Bezafibrate stimulates canalicular localization of NBD-labeled PC in HepG2 cells by PPARα-mediated redistribution of ABCB4

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Abstract **Fibrates, including bezafibrate (BF), upregulate the expression of ATP binding cassette protein B4 (ABCB4) through gene transcription in mice. To determine the effects of BF on the expression levels of ABCB4 and on the stimulation of biliary phosphatidylcholine (PC) transport in human HepG2 hepatoblastoma cells, mRNA and protein levels as well as subcellular localization were investigated in the cells treated with BF. The canalicular accumulation of a fluorescent PC was assessed by confocal laser scanning microscopy. Treatment with 300 μmol/l BF for 24 h increased levels of ABCB4 mRNA but not protein by up to 151%. BF caused redistribution of ABCB4 into pseudocanaliculi formed between cells. In association with this redistribution, BF accelerated the accumulation of fluorescent PC in bile canaliculi (up to 163% of that in nontreated cells). Suppression of peroxisome proliferator-activated receptor α (PPARα) expression by either a small interfering RNA duplex or morpholino antisense oligonucleotide attenuated the BF-induced redistribution of ABCB4. These findings suggest that BF may enhance the capacity of human hepatocytes to direct PC into bile canaliculi via PPARα-mediated redistribution of ABCB4 to the canalicular membrane. This provides a rationale for the use of BF to improve cholestasis and/or cholangitis that is attributable to hypofunction of ABCB4.**—Shoda, J., Y. Inada, A. Tsuji, H. Kusama, T. Ueda, T. Ikegami, H. Suzuki, Y. Sugiyama, D. E. Cohen, and N. Tanaka. **Bezafibrate stimulates canalicular localization of NBD-labeled PC in HepG2 cells by PPARα-mediated redistribution of ABCB4.** ***J. Lipid Res.*** **2004.** 45: **1813–1825.**

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Phosphatidylcholine (PC), the major class of biliary phospholipids, cholesterol, and bile acids form mixed micelles in bile. This protects the biliary epithelium from the detergent action of hydrophobic bile acids (1), which are present at high concentrations in the biliary tree (2, 3).

The PC-translocating activity of ATP binding cassette protein B4 (ABCB4), formerly known as the multidrug-resistance 2 P-glycoprotein (mdr2), the murine homolog of human *ABCB4* (*MDR3*), in the canalicular membranes of hepatocytes is considered the rate-limiting step in biliary phospholipid secretion in vivo (4–6). Evidence in support of ABCB4 as a PC translocator has been provided using membranes of yeast secretory vesicles (7, 8).

In mice, the phospholipid deficiency attributable to homozygous disruption of ABCB4 results in liver injury associated with chronic nonsuppurative destructive cholangitis (9) as well as spontaneous cholesterol cholecystolithiasis and hepatolithiasis (10). Decreased or even absent expression of ABCB4 has been observed in several clinical forms of cholestasis, such as progressive familial intrahepatic

Abbreviations: ABC, ATP binding cassette protein; BF, bezafibrate; CD26, cluster of differentiation 26; CLSM, confocal laser scanning microscopy; C6-NBD-PC, nitrobenzoxadiaole-labeled 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-y)amino]caproyl]-*sn*-glycero-3-phosphatidylcholine; PAb, polyclonal antibody; PC, phosphatidylcholine; PCTP, phosphatidylcholine transfer protein; PPARα, peroxisome proliferator-activated receptor α; siRNA, small interfering RNA.

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cholestasis type 3 (11), intrahepatic cholestasis during pregnancy (12), primary hepatolithiasis (intrahepatic bile duct stones) (13, 14), and low phospholipid-associated cholelithiasis (15). These genetic abnormalities provide an important link between the defective canalicular transport of phospholipids and the development of hepatocholangiopathies and indicate that ABCB4 may be a target for novel therapeutic strategies.

Fibrates are antihyperlipidemic drugs that upregulate the expression of ABCB4 through gene transcription and promote biliary phospholipid secretion (16). Biliary PC secretion is limited by the amount of ABCB4 present in the canalicular membrane of the hepatocytes (5, 11, 17, 18). Clinically, treatment with bezafibrate (BF) improves the increased serum levels of biliary enzymes in patients with primary biliary cirrhosis (19, 20), a chronic cholestatic liver disease characterized by progressive inflammatory destruction of the intrahepatic bile ducts. The beneficial therapeutic effects of BF in patients with primary biliary cirrhosis suggests the possibility that the drug promotes the PC-translocating function of ABCB4 by increasing the protein amount in the bile canaliculi and improves the bile duct injury through protecting the cholangiocytes from hydrophobic bile acid toxicity by forming mixed micelles with the increased biliary PC. Moreover, because the expression and function of ABCB4 is regulated by peroxisome proliferator-activated receptor α (PPARα) in mice (21), and because BF, acting as a ligand for PPARα (22), upregulates the transcriptional level of PPARα (22), we explored whether BF-induced changes in ABCB4 expression and function are mediated by PPARα.

Well-differentiated human HepG2 hepatoblastoma cells express canalicular ABCB4 (23). The functional activity of ABCB4 was demonstrated by the movement of fluorescent PC into pseudocanaliculi of the cells (23). Because retrieval of transporters from and insertion into the canalicular membrane play important roles in bile formation (24, 25), we used HepG2 cells as a model to study the regulation of transporter localization.

In this study, we investigated the effects of BF and PPARα on the expression levels and localization of ABCB4 and on PC distribution in HepG2 cells. Our results suggest that BF may enhance the capacity of human hepatocytes to direct PC into bile canaliculi via PPARα-mediated redistribution of ABCB4 to the canalicular membrane.

MATERIALS AND METHODS

Cell cultures

Human HepG2 hepatoblastoma cells (Riken Genebank, Ibaraki, Japan) were cultured in DMEM (Sigma-Aldrich) containing 10% fetal calf serum (Invitrogen Japan, Tokyo, Japan). Cells were kept in tissue culture flasks (Falcon, Heidelberg, Germany) in a humidified 5% CO_2 atmosphere at 37°C. HepG2 cells were subcultured and seeded in flasks, on collagen I-coated two-well chamber slides (BIOCOAT; Falcon), or on glass-bottom dishes (Asahi Techno Glass, Tokyo, Japan). HepG2 cells were grown to confluence for use in experiments in which vehicle (distilled water) or BF (100 or 300 μmol/l) was added to the medium at 37°C for up to 24 h.

Determination of steady-state mRNA levels

Total RNA was isolated from cell pellets using ISOGEN (Nippon Gene, Tokyo, Japan). Steady-state mRNA levels in cells were determined by real-time quantitative PCR using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probes of ABCB4, ABCC2 (formerly known as the multidrug resistance-associated protein 2, or MRP2), ABCB11 (formerly known as the bile salt-exporting pump), phosphatidylcholine transfer protein (PCTP), and PPARα were designed using Primer Express (Applied Biosystems) (**Table 1**). For GAPDH, the primers and probe in PreDeveloped TaqMan Assay Reagents (Applied Biosystems) were used. Reverse Transcriptase qPCR Master Mix (Eurogentec SA, Seraing, Belgium) was used in the assay.

