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Liver X Receptor alpha Mediated Genistein Induction of Human Dehydroepiandrosterone Sulfotransferase (hSULT2A1) in Hep G2 Cells

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Abstract

Cytosolic sulfotransferases are one of the major families of phase II drug metabolizing enzymes. Sulfotransferase-catalyzed sulfonation regulates hormone activities, metabolizes drugs, detoxifies xenobiotics, and bioactivates carcinogens. Human dehydroepiandrosterone sulfotransferase (hSULT2A1) plays important biological roles by sulfating endogenous hydroxysteroids and exogenous xenobiotics. Genistein, mainly existing in soy food products, is a naturally occurring phytoestrogen with both chemopreventive and chemotherapeutic potential. Our previous studies have shown that genistein significantly induces hSULT2A1 in Hep G2 and Caco-2 cells. In this study, we investigated the roles of liver X receptor (LXR α) in the genistein induction of hSULT2A1. LXRs have been shown to induce expression of mouse *Sult2a9* and hSULT2A1 gene. Our results demonstrate that LXR α mediates the genistein induction of hSULT2A1, supported by Western blot analysis results, hSULT2A1 promoter driven luciferase reporter gene assay results, and mRNA interference results. Chromatin immunoprecipitation (ChIP) assay results demonstrate that genistein increase the recruitment of hLXR α binding to the hSULT2A1 promoter. These results suggest that hLXR α plays an important role in the hSULT2A1 gene regulation. The biological functions of phytoestrogens may partially relate to their induction activity toward hydroxysteroid SULT.

Keywords

Sulfotransferase; genistein; liver X receptor; SULT2A1; gene regulation

Introduction

Cytosolic sulfotransferases (SULTs) are one of the major super families of phase II drug-metabolizing enzymes. They catalyze the sulfonation (sulfuryl group transfer) of hydroxyl-

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containing compounds (Duffel *et al.*, 2001; Coughtrie, 2002; Gamage *et al.*, 2006; Suzuki *et al.*, 2011; Dong *et al.*, 2012). The co-substrate (sulfuryl group donor) for all SULTs is adenosine 3'-phosphate 5'-phosphosulfate (PAPS) (Klaassen and Boles, 1997). Sulfonation is an important reaction in the metabolism/detoxification of xenobiotics (Duffel *et al.*, 2001). Some SULT isoforms have a broad range of substrate specificities and catalyze the sulfation of many xenobiotics. Sulfonation of drugs and xenobiotic is mainly associated with detoxification: sulfonation of a relatively hydrophobic xenobiotic into a more water-soluble sulfuric ester that is readily excreted. However, there are numerous important exceptions wherein the formation of chemically reactive sulfuric esters is an essential step in the metabolic pathways leading to carcinogenic responses (Falany and Wilborn, 1994; Hengstler, 1998). Sulfonation is also widely observed in various biological processes. Bio-signaling molecules including hormones, neurotransmitters, bile acids, and peptides can be sulfated to alter their biological activities. Sulfonation usually leads to the inactivation of biological signaling molecules, as the sulfated forms are usually unable to bind to receptors (Coughtrie, 2002).

Dehydroepiandrosterone sulfotransferase (SULT2A1) shows substrate predilection for hydroxysteroids, bile acids and certain medicinal compounds. The enzyme is expressed at high abundance in the first-pass tissues (liver and intestine) and in the steroidogenic tissue of adrenal gland. Xenobiotic-mediated regulation of SULT2A1 expression is primarily through nuclear receptors (Runge-Morris and Kocarek, 2005). Multiple nuclear receptors including the pregnane X receptor (PXR), constitutive androstane receptor (CAR), vitamin D receptor (VDR), farnesol X receptor (FXR), liver X receptors (LXRs), and retinoid X receptor α (RXR α), were reported to be responsible for induction of SULT2A1 in cultured human cells and *in vivo* in the mouse liver (Song *et al.*, 2001; Sonoda *et al.*, 2002; Echchgadda *et al.*, 2004; Song *et al.*, 2006; Chen *et al.*, 2007a; Echchgadda *et al.*, 2007b; Seo *et al.*, 2007; Uppal *et al.*, 2007). Most of these receptors form heterodimers with RXR α before binding to the xenobiotic responsive elements (XREs) in the promoter regions of drug metabolizing enzyme genes.

