

A Nuclear Receptor-mediated Choleric Action of Fibrates is Associated with Enhanced Canalicular Membrane Fluidity and Transporter Activity Mediating Bile Acid-independent Bile Secretion

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Fibrates are commonly used lipid-lowering agents that act via PPAR α , a member of the nuclear hormone receptor superfamily. The mechanism(s) of fibrate-induced changes in the hepatic canalicular membrane and bile lipids are still unknown. Therefore, the aim of this study was to investigate the influence of fibrates on hepatic lipid metabolism and to assess the hepatocellular cytoprotective effect on hepatocyte canalicular membrane. Male ICR mice were fed standard chow with or without bezafibrate (100 mg/kg) for 6 days. The expression of canalicular membrane transporters (Mdr2 and Mrp2) was evaluated by RT-PCR and Western blotting. Canalicular membrane fluidity was also investigated. Canalicular membrane fluidity was markedly increased by fibrates. The expression of mdr 2 and mrp2 mRNA and protein showed a significant increase in fibrate-treated mice. These results suggested that fibrates improve liver function by enhancing bile secretion. The mechanism of the choleric action of fibrate therapy might involve the enhancement of bile acid-independent bile secretion, since increased expression of Mdr2 and Mrp2 was found in fibrate-treated animals. These changes were very likely mediated by PPAR α , and the increase of canalicular membrane fluidity may have been partly associated with enhancement of this transporter activity. *J Atheroscler Thromb*, 2005; 12: 211–217.

Key words: ABC transporter, Fibrate, Membrane fluidity, Steatohepatitis

Abbreviations: ABC; ATP binding cassette, DPH; 1, 6-diphenyl-1,3,5-hexatriene, GAPDH; glyceraldehyde-3-phosphate dehydrogenase, PBC; primary biliary cirrhosis, PPAR; peroxisome proliferator-activated receptor

Introduction

Fibrates are widely used in the treatment of hyperlipidemia (1–3). These drugs decrease serum triglyceride lev-

els and increase HDL cholesterol levels in hyperlipidemic patients (1), as well as reducing the risk of coronary heart disease in patients with a low HDL cholesterol level (4).

Fibrates act via nuclear receptors known as peroxisome proliferator-activated receptors (PPARs), which belong to the nuclear hormone receptor gene superfamily. After heterodimerization with the retinoid X receptor (RXR), PPARs alter the transcription of specific genes controlling lipoprotein metabolism by binding to a distinct response element, which consists of a direct repeat of the

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AGGTCA consensus sequence separated by 1 or 2 nucleotides (DR-1, DR-2) (2, 5). Three different types of PPARs have been identified, which are termed α , δ (or β), and γ ; each is encoded by a separate gene and shows a distinct pattern of distribution (5). PPAR α shows a high level of expression in the liver, and controls the expression of genes involved in the β -oxidation of fatty acids as well as genes encoding apolipoprotein A-I, A-II, and C-III (6).

Fibrate therapy has an adverse influence on biliary lipid composition and decreases the excretion of bile acids, leading to the supersaturation of bile acids in gallbladder bile and a consequent increase in the incidence of cholesterol gallstones among patients on long-term therapy (7, 8).

Recently, it has been reported that fibrates are effective for the treatment of cholestatic diseases such as primary biliary cirrhosis (PBC) (9–11). Several lines of evidence indicate that fibrates have beneficial effects in PBC patients, but their mechanism of action is not known.

The formation of bile critically depends on the active secretion of osmotically active compounds from the liver into the bile canalicular lumen. Several transporter proteins at the hepatocyte canalicular membrane are involved in the secretion of biliary constituents in an active, ATP-dependent manner. We speculated that the administration of a fibrate influences hepatocyte membrane activity and bile composition.

The aim of this study was to investigate the effects of fibrates on hepatocytes, membrane fluidity and phospholipids (Mdr2), and organic anion (Mrp2) transporter activity. We demonstrated that the administration of fibrate led to changes in canalicular membrane fluidity and increased the activity of both transporters. This choleric action of fibrate therapy might involve the enhancement of bile acid-independent bile secretion, since increased expression of Mdr2 and Mrp2 has been found in fibrate-administered animals. These effects are very likely mediated by PPAR α , and the increase in canalicular membrane fluidity may be partly associated with enhanced transporter activity.