Immunoblot analysis

Cell lysates were prepared using RIPA buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1% protease inhibitor cocktail). Plasma membranes of the cells were prepared in a lysis buffer (10 mM Tris-HCl, 10 mM

TABLE 1. Primers and double dye probes used for real-time quantitative PCR

Name		Sequence
ABCB4 forward primer	:	5′-CAGTACTGGTGCACTTTCTACAAGACTT-3′
ABCB4 reverse primer	:	5′-TGCAATTAAAGCCAACCTGGTT-3′
ABCB4 probe	:	FAM5′-CACAGATGCTGCCCAAGTCCAAGGA-3′TAM
ABCC2 forward primer	:	5′-ATGCAGCCTCCATAACCATGA-3′
ABCC2 reverse primer	:	5′-CTTCGTCTTCCTTCAGGCTATTCA-3′
ABCC2 probe	:	FAM5′-TCGAACACTTAGCCGCAGTTCTAGGTCCA-3′TAM
ABCB11 forward primer	:	5′-GGCCATGACATTCGCTCTCT-3′
ABCB11 reverse primer	:	5′-TGGCAGCTTGGACTATGTCTTC-3′
ABCB11 probe	:	FAM5′-TTCAGTGGCTTAGAGATCAGATTGGGATAGTGGA-3′TAM
PCTP forward primer	:	5′-CAAATTCCGTCCTGGCTCAT-3′
PCTP reverse primer	:	5′-CAACCCATGGATGCAATGTTC-3′
PCTP probe	:	FAM5′-ACATGGCAAGAGCCTGTCAGAACTACCTCA-3′TAM
PPARα forward primer	:	5′-GAATCTACGAGGCCTACTTGAAGAAC-3′
PPARα reverse primer	:	5′-CAGCCATACACAGTGTCTCCATATC-3′
PPARα probe	:	FAM5′-CATCCTCTCAGGAAAGG-3′TAM

ABCB4, ATP binding cassette protein B4; FAM, 6-carboxyfluorescein; PCTP, phosphatidylcholine transfer protein; PPARα, peroxisome proliferator-activated receptor α; TAM, 6-carboxy-tetramethyl-rhodamine.

KCl, 1.5 mM $MgCl_2$, 0.5% SDS, and 1% protease inhibitor cocktail, pH 7.4) (26). Immunoblotting of ABCB4, ABCC2, ABCB11, and cluster of differentiation 26 (CD26) was performed using 50 μg of plasma membrane proteins. Immunoblotting of PCTP, PPARα, and GAPDH was performed using 15, 20, and 20 μg of protein of cell lysates, respectively. Proteins separated by SDS-PAGE were electrophoretically transferred onto polyvinylidene difluoride membranes (Hybond P; Amersham, Buckinghamshire, UK). The membrane was probed with a monoclonal antibody raised against human ABCB4 (6/1G from Chemicon, Temecula, CA), a polyclonal antibody (PAb) raised against the carboxy-terminal end of human ABCC2 (27), a PAb raised against the carboxy-terminal end of human ABCB11 (28), a PAb against human PPARα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or a PAb against human PCTP (29). The membrane was also probed with a PAb against human CD26, a reference marker of canalicular membrane (Santa Cruz Biotechnology, Inc.) or a PAb against human GAPDH (Trevigen, Gaithersburg, MD). Immunoreactive bands were visualized, quantified, and normalized to levels of CD26 or GAPDH.

Immunohistochemical localization of canalicular transporter proteins

HepG2 cells were treated with vehicle or BF (300 μmol/l) for 24 h (four experiments in each group). After fixation in 3.7% formaldehyde, the cells were incubated for 2 h with the monoclonal antibody of ABCB4, the PAb of ABCC2, the PAb of ABCB11, or the PAb of CD26. The cells were subsequently incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG plus IgM or Cy3-conjugated donkey rabbit IgG antibodies. Images were obtained using confocal laser scanning microscopy (CLSM) (TCS-SP2; Leica Lasertechnik GmbH, Wetzlar, Germany). After washing with PBS, the cells were fixed using a Slow Fade Antifade kit (Molecular Probes, Eugene, OR). The slides were analyzed using CLSM. Images were acquired from two channels at wavelengths of 488 and 543 nm. Cross-talk of fluorochromes was excluded by the use of a tunable optical filter.

Localization of fluorescent PC

HepG2 cells were seeded onto glass-bottom dishes (Asahi Techno Glass). Confluent HepG2 cells variably formed pseudocanaliculi (i.e., bile canaliculus-like structures) of different sizes between adjacent cells. After the confluent cells had been incubated with vehicle or BF (300 μmol/l) for 24 h, the fluorescent substrate, nitrobenzoxadiazole-labeled 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-y)amino]caproyl]*sn*-glycero-3-phosphatidylcholine (C6-NBD-PC) dissolved in ethanol, was added to the cells to a final concentration of 1 μmol/l (final ethanol concentration was less than 0.03%, v/v) (30). Fluorescent images were acquired every minute for 20 min using a CLSM apparatus equipped with a HCX PL Apo 40S/0.85 COPR objective and an argon ion laser with a 488 nm excitation wavelength. Changes in the intensity of C6-NBD-PC in the pseudocanaliculi were calculated according to the ratio of fluorescence of pseudocanaliculus/fluorescence of visual field.

Suppression of PPARα expression

Suppression of PPARα expression in HepG2 cells was accomplished using either a small interfering RNA (siRNA) duplex or morpholino antisense oligonucleotide targeted to PPARα mRNA sequences. The designs of the individual siRNA duplex and morpholino antisense oligonucleotide are summarized in **Table 2**. Cells were cultured on 24-well plates in 1 ml of DMEM with 10% fetal calf serum. When the cells were ~50–60% confluent, they were transfected with either a siRNA duplex (60 pmol/well; Qiagen-Xeragon, Germantown, MD) or morpholino antisense oligonucleotide (0.7 nmol/well; Gene Tools, Philomath, OR) using Oligofectamin reagent (Invitrogen) or ethoxylated polyethylenimine (Gene Tools), respectively. The cells were retransfected with the siRNA duplex or morpholino antisense oligonucleotide again 24 h after the initial transfection. Four hours after the second transfection, the cells were treated with vehicle or BF (300 μmol/l) for 24 h. Total RNA and cell lysates were then prepared, and mRNA and protein levels of PPARα were determined as described above. To investigate the subcellular localization of ABCB4, the cells were fixed in 3.7% formaldehyde and then immunolabeled as described above.

To assess transfection efficacy, the cells were transfected with a fluorescein-labeled control siRNA duplex in which the nucleotide sequence was scrambled (Qiagen-Xeragon). Seven hours after the treatment, transfected cells were washed with PBS and analyzed by CLSM.

Statistics

Statistical analysis was performed using the Duncan multiple statistical analysis method for Excel statistics program version 5 (Esumi, Tokyo, Japan). For experiments on PC distribution, the comparison of vehicle- and BF-treated groups was made using repeated-measures ANOVA (StatView; SAS Institute, Tokyo, Japan). At each point, comparisons between the two groups were made using Student's *t*-test (Excel 2000; Microsoft, Tokyo, Japan). $P < 0.05$ was considered statistically significant.

RESULTS

Effects of BF on steady-state expression levels of ABCB4 and PCTP

Steady-state mRNA levels of ABCB4 and PCTP, an intracellular PC transporter (31), were determined in HepG2 cells treated with vehicle or BF by real-time quantitative PCR. As shown in **Fig. 1A**, treatment with 100 μmol/l BF for 16 and 24 h and with 300 μmol/l BF for 24 h significantly increased the mRNA level of ABCB4 in the cells (up to 151% of the level in vehicle-treated cells). However, BF treatment did not result in any significant changes in the mRNA level of PCTP in the cells (Fig. 1B). Protein levels of ABCB4 and PCTP in cells treated with vehicle or BF (100 or 300 μmol/l) (Fig. 1C) showed no significant changes when normalized for the expression of CD26 (Fig. 1D).