LXR was initially isolated from a human liver cDNA library as an orphan receptor (Xiao *et al.*, 2010). There are two LXR isoforms in mammals, named LXR α (NR1H3) and LXR β (NR1H2). LXR α is highly expressed in the spleen, liver, adipose tissue intestine, kidney and lung, whereas LXR β is expressed in all tissues examined (Repa, 2002). Both LXR α and LXR β function as heterodimers with RXR α . The hLXRs/hRXR α heterodimers preferentially bind to LXR responsive element (LXRE) that contains two direct hexanucleotides repeats separated by four nucleotides (DR4) (Chawla, 2001). LXRs act as sterol hormone nuclear receptors and cholesterol sensor, protecting the cells from cholesterol overload by stimulating reverse cholesterol transport and activating its conversion to bile acids in the liver. Some studies have demonstrated that mouse Sult2a9, mouse Sult1e1 and human SULT2A1 can be induced by LXRs overexpression or synthetic LXRs agonist T0901317 treatment (Song, 2001; Gong *et al.*, 2007; Uppal *et al.*, 2007). The activation of Sult2a9/SULT2A1 by LXRs was associated with increased bile acid detoxification and alleviation of cholestasis.

Genistein (GEN), a natural isoflavonone found in soybean products, has been reported to have both chemo preventive and chemotherapeutic potential against estrogen-responsive diseases, including inhibition of tumor cell growth (Mitchell *et al.*, 2000; Kousidou *et al.*, 2006). In recent years, isoflavones have been reported to induce phase I and phase II drug-metabolizing enzymes and to interact with nuclear receptors, such as those that are well known to mediate the induction of drug-metabolizing enzymes. Our previous studies have shown that GEN significantly induces human simple phenol sulfotransferase (hSULT1A1) and hSULT2A1 in human Hep G2 cells and Caco-2 cells (Chen *et al.*, 2008). Because

SULT1A1 and SULT2A1 are the major SULTs responsible for drug metabolism, Hep G2 cells have been commonly used for SULT1A1 and SULT2A1 studies (Shwed *et al.*, 1992; Otterness *et al.*, 1995; Wilson *et al.*, 1997; Song *et al.*, 1998; Galijatovic *et al.*, 1999; Chen *et al.*, 2005; Chen *et al.*, 2006; Huang *et al.*, 2006; Chen *et al.*, 2008; Huang *et al.*, 2011).

To the best of our knowledge, the molecular mechanism involving in the GEN induction of hSULT2A1 has not been reported. In this study, the experimental focus is to elucidate the roles of hLXR α in GEN induction of hSULT2A1 in Hep G2 cells. Our results suggest for the first time that hLXR α mediates the GEN induction of hSULT2A1. The ChIP assay results demonstrate that GEN increases the recruitment of LXR α to the SULT2A1 promoter. Phytoestrogens induction of hydroxysteroid SULT may contribute to their biological functions.

Materials and Methods

Materials

Genistein, 9-cis-Retinoic acid, LXR agonist T0901317, Hep G2 cell culture medium (Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham) and 0.25% trypsin-EDTA were purchased from Sigma-Aldrich (St. Louis, MO). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reagents and protein assay reagent were obtained from Bio-Rad (Hercules, CA). Western blot chemiluminescence reagent kits (Super Signal West Pico Stable Peroxide and Super Signal West Pico Luminol/Enhancer solutions) were purchased from Pierce Chemical (Rockford, IL). Nitrocellulose membranes (Immobilon-P; Millipore Corporation, Bedford, MA) used for Western blots were purchased from Fisher Scientific (Fair Lawn, NJ). Total RNA extraction Kit (TRIZOL reagent) was purchased from Molecular Research Center, Inc. (Cincinnati, OH). Real-time PCR Kits (qPCR MasterMix Plus for SYBR Green I dNTP) were from EUROGENTEC (San Diego, CA). Rabbit primary antibody against human DHEA sulfotransferase (hSULT2A1) was a gift from Dr. David Ringer (American Cancer Society). RQI DNase, DNA restriction enzymes, and Wizard[®] SV genomic DNA purification system were from Promega (Madison, WI). SuperScript[™]III Reverse Transcriptase and Lipofectamine[™] 2000 were from Invitrogen (Carlsbad, CA). Characterized fetal bovine serum (FBS) was provided by HyClone (Logan, UT). The plasmid extraction kit was from QIAGEN (Valencia, CA) and a DNA gel purification kit was purchased from Q-Biogene (Carlsbad, CA). hLXR α expression plasmids were kind gifts from Dr. David J. Mangeldorf (University of Texas Southwestern Medical Center, Dallas, TX). hRXR α and pCMX expression plasmids were obtained from Dr. Ronald M. Evans (Howard Hughes Medical Institute, La Jolla, CA).

