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Involvement of SULT1A3 in elevated sulfation of 4-hydroxypropranolol in Hep G2 cells pretreated with β-naphthoflavone

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Abstract

Pretreatment of Hep G2 cells with β-naphthoflavone (BNF 1–25 μM) significantly increased cytosolic sulfation activities of 4-hydroxypropranolol (4-OH-PL) racemate. The profile was similar to those of sulfations towards dopamine and triiodothyronine in the same cytosolic fractions. Kinetic studies of 4-OH-PL sulfation in Hep G2 cytosolic fractions revealed that V_{max} values increased but apparent K_m values remained unchanged following the BNF pretreatment. Among five recombinant human SULT isoforms (SULT1A1, -1A3, -1B1, -1E1 and -2A1) examined, only SULT2A1 did not show 4-OH-PL sulfation activities under the conditions used. SULT1A3 and -1E1 exhibited an enantioselectivity of 4-OH-*R*-PL sulfation > 4-OH-*S*-PL sulfation, which agreed with that of BNF-pretreated Hep G2 cells as well as of nontreated cells, whereas SULT1A1 and -1B1 showed a reversed enantioselectivity ($R < S$). In kinetic studies of 4-OH-PL sulfations by four kinds of human SULT isoforms, apparent K_m values for SULT1A3 were the lowest, and the parameters were close to those of Hep G2 cytosolic fractions. Real time RT-PCR using TaqMan probes demonstrated that the mRNA levels of SULT1A3 increased following BNF pretreatment, which paralleled the results from Western blotting showing the elevated levels of SULT1A3 proteins. These results suggest that the induction of SULT1A3 is mainly responsible for the elevated 4-OH-PL sulfation activities following the pretreatment of Hep G2 cells with BNF.

Keywords: SULT1A3; Hep G2 cell line; 4-Hydroxypropranolol; β-Naphthoflavone

1. Introduction

Propranolol (PL) is one of the β-adrenoceptor blocking agents often prescribed clinically. PL is a chiral compound having an asymmetric carbon atom in its side chain yielding enantiomers *R*-PL and *S*-PL. The potency of *S*-PL as a β-blocker is much higher than that of *R*-PL [1,2]. Extensive studies demonstrated that PL is biotransformed to oxidized metabolites such as aromatic 4-, 5- or 7-hydroxypropranolol (OH-PL) and side-chain *N*-desisopropyl propranolol (NDP) in various animal species including humans [3–6]. In humans, 4-OH-PL and NDP are the major PL metabolites, and PL and its metabolites are known to undergo conjugation, forming mainly sulfates and partially glucuronides [7,8].

4-OH-PL is a pharmacologically active metabolite, which is equipotent to the parent compound as a β-blocker

Abbreviations: BNF, β-naphthoflavone; CYP, cytochrome P450; 4-OH-BTL, 4-hydroxybunitrolol; DHEA, dehydroepiandrosterone; 4-OH-PL, 4-hydroxypropranolol; IPTG, isopropyl-β-d-thiogalactopyranoside; NDP, *N*-desisopropyl propranolol; PL, propranolol; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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[9]. In this context, aromatic 4-hydroxylation is not only one of the major metabolic pathways, but also a metabolically active pathway of PL in humans. The oxidations of PL are mediated by hepatic cytochrome P450 (CYP) enzymes. Our previous in vitro studies using human liver microsomal fractions indicated that PL 4-hydroxylation is mediated mainly by CYP2D6 and *N*-desisopropylation is mainly by CYP1A2 in human livers [10]. After its formation, 4-OH-PL is biotransformed to 4-OH-PL sulfate by cytosolic sulfotransferases [7].

The properties of sulfotransferases catalyzing the sulfation of PL and its metabolites in human liver cytosolic fractions were characterized by Walle and Walle [11,12]. Using partially purified sulfotransferases, they demonstrated that two kinds of sulfotransferases (thermolabile and thermostable sulfotransferases) are involved in the sulfation of 4-OH-PL in human livers [11,12]. Many isoenzymes of sulfotransferase have been characterized at the mRNA level, and categorized into several gene families [13]. According to the category, the thermolabile and thermostable sulfotransferases catalyzing the sulfation of 4-OH-PL described above correspond to SULT1A3 and SULT1A1, respectively.

It was reported that 4-OH-PL was efficiently sulfated in Hep G2 cells, and demonstrated that the level of thermolabile sulfotransferase (SULT1A3) was considerably high, resulting in the high sulfation activities in this cell line [14,15]. In Hep G2 cells, CYP1A2 can be induced following pretreatment with 3-methylcholanthrene [16]. We have also been studying the properties of enzymes involved in the metabolism of PL and 4-OH-PL in mammals including humans, and found that 4-OH-PL inactivates mammalian CYP2D enzymes [17–19]. In the course of our studies, we thought that Hep G2 cell lines might be useful to examine the possible toxicities of PL and 4-OH-PL, because the Hep G2 cells have constitutively high sulfotransferase activity towards 4-OH-PL and are responsive to induction of CYP enzymes by some inducers [14–16].

Recently, we found that sulfation activities towards 4-OH-PL were increased in Hep G2 cells by pretreatment with β-naphthoflavone (BNF), a known inducer of CYP1A enzymes, and cell toxicities of 4-OH-PL were attenuated in BNF-pretreated Hep G2 cells compared to those in the vehicle (0.5% DMSO)-pretreated control cells [20]. In this study, we investigated the changes in 4-OH-PL sulfation activities in Hep G2 cells in response to various inducers, and identified the species of SULTs involved in the elevation of 4-OH-PL sulfation following the pretreatment of Hep G2 cells with inducers at the protein and mRNA levels.