TABLE 2. An siRNA duplex and a morpholino antisense oligonucleotide for PPARα

Name		Sequence
PPARα siRNA duplex	:	5′-GGCCAGUAACAAUCCACCUTT-3′ 3′-TTCCGGUCAUUGUUAGGUGGA-5′
PPARα morpholino antisense oligonucleotide	:	5′-GAGTGGGCTTTCCGTGTCCACCATC-3′

siRNA, small interfering RNA.

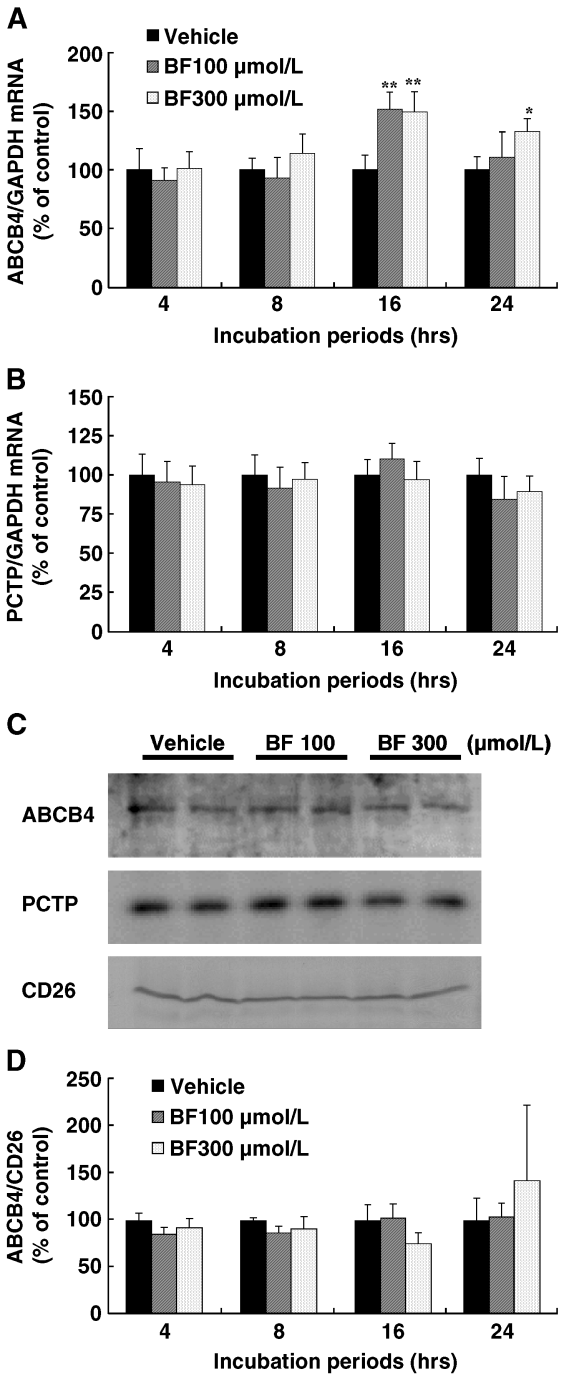

Fig. 1. Effects of bezafibrate (BF) on expression levels of ATP binding cassette protein B4 (ABCB4) and phosphatidylcholine transfer protein (PCTP). A and B: Steady-state mRNA levels of ABCB4 (A) and PCTP (B) in HepG2 cells treated with vehicle or BF. mRNA levels were quantified by real-time quantitative PCR using specific primers and double-dye probes (Table 1). ABCB4 mRNA levels were significantly increased in the cells treated with BF (100 or 300 μmol/l) for 16 and 24 h. All experiments were performed in duplicate. Data were normalized to the amounts of GAPDH mRNA present in each specimen. Data are presented as means ± SEM of four to six experiments. * $P < 0.05$, ** $P < 0.01$ versus vehicle-treated cells. C: Representative bands of ABCB4, PCTP, and cluster of differentiation 26 (CD26) proteins in immunoblot analysis of the lysates of HepG2 cells treated with vehicle or BF for 24 h. D: Densitometric analysis of ABCB4/CD26 and PCTP/CD26 ratios in HepG2 cells treated with vehicle or BF for 24 h. Data are presented as means ± SEM of four experiments.

Immunohistochemical localization of canalicular transport proteins

As demonstrated by contrast light microscopy, confluent HepG2 cells formed pseudocanaliculi of different sizes between adjacent cells (**Fig. 2A**). Immunohistochemistry using antibodies raised against human ABCB4, ABCC2, and ABCB11 showed staining of equivalent structures in HepG2 cells (Fig. 2B). Higher microscopic magnification of the cells revealed that the predominant staining of ABCB4, ABCC2, and ABCB11 was in the pseudocanaliculi, with some additional intracellular staining noted as well. Intracellularly, these transporters were confined to the apical compartment and were colocalized with the canalicular marker protein CD26 (Fig. 2B), and their subcellular localization in HepG2 cells resembled the transporter distribution in intact liver (32).

Effects of BF on the subcellular localization of ABCB4

The number of detectable ABCB4-positive pseudocanaliculi was far lower than the number of cells, indicating apical accumulation of ABCB4 in only a subpopulation of HepG2 cells. The ratio of ABCB4-positive pseudocanaliculi to cell nuclei in the vehicle-treated cells was 0.024 ± 0.003 (mean ± SEM; n = 6). The addition of 300 μmol/l BF to confluent HepG2 cells did not affect this ratio (0.024 ± 0.002; n = 6). In cells treated with 100 μmol/l BF for 16 and 24 h, the immunostaining intensity of ABCB4 in the pseudocanaliculi was increased compared with that in vehicle-treated cells by up to 134% (**Fig. 3A**), as was the intensity ratio of ABCB4/CD26 (Fig. 3B). Similar results were also obtained for the cells treated with 300 μmol/l BF for 8, 16, and 24 h (Fig. 3B).

Effects of BF on localization of C6-NBD-PC to pseudocanaliculi

Fluorescent C6-NBD-PC was used to examine the functional activity of ABCB4 in HepG2 cells. Strong accumulation of fluorescence in pseudocanaliculi of the cells was detectable within 5 to 20 min by CLSM, and the localization of the fluorescent PC reflected the distribution of ABCB4 staining (**Fig. 4A**). Considering the colocalization of C6-NBD-PC and ABCB4, we examined the effects of BF on the time course of PC localization to pseudocanaliculi using CLSM.

