Special Section on Transporters in Drug Disposition and Pharmacokinetic Prediction

Identification of a NF κ B Inhibition Site on the Proximal Promoter Region of Human Organic Anion Transporting Polypeptide 1A2 Coding Gene *SLCO1A2*

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ABSTRACT

Organic anion transporting polypeptides (OATPs; gene symbol *SLCO*) are membrane transporters that mediate the transport of wide ranges of compounds. The expression of different OATP members has been reported in the kidney, liver, placenta, brain, and intestine. Because of their broad substrate spectra and wide distribution within the human body, these transporters have been proposed to play key roles in the influx transport of many oral drugs. Inflammation is known to regulate the expression and functions of many drug-metabolizing enzymes and drug transporters. As a proinflammatory cytokine, tumor necrosis factor- α (TNF α) has been shown to affect the expression of different drug transporters, including OATP family members. In the present study, a putative nuclear factor- κ B (NF κ B) binding site ranging from –1845 to –1836 was identified at the proximal promoter region of OATP1A2 coding

gene *SLCO1A2*. Electrophoretic mobility shift assays and chromatin immunoprecipitation showed that nuclear extracts from both breast cancer cell MCF7 and liver cancer cell HepG2 interacted with an oligonucleotide probe containing the putative NF κ B binding site and that the DNA-protein complexes contained both p65 and p50 subunits of NF κ B. Further study revealed that the binding site may be responsible in part for the suppression effect of TNF α toward *SLCO1A2* expression because the treatment of TNF α significantly increased. Treatment of TNF α significantly increased formation of the DNA-protein complexes and mutations at essential bases of the putative NF κ B binding site abolished responsiveness to the TNF α neutralizing antibody, suggesting that the binding site may be responsible in part for the suppression effect of TNF α toward *SLCO1A2* expression.

Introduction

The organic anion transporting polypeptides [OATPs (human); Oatps (rodents); gene symbol *SLCO*] are membrane transporters that mediate sodium-independent transport of a wide spectrum of structurally independent compounds (Hagenbuch and Gui, 2008). Substrates of OATPs include bile salts such as cholate, taurocholate, taurochenodeoxycholate, tauroursodeoxycholate, and glycocholate (Kullak-Ublick et al., 1994; Eckhardt et al., 1999); bromosulfophthalein (Kullak-Ublick et al., 1994); hormones and their conjugates (estrone-3-sulfate, estradiol-17 β -glucuronide, dehydroepiandrosterone sulfate, aldosterone, and cortisol) (Bossuyt et al., 1996a; Kanai et al., 1996; Eckhardt et al., 1999); thyroid hormones (T₃, rT₃, and T₄) (Abe et al., 1998; Pizzagalli

et al., 2002); and eicosanoids (prostaglandin E₂ and leukotriene C₄) (Li et al., 1998; Cattori et al., 2001) as well as a broad range of drugs. In addition to these charged compounds, OATPs also transport uncharged drugs such as the glycosides digoxin (Noé et al., 1997) and ouabain (Bossuyt et al., 1996b). Some OATP family members are expressed ubiquitously; whereas, others, such as OATP1B1 and OATP1B3, are predominantly found in certain organs or tissues. In recent years, these transporter proteins have been proposed to play key roles in the influx transport of various oral drugs (Shitara et al., 2005; Poirier et al., 2007; Tamai, 2012), and the regulation of OATP functions may have a profound effect on the absorption and bioavailability of drugs. So far, 12 members of the human OATP family have been discovered: OATP1A2, OATP1B1, OATP1B3, OATP1B7, OATP1C1, OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP4C1, OATP5A1, and OATP6A1 (Hagenbuch and Meier, 2003; Nakanishi and Tamai, 2012), although OATP1B7 is considered to be nonfunctional and *SLCO1B7* was proposed as a pseudogene (Stieger and Hagenbuch, 2014).

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ABBREVIATIONS: BAY117082, 3-[[4-methylphenyl]sulfonyl]-(2E)-propenenitrile; bp, base pair; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; IL, interleukin; MRP2, multidrug resistance-associated proteins 2; NF κ B, nuclear factor- κ B; OATP, Oatp, organic anion transporting polypeptide; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; *SLCO*, organic anion transporting polypeptide gene; TNF α , tumor necrosis factor- α .

OATP1A2 was the first OATP family member cloned from humans (Kullak-Ublick et al., 1995). It is widely distributed throughout the body, and the highest mRNA expression was detected in the brain, liver, lung, kidney, and testes (Kullak-Ublick et al., 1995; Steckelbroeck et al., 2004). OATP1A2 has a broad substrate specificity, and studies on the substrates transported by other human OATPs demonstrated that most of them showed a substrate spectrum at least partially overlapping with that of OATP1A2 (König, 2011). Due to the extent of its substrate specificity and wide tissue distribution, OATP1A2 is believed to be a key determinant for drug absorption, distribution, and excretion (Lee et al., 2005).