Materials and Methods

Materials

Bezafibrate was kindly provided by Kissei Pharmaceuticals (Tokyo, Japan). 1,6-diphenyl-1,3,5-hexatriene (DPH) was purchased from Molecular Probes (Oregon, USA). The materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a Western blotting kit were obtained from Bio-Rad Laboratories (Japan). Mouse anti-P-glycoprotein, MDR3 monoclonal antibody was purchased from Chemicon International, Inc (Temecula, CA). K4 antibody to detect Mrp2 was kindly provided by Dr. M. Muller (The Netherlands).

Animals

Male ICR mice weighing 25–30 g were obtained from Hiroshima Jikken Doubutsu (Hiroshima, Japan). All animals were kept in an environmentally controlled facility with a diurnal light cycle. Food was provided ad libitum and the animals had free access to water. The experimental protocol was approved by the Ethics Committee for Animal Experiments (Faculty of Medical Sciences, Hiroshima University).

Experimental design

All of the mice ($n = 10$) were fed standard chow for a 4-week run-in period. Then the animals were randomly assigned to be fed either a control diet or the same diet containing bezafibrate (100 mg/kg body weight dissolved in dimethyl sulfoxide (DMSO)) by a gastric tube for 6 days. The control group was given DMSO alone via the same technique. After the completion of treatment, mice were anesthetized with an intraperitoneal injection of Hypnorm (fentanyl/fluanisone) and diazepam. After the animals were sacrificed, their livers were excised, weighed, cut into small pieces, snap-frozen in liquid nitrogen, and stored at -80°C until further analysis.

Analytical Procedures

Isolation of liver plasma membranes

Liver plasma membranes were isolated by density gradient ultracentrifugation as described elsewhere (12). Aliquots of the membranes were frozen in 10 mmol/l Tris-HCL (pH 7.4) and 250 mM sucrose supplemented with complete protease inhibitor cocktail (Roche, Mannheim, Germany) and stored prior to use at -80°C . Protein concentrations were determined according to the method of Lowry *et al.* (13) using bovine serum albumin as the standard. Enrichment of Na⁺/K⁺-ATPase (a marker enzyme for the canalicular fraction) was assessed as the specific activity of the enzyme in the isolated plasma membranes divided by the activity in the homogenate, and this parameter was used to determine the purity of the isolated membranes.

Membrane fluidity

Membrane fluidity was estimated by the DPH fluorescence depolarization method. In brief, 2×10^{-3} M DPH in tetrahydrofuran was diluted 1,000-fold with vigorous stirring for 10 min at 25°C to create a stable aqueous suspension of DPH (2×10^{-6} M). Then a membrane suspension (100 μg protein/ml) was mixed with an equal volume of the DPH suspension and incubated at 37°C in the dark for 45 min (the time required to reach the maximum fluorescence intensity) with gentle shaking. Steady state fluorescence polarization measurements were performed using a Hitachi F2000 spectrofluorometer (Hitachi, Tokyo, Japan) equipped with a circulating water bath that

was set at 37°C. The excitation and emission wavelengths were 360 and 430 nm, respectively. Polarization (P) values were corrected for light scatter according to the method of Lentz *et al.* (14). The polarization value of DPH fluorescence was used as a measure of membrane fluidity because increased anisotropy indicates a decrease of phospholipid acyl chain motion within the membrane (15). All determinations were performed in triplicate.

Western blot analysis

Canalicular membrane proteins (50 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA) using a tank-blotting system according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). After the transfer, the membranes were blocked for 2 h at room temperature with 5% powdered skim milk dissolved in Tris-buffered saline containing 0.05% Tween 20, and then incubated overnight at 4°C with the primary antibody. Immune complexes were detected using alkaline phosphatase-conjugated anti-rabbit IgG or rabbit anti-mouse IgG (1:3,000) according to the manufacturer's instructions (Bio-Rad Laboratories). The detection of membrane transporters was achieved through comparison with the following standards: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (43 kDa). Then the immunoreactive bands on the membranes were quantified by densitometry.

