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Identification of a NFκB 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 family 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 (TNFα) has been shown to affect the expression of different drug transporters, including OATP family members. In the present study, a putative nuclear factor-κB (NFκB) 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 NFκB binding site and that the DNA-protein complexes contained both p65 and p50 subunits of NFκB. Further study revealed that the binding site may be responsible in part for the suppression effect of TNFα toward SLCO1A2 expression because the treatment of TNFα significantly increased. Treatment of TNFα significantly increased formation of the DNA-protein complexes and mutations at essential bases of the putative NFκB binding site abolished responsiveness to the TNFα neutralizing antibody, suggesting that the binding site may be responsible in part for the suppression effect of TNFα 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-17β-glucuronide, dehydroepiandrosterone sulfate, aldosterone, and cortisol) (Bossuyt et al., 1996a; Kanai et al., 1996; Eckhardt et al., 1999); thyroxine (3, L-thyroxine) and triiodothyronine (L-triiodothyronine) (Abe et al., 1998; Pizzagalli and Meier, 2003; Nakanishi and Tamai, 2012), although OATP1B7 is considered to be nonfunctional and a pseudogene (Stieger and Hagenbuch, 2014). 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; Nakashiki and Tamai, 2012), although OATP1B7 is considered to be nonfunctional and SLCO1B7 was proposed as a pseudogene (Stieger and Hagenbuch, 2014).

AABBREVIATIONS: BAY117082, 3-[(4-methylphenyl)sulfonyl]-2(E)-propene nitrite; bp, base pair; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; IL, interleukin; MRP2, multidrug resistance-associated proteins 2; NFκB, nuclear factor-κB; OATP, Oatp, organic anion transporting polypeptide; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; SLCO, organic anion transporting polypeptide gene; TNFα, tumor necrosis factor-α.
OATP1A2 was the first OAT 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)-1β, tumor necrosis factor-α (TNFα), 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 metabolizing enzymes and drug transporters. A consensus sequence derived from these sequences was also shown.

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′-GTTACCTGGAAGCATTACCTGTTCCC-3′ and reverse 5′-GAGCTCCCTGGAAGCCTTATACAGA-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 Ota-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 pG3 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 μl of Stop & Glo Reagent (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 μl of Stop & Glo Reagent (Promega).

Electrophoretic Mobility Shift Assay. Oligonucleotides corresponding to the putative NFκB binding sequence or mutants were labeled with biotin with Biotin-11-DATP (Roche Diagnostics) and immobilized DNA-Sepharose 4B (Amersham Pharmacia Biotech) and hybridized with the 32P-labeled NFκB consensus probe. The samples were then incubated with a 100 μl mixture of 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 50 mM KCl, 1% NP40, 0.5 mg/ml BSA, 50 μg/ml poly(dI-dC), 2 μg/ml poly(dI-dC), and 5 μg/ml poly(dI-dC). The DNA-protein complexes were separated on a 5% nondenaturing polyacrylamide gel. The gel was dried, and bands were visualized by autoradiography.

Materials.

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.

Fig. 1. Putative NFκB 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 NFκB 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.

Fig. 2. Consensus sequences of NFκB binding site. The 10-bp putative NFκB binding segment was compared with NFκB binding sites of metabolizing enzymes and transporters. A consensus sequence derived from these sequences was also shown.
was loaded onto a 4% nondenaturing polyacrylamide gel, electrophoresed for 1.5 hours at 4°C at 90 V in 0.5 x 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

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**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 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 NFκB 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.

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**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 experiment. The oligonucleotide 5'-GCAACGAAATTTCTCTGA TTGGG-3' and its complement, which corresponds to the −1850 to −1827-bp upstream region of the human SLCO1A2 gene (GenBank Accession No. Y08082) 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 μg/ml, and leupeptin, 3 μg/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 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 preclar the sample for 1 hour at 4°C. Twenty microliters of the supernatant was taken out in the following PCR, and p50 or p50 NFκB 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% Triton X-100, 500 mM NaCl, 2 mM EDTA, and 20 mM Tris-HCl, pH 8.0) overnight. The supernatant was taken out as input in the following PCR, and p65 or p50 NFκB were shown. Asterisks indicate a significant difference compared with untreated control (P < 0.05). The results shown are expressed as the mean ± S.D. (n = 3). (C) NFκB binding partially released the inhibitory effect on SLCO1A2 expression.