2. Materials and methods

2.1. Materials

4-Hydroxybunitrolol (4-OH-BTL) was supplied by Nippon Boehringer Ingelheim Co. (Hyogo, Japan). 4-OH-PL enantiomers (as hydrochlorides) were synthesized as reported previously [21]. Adenosine 3′-phosphate 5′-phosphosulfate (PAPS), penicillin–streptomycin solution and Williams' Medium E were obtained from Sigma Chemical Co. (St. Louis, MO, USA); phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT) were from Nacalai Tesque Co. (Kyoto, Japan); fetal bovine serum (FBS) was from Gibco BRL (Grand Island, NY, USA); BNF was from Wako Pure Chemical Ind. (Osaka, Japan); RNeasy Mini Kit and QIAshredder were from QIAGEN (Hilden, Germany); yeast tRNA was from Life Technologies, Inc. (Rockville, MD, USA). [2,4,6,7-^{3}H]-β-Estradiol (2.96 TBq/mmol) and [^{35}S]-PAPS (50.5 GBq/mmol) were obtained from NEN Life Science Products, Inc. (Boston, MA). TaqMan one-step RT-PCR master mix reagents, TaqMan GAPDH control reagents, TaqMan β-actin control reagents, micro amp optical 96-well reaction plates, optical adhesive covers and optical cover compression pads were purchased from Applied Biosystems (Foster City, CA, USA). Other reagents, enzymes and organic solvents used were of the highest quality commercially available.

2.2. Cell culture

Hep G2 cells were obtained from Cell Bank, Riken BioResource Center (Ibaraki, Japan). The cells were cultured in Williams' Medium E supplemented with 10% FBS, and penicillin (100 units/ml)–streptomycin (100 mg/ml) at 37 °C in a humidified 5% CO_2 atmosphere. When the cells in 150 mm dishes were about 50% confluent, they were treated with BNF (0.5–100 μM final concentrations) dissolved in DMSO (0.5% final volume) or the same volume of DMSO for an appropriate time (24, 48, 72 or 96 h). Following the treatment, the cells were washed with the culture medium containing no drugs, and further cultured for 24 h without drugs. The cells were then suspended in ice-cold 20 mM Tris–HCl buffer (pH 7.4) containing 1 mM PMSF, frozen in liquid nitrogen and thawed in a 37 °C water bath followed by homogenization with a Potter Elvehjem-type glass homogenizer and a teflon pestle on ice. This process (freezing and homogenizing) was repeated five times, and the final homogenate was centrifuged at 9000 × *g* for 20 min at 4 °C, and the supernatant was centrifuged at 105,000 × *g* for 1 h at 4 °C. The supernatant obtained was used as the cytosolic fraction. Protein concentrations were measured by the method of Bradford [22].

2.3. Cloning and expression of recombinant human SULTs

The entire coding regions of human SULT1A1 (GenBank accession no. X78283), SULT1A3 (L19956), SULT1B1 (D89479), SULT1E1 (L25275) and SULT2A1 (U08025) were amplified by PCR from a human liver cDNA library [23]. The amplified SULT1A1 cDNA

sequence in the present study was identical to SULT1A1*1 reported by Ozawa et al. [24], which was the most abundant of three reported SULT1A1 alleles in the human population [25]. The cDNAs were subcloned into the NdeI/XhoI sites (SULT1A1, SULT1A3 and SULT1E1), NdeI/BamHI sites (SULT1B1) and NdeI site (SULT2A1) of the pET-14b expression vector. The resulting expression plasmid was used for transformation of *E. coli* BL21(DE3)pLysS. Cultures (100 mL) of the transformed bacteria were grown at 37 °C in Luria-Bertani medium containing ampicillin (100 μg/ml) to an optical density at 660 nm of 0.4–0.6 in the absence of IPTG and then at 37 °C for 5 h, following the addition of IPTG (1 mM). A bacterial cytosolic fraction was obtained by sonication of the harvested bacteria in 20 mM Tris–HCl buffer, pH 8.0, containing 5 mM imidazole, 500 mM NaCl and 1 mM PMSF followed by centrifugation in the usual manner.

2.4. Purification of recombinant human SULTs:

Purification of His-tagged recombinant human SULTs was performed by one-step column chromatography using HiTrap Chelating HP column (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the previous methods with a slight modification [26]. Briefly, each of His-tagged recombinant human SULTs was loaded to the HiTrap Chelating HP column pre-equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 5 mM imidazole, 500 mM NaCl, 1 mM PMSF, 0.025% (w/v) Tween 20 and 10% (w/v) glycerol (buffer A). The recombinant protein was eluted with a step-wise gradient of 10 ml of 100, 200 and 500 mM imidazole in buffer A. The purified enzyme solution was desalted on a PD-10 column (Amersham Pharmacia Biotech) pre-equilibrated with 50 mM sodium phosphate buffer (pH 6.8) containing 8 mM dithiothreitol, 0.025% (w/v) Tween 20 and 10% (w/v) glycerol. The purified SULTs were stored at −80 °C after adding BSA (10%, w/v) as a stabilizer.

2.5. Assay of 4-OH-PL sulfation

The sulfation activity of 4-OH-PL was measured according to the reported HPLC method [27] with a slight modification. Briefly, a reaction medium (a final volume of 200 μl) in a 1.5 ml Eppendorf-type tube consisted of 0.15 M 2-mercaptoethanol, 0.05 mM ascorbic acid, cytosolic fraction from Hep G2 cells (0.05–0.2 mg protein) or purified recombinant SULT isoform (0.7–8.8 μg as SULT protein), 4-OH-PL enantiomers or racemate in 150 mM Tris–HCl buffer (pH 7.4). After pre-incubation at 37 °C for 3 min, the reaction was started by adding PAPS (100 μM) and continued for an appropriate time (2 or 10 min). The reaction was terminated by adding ice-cold methanol (200 μl), vortex mixing and chilling in an icebox. A 5 min incubation was employed for the cytosolic fractions from Hep G2 cells, and 2 or 10 min incubation was used for those from *E. coli* cells.

In the assay of the sulfation of 4-OH-PL enantiomers, 4-OH-BTL (100 pmol) was added to the methanolic reaction medium as internal standard (i.s.), and procaine (1 nmol) was used as i.s. for the assay of the sulfation of 4-OH-PL racemate, followed by vortex mixing. The methanolic reaction medium was then centrifuged at 13,000 × *g* at 4 °C for 15 min, and the supernatant was passed through a membrane filter (Millex-LH, 0.45 μm porosity, 4 mm, Millipore, Bedford, MA). A portion (10 μl) of the filtrate was subjected to HPLC using the conditions described below.

In kinetic experiments for Hep G2 cell cytosolic fractions or purified SULTs, the following substrate concentration ranges were employed: Hep G2 cell cytosol, 4-OH-PL racemate and enantiomers (0.25–10 μM); SULT1A1, 4-OH-PL racemate (10–1000 μM) and enantiomers (10–500 μM); SULT1A3, 4-OH-PL racemate and enantiomers (1–25 μM); SULT1B1, 4-OH-PL racemate and enantiomers (10–1500 μM); SULT1E1, 4-OH-PL racemate (10–500 μM) and enantiomers (5–250 μM). Enzyme kinetic parameters (apparent K_m and V_{max} values) were analyzed by non-linear least square regression based on a simplex method [28].