Analysis of mRNA using the reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from frozen mouse liver tissues using an RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was performed with a GeneAmp RNA PCR Core kit (Roche, Branchburg, NJ) according to the manufacturer's directions. Single-stranded complementary DNA (cDNA) was synthesized from 2 µg of total RNA using M-MuLV reverse transcriptase (Roche), and then subjected to PCR as described previously (16). Specific primer sets were designed for the various hepatic ABC proteins. Using each primer set, an increasing number of PCR cycles was performed with other conditions being fixed in order to determine the optimal number of cycles. In every reaction, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. Sequence analysis confirmed the specificity of the PCR primers for each product. In each experiment, water was used as a negative control. The PCR products (4-µL aliquots) were run on 2% agarose gels, which were stained with ethidium bromide.

Statistical analysis

Differences between the groups were determined by

ANOVA and the level of significance for all analyses was set at $p < 0.05$.

Results

DPH fluorescence polarization

To determine whether the administration of bezafibrate had an influence on membrane fluidity, DPH fluorescence polarization was analyzed by spectrofluorometry. In fibrate-treated mice, the fluorescence polarization value was significantly low compared with that in control mice (fibrate-treated: 0.196 ± 0.003 , control: 0.214 ± 0.001 , $p < 0.01$), indicating that the fibrate caused an increase of canalicular membrane fluidity (Fig. 1). The mechanism of this beneficial effect on canalicular membrane fluidity might be closely associated with alterations of phospholipid and cholesterol metabolism that cause an increase in the cholesterol to phospholipid molar ratio of the hepatocyte membrane.

Western blotting

The expression of hepatic ATP-binding cassette (ABC) transport proteins (Mdr 2 and Mrp 2) during bezafibrate treatment was examined by immunoblotting. The changes of membrane transporter proteins were quantified by densitometry and expressed as a ratio relative to DPPIV, which is constitutionally expressed in the hepatocyte membrane. The expression of both Mdr2 and Mrp2 was significantly increased compared with that in vehicle-treated mice (Fig. 2).

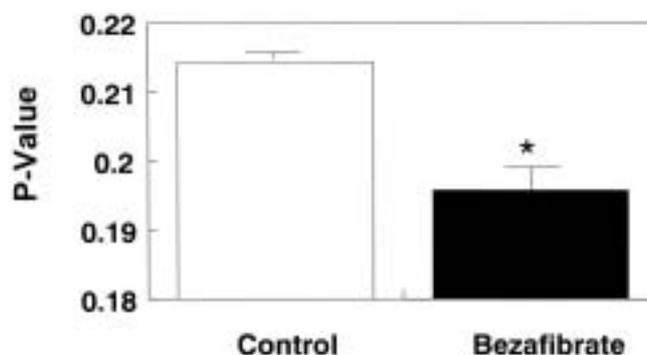


Fig. 1. Effects of fibrate on hepatocyte membrane fluidity. Plasma membranes were prepared from control mice ($n = 4$) and from bezafibrate-treated mice. Membrane fluidity was measured as 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence polarization. In fibrate-treated mice, the fluorescence polarization value was significantly low compared with that in control mice (fibrate: 0.196 ± 0.003 , control: 0.214 ± 0.001 , $p < 0.01$), indicating that the fibrate increased the canalicular membrane fluidity. * $p < 0.05$ compared with controls.

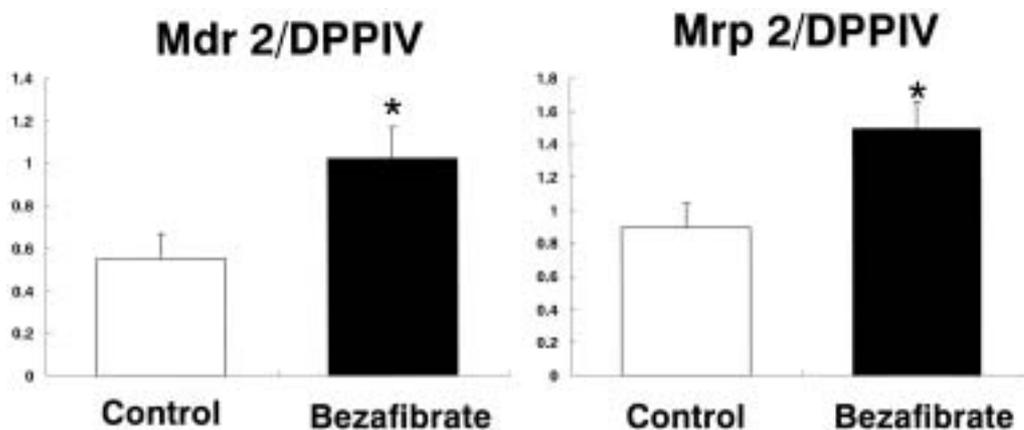


Fig. 2. Western blot analysis of mrp2 and mdr2 protein levels in liver plasma membrane fractions from untreated and bezafibrate-treated mice.