Although the background fluorescence intensity was slightly increased in vehicle-treated cells after the addition of the fluorescent PC, C6-NBD-PC was initially distributed in membranes other than pseudocanaliculi and subsequently accumulated in pseudocanaliculi (Fig. 4A). As shown in Fig. 4B, treatment with 300 μmol/l BF caused a significant increase in the movement of C6-NBD-PC to pseudocanaliculi compared with the vehicle-treated cells ($P < 0.01$, repeated-measures ANOVA). The fluorescence intensities of C6-NBD-PC in pseudocanaliculi were significantly higher in the BF-treated cells than in the vehicle-treated cells for the first 3 min after addition of the fluorescent PC (Fig. 4B). Moreover, the area under the curve of fluorescence intensity (corresponding to the amount of PC distributed) within 5 min was significantly higher in

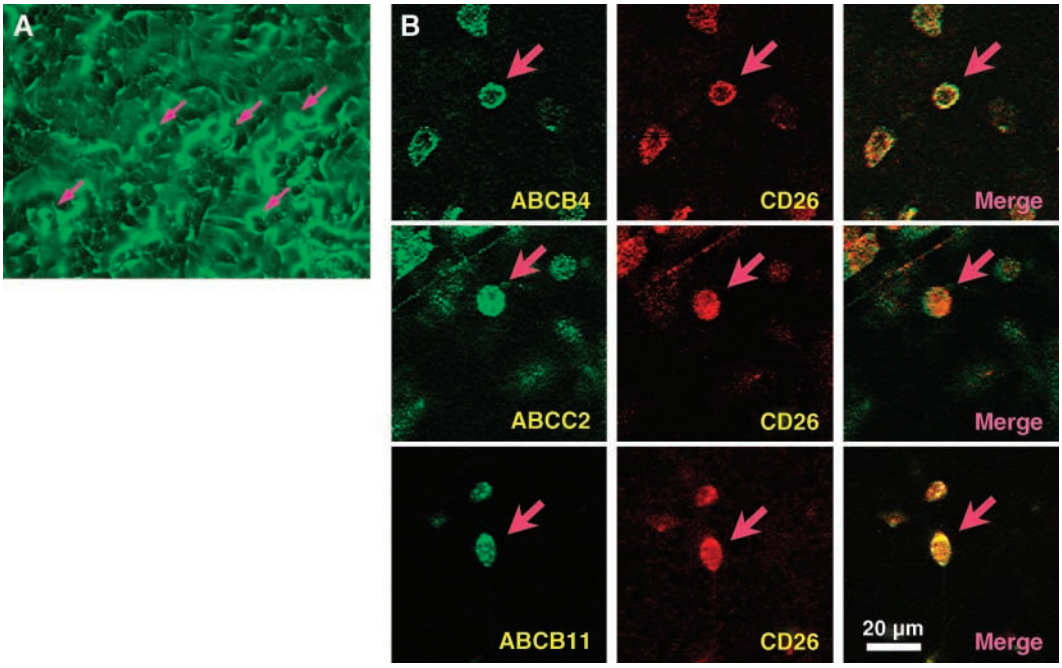

Fig. 2. Localization of transporter proteins in pseudocanaliculi (bile canaliculus-like structures) formed by cultured HepG2 cells. A: Phase-contrast image of the pseudocanaliculi of confluent HepG2 cells indicated by arrows. B: Immunofluorescent images of ABCB4, ABCC2, ABCB11 (green), and CD26 (red) in pseudocanaliculi (indicated by arrows). Each transporter was colocalized (yellow) with CD26, a canalicular marker protein, in the pseudocanaliculi.

the BF-treated cells than in the vehicle-treated cells (163 ± 21%; $P < 0.05$) and tended to be higher within 10 min (120 ± 15%; $P = 0.07$) (Fig. 4C). Pretreatment of the cells with verapamil (50 μmol/l), an inhibitor of ABC transporters (33, 34), abolished the BF-induced localization of C6-NBD-PC to pseudocanaliculi (Fig. 4B). Therefore, these results revealed that the increase in immunoreactive ABCB4 in the pseudocanaliculi of BF-treated cells was accompanied by an increase in the movement of PC to the canalicular pole.

Suppression of PPARα expression by transfection with a siRNA duplex or morpholino antisense oligonucleotide

HepG2 cells transfected with a fluorescein-labeled scrambled control siRNA duplex are shown in **Fig. 5A**. In these experiments, the transfection efficiency of the control siRNA duplex was estimated to be 35 ± 6% (mean ± SEM; four experiments). The efficiency was increased by repeat transfection. In cells transfected twice with the siRNA duplex targeted to PPARα, the levels of PPARα mRNA were markedly reduced by 64% compared with the levels in the cells transfected with the control siRNA duplex (Fig. 5B). Protein levels of PPARα in the transfected cells were also reduced by 64% (Fig. 5C, D). Transfection with a morpholino antisense oligonucleotide targeted to PPARα also suppressed PPARα protein expression by 57% (Fig. 5C, D).

To investigate the effects of PPARα suppression on the BF-induced redistribution of ABCB4 into pseudocanaliculi, immunofluorescent staining of ABCB4 was performed in the cells transfected with either the siRNA duplex or morpholino antisense oligonucleotide targeted to PPARα (**Fig. 6**). On the immunoblot analysis, transfection of the siRNA duplex or morpholino antisense oligonucleotide itself did not affect protein levels of ABCB4 in HepG2 cells (data not shown). In addition, the transfection did not affect the protein levels of ABCB4 in pseudocanaliculi, because there was no difference between the ABCB4/CD26 ratios in the pseudocanaliculi of vehicle-treated cells transfected with the control siRNA duplex (100 ± 2%) or control morpholino antisense oligonucleotide (100 ± 5%) and in the vehicle-treated cells transfected with the PPARα siRNA duplex (94 ± 4%) or PPARα morpholino antisense oligonucleotide (91 ± 3%) (Fig. 6). Treatment with 300 μmol/l BF for 24 h resulted in a significant increase in the ABCB4/CD26 ratio in the cells transfected with the control siRNA duplex (134 ± 6%), whereas the BF-induced increase in the ABCB4/CD26 ratio was significantly attenuated in the cells transfected with the PPARα siRNA duplex (109 ± 8%) ($P < 0.05$) (Fig. 6A, B). The BF-induced redistribution of ABCB4 into pseudocanaliculi was also inhibited in the cells transfected with the PPARα morpholino antisense oligonucleotide (Fig. 6B); the ABCB4/CD26 ratios were 128 ± 8% in the BF-treated cells transfected with the control morpholino antisense oligonucleotide and 108 ± 2% in the BF-treated cells transfected with the PPARα morpholino antisense oligonucleotide ($P < 0.05$). Therefore, the BF-induced redistribution of ABCB4 into pseudocanaliculi was mediated at least in part by a PPARα-dependent mechanism.