2.6. Sulfation assay of typical substrates for human SULT isoforms

Sulfation activities of cytosolic fractions from BNF-pretreated or vehicle-pretreated Hep G2 cells were estimated for typical substrates as follows: *p*-nitrophenol (5 μM as a substrate concentration) for SULT1A1 [26]; dopamine (25 μM) for SULT1A3 [26], 3,3′,5-triiodothyronine (500 μM) for SULT1B1 [29], β-estradiol (50 μM) for SULT1E1 [30] and dehydroepiandrosterone (DHEA, 2.5 μM) for SULT2A1 [26].

2.7. HPLC conditions

Instrument, a Shimadzu LC-10A liquid chromatograph equipped with a Shimadazu RF-10A fluorescence detector, Rheodyne 7125 injector, and a Shimadzu C-R6A Chromatopac data procecssor. For the determination of 4-OH-PL sulfate, formed from 4-OH-PL enantiomers or racemate: column, Inertsil ODS (4.6 mm i.d.×250 mm; GL Science, Tokyo, Japan); mobile phase, acetonitrile/methanol/water/acetic acid (13:13:74:1, by volume); flow rate 1.0 ml/min; fluorescence detection, excitation/emission wavelengths at 310/380 nm. For the determination of the enantiomers of 4-OH-PL sulfate formed from 4-OH-PL racemate: column, ULTRON ES-Ph (6.0 mm × 100 mm, Shinwa Kako, Tokyo, Japan); mobile phase, 20 mM KH_2PO_4/acetonitrile (77:23, by volume); flow rate 1.0 ml/min; fluorescence detection, excitation/emission wavelengths at 310/380 nm.

Table 1
The pairs of forward and reverse primers, TaqMan probes and the nucleotide positions of the oligonucleotides used

Enzyme	Forward primer	Reverse primer	Probe
SULT1A1 (NM-001055)[a]	5′-AACGCAAAGGATGTG-GCA-3′ (391–408)[b]	5′-TCCGTAGGACACTTC-TCCGA-3′ (510–491)[b]	5′-ACATGGCCAAGGTGCA-CCCTGA-3′ (431–452)[b]
SULT1A2 (NM-001054)	5′-ACTACCACTTCTACCA-CATGGCC-3′ (416–438)	5′-GGACCCATAGGACAC-TTCTCCA-3′ (513–492)	5′-AGTGTACCCTCACCCT-GGGACCTGG-3′ (441–465)
SULT1A3 (NM-003166)	5′-AGGTCAATGATCCAGG-GGAA-3′ (248–267)	5′-GGCAGGTGTGACTTG-ATGAGC-3′ (329–309)	5′-CTGGAGACTCTGAAAG-ACACACCGCC-3′ (277–302)
SULT1B1 (NM-014465)	5′-TGGCTCGTAATGCCA-AGGA-3′ (386–404)	5′-CAGGAACCATAGGCC-ACTTTT-3′ (518–498)	5′-CAGCCTTTTCCTGGTACCT-GGGAAGAATATCT-3′ (448–479)
SULT1E1 (NM-005420)	5′-TGGTGGCTGGTCAT-CCAAA-3′ (431–449)	5′-ACACGTGGACTCTTTC-CCTTTT-3′ (560–539)	5′-CCTGGATCCTTTCCAGAG-TTTGTGGAGA-3′ (451–478)
SULT2A1 (NM-003167)	5′-CCACGTTTATTCTCC-TCCCAC-3′ (277–297)	5′-AGCACAGTTCCTTGAC-AAAACC-3′ (476–455)	5′-TCCCCATCCAGTTATTCC-CCAAGTCTT-3′ (299–325)

[a] GenBank accession number.
[b] Nucleotide position.

2.8. Oligonucleotides

The pairs of primers and the TaqMan probes for sulfotransferase mRNAs were designed using Primer Express software (Applied Biosystems). The nucleotide positions of the oligonucleotides are shown in Table 1. The primers and TaqMan probes were synthesized by Sawady Technology Co. Ltd. (Tokyo, Japan). The TaqMan probes contained 6-carboxyfluorescein (FAM) at the 5′ end and 6-carboxytetramethylrhodamine (TAMRA) at the 3′ end and were designed to hybridize to a sequence located between the PCR primers.

2.9. TaqMan RT-PCR conditions

Total RNA from Hep G2 cells was diluted with yeast tRNA at 50 μg/ml. The RT-PCR assay was performed in 50 μl of TaqMan one-step RT-PCR master mix reagents containing 300 nM forward primer, 900 nM reverse primer, 200 nM TaqMan probe and about 20 ng of total RNA. Amplification and detection were performed using the ABI PRISM 7700 sequence detector system (Applied Biosystems) with the following profile: 1 cycle at 48 °C for 30 min, 1 cycle at 95 °C for 10 min and 40 cycles each at 95 °C for 15 s and 60 °C for 1 min. For GAPDH, 200 nM forward primer, 200 nM reverse primer, and 100 nM TaqMan probe were used, and for β-actin, 300 nM forward primer, 300 nM reverse primer and 200 nM TaqMan probe were used.

2.10. Western blot analysis

Appropriate portions of cytosolic fractions from Hep G2 cells pretreated with BNF were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% slab gels. Following the electrophoresis, proteins on the gels were electroblotted to a PVDF membrane (Bio-Rad, Richmond, CA). The protein levels of SULTs were examined by Western blotting according to the method of Guengerich et al. [31] using rabbit antiserum fractions raised against SULT1A1, -1A3 and -1B1 [26] and a commercially available rabbit antiserum fraction raised against SULT1E1 and -2A1 (PanVara LLC., Madison, WI).

2.11. Statistical analysis

Analysis of the data in TaqMan RT-PCR was performed with the ABI PRISM sequence detection software. Relative expression of each mRNA was calculated by the ΔCt method and normalized to that of β-actin [32,33]. Experiments involving human hepatocyte cultures were performed in duplicate, and the results relative to controls are shown as mean values. Enzyme activities were analyzed by the Student's *t*-test. A *p*-value of 0.05 was considered significant.

