic acid.

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dase gene (33) and the CYP4A6 gene (34). Although no ligand has yet been identified for PPAR, these results suggest that key enzymes in fatty acid metabolism may be autoregulated at the transcriptional level by fatty acids through PPAR responsive *cis*-regulatory elements.

Although peroxisomal proliferators are very good inducers of P4504A1, the function and regulation of P4504A1 under normal physiological conditions is not well understood. It is known, however, that P4504A1 mRNA is increased in diabetic animals (10), a condition associated with high serum levels of fatty acids. Furthermore, we have recently shown that the levels of P4504A1 mRNA and enzymatic activity are increased in the livers of rats treated with a single dose of sulfur-substituted fatty acids with different ability to undergo β-oxidation (35). In the present study we have investigated the direct effect of fatty acids on P4504A1 mRNA levels in primary cultures of rat hepatocytes. By culturing primary liver cells on a basement membrane matrix in a serum-free medium, the cells maintain their liver-characteristic phenotype and respond to various inducers of P450 enzymes (36). In this system, several fatty acids known to activate the rat PPAR to different degrees, including sulfur-substituted fatty acids, were compared with a peroxisomal proliferator in terms of potency of elevating P4504A1 mRNA and protein levels.

MATERIALS AND METHODS

Animals and materials

Adult male Sprague-Dawley rats (Alab, Stockholm, Sweden), 8 weeks old, were maintained under standardized conditions of light and temperature, with free access to animal chow and water. Collagenase (type XI), insulin (24.4 U/mg), clofibrate, docosahexaenoic acid, arachidonic acid, linolenic acid, lauric acid, 1,16-hexadecanedioic acid, and salmon sperm DNA were purchased from Sigma Chemical Co. (St. Louis, MO). Wy 14,643 was obtained from Chemsyn Science Laboratories (Lenexa, KS). Sulfur-substituted fatty acids were synthesized as described (35). Glass-fiber filters (Whatman GF/C) were obtained from Whatman Ltd. (Madisone, Kent, UK), and tRNA, proteinase K, RNase-A, and RNase-T_1 were from Boehringer-Mannheim (Mannheim, Germany). Reagents for in vitro transcription of cRNA probes were purchased from Promega Biotech (Madison, WI). The sheep anti-P4504A1 antibody was a generous gift from G. G. Gibson (Guildford, UK). The rabbit anti-sheep antibody and the alkaline phosphatase-conjugated porcine anti-rabbit antibody were obtained from Dako AS (Copenhagen, Denmark). α-Naphthylphosphate and Fast Blue were purchased from Merck AG (Darmstadt, Germany).

Hepatocyte isolation and cell culture

Matrigel was prepared from Engelbreth-Holm-Swarm sarcoma propagated in C57BL/6 female mice and stored at −20°C, as described (36). After thawing on ice, 200 μl was evenly inoculated onto 60-mm plastic dishes and allowed to form a gel at room temperature before cell isolation. Hepatocytes were prepared by nonrecirculating collagenase perfusion through the portal vein of ether-anesthetized rats, according to the method of Bissell and Guzelian (37). Cells were seeded at a density of 3.5×10^6/dish in 3 ml of a standard serum-free medium. The medium was renewed daily. This medium is a modification of Waymouth's medium 752 containing amino acids, salts, vitamins, minerals (zinc and selenium), and insulin (0.1 μg/ml) (37). All cell-medium constituents were of cell culture grade. Cultures were maintained in a humidified incubator at 37°C in an atmosphere containing 5% CO_2. When harvesting the cells, the medium was aspirated from the plates, and cells were washed and scraped with a rubber spatula in ice-cold phosphate-buffered saline–5 mM EDTA. The cell-suspension was incubated on ice for 60 min in order to dissolve the matrigel. Thereafter, cells were pelleted at 750 *g* for 5 min. Treatment of the cells was carried out between 66 and 138 h of cell culture age, a time period when the cells have adapted to the in vitro environment and show stable RNA/DNA ratios. Fatty acids were added to the cell culture medium, containing 0.1% fatty acid-free bovine serum albumin (Sigma), as 300- to 1000-fold concentrated stock solutions in dimethyl sulfoxide (DMSO), and suspended by sonication.