Inflammation is known to regulate the expression and functions of many drug-metabolizing enzymes and drug transporters. Proinflammatory cytokines such as interleukin (IL)-1 β , tumor necrosis factor- α (TNF α), and IL-6 have been shown to affect the expression of rodent (Green et al., 1996; Geier et al., 2003, 2005; Siewert et al., 2004) and human hepatic drug transporters such as OATP1B1 and OATP1B3 (Le Vee et al., 2008, 2009). Transporters in extrahepatic tissue were also found to be regulated under inflammatory conditions (Morgan et al., 2008). However, compared with the hepatic OATP members, little is known about the regulation of *SLCO1A2* in response to inflammation, although a minimal promoter region within 91 bp upstream of the transcription initiation site was identified, and a putative silencer element was suggested in the -662- to -440-bp region (Kullak-Ublick et al., 1997). In the present study, we analyzed the promoter sequence of *SLCO1A2* further upstream of the transcription initiation site and found a putative nuclear factor- κ B (NF κ B) binding site. NF κ B is known to be induced by the proinflammatory cytokine TNF α (Fitzgerald et al., 2007) and is considered to be a major regulatory factor responsible for the regulation of genes under

inflammation conditions (Morgan et al., 2008). Further investigation with breast cancer cell line MCF7 and liver cancer cell line HepG2 revealed that this binding site interacts with NF κ B and may be responsible for the suppressive effect of TNF α on *SLCO1A2* gene expression. In addition, the inhibition of NF κ B significantly elevated the suppressive effect of TNF α on *SLCO1A2* expression in multiple cancer cell lines.

Materials and Methods

Materials. Reagents and enzymes for molecular biology and cell culture were obtained from Thermo Fisher Scientific (Waltham, MA). All other chemical compounds were purchased from Sigma-Aldrich (St. Louis, MO) except where otherwise stated.

Construction of Plasmid DNA. The 2160-bp segment of the *SLCO1A2* promoter region was obtained by polymerase chain reaction (PCR) amplification with the following primer set: forward 5' GGTACCCTGAAGCATTACCTGTTC 3' and reverse 5' GAGCTCCCTGGAACGCTTTAATACAGA 3'. The underlined sequences are restriction sites for KpnI and SacI, which were used to ligate the fragment into the pGL3 basic vector (Promega, Fitchburg, WI). Segments with other lengths were generated with the Ω -PCR technique (Chen et al., 2013); whereas, site-directed mutagenesis was carried out with the QuikChange Lightning Site-Directed Mutagenesis Kit from Agilent (Santa Clara, CA), using the pGL3 2160-bp plasmid as the template. All mutant sequences were confirmed by full-length sequencing.

Cell Culture and Transfection of Plasmid Constructs into Cells. MCF7 and HepG2 cells (American Type Culture Collection, Manassas, VA) were grown at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum; whereas T-47D (American Type Culture Collection) cells were grown in RPMI 1640 medium supplemented with 0.2 U/ml insulin and 10% fetal bovine serum at 37°C and 5% CO₂. Confluent cells in a 48-well plate were transfected with DNA plasmid using Lipofectamine 2000 reagent (Thermo Fisher Scientific) following manufacturer's instructions. Transfected cells were incubated for 48 hours at 37°C and then used for luciferase assay analysis.

Luciferase Assay. The luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer instructions. Briefly, 48 hours after transfection, cells in a 48-well plate were lysed with the passive lysis buffer for 20 minutes at room temperature. Cell debris was then spun down, and the supernatant was transferred to a clean centrifuge tube. Twenty microliters of sample was added to 100 μ l of Luciferase Assay Reagent II (Promega) and then subjected to measurement with a GloMax-Multi Jr Single Tube Multimode Reader (Promega). The quenching of firefly luciferase luminescence and concomitant activation of *Renilla* luciferase are accomplished by adding 100 μ l of Stop & Glo Reagent (Promega).

Electrophoretic Mobility Shift Assay. Oligonucleotides corresponding to the putative NF κ B binding sequence or mutants were labeled with biotin with Beyotime EMSA Biotin-Labeling Kit according to the manufacturer's instruction (Beyotime Biotechnology Inc., Jiangsu, People's Republic of China). The incubations, which consist of 100 nM labeled oligonucleotide and 3 μ g of nuclear extracts in a final volume of 10 μ l, were carried out with the Beyotime EMSA/Gel-Shift Kit (Beyotime Biotechnology Inc.). After incubation for 30 minutes at room temperature, loading buffer was added, and the mixture