Cell culture and drug treatment

The Hep G2 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA). Hep G2 cells were grown and maintained in Dulbecco's Modified Eagles's Medium / Nutrient Mixture F-12 Ham (DMEM/F-12) (Sigma) supplemented with 10% fetal bovine serum (FBS). For induction studies, Hep G2 cells were seeded in 100 × 20 mm dishes at a density of 1.0×10^7 cells per dish on day 0. After 24 hours, GEN or T0901317 (dissolved in sterile DMSO) were added to the medium. The same volume of sterile DMSO (less than 0.1% V/V) was added to the controls. Medium was replaced every 2 days and replenished with freshly dissolved GEN or T0901317. After treatment for required time, the cells were harvested. Total RNA was extracted from cells with TRIZOL reagent and subjected to real-time RT-PCR. Cell cytosols were prepared for Western blot analyses.

Cytosol preparation

Hep G2 cells were harvested from their culture dishes using 0.25% trypsin-EDTA solution (Sigma), washed with phosphate-buffered saline, and then homogenized in 1 ml of cell lysis buffer (3 mM 2-mercaptoethanol; 0.1 mM phenylmethylsulfonyl fluoride; 20 mM Tris, pH 7.4; 160 mM NaCl; 0.03% Tween-20; and 0.1 mM EDTA). The homogenate was then centrifuged at $12,000 \times g$ for 30 min, and the supernatant was used in Western blot studies.

Western Blot

The specific Western blot procedure has been described previously (Maiti and Chen, 2003; Maiti *et al.*, 2005). Rabbit anti-hSULT2A1 primary antibody was a gift from Dr. David Ringer (American Cancer Society).

Quantitative real-time RT-PCR

Total RNA was prepared from treated Hep G2 cells using TRIZOL reagent according to the manufacturer's protocol. RNA samples were incubated with RQ1 DNase at 37°C for 30 min and then inactivated at 65°C for 10 min. Superscriptase II (Invitrogen) reverse transcriptase with 100 ng of total RNA was used to synthesize the first strand cDNA, and 1 µl of reverse-transcribed product served as the template in polymerase chain reactions. Real-time PCR was performed using qPCR MasterMix Plus for SYBR Green I dNTP Kit (EUROGENTEC) following the manufacturer's instructions. Primers used are as follows: ACTBF321: 5'-AGAAAATCTGGCACCACACC-3'; ACTBR462: 5'-GGGGTGTGTAAGGTCTCAAA-3', GI, L5016088; hSULT2A1F163: 5'-TGAGTTCGTGATAAGGG ATGAA-3'; hSULT2A1R294: 5'-CAGATGGGCAGATTGGAT-3', GI, L29540544; hLXRα F: 5'-AAGCCCTGCATGCCTACGT-3'; hLXRα R: 5'-TGCAGACGCAGTGCAAACA-3', GI, 10062.

Real-time PCR was performed using an ABI PRISM 7500 Fast System (Applied Biosystems). Initially, regular PCR product DNA was purified with GENECLEAN Turbo (Q-Biogene, Carlsbad, CA) for constructing standard curves (10^3 - 10^8 copies gene). A standard curve was plotted with the threshold cycle (C_T) vs. the logarithmic value of the gene copy number. The gene copy number of unknown samples was generated directly from the standard curve by Sequence Detector (ver. 1.7) software (Applied Biosystems). At least two duplications were run for each experiment and each experiment was repeated at least three times. All gene copy numbers were normalized to human β-actin mRNA.

hSULT2A1 promoter reporter construction

Luciferase reporter constructs were used in the transfection studies. Primer design for the hSULT2A1 promoter sequence was based on previously published papers (Otterness DM, 1995; Duanmu Z, 2002; Chen *et al.*, 2006; Chen *et al.*, 2007b). Briefly, a fragment containing the 5-flanking region (from -1463 to +48) of hSULT2A1 was generated by PCR using genomic DNA extracted from Hep G2 cells. The fragment was inserted into the luciferase reporter vector pGL3-Basic (Promega, Madison, WI) at the *MluI* and *XhoI* sites to drive the promoterless firefly luciferase gene. DNA sequencing at the Oklahoma State University core facility verified the construct.