Preparation of total nucleic acids and solution hybridization

Total nucleic acids (TNA) were prepared from the pooled cells from four culture dishes by lysis of the cells in 1% (wt/vol) sodium dodecyl sulfate, 10 mM EDTA, and 20 mM Tris-HCl (pH 7.5). Digestion of samples with proteinase-K and subsequent extraction with chloroform and phenol have been described (38). The concentration of nucleic acids in TNA samples was measured spectrophotometrically.

A synthetic oligonucleotide corresponding to nucleotides 1719–1768 of P4504A1 cDNA (6) was cloned into the Eco RI-Bam HI site of the polylinker of plasmid pSP72. After linearization with Eco RI, the plasmid was used as a template for synthesis of a [^{35}S]UTP-labeled cRNA probe, with an approximate specific activity of 2×10^9 cpm/μg RNA, using SP6 RNA polymerase. Levels of P4504A1 mRNA in TNA samples were analyzed by hybridization to this cRNA probe in solution. Hybridizations were performed at 65°C in a total volume of 40 μl, containing 75 μg/ml tRNA, 0.75 mM dithiothreitol, 25% formamide, 0.6 M NaCl, 5 mM EDTA, 22 mM Tris-HCl (pH 7.5), 0.1% (wt/vol) sodium dodecyl sulfate, and ap-

proximately 20,000 cpm labeled cRNA probe. After overnight incubation, samples were treated with RNase for 45 min at 37°C by the addition of 1 ml solution containing 40 μg RNase A, 500 U RNase T_1, 100 μg salmon sperm DNA, 0.3 M NaCl, 10 mM Tris-HCl (pH 7.5), and 20 mM EDTA. Radioactivity protected from RNase digestion was precipitated by the addition of 100 μl trichloroacetic acid (6 M), collected on a glass-fiber filter, and analyzed by scintillation counting.

Quantitations of P4504A1 mRNA levels were achieved by comparison with a standard curve obtained from hybridizations to in vitro synthesized mRNA. This mRNA was synthesized using a BamHI-linearized plasmid and T7 RNA polymerase, which generates an RNA molecule complementary to the probe. When analyzing β-actin mRNA levels, a plasmid containing a 50 bp insert corresponding to amino acid 331–347 of rat β-actin was used in a way similar to that described for P4504A1. Samples were analyzed in triplicate, and the results are expressed as attomoles of mRNA per μg TNA. The interassay variation averaged 10%. All experiments were performed at least twice, with cells obtained from different rats. Results are expressed as the average of two experiments or, when more than two identical experiments were carried out, as the mean ± SEM.

Preparation of microsomes and Western blotting

Hepatocytes pooled from six culture plates were lysed by sonication for 5 sec, 10 μm amplitude (MSE ultrasonic disintegrator, MSE Scientific Instruments, Crawley, UK), in 0.5 ml of a buffer consisting of 10 mM potassium phosphate (pH 7.4), 1 mM EDTA, and 0.25 M sucrose, and the volume was made up to 3 ml with the same buffer. Microsomes were prepared from cell lysates by sequential centrifugation: 10 000 *g* for 20 min followed by 105 000 *g* for 60 min using a Beckman TL-100 benchtop ultracentrifuge and a TL-100.3 rotor (Beckman Instruments Inc.). The microsomal pellet was resuspended in 0.2 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol. The concentration of proteins in the microsomal samples was determined by the method of Bradford (39).

Western blotting was used to measure immunoreactive P4504A1. Twenty μg of microsomal proteins was reduced and separated on 7.5% sodium dodecyl sulfate/polyacrylamide gels, electrophoretically transferred to nitrocellulose filters, and probed with a polyclonal sheep antibody against P4504A1. As secondary and tertiary antibodies rabbit anti-sheep and alkaline phosphatase-conjugated porcine anti-rabbit antibodies, respectively, were used. The alkaline phosphatase reaction was developed in 100 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$, 0.02 mg/ml α-naphthylphosphate, and 0.02 mg/ml Fast Blue.