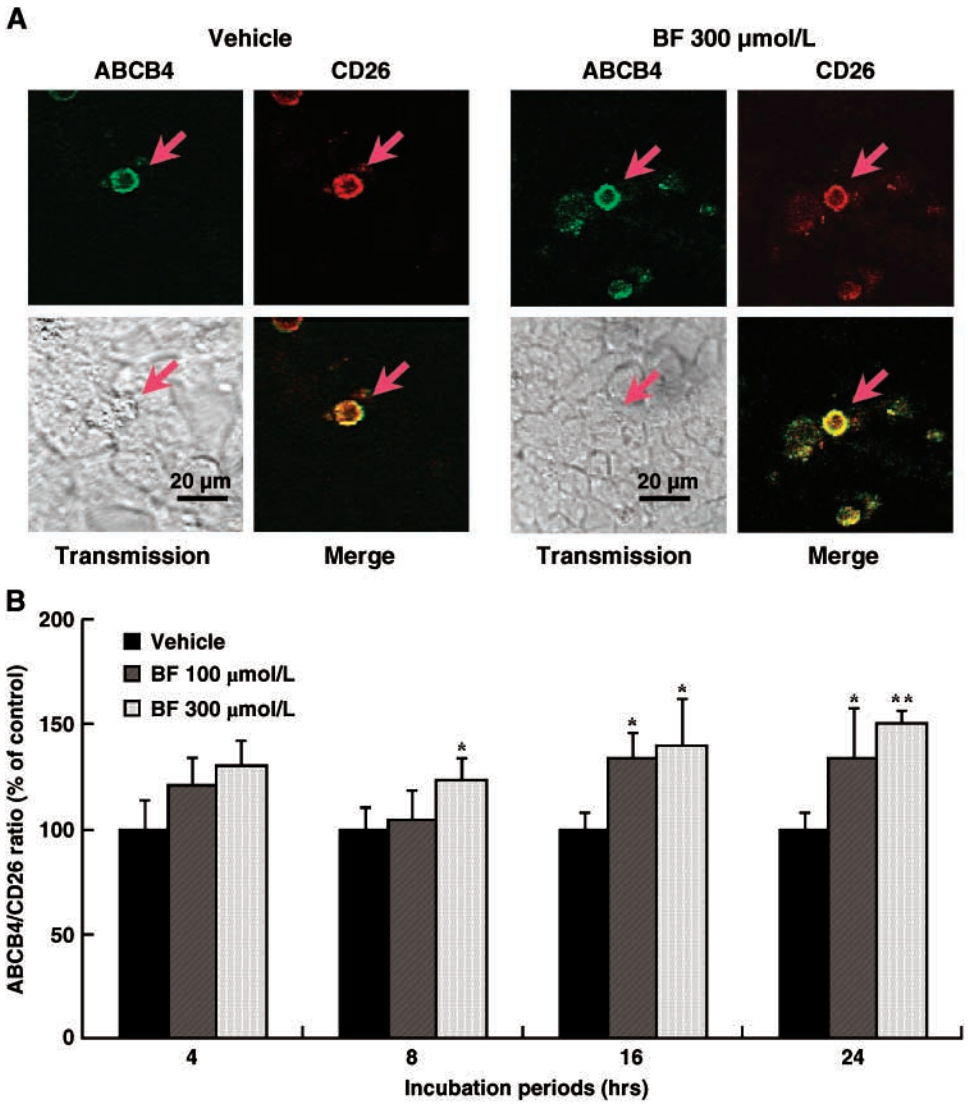

Fig. 3. Effects of BF on the redistribution of ABCB4 in HepG2 cells. A: Immunofluorescent images were obtained by confocal laser scanning microscopy (CLSM) of ABCB4 and CD26 in HepG2 cells treated with vehicle or BF (300 μmol/l) for 24 h. BF treatment increased the immunostaining intensity of ABCB4 in pseudocanaliculi. B: Time-course changes in the ratios of ABCB4/CD26 in the pseudocanaliculi of HepG2 cells treated with vehicle or BF. The cells were treated with vehicle or BF (100 or 300 μmol/l) for 4–24 h. Immunofluorescent staining was performed using the monoclonal antibody against ABCB4 and the polyclonal antibody against CD26 as described in Materials and Methods. The fluorescence intensity of ABCB4 in pseudocanaliculi was determined by calculating the ratio of ABCB4/CD26. Data are presented as means ± SEM of four experiments. * $P < 0.05$, ** $P < 0.01$ versus vehicle-treated cells.

Effects of BF on expression levels of other major transporters, ABCC2 and ABCB11

Figure 7 summarizes the effects of BF on expression levels of ABCC2, functioning as the canalicular multispecific organic anion transporter, and ABCB11, functioning as the canalicular bile acid transporter, in HepG2 cells. ABCC2 mRNA levels were significantly increased in cells treated with 300 μmol/l BF for 24 h (Fig. 7A). ABCB11 mRNA levels were significantly increased in cells with 300 μmol/l BF for 8 h, whereas the levels were significantly decreased in cells treated with 300 μmol/l BF for 24 h (Fig. 7B). Protein levels of ABCC2 and ABCB11 in cells treated with 300 μmol/l BF for 24 h showed no significant changes when normalized for expression of CD26 (Fig. 7B). For the effects of BF on subcellular localization of ABCC2 and ABCB11 in the cells, the immunostaining intensity of ABCC2 and ABCB11 in pseudocanaliculi was increased compared with vehicle-treated cells by up to 141% and 149%, respectively, as were the intensity ratios of ABCC2/CD26 and ABCB11/CD26 (Fig. 7C).

DISCUSSION

This study demonstrates that BF treatment of HepG2 cells translocates ABCB4 to pseudocanaliculi by a PPARα-