-2087
CTGAGCATTACCTGTTCCCAATTGAATGCTGAGAAAGATTAGGGAATACATGATTCTCCACTTAATAAACTTCAGCATG
TCTTTTTCATTAGAGTGATGATTGACAGGATTGTTGGTGACACTGTGGTCTGGGAGCAGAGTCACCTGTGTAAACAAC
ACTGTGCTAACAAAAGAGGACTGTTCCACATGGCTGGCACTAGCACAGCTGAGAAAGATGAAAGAGCGGCACACCAATTTCTG
-1826
GATTGGGGCCAGATGGACAACATGACTATGATATAACCGTGGGATTTTATGGATTTTATTAATAAATGGAATAGATT
-1845
TGTAAATTTGTTTATTTCTCCATGAAGATGTTTGAATGATCTGAAATATAAAATATTTTCATCATATAAGTTGGAAAC
TAGGGAAAGTTTAGGGTAAATTAATCCATGTAATTCTTTCAAGTGCTAGCCTAAATATTACCTCTTACTCTCAGTTAAGA
TAATAGTTTTTAAACCCCTTTTTCATATGTTAAACAATTTGAAGCAATCTCCAGACATAGTAAAAAACCCTGCTCTAGA
TGGTTACTAGTGTCACAAATTCAGGAGGAACATATAGAAATCCCTATATGGTGATTTTATGCCACAAGAGAGTGAAGGCAATTTGAC
TATGCCCTCTGGCTATATAGAAAAATAGACATTTAATATAGTAAATGTTTATGCCACAAGAGAGTGAAGGCAATTTGAC
GTGGGAGAAGAATAAGTTCTCCCTTGTGTTGATACGGTGACTATATGGGCTAAGATAATGGTTAGGAGAGAGAGAGAG
AAAAGGGAGAGAGATGGGTCTAATGACAGAGAAGAAAGAAAGAGGGCAGTTCAGAACAAATAACCTGTGAATTTTTCAC
CACTTACATACCAAGCAAAATGCAATTTCTTACACTGTCTGGTTCTATCCAAATGTTTATTTAAAGATTTTATCTTCC
CAATGAAAGTTTAAAGTCTCTGGAATCTGGGTCCAGATCTCTOCACAGAAACACTTCTCTCGCTAGTTTATACCTCTCTGTA
GGCAGTACTTGGCTTTCTGCTCACTGCTGAATGCTGGTGCTGACACCATGACTGGCAGAGAGTAGATGTTCAATAAGTATT
TGTGTATCTGTAGAGAGATGTTTAAATAACAAACTCAACATAATATGCGAGACTATGCCCTGATGTGTGTGTGTTG
GGATTACGACATTTGTAGAGGTAAAGCAGTTGTTTAAAAAATAGTGCATTCAGATCACTGTTAAATGGATATTGT
-710
TTAAAGGACTTAATTAATTAATGCTGGGAGACTGTTAAGAAATCACTTGAAGAACTAGAATAATATGCCAAAGTGAACAC
TTTGGTTTGTATACAAATATTGCTGATTTCAAGATGTAACAAACAAACAACTCTTGATGGGATTAGATAATTAATTGAG
TTTAATTCACATTTTGTGTTTACATATAAAATGTGCGCTGTATGTTATACATCTGTACATATAATTTCCACATATATCTATAC
CTTATTACGTATAGCTACATATAGATACCAATTTATATGTTTAACTCTCAAGCTTACATCTTATGTTTGAATCTGGGATATT
TTCCAGATCATAGAAATTTGTTTATTTTACTAGTTGTTTTCATGATTAATAATTCGGTGTCTCCGAGGTTTCTCATGTATC
AGCAGTGCCCCAGAAAGTGTTCCTCTGAGACTTAGGGAAGCTTGAATGCAAAATACTTGAAGAGAGAGCTGCCCA
-293
AAAAGATTTTGAAGCTTGGTAGGGTTAGTGAAGCAGTTTATAGTACAAACACAACCTCAAGATGTGTGTTTAAAGTA
GCTATTTTCATGGAAGTCTTTTGGTACTATTGTTTGGCTAGAGAGTGAAGGCTTTCTTTAAACATGTGACCATTTT
GCAAGATCATAACAAACATCTAATATCTTAATCTCACTAAAGAGTCACTAAAGTTATTGATGTACCTTTTGAATAGAA
-1
ACCAACAACATAATCTCTAACCAATGATTCTTAATCTGTATTAAAGGTTCCAG

Fig. 1. Putative NF κ B binding sequence at the promoter region of *SLCO1A2*. The promoter region of *SLCO1A2* was extracted from GenBank of the National Center for Biotechnology Information and analyzed with MatInspector (Genomatix AG, Munich, Germany) for the corresponding motifs. The putative NF κ B binding site from -1845 to -1836 bp was underlined. Start positions for promoter regions with different lengths and transcription start sites were indicated and marked in bold.

Mouse <i>slc2a4</i>	GGGCGTGTCC (Furuya et al., 2013)
	GGGCGTGGCC
ABCC6	GGGACCCCC (Jiang et al., 2006)
ABCG2	TGGGGAAACC (Pradhan et al., 2010)
Rat <i>mdr1b</i>	GGGGAATTC (Yu et al., 2008)
CYP2C11	AGGAGTCTCC (Iber et al., 2000)
Consensus NF- κ B binding sequence	NGGAMTNYCC
<i>SLCO1A2</i>	GCACACGGAATTCCTGATT
(N=any nucleotide, M=A or T; Y=C or T)	

Fig. 2. Consensus sequences of NF κ B binding site. The 10-bp putative NF κ B binding segment was compared with NF κ B binding sites of metabolizing enzymes and transporters. A consensus sequence derived from these sequences was also shown.