RESULTS AND DISCUSSION

Studies both in vivo (14, 21, 35, 40) and in vitro (15, 41, 42) have earlier demonstrated that administration of hypolipidemic drugs, including clofibrate and Wy 14,643, causes peroxisomal proliferation and induction of lipid

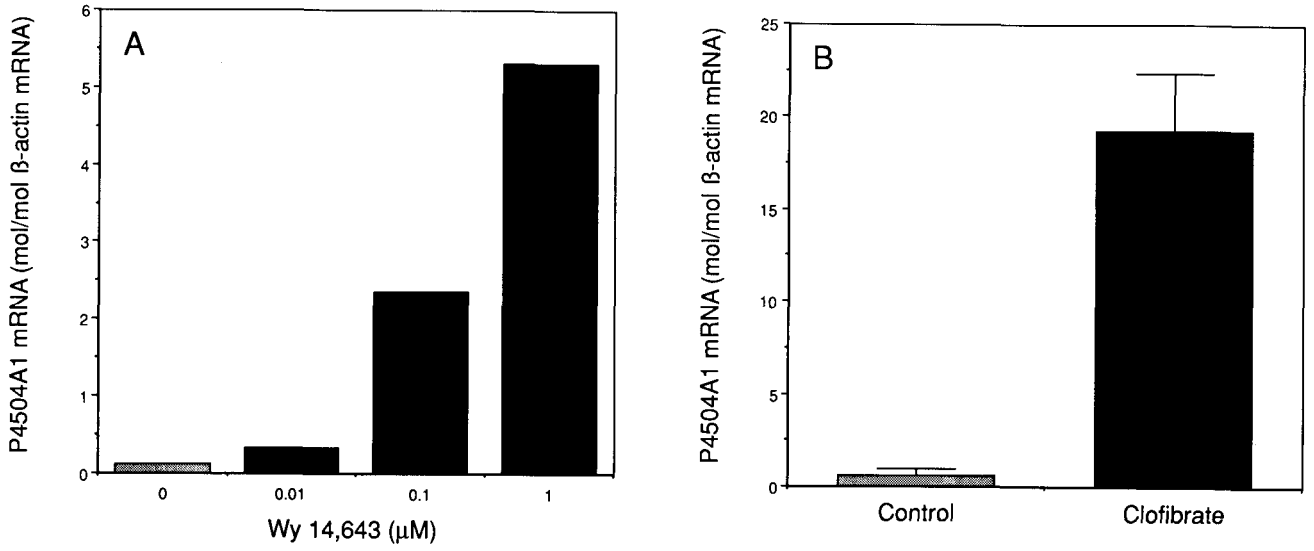

Fig. 1. Effects of peroxisomal proliferators on P4504A1 mRNA levels in vitro and in vivo. A) Primary hepatocytes from adult male rats were kept in culture for 66 h and then treated for 24 h with the indicated amount of Wy 14,643. The cells were harvested, TNA was prepared, and P4504A1 and β-actin mRNA were quantitated by solution hybridization assay as described in Materials and Methods. B) Adult male rats received a single intra peritoneal injection of clofibrate (500 mg/kg) and were killed 24 h later together with untreated rats. Liver RNA was prepared and P4504A1 and β-actin mRNA were quantitated by solution hybridization assay. The results are expressed as mol P4504A1 mRNA/mol β-actin mRNA. Data represent mean ± SEM of three experiments (B), or the average of two experiments (A).

metabolism in rat liver. Cytochrome P4504A1 is responsible for at least part of the fatty acid ω-hydroxylase activity induced by clofibrate (6). Here we show that P4504A1 is increased at the mRNA level to a similar extent (about 30-fold) in rat hepatocytes, maintained on matrigel in a minimal serum-free medium, as in the liver after the administration of Wy 14,643 and clofibrate, respectively (**Fig. 1**). Although the basal expression of P4504A1 is reduced in the hepatocytes, possibly because of the absence of the endogenous inducers, the responsiveness to peroxisomal proliferators is maintained. This cell system is therefore suitable for studying the regulation of P4504A1. The steady state mRNA levels were measured by a solution hybridization assay, using a cRNA probe specific for P4504A1. **Fig. 2** shows a denaturing polyacrylamide gel, demonstrating that one fragment of the expected size (50 bp) was protected from RNase digestion, when total nucleic acids extracted from hepatocytes treated with 300 μM 3,14-dithiahexadecanedioic acid (see below) were hybridized with the ^{35}S-labeled probe.