3. Results

3.1. Effects of the pretreatment of Hep G2 cells with BNF on cytosolic sulfation of 4-OH-PL racemate and five typical substrates

Hep G2 cells were cultured in the medium containing BNF (0.5, 1, 5, 10, 25, 50 or 100 μM) or the vehicle (0.5% DMSO) and washed with fresh medium without BNF, followed by further cultivation for 24 h. The viability of the cells was between 90 and 70% at BNF concentrations from 25 μM or less, but decreased to less than 50% at above 25 μM. The cell toxicity of BNF at above 25 μM was thought to cause the low viability. Cytosolic fractions were prepared from the Hep G2 cells, and sulfation activities for 4-OH-PL racemate (10 μM) were determined using PAPS (100 μM). As shown in Fig. 1(A), the pretreatment of Hep G2 cells with BNF significantly increased 4-OH-PL sulfation activities 1.6- to 1.7-fold that of control (120.2 ± 4.0 pmol/(min mg protein), $n = 4$).

Sulfation activities towards typical five substrates are summarized in Fig. 1. The activities were categorized into

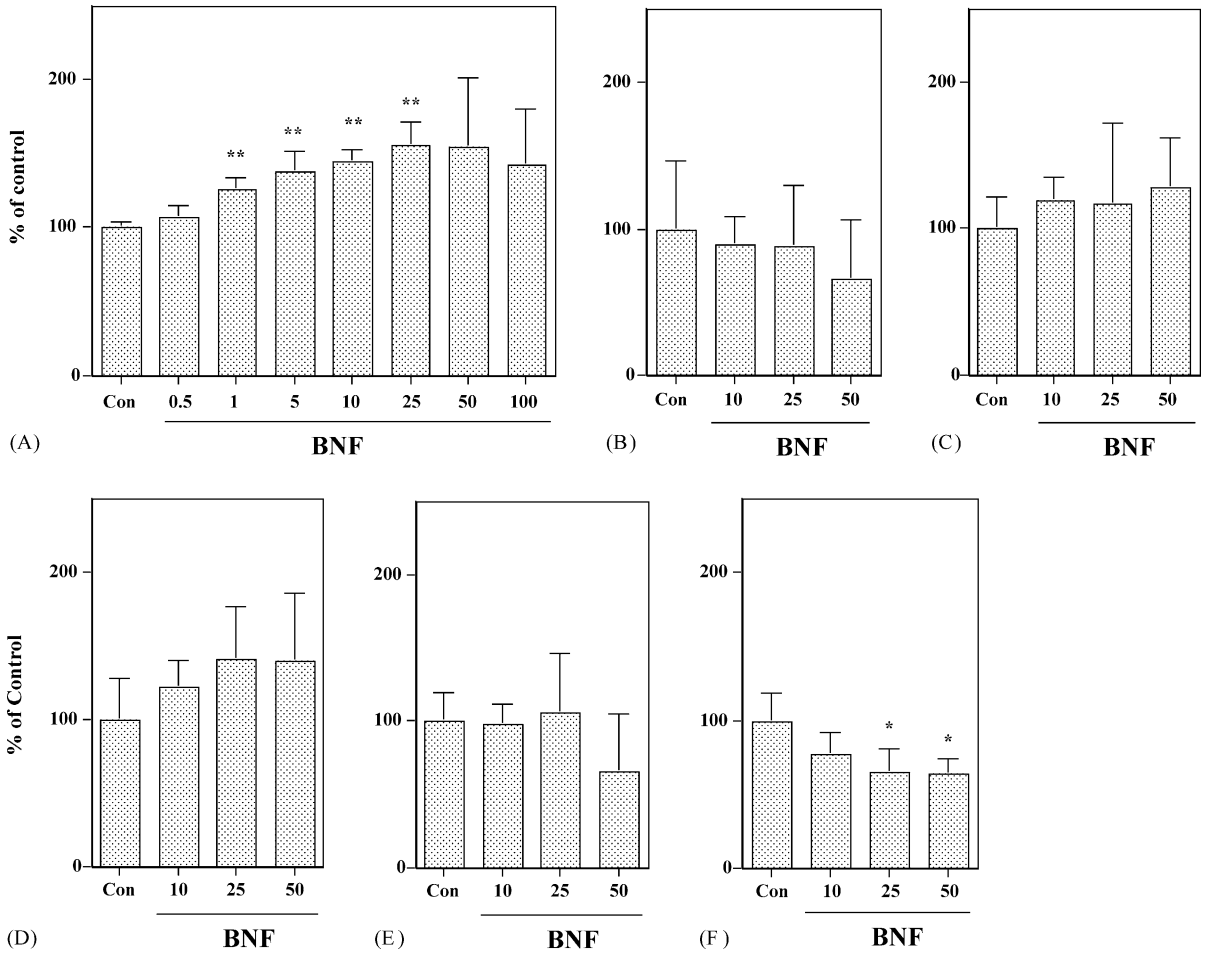

Fig. 1. Effects of BNF pretreatment on the sulfation towards various substrates in cytosolic fractions from Hep G2 cells: (A), 4-OH-PL racemate (10 μM); (B), *p*-NP (5 μM); (C), dopamine (25 μM); (D), triiodothyronine (500 μM); (E), β-estradiol (50 μM); (F), DHEA (2.5 μM). Con, Control; BNF, pretreatment with BNF (0.5–100 μM for A; 10–50 μM for B–F). Each value represents the mean ± S.D. ($n = 4$). Control values for each substrate were: 4-OH-PL, 120.2 ± 4.0 pmol/(min mg protein); *p*-NP, 152.0 ± 70.6 pmol/(min mg protein); dopamine, 119.2 ± 26.3 pmol/(min mg protein); triiodothyronine, 38.0 ± 10.6 pmol/(min mg protein); *β*-estradiol, 74.6 ± 14.8 pmol/(min mg protein); DHEA, 80.1 ± 15.0 pmol/(min mg protein). *Significantly different from the control ($p < 0.05$); **significantly different from the control ($p < 0.01$).

three patterns: the sulfation activities of dopamine (Fig. 1C) and triiodothyronine (Fig. 1D) tended to increase; those of *p*-nitrophenol (Fig. 1B) and DHEA (Fig. 1F) to decrease; those of *β*-estradiol (Fig. 1E) to be unchanged. Therefore, SULT1A3 (dopamine sulfotransferase) and/or SULT1B1 (triiodothyronine sulfotransferase) could be affected by the pretreatment of Hep G2 cells with BNF.