Fig. 3. Putative NFκB binding site at *SLCO1A2* promoter interacts with NFκB in MCF7 cells. (A) Nuclear extracts specifically bind to the putative NFκB binding site. EMSA was performed as described in the *Materials and Methods* section. Nuclear extracts from MCF7 cells were incubated with 0.1 μM biotin-labeled oligonucleotides. Increasing concentrations of the unlabeled probe, which was 1-, 10-, and 100-fold molar excess over the labeled probe was added into the incubation system. (B) Wild-type or mutated unlabeled oligonucleotides that were 10-fold molar excess over the labeled probe were incubated with the labeled NFκB binding segment. The mutations were underlined. (C) Supershift analysis of the DNA/protein complexes. Antibodies to the p50 and p65 subunits of NF-κB were incubated with the nuclear extracts for 0.5 and 2 hours, respectively, before the labeled oligonucleotides were added; or the biotin-labeled segments were added 30 minutes before the antibodies were added into the incubation system. (D) ChIP analysis for the interaction of NFκB p50 and p65 subunits with the putative binding sequence. The cross-linked DNA/protein complexes were precipitated with p50 or p65 antibodies, with rabbit IgG (IgG) as the nonspecific binding control, pulled down by protein G agarose/salmon sperm DNA, and subjected to PCR for detection of the *neg*-NFκB region. For all of the analyses, three independent experiments were performed and a representative figure was presented. M, molecular marker; Mu, mutated probes; WT, wild-type probe.

was loaded onto a 4% nondenaturing polyacrylamide gel, electrophoresed for 1.5 hours at 4°C at 90 V in 0.5 × Tris/borate/EDTA buffer, and subsequently transferred to a positively charged nitrocellulose membrane. The membrane was cross-linked with UV light at 254 nm, then blotted with blotting buffer and incubated with streptavidin-horseradish peroxidase conjugate for 30 minutes at

room temperature. The membrane was washed, reacted with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA) and exposed to X-ray film for detection. In competition experiments, a 1-fold to 100-fold excess of unlabeled oligonucleotide was added to the labeled oligonucleotide in buffer before the addition of nuclear extracts. Nuclear extracts were incubated

Fig. 4. Putative NFκB binding site at *SLCO1A2* promoter interacts with NFκB in HepG2 cells. (A) Wild-type (WT) or mutated (M) unlabeled probes that had a 10-fold molar excess over the labeled NFκB binding segment compete for binding with NFκB. (B) Analysis for the interaction of NFκB subunits with the putative binding sequence was carried out with the ChIP assay. The cross-linked DNA/protein complexes were precipitated with p50 or p65 antibodies and subjected to PCR for detection of the *neg*-NFκB region, as described in Fig. 3. For all of the analyses, three independent experiments were performed and a representative figure was presented.

in buffer with p65 or p50 antibody (1:10 dilution) for 2 hours at room temperature before the labeled DNA was added in the supershift experiments. The oligonucleotide 5'-GCACACGGAATTCCTGA TTTGGG-3' and its complement, which corresponds to the -1850- to -1827-bp upstream region of the human *SLCO1A2* gene (GenBank Accession No. Y08062) as well as oligonucleotides for the mutants were synthesized by Thermo Fisher Scientific.

Chromatin Immunoprecipitation. Chromatin immunoprecipitation (ChIP) was carried out with the ChIP Assay Kit (Beyotime Biotechnology Inc.) following manufacturer instructions. Briefly, cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature. The cross-linking reaction was then quenched with 0.125 M glycine, scraped down, and collected in an Eppendorf tube. Cells were washed with cold phosphate-buffered saline and lysed with SDS lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS with protease inhibitors phenylmethylsulfonyl fluoride, 500 μ g/ml, and leupeptin, 3 μ g/ml), subjected to sonication with a Branson S450-D Digital Sonifier (Branson Ultrasonic, Danbury, CT). The sonication procedure was as follows: seven rounds of 5-second sonication on ice, with 10-second intervals in between at 50 amplitude microns power. Cell debris was removed by centrifugation, and the supernatant was transferred to a clean Eppendorf tube, in which protein G agarose/salmon sperm DNA was added to pre-clean the sample for 1 hour at 4°C. Twenty microliters of the supernatant was taken out as input in the following PCR, and p65 or p50 NF κ B antibody (1:100 dilution) was added to the remaining solution, incubated overnight at 4°C, then pulled down by protein G agarose/salmon sperm DNA. The agarose beads were washed sequentially with high-salt wash buffer (0.1% SDS, 0.1% Triton X-100, 500 mM NaCl, 2 mM EDTA, and 20 mM Tris-HCl, pH 8.0) and TE Buffer (Thermo Fisher Scientific), and released in elution buffer (0.1% SDS and 0.1 M NaHCO_3 , pH 8.0), and the cross-linking of DNA-protein was reversed by adding 200 mM NaCl and incubating the solution at 65°C overnight. The DNA was purified with the High Pure PCR Product Purification Kit (Roche Applied Science, Penzberg, Germany) and analyzed with PCR or real-time quantitative PCR (qPCR) with the following primers: forward, 5'-GCAGAAGT-CACGTGTAAAC-3'; and reverse, 5'-GTCATTGTTGTCCATCTTGG-3'. For

qPCR, the fluorescent dye SYBR Green methodology and an ABI Prism 7000 Detector (Applied Biosystems, Foster City, CA) were used as described previously (Jigorel et al., 2005). Specificity of amplification was verified at the end of each reaction by the analysis of dissociation curves of the PCR products.

Isolation of Nuclear Proteins and Western Blotting. Proteins were extracted with cell nucleic and cytosolic proteins extraction kit (Beyotime Biotechnology Inc.) following manufacturer instructions. Proteins were denatured with 4 $\times$ Laemmli buffer and loaded onto a 7.5% SDS-PAGE gel, then transferred electrophoretically to a polyvinylidene difluoride membrane (Merck Millipore). Different subunits of NF κ B were detected with corresponding antibodies (Beyotime Biotechnology Inc.).