Hepatocyte membrane fractions were isolated from control mice ($n = 4$) and from bezafibrate-treated mice. The changes of membrane transporter proteins were quantified by densitometry and expressed as a ratio relative to DPPIV protein, which is constitutively expressed in the hepatocyte membrane. Data (mean \pm SD) are expressed as a percentage of the control bands intensity. * $p < 0.05$ compared with the control

mRNA analysis by RT-PCR

The relative mRNA levels of selected hepatic ABC transport proteins were analyzed by RT-PCR. As shown in Fig. 3, the steady-state level of Mrp2 mRNA was clearly higher in the livers of treated mice than in the livers of control mice and the Mdr2 mRNA level was also increased by fibrate treatment. These results were consistent with the changes in expression of these proteins on the canalicular membrane (Fig. 2) and were also closely associated with the membrane fluidity data (Fig. 1). In contrast, GAPDH levels were not influenced by fibrate treatment. The expression of PPAR α mRNA was significantly increased by the administration of its ligand, bezafibrate (Fig. 3).

Discussion

Fibrates are commonly used lipid-lowering agents that act via PPAR α , a member of the nuclear hormone receptor superfamily. PPAR α shows high levels of expression in the liver and plays a pivotal role in lipoprotein metabolism.

Recently, Kurihara *et al.* has reported that the treatment of PBC with bezafibrate led to a sustained improvement in the levels of hepatobiliary enzymes and IgM during a relatively long period of monotherapy (10). We have speculated about the beneficial effects of fibrates with regard to the activation of PPAR α . Because bezafibrate is a ligand with a strong affinity for PPAR α , it may im-

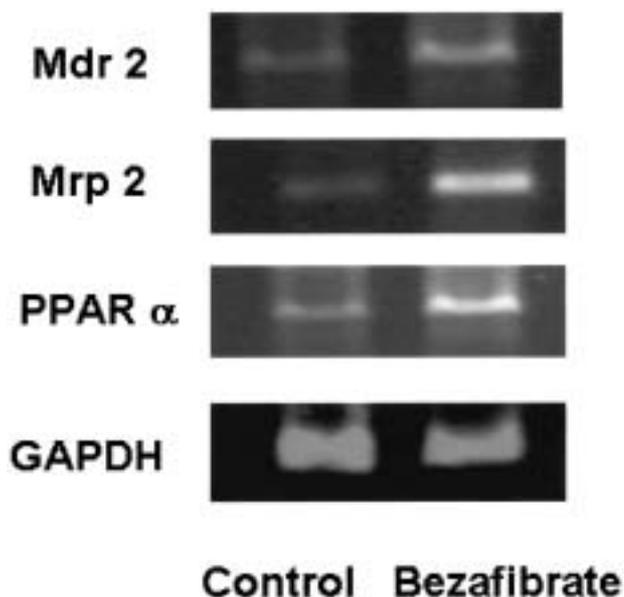


Fig. 3. Expression of mdr2, mrp2, PPAR α , and GAPDH mRNA in the livers of control and bezafibrate-treated mice.

Mice were fed either a control diet or the same diet containing bezafibrate (100 mg/kg) dissolved in dimethyl sulfoxide (DMSO) via a stomach tube for 6 days. The control group was administered DMSO alone using the same technique. PCR products were separated on 2% agarose gel. A representative result for each group ($n = 4$) is shown.

prove symptoms of PBC and steatohepatitis by eliciting several responses via PPAR α . First, it causes the inactivation of leukotriene B₄ by activating the β and ω oxidation of fatty acids (17). Second, it decreases the levels of interleukin-6 and cyclo-oxygenase by suppressing the activation of nuclear factor κ B, a transcription factor (18). Third, apolipoprotein A-II levels are increased via PPAR α , suppressing the expression of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 (19). In addition, Inoue *et al.* have reported that bezafibrate had an antioxidative effect through activation of the PPAR α pathway in the liver (20, 21). They found that the administration of bezafibrate significantly reduced the level of plasma thiobarbituric acid-reactive substances and was closely associated with expression of the superoxide dismutase gene and PPAR α in the liver.