When the hepatocytes were treated with different endogenous fatty acids, increased levels of P4504A1 mRNA were observed (**Fig. 3**). Most potent were docosahexaenoic acid (22:6) and arachidonic acid (20:4) which both gave a dose-dependent induction of P4504A1 mRNA levels, with a 6-fold increase 24 h after the administration of 300 μM of the fatty acid. Linolenic acid (18:3) at the same concentration, and lauric acid (12:0) at 1 mM, caused 4- and 3-fold inductions of P4504A1 mRNA, respectively. This could be compared to the 30-fold induction of P4504A1 mRNA seen after treating the cells for 24 h with 1 μM of the potent peroxisomal proliferator Wy 14,643 (Fig. 1A). Based on these results, it could be suggested that fatty acids constitute the endogenous activators of P4504A1 expression in rat hepatocytes, whereby

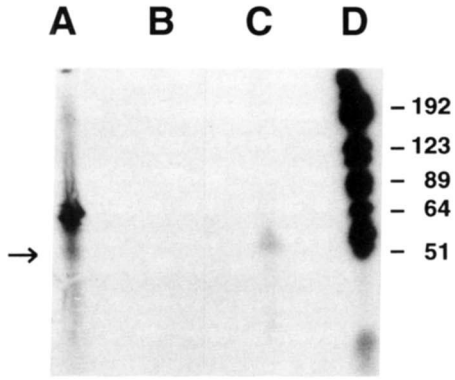

Fig. 2. RNase protection analysis of the P4504A1 riboprobe used in the solution hybridization assay. A) Intact probe; B) probe digested with RNase in the absence of protecting TNA; C) fragment of probe protected from RNase degradation by the presence of TNA prepared from hepatocytes treated with 300 μM TD; and D) size markers, run on a denaturing polyacrylamide gel. The arrow indicates the position of the 50 nucleotides-long protected fragment.

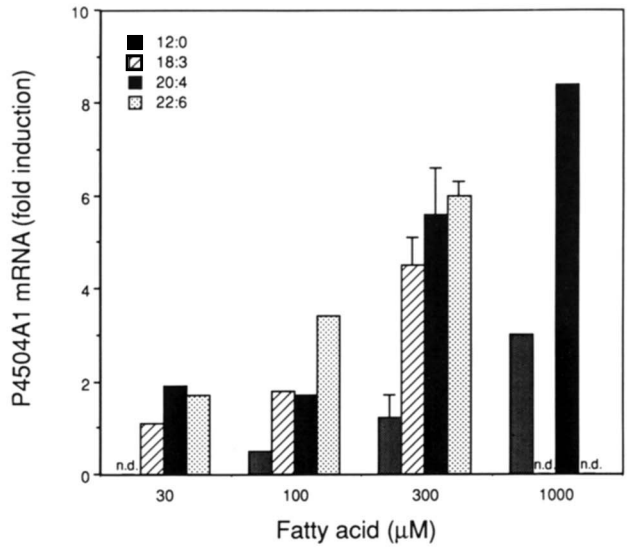

Fig. 3. Effects of fatty acids on P4504A1 mRNA levels. P4504A1 mRNA levels were determined in TNA samples from hepatocytes treated for 24 h with the indicated amounts of lauric acid (12:0), linolenic acid (18:3), arachidonic acid (20:4), and docosahexaenoic acid (22:6) using the solution hybridization assay. Linolenic acid and docosahexaenoic acid were toxic to the cells at the 1 mM concentration. The results are expressed as fold induction compared to control cells receiving vehicle only (0.3% DMSO). Data represent mean ± SEM of three experiments, or the average of two experiments; n.d., not determined.

the degree of fatty acid ω-hydroxylation and further metabolism could be autoregulated in the cell. Interestingly, high concentrations of long chain fatty acids have been shown to cause a limited (150–170%) increase in activities related to peroxisomal β-oxidation in rat hepatocytes, e.g., palmitoyl-CoA oxidase and enoyl-CoA hydratase (43).

It is well established that administration of a high-fat diet to rats causes peroxisomal proliferation and increased lipid metabolism in the liver (22, 23). Not only the amount of lipids, but also the composition of fatty acids in the diet has been shown to be of importance. Particularly, long-chain monounsaturated fatty acids, which are poorly metabolized by mitochondria and therefore dependent on the peroxisomes for chain shortening, seem to be effective in this context (43–45). The difference in potency of inducing P4504A1 mRNA observed between the different fatty acids (Fig. 3) may, in a similar way, be explained by their susceptibility to mitochondrial β-oxidation. This hypothesis is based on the notion that fatty acids are poorly β-oxidized in the mitochondria when they contain more than 20 carbon atoms (24), and the observation that the ability of the fatty acids to induce P4504A1 mRNA increased with increasing chain-lengths, docosahexaenoic acid (22:6) and arachidonic acid (20:4) being the most potent.