Quantitative PCR to Analyze *SLCO1A2* Expression. Total RNA was isolated from cells with TRIzol reagent (Thermo Fisher Scientific). The same amount of RNA was subjected to reverse transcription reaction. The resulting cDNA was then used for qPCR analysis as described above with the primer set as follows: forward, 5'-TTGTTGGCATCATTCTGTGC-3'; and reverse, 5'-CGACCAACGAGTGTCACT-3'. Relative quantification of *SLCO1A2* mRNA level was calculated by normalizing the total cDNA amount tested to an *actin* endogenous control.

Data Analysis. Statistical analysis was carried out with one-way analysis of variance with Bonferroni's post hoc test. the differences between means are regarded as significant at $P < 0.05$.

Results

Identification of a Putative NF κ B Binding Site at the Promoter Region of *SLCO1A2*. It has been reported that the *SLCO1A2* gene has a minimal promoter region of 91 bp related to the transcription start site and that a putative suppressive element was localized in the -662- to -440-bp region (Kullak-Ublick et al., 1997). In the study, although an inhibitory effect was observed in the -662- to -440-bp region and up to -1640 bp

Fig. 5. The putative NF κ B binding site has a suppressive effect on *SLCO1A2* expression. (A) Analysis of the promoter function of *SLCO1A2* constructs containing different lengths of the promoter region relative to transcription start site. The ratio of the firefly luciferase activity to *Renilla* luciferase activity was measured and calculated to represent promoter activity. The results were expressed as fold changes compared with that of pGL3 empty vector. Three independent experiments were performed, each with triplicate measurements. The data were expressed as the mean $\pm$ S.D. ($n = 3$). (B) Interference of NF κ B binding partially released the inhibitory effect on *SLCO1A2* promoter function. Fold changes of mutant promoter activity compared with wild-type activity was shown. Different letters indicate a significant difference between treatments ($P < 0.05$). The results shown are shown as the mean $\pm$ S.D. ($n = 3$). (C) NF κ B inhibitor relieved the suppressive effect of wild-type promoter but showed no effect on mutants. Cells were pretreated with 5 μ M BAY117082 (BAY) for 16 hours before luciferase activity was measured. Fold changes of promoter activity compared with untreated control were shown. Asterisks indicate a significant difference compared with untreated control ($P < 0.05$). The results shown are expressed as the mean $\pm$ S.D. ($n = 3$).

upstream of the *SLCO1A2* transcription start site was analyzed, a substantial amount of promoter activity was still observed. When we analyzed further upstream of the sequence, a region ranging from -1845 to -1836 bp (Fig. 1) contains the sequence highly homologous to that of the consensus NF κ B binding sequence described in the literature (Fig. 2). We therefore furthered our studies on this 10-bp region.

The Putative NF κ B Binding Site Interacts with Different Subunits of the Transcription Factor. It has been reported that *SLCO1A2* is expressed in breast cancer cell line MCF7 (Banerjee et al., 2012). In addition, our previous study on radiation-induced responses of OATP expression found out that OATP1A2 was the major transporter that responded to low-dose, high-linear energy transfer irradiation in MCF7 cells (Zhou et al., 2017). The alteration of cytokines such as TNF α by radiation is well documented, and our preliminary data suggested that low-dose, high-linear energy transfer irradiation may suppress the expression of TNF α (unpublished data). Hence, we speculate that radiation may affect the expression of *SLCO1A2* through a TNF α -related pathway. Therefore, we used MCF7 as a cell model for the present study. To examine whether NF κ B binds to the putative NF κ B binding site at the *SLCO1A2* promoter sequence, electrophoretic mobility shift assay (EMSA) was performed with biotin-labeled oligonucleotides that contained the 10-bp putative NF κ B binding segment (-1845 to -1836 bp) plus 3 bp at the 5' end and 6 bp at the 3' end. As shown in Fig. 3A, the labeled sequence formed two complexes (complex 1 and complex 2) with nuclear extracts from MCF7 cells. The intensity of both complex bands was reduced gradually when competed with increasing concentrations of the unlabeled specific

NF κ B binding segment. To further evaluate the specificity of the interaction, we mutated two critical regions for NF κ B binding within the sequence (Iber et al., 2000) as well as a mutant that contained a mutation region outside the putative binding site (mu3). As shown in Fig. 3B, the addition of the unlabeled 5'GG (mu1) mutant partially affected the formation of DNA/protein complexes, whereas the presence of the unlabeled 3'CC (mu2) or double mutant showed no effect on the formation of the DNA/protein complexes. Mu3 exhibited a similar effect to that of the wild-type unlabeled oligonucleotides.

To investigate the participation of NF κ B subunits in the observed complexes, antibodies for p50 and p65 NF κ B subunits were used for the supershift experiments. Unexpectedly, when nuclear extracts were incubated with the p65 and p50 subunit antibodies, we could not observe any band that was shifted to a lower mobility. However, the addition of p65 or p50 antibodies prior to addition of the probe significantly reduced the intensity of both bands, whereas the addition of the biotin-labeled probe before the antibodies disrupted the formation of these complexes. The addition of p50 antibody appeared to exert a more significant effect than addition of the p65 antibody (Fig. 3C). To confirm the interaction of the putative NF κ B binding sequence with different NF κ B subunit exists in endogenous DNA, the ChIP assay was performed. Figure 3D showed that both p65 and p50 antibodies could precipitate the corresponding fragment that contains the NF κ B binding sequence.