Furthermore, it is well known that fibrates affect biliary lipid excretion via PPAR α and lead to alterations of bile components. For example, fibrate treatment causes adverse changes in biliary lipid composition and decreases the excretion of bile acids, leading to the supersaturation of gallbladder bile and consequently to an increased incidence of cholesterol gallstones in patients on long-term therapy (4, 5). Post *et al.* have recently reported that fibrates inhibit bile acid synthesis via PPAR α -mediated down-regulation of cholesterol 7 α -hydroxylase and sterol 27-hydroxylase, which may contribute to the formation of gallstones in patients on long-term therapy (22).

It has been reported that fibrates induce the expression of *mdr2*, which encodes the canalicular phospholipid translocator Mdr2 Pgp in rodents (23). Kok *et al.* recently investigated the physiological role of PPAR α using PPAR α null mice (24). They clearly demonstrated that the induction of *Mdr2* mRNA and protein expression by fibrates is mediated by PPAR α (24). In this study, we confirmed that *Mrp2* protein and mRNA expression were up-regulated in fibrate-treated mice as well as *Mdr2* protein and mRNA expression. The enhancement of these proteins might be regulated by the PPAR α pathway. Production of *Mdr2* protein in the canalicular membrane leads to the secretion of phospholipids into the bile and the formation of micelles with hydrophobic bile salts, thereby having a cytoprotective effect on biliary epithelial cells (25). *Mrp2* expression is closely associated with bile acid-independent bile flow (BAIF), which is regulated by organic anions such as glutathione (26). Bile formation in the liver is driven by an osmotic gradient that is generated by the active secretion of relatively membrane-impermeable solutes into the canalicular lumen (25, 27). The lumenal accumulation of such osmotically active solutes promotes the movement of water and electrolytes through tight junctions and across the canalicular membrane. The active secretion of bile acids and their conjugates has been shown to account for 40–50% of canalicular bile flow in all species examined (28). In this study,

we demonstrated that expression of *Mrp2* was significantly up-regulated in fibrate-treated mice. Enhancement of *Mrp2* activity by over-expression might contribute to an increase of BAIF and thus lead to improved liver function. Kok *et al.* have reported that hepatobiliary bile salt flux was unaffected, but bile flow was strongly increased, in fibrate-treated mice and this latter effect was not seen in fibrate-treated PPAR α null mice. They concluded that the increased bile flow might be related to the PPAR α -dependent excretion into bile of fibrate metabolites or other metabolites/solutes secondary to the activation of PPAR α .

Another interesting finding was the alteration of hepatocyte membrane fluidity in fibrate-treated mice. To confirm these changes, we directly measured the membrane fluidity using fluorescence polarization. Fluidity was increased significantly after the administration of bezafibrate (Fig. 1), and this would represent a cytoprotective response to cholestasis. Taken together, these results indicate that changes to the membrane may confer on hepatocytes a resistance to increased intra-ductal pressure in the bile canaliculi and bile reflux. Moreover, these changes were tightly associated with the transporter activity of the canalicular membrane (*Mdr2* and *Mrp2*). Several reports have suggested a connection between membrane lipid fluidity and membrane proteins (29–32). Simon *et al.* have reported that decreased sinusoidal membrane lipid fluidity was correlated with altered Na⁺-K⁺-ATPase activity in cholestasis induced by ethinyl estradiol (29), while Davis *et al.* demonstrated that this decrease of activity was reversed *in vitro* by addition of detergents that are known to increase membrane fluidity (32). Similar results correlating liver plasma membrane fluidity with membrane protein function have been reported by Storch and Schachter (30, 31). Taken together, these investigations indicate that the physiological function of membrane transporters might be influenced by membrane fluidity, which in turn is modulated by the fatty acid composition of phospholipids of the membrane lipid bilayer. We clearly demonstrated that the administration of a fibrate increased the membrane fluidity of hepatocytes and transporter activity.

The mechanism of the choleric action of fibrate therapy might be enhancement of bile acid-independent bile secretion, since increased expression of *Mdr2* and *Mrp2* was found in fibrate-treated mice. These effects would probably be mediated by PPAR α , and the increase of canalicular membrane fluidity may be partly associated with enhancement of transporter activity.

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