3.2. Kinetics for the sulfation of 4-OH-PL racemate and enantiomers by Hep G2 cell cytosolic fractions

PL is a chiral compound having an asymmetric carbon atom at the isopropylethanolamine side-chain, yielding the enantiomers *R*-PL and *S*-PL. We previously established an HPLC method for the determination of the enantiomers of 4-OH-PL sulfate formed from 4-OH-PL racemate in cytosolic fractions from primates [27]. Employing the HPLC method, kinetics was then examined for cytosolic fractions from Hep G2 cells pretreated with BNF (25 μM) and the vehicle (0.5% DMSO) using a concentration range of 4-OH-PL racemate or enantiomers from 0.25 to 10 μM. The kinetic parameters are summarized in Table 2. Apparent K_m values for the sulfation of 4-OH-PL racemate were similar between the control and BNF-pretreated groups, but the V_{max} value of the BNF-pretreated group was 1.6- to 1.8-fold higher than that of the control group, resulting in a 1.6- to 1.7-fold increase in the sulfation efficiency (V_{max}/K_m values) by the pretreatment (Table 2, upper lines). Similar results were observed for the sulfation of 4-OH-PL enantiomers (Table 2, lower lines).

3.3. Comparison of sulfation of 4-OH-PL by Hep G2 cell cytosolic fractions and purified recombinant human SULTs

Cytosolic fractions from BNF-pretreated Hep G2 cells as well as from control Hep G2 cells showed enantiomer selectivity of $R > S$ in the sulfation of 4-OH-PL (a sub-

Table 2
Kinetic parameters for the formation of enantiomeric 4-OH-PL sulfate from 4-OH-PL racemate or enantiomers by cytosolic fractions from Hep G2 cells with or without BNF pretreatment

Pretreatment	Product	K_m (μM)	V_{max} (pmol/(min mg protein))	V_{max}/K_m (μl/(min mg protein))
4-OH-PL racemate as a substrate				
Control	4-OH-*R*-PL sulfate	4.3 ± 1.4	89.6 ± 22.3	21.1 ± 2.3
	4-OH-*S*-PL sulfate	3.7 ± 0.8	19.0 ± 4.6	5.5 ± 2.7
		(1.16)[a]	(4.72)	(3.84)
25 μM BNF	4-OH-*R*-PL sulfate	4.5 ± 0.1	150.8 ± 13.8	33.9 ± 2.8
	4-OH-*S*-PL sulfate	4.4 ± 0.4	37.2 ± 5.8	9.1 ± 1.5
		(1.02)	(4.11)	(3.72)
4-OH-PL enantiomer as a substrate				
Control	4-OH-*R*-PL sulfate	3.0 ± 0.4	152.8 ± 24.1	51.1 ± 4.1
	4-OH-*S*-PL sulfate	7.4 ± 0.91	84.5 ± 19.6	11.3 ± 1.5
		(0.41)	(1.81)	(4.56)
25 μM BNF	4-OH-*R*-PL sulfate	2.7 ± 0.5	244.4 ± 77.0	88.9 ± 9.6
	4-OH-*S*-PL sulfate	10.0 ± 0.3	177.8 ± 19.2	17.7 ± 1.3
		(0.27)	(1.37)	(5.02)

Values are the mean of three determinations ± S.D.
[a] Numbers in parentheses are *R/S* ratio values.

strate concentration of 10 μM) as shown in Fig. 2A. That is, the sulfation activity of 4-OH-*R*-PL was 3.7-fold higher than that of 4-OH-*S*-PL in the control group (Fig. 2A, left), and 4.0-fold higher in the BNF-pretreated group (Fig. 2A, right). This means that the enantioselectivity favoring *R*-configuration over *S*-configuration in the formation of 4-OH-PL sulfate in Hep G2 cytosolic fraction was not affected by the pretreatment of the cells with BNF.

To determine what kinds of SULT isoforms are responsible for the increased 4-OH-PL sulfation following the treatment of Hep G2 cells with BNF, sulfation capacities toward 4-OH-PL of five kinds of purified recombinant human SULTS, SULT1A1, -1A3, -1B1, -1E1 and -2A1 expressed in *E. coli* (Fig. 3) were examined in the next step. Taking K_m values into account, two substrate concentrations, 20- to 100-fold different to each other, were