To rule out the possibility that interactions between the putative binding site and NF κ B subunits were cell-type specific, we also analyzed the above-mentioned effects with liver cancer cell line HepG2,

Fig. 6. TNF α affects nuclear translocation of NF κ B. (A) MCF7 cells were pretreated with TNF α (20 ng/ml) or TNF α -neutralizing antibody (TNF α -neu) (50 ng/ml) for the indicated time periods and were subjected to nuclear protein extraction. (B) MCF7 cells were pretreated with TNF α (0.5 hour) or TNF α -neutralizing antibody (1 hour) with the indicated concentrations and were subjected to nuclear protein extraction. (C) HepG2 cells were pretreated with TNF α (20 ng/ml) for 0.5 hour or TNF α -neutralizing antibody (50 ng/ml) for 1 hour and were subjected to nuclear protein extraction. Fifteen micrograms of proteins were loaded on a 7.5% SDS-PAGE gel, followed by Western blotting with anti-p50 or anti-p65 antibody (1:1000 dilution). The same blot was probed with anti-Histone H3 antibody (1:1000 dilution) as the loading control. Three independent experiments were carried out, and a representative blot was shown.

another cell line that was demonstrated to express *SLCO1A2* (Kullak-Ublick et al., 1996). As shown in Fig. 4A, the putative NF κ B binding site at the *SLCO1A2* promoter sequence also specifically formed DNA/protein complexes with the nuclear extracts from HepG2 cells. In addition, ChIP analysis demonstrated that the putative NF κ B binding sequence interacts with both subunits of NF κ B in HepG2 cells as well (Fig. 4B).

The Putative NF κ B Binding Site Has a Suppressive Effect on *SLCO1A2* Expression. Next, we wanted to see whether this putative NF κ B binding sequence has an effect on the expression of *SLCO1A2*. For such a purpose, we further generated three additional promoter constructs that contained the -293-, -710-, and -1826-bp regions relative to the *SLCO1A2* transcription start site and expressed them in MCF7 cells (Fig. 1). Consistent with a previous report (Kullak-Ublick et al., 1997), the construct with the -293-bp region exhibited significant promoter activity, whereas those of the -710- and -1826-bp regions showed reduced function (Fig. 5A). Interestingly, promoter function of the construct that contained the -1845- to -1836-bp segment was further suppressed, suggesting the presence of an inhibitory element. To evaluate the effect of NF κ B binding with the -1845- to -1836-bp region on *SLCO1A2* promoter activity, we analyzed the *SLCO1A2* promoter activity in the 5'GG or 3'CC mutants as well. Figure 5B showed that both mutations partially increased promoter function. The double mutation of both sites did not seem to release the suppressive effect more significantly, suggesting that these two sites are important

for the inhibitory effect, and that the mutation of either one interferes with NF κ B inhibition of *SLCO1A2* promoter activity. When cells were pretreated with NF κ B inhibitor 3-[(4-methylphenyl)sulfonyl]-(2E)-propenenitrile (BAY117082) (Pierce et al., 1997), suppression of the promoter activity was partially relieved, whereas those of the mutants remained unchanged (Fig. 5C). These results suggested that the -1845- to -1836-bp region interacts with NF κ B and that such an interaction has a suppressive effect on *SLCO1A2* expression. We thus named this element as *neg*-NF κ B.

***SLCO1A2* Responses to TNF α Treatment Is Mediated through *Neg*-NF κ B.** Since TNF α is a well-known cytokine that affects NF κ B translocation and activation (Schütze et al., 1995), and cytokines have been demonstrated to inhibit the expression of OATPs (Geier et al., 2003; Le Vee et al., 2009), we next wanted to see whether TNF α affects the expression of *SLCO1A2* through the interaction of NF κ B with *neg*-NF κ B. To first test the effect of TNF α on NF κ B activation, MCF7 cells were treated with TNF α or TNF α -neutralizing antibody and the nuclear fraction of NF κ B level was analyzed. As shown in Fig. 6, A and B, both the p65 and the p50 subunits of NF κ B increased in the nuclear fraction after TNF α treatment, whereas they were reduced with the treatment of neutralizing antibody. A similar effect exerted by TNF α modulators was observed in HepG2 cells (Fig. 6C). These results suggested that NF κ B is activated by TNF α and that both the p65 and p50 subunits were translocated into the nuclear fraction in MCF7 and HepG2 cells.