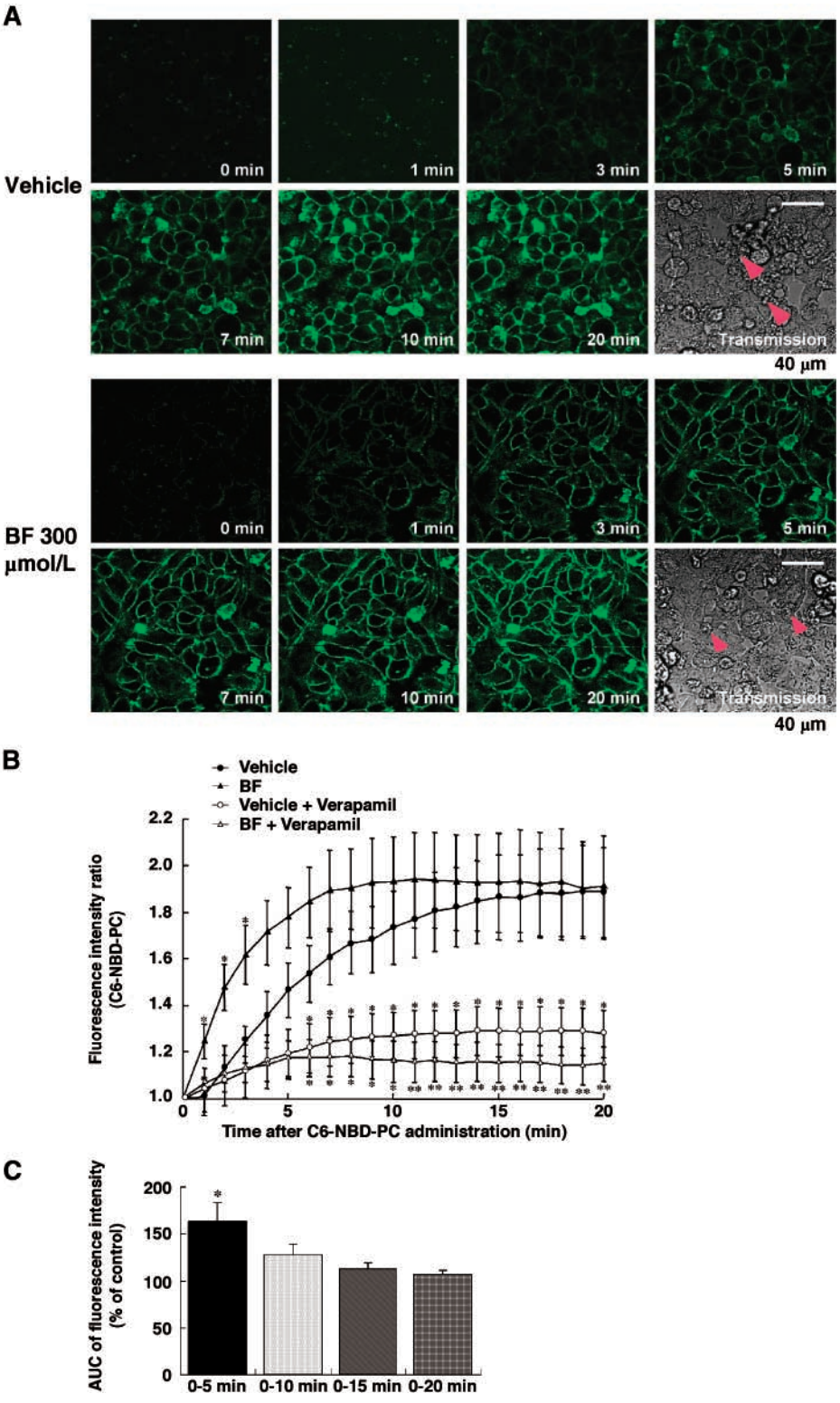

Fig. 4. Localization of nitrobenzoxadiaole-labeled 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-y)amino]caproyl]-*sn*-glycero-3-phosphatidylcholine (C6-NBD-PC) to the pseudocanaliculi of HepG2 cells. A: Representative time-dependent changes in C6-NBD-PC localization to the pseudocanaliculi of cells treated with vehicle or BF (300 μmol/l) for 24 h. B: Effects of BF on time-dependent movement of C6-NBD-PC to pseudocanaliculi. Cells were treated with vehicle or BF (300 μmol/l) for 24 h. C6-NBD-PC was added to the cells (at a final concentration of 1 μmol/l). Fluorescence images were obtained using CLSM every 1 min over a period of 20 min. The fluorescence intensity of C6-NBD-PC in pseudocanaliculi was determined by calculating the ratio of fluorescence of pseudocanaliculi/fluorescence of visual field. Cells were also treated with verapamil (50 μmol/l), an inhibitor of ABC transporters. Data are presented as means ± SEM of six experiments. C: The area under the curve (AUC) of the fluorescence intensity ratio of C6-NBD-PC was determined at 5, 10, 15, and 20 min. The AUC of BF-treated cells was expressed as a percentage of the corresponding AUC of vehicle-treated cells. Data are presented as means ± SEM of six experiments. * $P < 0.05$ versus vehicle-treated cells.

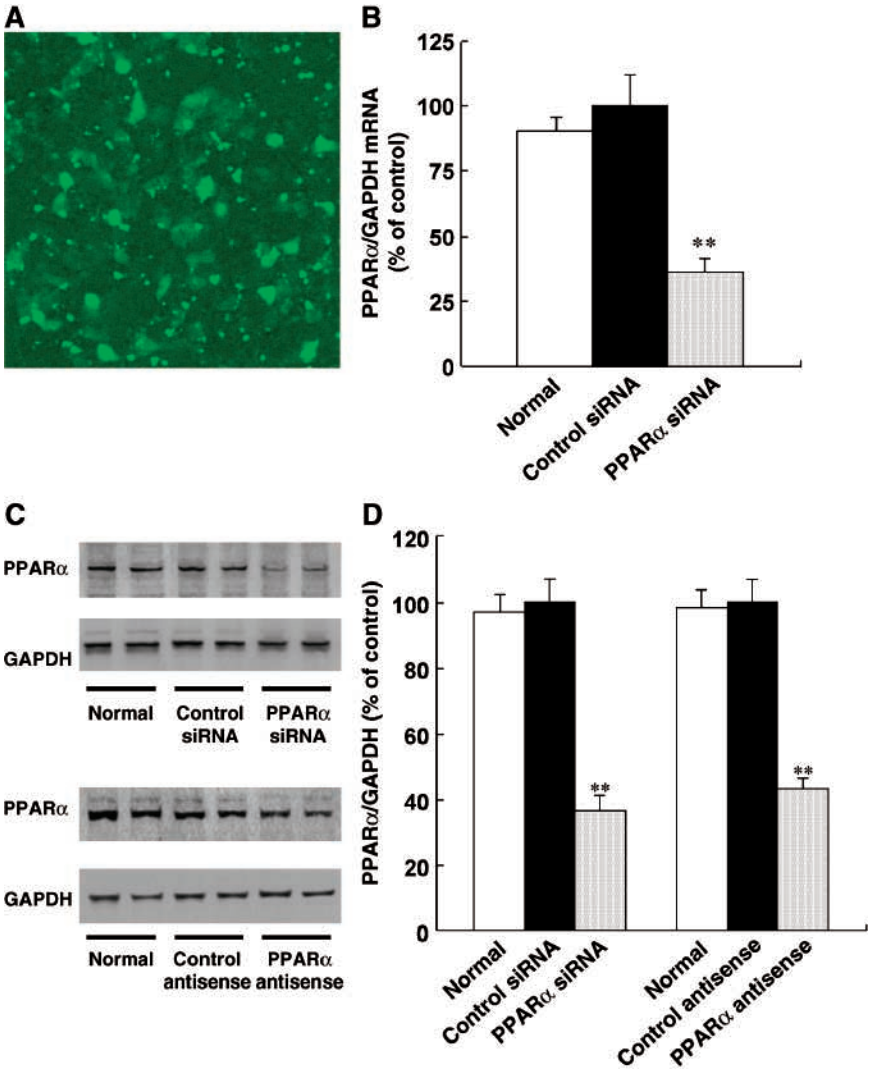

Fig. 5. Suppression of peroxisome proliferator-activated receptor α (PPARα) expression in HepG2 cells by transfection with a small interfering RNA (siRNA) duplex or morpholino antisense oligonucleotide. A: Distribution of a FITC-labeled control siRNA duplex transfected in HepG2 cells (7 h after transfection). B: Expression levels of PPARα mRNA in HepG2 cells. The mRNA levels were quantified by real-time quantitative PCR using specific primers and a double-dye probe (as shown in Table 1). Data are presented as means ± SEM of four experiments. ** $P < 0.01$ versus the cells transfected with a control siRNA duplex. C: Representative bands of PPARα protein and GAPDH in immunoblot analysis of the lysates of HepG2 cells treated with vehicle or BF for 24 h. D: Expression levels of PPARα protein and GAPDH in lysates of HepG2 cells. Data are presented as means ± SEM of six experiments. ** $P < 0.01$ versus the cells transfected with a control siRNA duplex or a control morpholino antisense oligonucleotide.

dependent mechanism and enhances the apical localization of PC within the cells.

BF was added to HepG2 cells at final concentrations of 100 and 300 μmol/l. In an in vivo experiment using rats, the concentration of BF in liver tissue was approximately four to five times higher than that in plasma after oral administration of BF (our unpublished observations). When BF at a dose of 10 mg/kg, corresponding to a commonly administered dose in human subjects, was orally administered to rats, the concentration of BF in the liver reached a maximum of 300 μmol/kg and the minimum concentration exceeded 100 μmol/kg. Therefore, the concentration of BF added to the HepG2 cells was likely to have been clinically relevant.