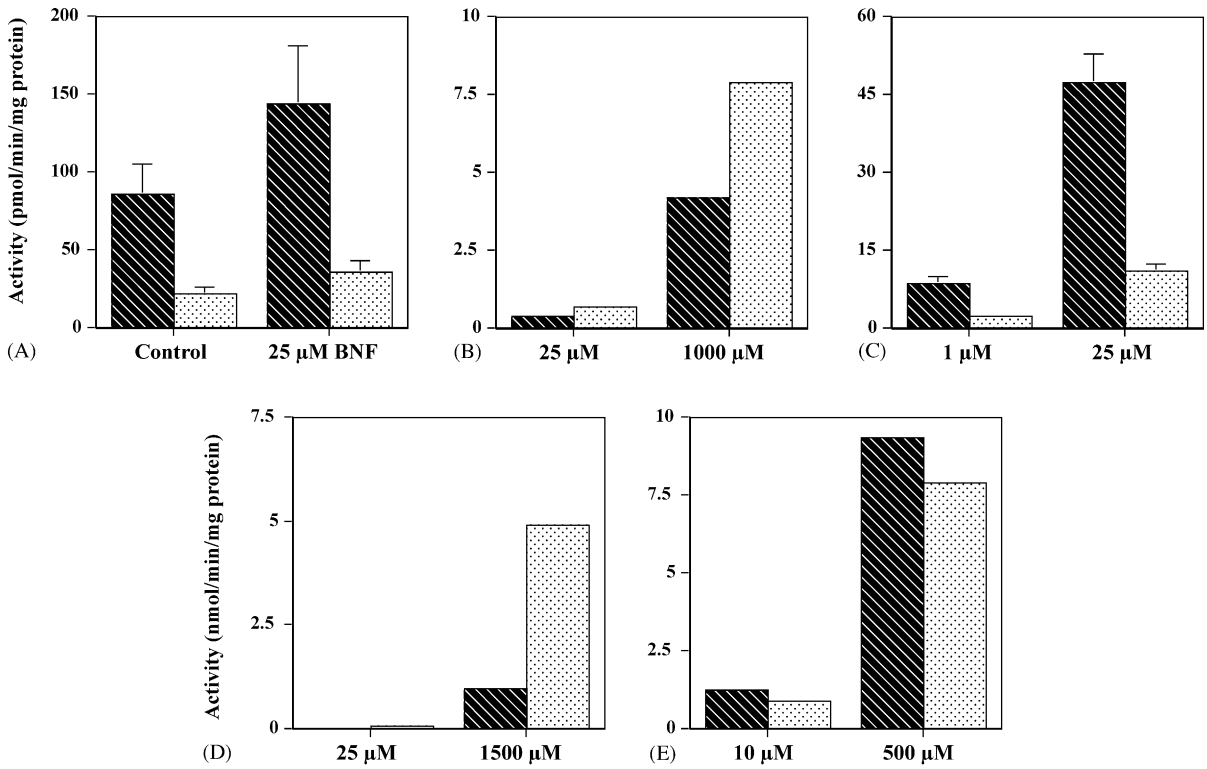

Fig. 2. Comparison of enantioselectivity in the sulfation of 4-OH-PL racemate between cytosolic fractions from Hep G2 cells and purified recombinant human SULT isoforms expressed in *E. coli*. The values for cytosolic fractions from Hep G2 cells (A) and for SULT1A3 (C) are the mean ± S.D. ($n = 4$). Other values for SULT1A1 (B), -1B1 (D) and -1E1 (E) are the mean of two determinations. Only the data of 4-OH-PL (A) are the results obtained using a single substrate concentration (10 μM). Hatched columns, 4-OH-*R*-PL sulfation; dotted column, 4-OH-*S*-PL sulfation.

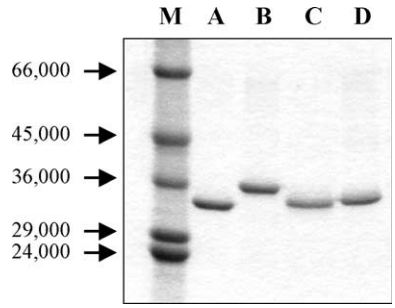

Fig. 3. SDS-PAGE of purified SULT isoforms. Small amounts (1 μg each) of purified SULT isoforms were subjected to SDS-PAGE using a 10% slab gel and electrophoresed. Proteins were then stained with Coumassie Brilliant Blue. M, molecular weight markers; A, SULT1A1; B, SULT1A3; C, SULT1B1; D, SULT1E1. Molecular weight markers: bovine serum albumin 66,000; egg albumin, 45,000; rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, 36,000; bovine carbonic anhydrase, 29,000; bovine pancreas trypsinogen, 24,000.

employed for each isoform. As shown in Fig. 2B–E, SULT1A3 and SULT1E1 exhibited the same enantioselectivity ($R > S$) in the sulfation of 4-OH-PL racemate as that of Hep G2 cells, and the selectivity of SULT1A1 and SULT1B1 was found to be reversed ($R < S$). SULT2A1 did not show any activities under the conditions employed. Similar results were obtained in the experiments using 4-OH-PL enantiomers instead of the racemate (data not shown).

3.4. Kinetic analysis for 4-OH-PL sulfation by purified recombinant human SULTs

On the basis of the results in Fig. 2, kinetic studies using 4-OH-PL racemate and enantiomers were performed for the four recombinant SULT isoforms (SULT1A1, -1A3, -1B1 and -1E1) having detectable 4-OH-PL sulfation activities under the conditions examined. Calculated kinetic parameters are listed in Tables 3 and 4. The K_m values for the sulfation of 4-OH-PL racemate by SULT1A3 were 8–10 μM (Table 3), which were close to those (around 4 μM) by cytosolic fractions from Hep G2 cells with or without the pretreatment by BNF (Table 2, upper lines). Similar results were obtained from the kinetic experiments using 4-OH-PL enantiomers (Tables 4 and 2, lower lines). The K_m values of other three SULT isoforms (SULT1A1, -1B1 and -1E1) were much higher than those of Hep G2 cell cytosolic fractions and SULT1A3.

3.5. The levels of mRNAs for SULT isoforms measured by real time RT-PCR

On the basis of the results obtained in preliminary experiments the mRNA levels of SULT isoforms were measured following the exposure of the cells to BNF 5 or 25 μM for 24 (Fig. 4A) and 48 h (Fig. 4B). In both cases,

Table 3
Kinetic parameters for the formation of enantiomeric 4-OH-PL sulfate from 4-OH-PL racemate by purified human recombinant SULT isoforms

Isoform	Product	K_m (μM)	V_{max} (pmol/(min mg protein))	V_{max}/K_m (μl/(min mg protein))
SULT1A1	4-OH-*R*-PL sulfate	231	3.52	0.02
	4-OH-*S*-PL sulfate	174 (1.33)[a]	5.88 (0.60)	0.03 (0.45)
SULT1A3	4-OH-*R*-PL sulfate	10.0 ± 3.8	94.2 ± 22.9	9.7 ± 1.7
	4-OH-*S*-PL sulfate	8.1 ± 2.2 (1.25)	20.6 ± 6.8 (4.60)	2.6 ± 0.4 (3.69)
SULT1B1	4-OH-*R*-PL sulfate	2465	1.53	0.0006
	4-OH-*S*-PL sulfate	1364 (1.81)	3.92 (0.39)	0.0029 (0.22)
SULT1E1	4-OH-*R*-PL sulfate	74.7	10.6	0.14
	4-OH-*S*-PL sulfate	96.5 (0.77)	9.9 (1.07)	0.10 (1.39)

Values for SULT1A3 are the mean of three determinations ± S.D. Values for other isoforms are the mean of two determinations.
[a] Numbers in parentheses are *R/S* ratio values.