**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 as described above with the primer set as follows: forward, 5'-TGTGGCATTCTGTGC-3'; and reverse, 5'-CGACCCCAACGATGTCGAT-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 NFκB 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 NFκB 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 ± S.D. (n = 3). (B) Interference of NFκB 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 as shown as the mean ± S.D. (n = 3). (C) NFκB inhibitor relieved the suppressive effect of wild-type promoter but showed no effect on mutants. Cells were pre-treated with 5 μM 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 ± 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 NFκB binding sequence described in the literature (Fig. 2). We therefore furthered our studies on this 10-bp region.

The Putative NFκB 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 TNFα by radiation is well documented, and our preliminary data suggested that low-dose, high–linear energy transfer irradiation may suppress the expression of TNFα (unpublished data). Hence, we speculate that radiation may affect the expression of SLCO1A2 through a TNFα-related pathway. Therefore, we used MCF7 as a cell model for the present study. To examine whether NFκB binds to the putative NFκB binding site at the SLCO1A2 promoter sequence, electrophoretic mobility shift assay (EMSA) was performed with biotin-labeled oligonucleotides that contained the 10-bp putative NFκB 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 NFκB binding segment. To further evaluate the specificity of the interaction, we mutated two critical regions for NFκB 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 NFκB subunits in the observed complexes, antibodies for p50 and p65 NFκB 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 NFκB binding sequence with different NFκB subunit exits 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 NFκB binding sequence.

To rule out the possibility that interactions between the putative binding site and NFκB subunits were cell-type specific, we also analyzed the above-mentioned effects with liver cancer cell line HepG2.
another cell line that was demonstrated to express SLCO1A2 (Kullak-Ublick et al., 1996). As shown in Fig. 4A, the putative NFκB 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 NFκB binding sequence interacts with both subunits of NFκB in HepG2 cells as well (Fig. 4B).

The Putative NFκB Binding Site Has a Suppressive Effect on SLCO1A2 Expression. Next, we wanted to see whether this putative NFκB 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 NFκB 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 NFκB inhibition of SLCO1A2 promoter activity. When cells were pretreated with NFκB 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 NFκB and that such an interaction has a suppressive effect on SLCO1A2 expression. We thus named this element as neg-NFκB.

SLCO1A2 Responses to TNFα Treatment Is Mediated through Neg-NFκB. Since TNFα is a well-known cytokine that affects NFκB 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 TNFα affects the expression of SLCO1A2 through the interaction of NFκB with neg-NFκB. To first test the effect of TNFα on NFκB activation, MCF7 cells were treated with TNFα or TNFα-neutralizing antibody and the nuclear fraction of NFκB level was analyzed. As shown in Fig. 6, A and B, both the p65 and the p50 subunits of NFκB increased in the nuclear fraction after TNFα treatment, whereas they were reduced with the treatment of neutralizing antibody. A similar effect exerted by TNFα modulators was observed in HepG2 cells (Fig. 6C). These results suggested that NFκB is activated by TNFα and that both the p65 and p50 subunits were translocated into the nuclear fraction in MCF7 and HepG2 cells.