Fig. 7. The effect of TNF α on *SLCO1A2* promoter function is mediated by NF κ B in MCF7 cells. (A) The treatment of TNF α -neutralizing antibody (TNF-neu) or TNF α affects the binding of NF κ B with the *neg*-NF κ B segment. Cells were treated with 50 ng/ml TNF α -neutralizing antibody for 1 hour or 20 ng/ml TNF α for 0.5 hour before EMSA was performed. Three independent experiments were performed, and a representative figure was shown. Different letters indicate a significant difference between treatments ($P < 0.05$). The results shown are the mean $\pm$ S.D. ($n = 3$). (B) The TNF α neutralization antibody partially released the inhibition of *SLCO1A2* promoter activity but showed no effect on *neg*-NF κ B mutants. Cells were pretreated with 50 ng/ml TNF neutralization antibody for 16 hours, and luciferase activity was measured as described in Fig. 5. Fold changes in promoter activity compared with untreated control were shown. Asterisks indicate a significant difference compared with untreated control ($P < 0.05$). The results shown are expressed as the mean $\pm$ S.D. ($n = 3$). (C) ChIP analysis for the interaction of p50 and p65 subunits with *neg*-NF κ B after cells were subjected to TNF α treatment. Cells were treated with 20 ng/ml TNF α for 0.5 hour, and ChIP analysis was carried out as described in Fig. 3. The DNA pulled down by p65 or p50 antibody was evaluated by qPCR. Three independent experiments were performed. Asterisks indicate a significant difference compared with untreated control ($P < 0.05$). The results are shown as the mean $\pm$ S.D. ($n = 3$).

Fig. 8. Effect of TNF α on *SLCO1A2* promoter function is mediated by NF κ B in HepG2 cells. (A) TNF α -neutralizing antibody (TNF α -neu) or TNF α affects the binding of NF κ B with *neg*-NF κ B segment. Cells were treated and analyzed as described in Fig. 7. Three independent experiments were performed, and a representative figure was shown. Different letters indicate significant differences between treatments ($P < 0.05$). The results are shown as the mean $\pm$ S.D. ($n = 3$). (B) TNF α neutralization antibody or NF κ B inhibitor BAY117082 partially released the inhibition of *SLCO1A2* promoter activity but showed no effect on *neg*-NF κ B mutants. Cells were pretreated with 50 ng/ml TNF α neutralization antibody or 5 μ M BAY117082 for 16 hours, and luciferase activity was measured as described in Fig. 5. Fold changes of promoter activity compared with untreated control were shown. Different letters indicate significant differences between treatments of the same construct ($P < 0.05$). The results are shown as the mean $\pm$ S.D. ($n = 3$). (C) ChIP analysis for the interaction of p50 and p65 subunits with *neg*-NF κ B after cells were subjected to TNF α treatment. Cells were treated with 20 ng/ml TNF α for 0.5 hour, and ChIP analysis was carried out as described in Fig. 3. The DNA pulled down by p65 or p50 antibody was evaluated by qPCR. Three independent experiments were performed. Asterisks indicate significant differences compared with untreated control ($P < 0.05$). The results are shown as the mean $\pm$ S.D. ($n = 3$).

To see the responses of cells toward TNF α , EMSA was carried out after TNF α treatment. As shown in Fig. 7A, when the nuclear extracts of MCF7 cells were incubated with the biotin-labeled probe that corresponds to *neg*-NF κ B, a more intense shifted band (50% increase, $P < 0.05$) was observed in the TNF α -treated sample compared with that of the untreated control. In addition, when cells were pretreated with TNF α -neutralizing antibody, the suppressive effect of *SLCO1A2* expression was partially released (Fig. 7B). On the other hand, when the critical regions of *neg*-NF κ B were mutated (mu1 and mu2 and double mu), the addition of TNF α -neutralizing antibody exerted no effect on the promoter activity of *SLCO1A2*, implicating that the binding of NF κ B with *neg*-NF κ B is involved in *SLCO1A2* responses to TNF α treatment. In addition, qPCR analysis coupled with ChIP showed that the treatment of TNF α increased the formation of endogenous DNA/protein complexes (Fig. 7C).

Similar results were obtained from HepG2 cells. A more intense shifted band composed of *neg*-NF κ B/NF κ B subunits (2-fold increase, $P < 0.05$) was observed under TNF α treatment, whereas the signal was reduced when the cells were treated with TNF α -neutralizing antibody (Fig. 8A). In addition, both TNF α -neutralizing antibody and NF κ B inhibitor BAY117082 partially relieved the suppressive effect on *SLCO1A2* promoter activity in HepG2 cells, whereas those of the mutants remained unchanged (Fig. 8B). qPCR analysis coupled with ChIP also demonstrated that the amount of endogenous DNA/protein complexes increased after TNF α treatment (Fig. 8C).

Finally, to see whether the NF κ B-mediated TNF α suppression occurs *in vivo*, we treated MCF7, HepG2, and another breast cancer cell line, T47-D, which has been reported to show a high level of *SLCO1A2* (Obaidat et al., 2012) with TNF α and BAY117082. As

Fig. 9. NF κ B inhibitor relieved the suppressive effect of *SLCO1A2* expression by TNF α . Cells were pretreated with 20 ng/ml TNF α , 5 μ M BAY117082 (BAY), or both for 16 hours. Total RNA was isolated from cells and subjected to reverse transcription reaction. The resulting cDNA was then used for qPCR analysis. The expression of *SLCO1A2* after treatment was compared relative to the untreated control. Three independent experiments were performed. Different letters indicate significant differences between treatments for the same cell line ($P < 0.05$). The results are shown as the mean $\pm$ S.D. ($n = 3$).

shown in Fig. 9, TNF α significantly decreased the expression of *SLCO1A2* in all three cell lines, whereas the inhibition of NF κ B relieved such a suppression.