Table 4
Kinetic parameters for the formation of enantiomeric 4-OH-PL sulfate from 4-OH-PL enantiomers by purified human recombinant SULT isoforms

Isoform	Product	K_m (μM)	V_{max} (pmol/(min mg protein))	V_{max}/K_m (μl/(min mg protein))
SULT1A1	4-OH-*R*-PL sulfate	133	4.93	0.04
	4-OH-*S*-PL sulfate	101 (1.32)[a]	5.63 (0.87)	0.06 (0.45)
SULT1A3	4-OH-*R*-PL sulfate	3.6 ± 3.8	88.3 ± 19.7	24.5 ± 1.8
	4-OH-*S*-PL sulfate	7.7 ± 2.2 (0.50)	59.0 ± 11.1 (1.49)	7.6 ± 0.4 (3.23)
SULT1B1	4-OH-*R*-PL sulfate	860	1.45	0.002
	4-OH-*S*-PL sulfate	737 (1.17)	4.03 (0.36)	0.005 (0.31)
SULT1E1	4-OH-*R*-PL sulfate	52.2	20.8	0.40
	4-OH-*S*-PL sulfate	70.1 (0.74)	16.9 (1.23)	0.24 (1.65)

Values for SULT1A3 are the mean of three determinations ± S.D. Values for other isoforms are the mean of two determinations.
[a] Numbers in parentheses are *R/S* ratio values.

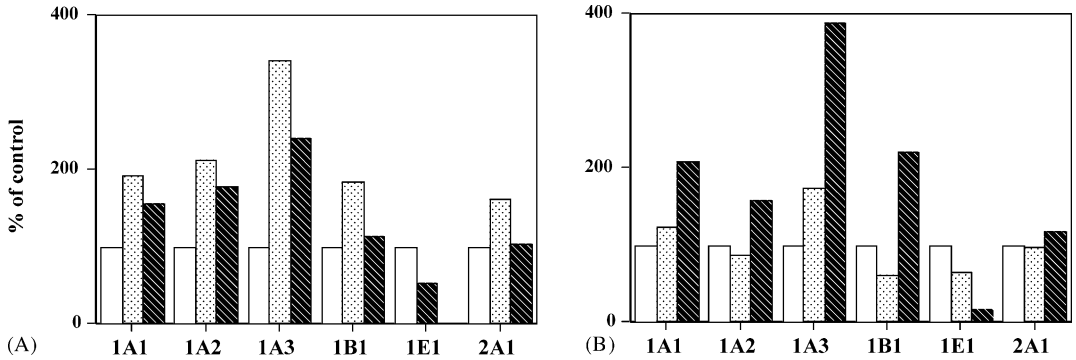

Fig. 4. Comparison of the mRNA levels of SULT isoforms in cytosolic fractions from Hep G2 cells with or without BNF-pretreatment Hep G2 cells were incubated with BNF (5 or 25 μM) for 24 or 48 h, followed by further culture for 24 h without drugs. Total RNA fractions were then extracted and the levels of mRNA for each SULT were determined by real time RT-PCR using TaqMan probes as described in Section 2. Each value is the mean of two determinations. A, exposure for 24 h; B, exposure for 48 h. Open column, control (0.5% DMSO); shaded column, 5 μM BNF; closed column, 25 μM BNF.

the levels of SULT1A3 mRNA were found to be higher than the control levels. The mRNA levels of SULT1A1, -1A2 and -1B1 also tended to be increased by the BNF-treatment, but to lesser extents. In contrast, the mRNA level of SULT1E1 showed a tendency to be decreased by the pretreatment of Hep G2 cells with BNF.

3.6. Western blot analysis of SULTS isoforms in Hep G2 cells pretreated with BNF

The results of immunoblot analyses using polyclonal antibodies raised against each SULT isoform are shown in Fig. 5. Among the four isoforms, only a protein band corresponding to SUL1A3 seemed to be increased by BNF-pretreatment. Quantitative analysis of the protein bands on the membranes by NIH Image also demonstrated that pretreatment of Hep G2 cells with BNF significantly increased SULT1A3 protein levels 1.5- to 2-fold compared with those of the corresponding control group following the exposure of the cells to BNF for 48, 72 and 96 h (Fig. 6).

4. Discussion

In the present study, pretreatment of Hep G2 cells with BNF was found to elevate 4-OH-PL sulfation, which paralleled the elevation of the levels of SULT1A3 proteins in Western blot analysis. Furthermore, real time RT-PCR demonstrated that the mRNA levels of SULT1A3 also increased following the BNF-pretreatment. These results indicate that the induction of SULT1A3 is responsible for the increased 4-OH-PL sulfation following pretreatment of Hep G2 cells with BNF. Dawson et al. [34] reported that pretreatment of Hep G2 cells with 3-methylcholanthrene increased the activities of 7-ethoxycoumarin *O*-deethylation (20- to 30-fold), 7-hydroxycoumarin glucuronidation (36-fold) and sulfation (7-fold) compared with those of nontreated control cells. However, no systematic studies have been reported on the mechanism involved in the increased enzyme activities in Hep G2 cells pretreated with these inducers.

Five human SULT isoforms expressed in *E. coli* were examined for their functions toward 4-OH-PL as a substrate. Among the isoforms tested, only SULT2A1 did not show any 4-OH-PL sulfation activities under the conditions used. SULT1A3 and SULT1E1 showed substrate enantioselectivity of 4-OH-*R*-PL > 4-OH-*S*-PL, which is the same as that of the cytosolic fraction from nontreated Hep G2 cells. In contrast, SULT1A1 and SULT1B1 exhibited a reversed selectivity (4-OH-*R*-PL < 4-OH-S-PL). Walle and Walle [11,12] reported that a thermostable phenol sulfotransferase, i.e. SULT1A1 did not show any substrate enantioselectivity, whereas a thermolabile sulfotransferase, i.e. SULT1A3 showed substrate enantioselectivity of 4-OH-*R*-PL > 4-OH-*S*-PL, which agreed with that of human liver cytosolic fractions. It is not clear at present what causes this inconsistency in the substrate enantioselectivity of SULT1A1 between Walle's results and ours.

From the kinetic results, SULT1A3 exhibited the smallest K_m values among the four enzymes examined (SULT1A1, -1A3, -1B1 and -1E1), and the values were close to the K_m values of Hep G2 cells with or without the pretreatment by BNF. These results suggest that SULT1A3 is responsible for the increased activities of 4-OH-PL sulfation following the pretreatment of Hep G2 cells with BNF.