The administration of BF at the concentrations of 100 and 300 μmol/l results in a modest increase in the mRNA levels of ABCB4 in HepG2 cells without changing cellular protein levels. This suggests that BF does not influence the protein expression of ABCB4 in human livers. This represents a major difference from the effect of BF on murine livers. Chianale et al. (16) reported that mRNA and protein levels of ABCB4 were both increased in livers of BF-treated mice. BF is a ligand for PPARα, a nuclear hormone receptor (22) that increases its transcriptional activity level (22). Because expression levels of PPARα are much lower in human than in rodent liver (35–37), it is likely that the responsiveness to BF is also lower in hu-

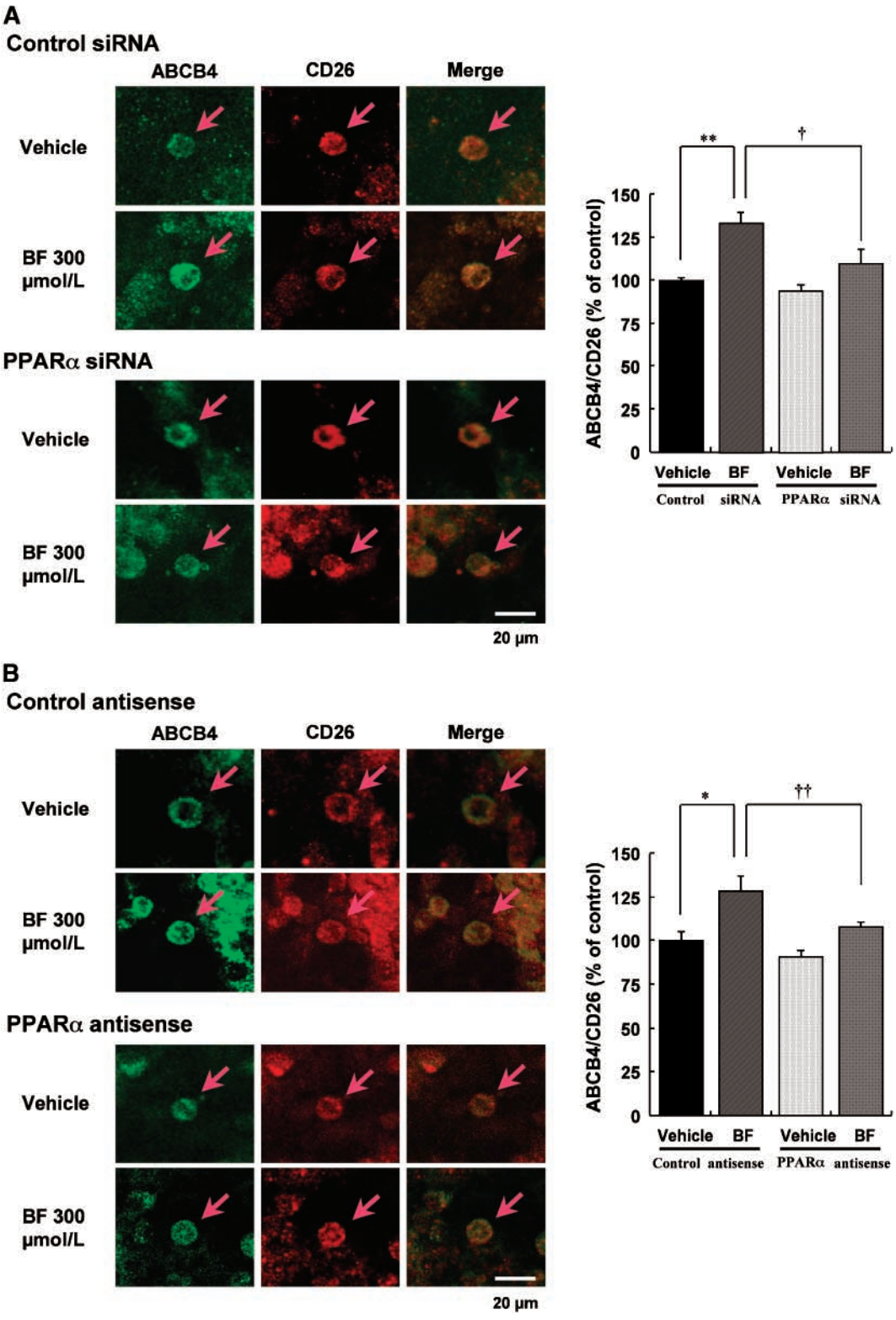

Fig. 6. Effects of suppression of PPARα expression on the BF-induced redistribution of ABCB4 in HepG2 cells. Cells were transfected with a PPARα siRNA duplex (A) or a PPARα morpholino antisense oligonucleotide (B). Data are presented as means ± SEM of five to six experiments. * $P < 0.05$, ** $P < 0.01$ versus vehicle-treated cells transfected with a control siRNA duplex or a control morpholino antisense oligonucleotide. † $P < 0.05$, †† $P < 0.01$ versus BF-treated cells transfected with a control siRNA duplex or a control morpholino antisense oligonucleotide.