Discussion

Inflammation cytokines have been reported to inhibit the expression of OATP members, and NF κ B is an important mediator in response to cytokines such as TNF α . However, whether NF κ B affects the promoter activity of OATP family members remains unclear. In the present study, we showed that NF κ B can bind to the promoter region of *SLCO1A2* and exert inhibitory effect on transcriptional activity of the gene in breast cancer cell line MCF7 and hepatocarcinoma cell line HepG2. In addition, our results demonstrated that TNF α , a well-known proinflammatory cytokine, may suppress *SLCO1A2* expression through a negative regulation element.

A previous study (Kullak-Ublick et al., 1997) demonstrated that constructs up to –1640 bp upstream of the *SLCO1A2* transcription start site exhibited substantial promoter activity. However, when a construct containing the putative NF κ B binding site located within –1845 to –1836 bp was generated and expressed within MCF7 cells, a significant suppressive effect on transcription activity of *SLCO1A2* was observed, which implicated that the binding of NF κ B to this element may negatively regulate the expression of the gene. EMSA analysis showed the presence of two shifted bands, both of which displayed lower intensity in the presence of unlabeled probes, suggesting that there is more than one DNA/protein complex formed during the incubation with nuclear extracts. The addition of p50 or p65 antibody revealed that the putative NF κ B binding segment interacts with both subunits because the presence of either antibody significantly reduced the intensity of both shifted bands. Interestingly, the reduction of band intensity was more dramatic for the p50 subunit, because it required only 0.5 hour of incubation with the antibody to obtain a similar result as that of 2 hours of incubation with the p65 antibody. These results suggested that p50 may be more readily interact with *neg*-NF κ B than the p65 subunits. The binding between NF κ B subunits with *neg*-NF κ B was further confirmed using a system containing whole genomic DNA through the ChIP assay, a technique that is used to investigate association of regulatory molecules to specific DNA regions in vivo. However, we could not rule out the possibility that other subunits of NF κ B interact with the sequence as well. The binding of p50 and/or p65 subunits of NF κ B to *neg*-NF κ B was also observed in HepG2 cells, suggesting such an interaction is not cell-type specific.

The result of the EMSA-supershift experiment is different from the analysis of many other transcription factors in which binding of the antibody increases the size of the DNA/protein complex and displays a classic supershift band (Furuya et al., 2013). In our experiments, the addition of either p65 or p50 antibody before incubation with the biotin-labeled probe did not result in a size change of the DNA/protein complex but showed intensity change instead. On the other hand, when the labeled probe was incubated with nuclear extracts before antibodies were added, the disruption of DNA/protein complex formation was abolished. Such a phenomenon was also observed in synovial cells (Juaranz et al., 2005), NIH 3T3 fibroblasts (Balasubramanian et al., 2011) as well as in the study of *slc2a4* interaction with NF κ B in white adipose tissue and 3T3-L1 adipocytes (Furuya et al., 2013). It was suggested that p50 or p65 antibody binds to their respective proteins in a location that is close to the DNA binding site and hence hinder the formation of the DNA/protein complex. On the other hand, the formation of the protein/DNA binding may lead to conformational changes that impair further antibody binding to the protein (Furuya et al., 2013).

Inflammation occurs in many acute and chronic disease states, and it has been shown to modulate pharmacokinetics by changing the expression of metabolizing enzymes and membrane-associated drug transporters. Many key transcription factors including NF κ B are believed to be involved in this kind of expression regulation (Cressman et al., 2012). NF κ B has been shown to increase the expression of transporters such as P-glycoprotein (Bentires-Alj et al., 2003), breast cancer resistance protein, and multidrug resistance-associated protein 2 (Ke et al., 2013) but also can be an inhibitory factor for metabolizing enzymes such as cytochrome P450 enzyme CYP2C11 (Iber et al., 2000) and transporters like organic anion transporter 3 (Phatchawan et al., 2014). OATP family members OATP1B1 and OATP1B3 as well as OATP2B1 were shown to be downregulated by TNF α and IL-6 (Le Vee et al., 2009). In the present study, although a reliable suppressive effect of *SLCO1A2* by TNF α treatment was hard to obtain due to the low activity of the untreated luciferase construct control, both MCF7 and HepG2 cells treated with TNF α -neutralizing antibody showed significantly increased promoter activity. The mutation of essential positions within *neg*-NF κ B abolished such an effect, and the fact that TNF α treatment increased the number of DNA/protein complexes formed between *neg*-NF κ B and the corresponding NF κ B subunits suggested that the putative binding site of NF κ B may in part be responsible for the suppressive effect of TNF α on *SLCO1A2* expression. Our qPCR results showing that NF κ B inhibitor BAY117082 relieved the suppressive effect of TNF α on *SLCO1A2* expression in different cancer cell lines seemed to support such a speculation.

In summary, we identified a functional NF κ B binding site located at –1845 to –1836 bp upstream of the transcription start site of *SLCO1A2*. Such an element exerts a repressive effect on *SLCO1A2* expression and may mediate the gene responses toward inflammation. To our knowledge, this is the first report demonstrating that the expression of OATP family members can be directly regulated by the transcription factor NF κ B. Such a regulatory mechanism may also be found in inflammation-related regulation of other OATP members.

Authorship Contributions

Participated in research design: Yi, Hong.

Conducted experiments: Xiang, Li, Wang, Chen.

Performed data analysis: Yi, Hong.

Wrote or contributed to the writing of the manuscript: Hong.

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