To investigate whether different susceptibility of fatty acids to β-oxidation affects their potency to induce P4504A1 mRNA, the effects of three differentially β-

oxidizable sulfur-substituted fatty acids were studied. As shown in **Fig. 4,** the fatty acid tetradecylthioacetic acid (ME), which is blocked for β-oxidation at one end, was more potent than tetradecylthiopropionic acid (MP), which can undergo one round of β-oxidation. Most potent was the dicarboxylic acid 3,14-dithiahexadecanedioic acid (TD), which is blocked for β-oxidation by sulfur-substitution at both ends. After 24 h of treatment with 300 μM ME, MP, or TD, the P4504A1 mRNA levels were increased 2-, 8-, and 31-fold, respectively. These results demonstrate that fatty acids that are blocked for β-oxidation (ME and TD) are more potent inducers of P4504A1 than those that are not (MP and the ones referred to above). This observation is in line with the results earlier reported by us and others (25, 26, 32, 35, 46), showing that inhibition of fatty acid degradation leads to strong induction of peroxisomal proliferation and fatty acid metabolism. Furthermore, Göttlicher et al. have recently demonstrated that ME and TD are more potent than MP as inducers of PPAR-mediated transcriptional activation in primary cultures of rat hepatocytes (47).

The experiments showing that the β-oxidation-blocked dicarboxylic fatty acid TD was much more potent than the monocarboxylic equivalent ME (Fig. 4), led us to investigate whether a non-blocked dicarboxylic fatty acid would be equally efficient in elevating P4504A1 mRNA levels. However, treatment of the cells for 8 h with 300 μM 1,16-hexadecanedioic acid caused the same increase (5-fold) of P4504A1 mRNA as the same dose of

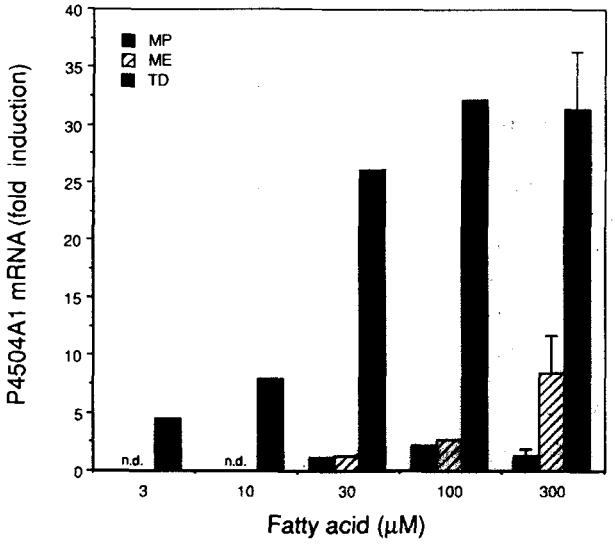

Fig. 4. Effects of sulfur-substituted fatty acids on P4504A1 mRNA levels. P4504A1 mRNA levels were determined in TNA samples from hepatocytes treated for 24 h with the indicated amounts of tetradecylthiopropionic acid (MP), tetradecylthioacetic acid (ME), or 3,14-dithiahexadecanedioic acid (TD). The results are expressed as fold induction compared to control cells receiving vehicle only (0.3% DMSO). Data represent mean ± SEM of three experiments, or the average of two experiments; n.d., not determined.