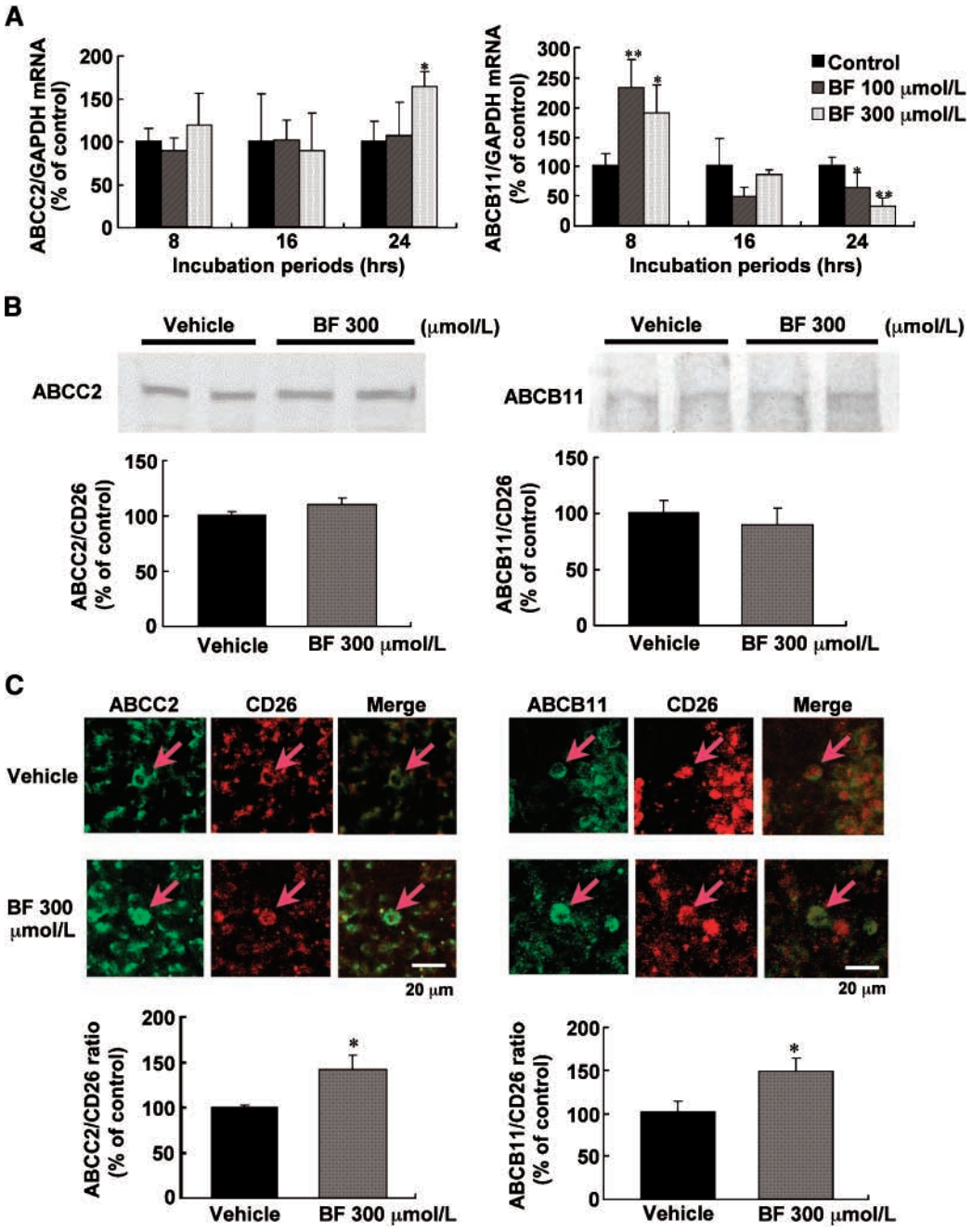

Fig. 7. Effects of BF on expression levels of ABCC2 and ABCB11. A: Steady-state mRNA levels of ABCC2 and ABCB11 in HepG2 cells treated with vehicle or 300 μmol/l BF for 24 h. mRNA levels were quantified by real-time quantitative PCR using specific primers and double-dye probes (Table 1). All experiments were performed in duplicate. Data were normalized to the amounts of GAPDH mRNA present in each specimen. Data are presented as means ± SEM of four to six experiments. * $P < 0.05$, ** $P < 0.01$ versus vehicle-treated cells. B: Representative bands of ABCC2, ABCB11, and CD26 proteins in immunoblot analysis of the lysates of HepG2 cells treated with vehicle or 300 μmol/l BF for 24 h and densitometric analysis of ABCC2/CD26 and ABCB11/CD26 ratios in the cells. Data are presented as means ± SEM of four experiments. C: Localization of ABCC2 and ABCB11 in HepG2 cells treated with vehicle or 300 μmol/l BF for 24 h. Immunofluorescent images were obtained by CLSM of ABCC2, ABCB11, and CD26 in the cells. BF treatment increased the immunostaining intensity of ABCC2 and ABCB11 in pseudocanaliculi of the cells. The fluorescence intensity of ABCC2 and ABCB11 in pseudocanaliculi was determined by calculating the ratios of ABCC2/CD26 and ABCB11/CD26. Data are presented as means ± SEM of four experiments. * $P < 0.05$ versus vehicle-treated cells.

mans than in rodents. Alternatively, it is also conceivable that the human ABCB4 promoter differs from that of the mouse with respect to BF sensitivity. This presumably explains why BF did not appreciably alter the expression levels of ABCB4 mRNA or protein in HepG2 cells. However, immunofluorescence staining of ABCB4 showed that BF increased the localization of ABCB4 in the pseudocanaliculi of HepG2 cells.

To determine whether increased ABCB4 protein in the canalicular membrane enhanced the movement of PC molecules to the bile canaliculi, we performed a functional analysis of ABCB4 using C6-NBD-PC, a fluorescent analog of PC and a translocation substrate for ABCB4 (34). Consistent with the observations of Ruetz and Gros (7), treatment with 300 μmol/l BF promoted the accumulation of PC in pseudocanaliculi (Fig. 4). Pretreatment with verapamil, an inhibitor of ABC transporters (33, 34), inhibited the accumulation of fluorescence-labeled PC in both the vehicle-treated cells and the BF-treated cells. This indicated that the localization of fluorescence-labeled PC in pseudocanaliculi was most likely attributable to increased ABCB4 activity. Taken together, our results suggest that BF enhances the localization of PC in pseudocanaliculi by promoting the redistribution of ABCB4 to the canalicular membrane.

The mechanism underlying the BF-induced change in the subcellular localization of ABCB4 is not well defined. Kok et al. (21, 38) reported that fibrates induced mdr2 mRNA and protein levels in the murine liver by a PPARα-mediated mechanism. PPARα could also regulate the redistribution of ABCB4 to the bile canaliculi. Recently, various techniques have become available to selectively knock down the expression of molecular targets, including siRNA duplexes and morpholino antisense oligonucleotides (39, 40). To determine whether changes in ABCB4 distribution induced by BF are mediated by PPARα activation, we designed a siRNA duplex and a morpholino antisense oligonucleotide specific to PPARα and introduced these into HepG2 cells. The redistribution of ABCB4 into the pseudocanaliculi of HepG2 cells induced by BF was inhibited by the knockdown of PPARα, even though expression of PPARα was not completely abolished (Fig. 6). Suppression of PPARα protein levels by either approach was estimated to be 60% (Fig. 5). This was roughly correlated with the reduction in the ABCB4/CD26 ratio. Therefore, the effect of BF on the redistribution of ABCB4 to the canalicular membrane was most likely mediated through the activation of PPARα

In terms of canalicular ABC transporter trafficking in hepatocytes, the newly synthesized transporters are directly targeted from the Golgi to the bile canaliculi or transiently sequestered in an intracellular pool(s) (a subapical compartment involved in sorting and/or apical recycling) from which they are delivered to the canalicular membrane (41, 42). The results of this study do not fully clarify the mechanisms by which BF enhances the redistribution of ABCB4 protein to pseudocanaliculi. The posttranslational regulations (e.g., the membrane targeting of canalicular ABC transporters) are mediated via classic second messengers (43). These second messengers and kinases are involved in various aspects of bile formation like solute transport and vesicular trafficking. Although these signal transduction pathways are complex and the details are still being worked out, we may exclude several possibilities. First, cAMP regulates PC transport activity at the canalicular membrane by stimulating the insertion of vesicles containing ABCB4 from intracellular stores (42, 44). This was unlikely in our experiments because treatment of HepG2 cells with BF had little effect on the intracellular concentrations of cAMP (data not shown), indicating that the effects of BF were not mediated by protein kinase A activation. Second, redistribution of ABCC2 protein into the canalicular membrane is stimulated in part via Ca^{2+}- and α-protein kinase C-dependent mechanisms (45). However, immunoblot analysis of protein kinase C isozymes (data not shown) did not reveal the translocation of protein kinase C isozymes to any particular membrane fraction, a key step for the activation of protein kinase C.

In addition to ABCB4, we examined the effects of BF on expression levels of PCTP and other major canalicular transporters, ABCC2 and ABCB11, in HepG2 cells. It has been suggested that PCTP may play a role in resupplying the canalicular membrane with biliary PC during bile formation (46) and that PCTP may be regulated by PPARα stimulation both transcriptionally (31) and posttranslationally (47). Moreover, coregulation of PCTP, ABCB4, and biliary PC secretion in humans (13) suggests that PCTP may play a role in bile formation. However, BF had no appreciable effect on PCTP expression in HepG2 cells (Fig. 1). On the other hand, for ABCC2 and ABCB11, BF induced the redistribution of ABCC2 and ABCB11 proteins into the pseudocanaliculi of the cells in a similar manner to ABCB4 without affecting their protein levels (Fig. 7). These results are quite important to address the specificity of BF effects on canalicular ABC transporters in human livers. This generalized mode of BF action may potentiate not only ABCB4 but also ABCC2 and/or ABCB11 function, which in turn would strongly support our proposal to use fibrates for the treatment of cholestatic liver diseases. Regulation of ABCC2 is mediated by the nuclear receptors pregnane X receptor, farnesoid X receptor, and constitutive androstane receptor (48). Therefore, further studies are needed to investigate whether such BF-induced redistribution could be explained by ligand-induced activation of these transcriptional factors for organic xenobiotics and bile acids.

In summary, BF may enhance the capacity of human hepatocytes to transport PC into bile canaliculi by promoting the insertion of ABCB4 molecules into the canalicular membrane. Moreover, vesicular targeting of ABCB4 may occur as a result of a PPARα-mediated effect. These observations provide a rationale for the use of BF to restore canalicular ABCB4 expression and function in various types of cholestatic hepatobiliary diseases.

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