In Western blot analysis, the protein levels of SULT1A3 most clearly increased by the pretreatment with BNF among the five SULT isoforms. However, the protein levels of SULT1B1 also showed a tendency to increase by the pretreatment although the extent was smaller than that of SULT1A3. In contrast, the protein levels of some SULTs, especially SULT1E1 seemed to decrease by BNF treatment. These results agreed well with the results obtained in RT-PCR. That is, the mRNA levels of SULT1A3 were increased, whereas those of SULT1E1 were decreased by BNF pretreatment.

Taken together, the results obtained in the present study indicate that SULT1A3 is mainly responsible for the

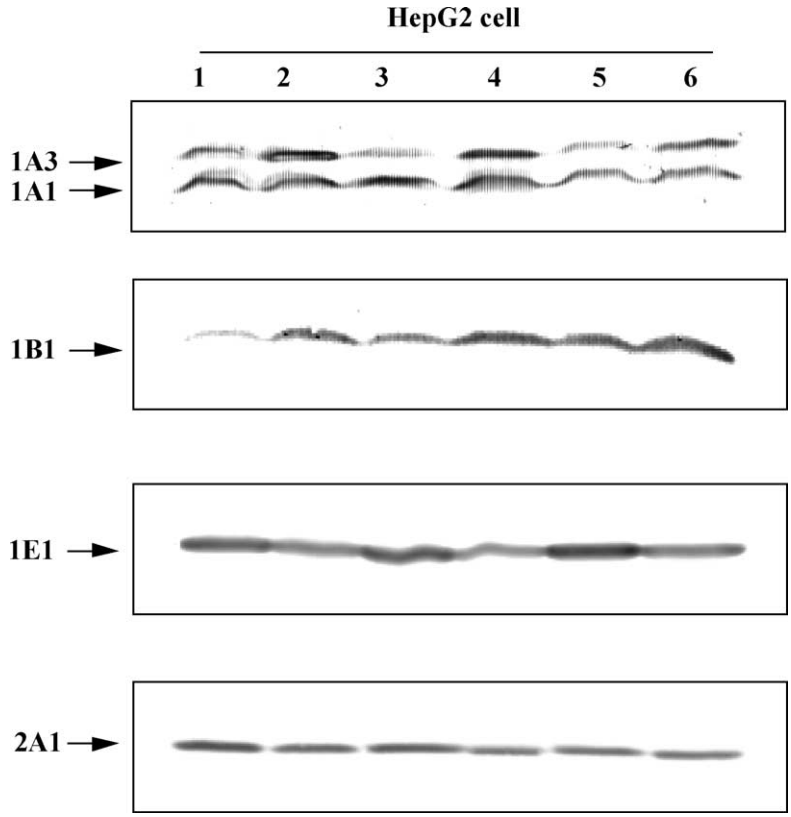

Fig. 5. Western blot analysis of SULT isoforms in Hep G2 cells pretreated with BNF Hep G2 cells were cultured with BNF (25 μM) or vehicle (0.5% DMSO) for 48, 72 and 96 h, followed by further culture for 24 h without drugs, and then cytosolic fractions were obtained. Appropriate portions of the cytosolic fractions (SULT1A1 and -1A3, 200 μg; SULT1B1, 100 μg; SULT1E1, 50 μg; SULT2A1, 50 μg) were subjected to SDS-PAGE using 10% slab gels. Proteins on the gels were electroblotted to a PVDF membrane and probed with antibodies raised against each human SULT isoforms. Lane numbers 1, 3 and 5, control; 2, 4 and 6, BNF-pretreated; 1, and 2, 48 h treatment; 3 and 4, 72 h treatment; 5 and 6, 96 h treatment.

elevation in 4-OH-PL sulfation following the pretreatment of Hep G2 cells with BNF. It has been extensively reported mainly in rats that CYP inducers affect the sulfation of various chemicals or drugs [35–38]. Recently, Duanmu et al. [39] demonstrated that the pretreatment of primary cultured human hepatocytes with dexamethasone increased SULT2A1 activities. Furthermore, Walle and Walle [40] presented the data showing that the sulfation of chrysin by SULT1A1 was elevated in Hep G2 cells pretreated with genistein. Therefore, the present paper is the first report showing that the levels of protein and mRNA of SULT1A3 are enhanced by the pretreatment with BNF in human cell lines.

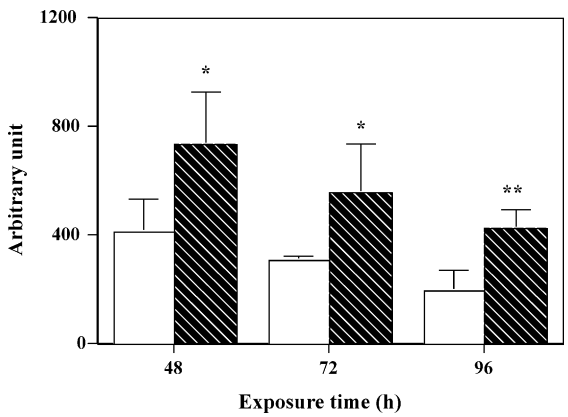

Fig. 6. Comparison of the protein levels of SULT1A3 on the PVDF membranes in Western blot analysis Following the pretreatment of Hep G2 cells with BNF (25 μM) as described in the caption of Fig. 5 staining intensities of SULT1A3 protein bands on the PVDF membranes were estimated using NIH image (version 1.5) on a Macintosh G4 computer equipped with a Canon CC550L scanner and printer. Open column, control (0.5% DMSO); hatched column, 25 μM BNF-pretreated. Each value represents the mean ± S.D. ($n = 4$). *Significantly different from the control ($p < 0.05$); **significantly different from the control ($p < 0.01$).

In summary, pretreatment of Hep G2 cells with BNF significantly increased the cytosolic sulfation activities of 4-OH-PL racemate, which was similar to the profile of sulfations towards dopamine and triiodothyronine in the same cytosolic fractions. Human recombinant SULT1A3 and -1E1 exhibited the enantioselectivity of $R > S$ in 4-OH-PL sulfation, which agreed with that of BNF-pretreated Hep G2 cells as well as of nontreated cells, whereas SULT1A1 and -1B1 showed a reversed selectivity ($R < S$). Real time RT-PCR demonstrated that the mRNA levels of SULT1A3 increased following BNF pretreatment, which paralleled the results from Western blotting showing the elevated levels of SULT1A3 proteins. These results suggest that the induction of SULT1A3 is mainly responsible for the elevated 4-OH-PL sulfation activities following the pretreatment of Hep G2 cells with BNF.

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