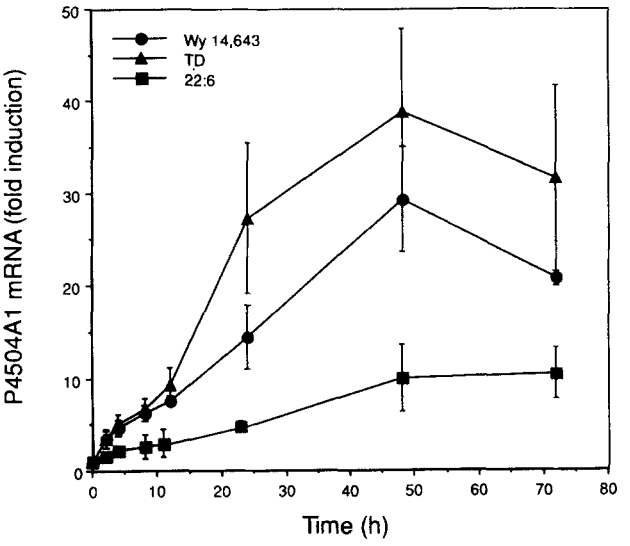

Fig. 5. Time-course of the induction of P4504A1 mRNA levels by Wy 14,643, TD, and docosahexaenoic acid. P4504A1 mRNA levels were determined in TNA samples from hepatocytes treated for the indicated time periods with docosahexaenoic acid (300 μM), TD (300 μM), or Wy 14,643 (10 μM). The results are expressed as fold induction compared to control cells receiving vehicle only. Data represent mean ± SEM of three or four experiments, or the average of two experiments.

docosahexaenoic acid (not shown). These results suggest that the higher potency of TD was due to its complete resistance to metabolism rather than to the presence of an additional carboxylic acid group. ME, although blocked for β-oxidation, may be ω-hydroxylated through the action of cytochrome P450 and then further metabolized to a dicarboxylic acid with one end accessible to β-oxidation. As inhibition of fatty acid degradation is known induce peroxisomal proliferation, these observations support the hypothesis that accumulated fatty acids, their CoA-esters, or any derivative thereof are the ultimate activators of peroxisomal proliferation as well as regulators of enzymes involved in lipid metabolism.

The time-course of induction of P4504A1 mRNA by TD, docosahexaenoic acid, and Wy 14,643 was investigated and found to be similar for the three different agents (**Fig. 5**). An effect of the inducers was already detectable 2 h after administration, and became maximal at 48 h. The increased mRNA levels were accompanied by increased expression of P4504A1 protein, as determined by Western blotting (**Fig. 6**). TD and Wy 14,643 clearly increased the amount of P4504A1 protein in the hepatocytes after 48 and 72 h of treatment. However, at 24 h the difference between untreated and treated cells was barely detectable (data not shown).

In summary, we have shown that endogenous fatty acids act as pretranslational regulators of P4504A1 when added to primary cultures of rat hepatocytes. Furthermore, blockage of their metabolism through β-oxidation led to significant enhancement of their activity, which is

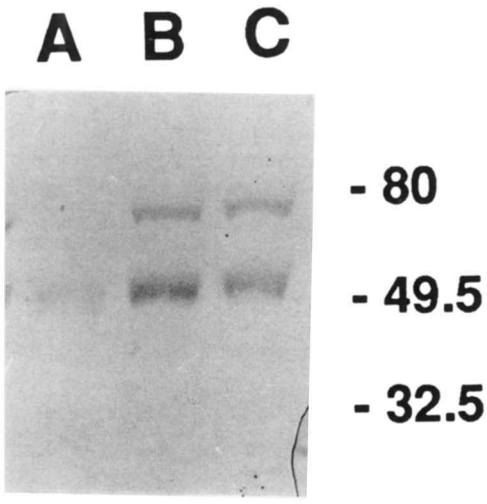

Fig. 6. Induction of P4504A1 protein levels by Wy 14,643 and TD. P4504A1 protein levels were determined in microsomal samples from A) untreated cells, or cells treated for 48 h with B) 300 μM TD or C) 10 μM Wy 14,643, by Western blotting technique, as described in Materials and Methods.

in line with the reported potency of sulfur-substituted fatty acids in activating PPAR-mediated transcription in vitro, as well as peroxisomal β-oxidation, microsomal ω-hydroxylation, and peroxisomal proliferation in vivo. The sulfur-substituted fatty acid TD, the endogenous fatty acid docosahexaenoic acid, and the peroxisome proliferator Wy 14,643 gave similar time-courses of induction of P4504A1 mRNA and protein (TD and Wy 14,643) levels, indicating similar mechanisms of action. The level of P4504A1 enzyme and the degree of fatty acid ω-hydroxylation and further metabolism in the hepatocyte may therefore, possibly via activation of PPAR, be autoregulated by endogenous levels of fatty acids.

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