Fig. 7. The effect of TNFα on SLCO1A2 promoter function is mediated by NFκB in MCF7 cells. (A) The treatment of TNFα-neutralizing antibody (TNFα-neu) or TNFα affects the binding of NFκB with the neg-NFκB segment. Cells were treated with 50 ng/ml TNFα-neutralizing antibody for 1 hour or 20 ng/ml TNFα 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 ± S.D. (n = 3). (B) The TNFα neutralization antibody partially released the inhibition of SLCO1A2 promoter activity but showed no effect on neg-NFκB 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 ± S.D. (n = 3). (C) ChIP analysis for the interaction of p50 and p65 subunits with neg-NFκB after cells were subjected to TNFα treatment. Cells were treated with 20 ng/ml TNFα for 0.5 hour, and ChIP analysis was carried out as described in Fig. 3. The DNA pulled down by p65 or p50 subunits were performed. Asterisks indicate a significant difference compared with untreated control (P < 0.05). The results are shown as the mean ± S.D. (n = 3).
To see the responses of cells toward TNFα, EMSA was carried out after TNFα treatment. As shown in Fig. 7A, when the nuclear extracts of MCF7 cells were incubated with the biotin-labeled probe that corresponds to neg-NFκB, a more intense shifted band (50% increase, P < 0.05) was observed in the TNFα-treated sample compared with that of the untreated control. In addition, when cells were pretreated with 50 ng/ml TNFα neutralization antibody or 5 μM 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 ± S.D. (n = 3). (C) ChIP analysis for the interaction of p50 and p65 subunits with neg-NFκB after cells were subjected to TNFα treatment. Cells were treated with 20 ng/ml TNFα 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 ± S.D. (n = 3).

Finally, to see whether the NFκB-mediated TNFα 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 TNFα and BAY117082. As shown in Fig. 8, the expression of SLCO1A2 in TNFα-treated MCF7 cells was significantly lower than that of untreated control (P < 0.05). To confirm these in vivo results, qPCR analysis coupled with ChIP was performed. Similar results were obtained from HepG2 cells. A more intense shifted band composed of neg-NFκB/NFκB subunits (2-fold increase, P < 0.05) was observed under TNFα treatment, whereas the signal was reduced when the cells were treated with TNFα-neutralizing antibody (Fig. 8A). In addition, both TNFα-neutralizing antibody and NFκB inhibitor BAY117082 partially relieved the suppressive effect of 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 TNFα treatment (Fig. 8C).

Fig. 8. Effect of TNFα on SLCO1A2 promoter function is mediated by NFκB in HepG2 cells. (A) TNFα-neutralizing antibody (TNFα-neu) or TNFα affects the binding of NFκB with neg-NFκB 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 ± S.D. (n = 3). (B) TNFα neutralization antibody or NFκB inhibitor BAY117082 partially released the inhibition of SLCO1A2 promoter activity but showed no effect on neg-NFκB mutants. Cells were pretreated with 50 ng/ml TNFα neutralization antibody or 5 μM 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 ± S.D. (n = 3). (C) ChIP analysis for the interaction of p50 and p65 subunits with neg-NFκB after cells were subjected to TNFα treatment. Cells were treated with 20 ng/ml TNFα 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 ± S.D. (n = 3).

Fig. 9. NFκB inhibitor relieved the suppressive effect of SLCO1A2 expression by TNFα. Cells were treated with 20 ng/ml TNFα, 5 μM 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 ± S.D. (n = 3).
shown in Fig. 9, TNFα significantly decreased the expression of SLC01A2 in all three cell lines, whereas the inhibition of NFκB relieved such a suppression.

**Discussion**

Inflammation cytokines have been reported to inhibit the expression of OATP members, and NFκB is an important mediator in response to cytokines such as TNFα. However, whether NFκB affects the promoter activity of OATP family members remains unclear. In the present study, we showed that NFκB can bind to the promoter region of SLC01A2 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 TNFα, a well-known proinflammatory cytokine, may suppress SLC01A2 expression through a negative regulation element.

A previous study (Kullak-Ublick et al., 1997) demonstrated that constructs up to −1640 bp upstream of the SLC01A2 transcription start site exhibited substantial promoter activity. However, when a construct containing the putative NFκB binding site located within −1845 to −1836 bp was generated and expressed within MCF7 cells, a significant suppressive effect on transcription activity of SLC01A2 was observed, which implicated that the binding of NFκB 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 NFκB 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-NFκB than the p65 subunits. The binding between NFκB subunits with neg-NFκB 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 NFκB interact with the sequence as well. The binding of p50 and/or p65 subunits of NFκB to neg-NFκB 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 interaction is not cell-type specific. On the other hand, the location that is close to the DNA binding site and hence hinder the interaction is not cell-type specific. This speculation.

In summary, we identified a functional NFκB binding site located at −1845 to −1836 bp upstream of the transcription start site of SLC01A2. Such an element exerts a repressive effect on SLC01A2 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 NFκB. 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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