Transfections and reporter gene assays in Hep G2 cells

Hep G2 cells at 5×10^5 /well were seeded onto a 24-well plate and transfected after 16 hours with 100 ng of reporter plasmid, 50 ng of nuclear receptor expression vector and 10 ng of pRL-TK plasmid (Promega, Madison, WI) with 5% charcoal stripped FBS. The transfection agents contained 98 µl of Opti-MEM and 1 µl of LipofectamineTM2000 (Invitrogen,

Carlsbad, CA). The pRL-TK plasmid, which expresses *Renilla* luciferase, was used as an internal standard for transfection. The pCMX vector DNA was used as an empty vector to keep the total transfected DNA at a fixed value of 210 ng. Agonists were added 6 hours after transfection with a final concentration of 5.0 μ M GEN, 0.05 μ M T0901317, 1 μ M 9-cis-retinoid acid or 0.1% (V/V) DMSO. Culture medium supplemented with drug was replaced 12 hours post-transfection to remove the Lipofectamine™ 2000. Cells were collected 48 hours after transfection and firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI). Each experiment was repeated three times with each performed in duplicate. Results are given as means $\pm$ S.D.

hLXR RNA interference in Hep G2 cells

The small interfering RNA (siRNA) transfection was performed using Lipofectamine™2000 as previously described (Chen *et al.*, 2007b). The siRNA targeting hLXR α (NR1H3, siRNA ID: s19569), the siRNA targeting hLXR β (NR1H2, siRNA ID: 14684) and the siRNA negative control (Cat# AM4611) were provided by Ambion (Austin, TX). The sequences of siRNAs are: hLXR α sense 5'-CGACUGAUGUCCACGGAtt-3', antisense 5'-UCCGUGGGAACAUCAGUCGgt-3'; hLXR β sense 5'-GAACAGAUCCGGAAGAAGAtt-3', antisense 5'-UCUUCUCCGGAUCUGUUCtt-3'. Briefly, the transfection agents containing 300 μ l of Opti-MEM and 3 μ l of Lipofectamine™2000 were added to Hep G2 cells according to the manufacturer's instructions in 6-well plates containing 125 nM siRNA per well. Negative control siRNA was used in control experiments to exclude the possibility of cytotoxicity caused by siRNA transfection. Cells were transfected for 6 hours before being replaced with medium containing 10% FBS. Forty eight hours after transfection, Hep G2 cells were harvested for real-time RT-PCR analysis. For dual luciferase assay, plasmid DNA was first transfected to Hep G2 cells and siRNA was transfected 6 hours later with refreshed 5% charcoal stripped FBS medium. Cells were collected 48 hours after transfection and firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI).

Chromatin immunoprecipitation (ChIP) assay

Formaldehyde cross-linking and chromatin immunoprecipitation assays using Hep G2 cells were performed as described by (Boyd, 1999) and Y. Shang et al. (Yongfeng Shang, 2000) with some modifications.

Briefly, Hep G2 cells were grown to 95% confluence in DMEM/F12 medium supplemented with 10% FBS for 24 hours. Hep G2 cells were transfected with LXR α expression plasmid or pCMX vector plasmid for 6 hours, then changed fresh DMEM/F12 medium and treated with 5 μ M GEN or 0.1% (V/V) DMSO. Following the addition of GEN for 48 hours, cells were washed twice with PBS and cross-linked with 1% formaldehyde at room temperature for 10 min. Cell cross-linking was stopped by adding glycine to a final concentration of 125 mM. Cells were then rinsed with ice-cold PBS twice and scraped into PBS buffer plus 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and collected by centrifugation for 4 min at 700 g. Cell pellets were resuspended in 3 ml of swelling buffer (5 mM Pipes [piperazine-N,N'-bis(2-ethanesulfonic acid), pH 8.0], 85 mM KCl, 0.5% NP-40, 0.5mM PMSF, and 100 ng of leupeptin and aprotinin per ml) and incubated on ice for 20 min. Nuclei were collected by microcentrifugation at 6,000 g for 5 min, resuspended in 1–2 ml of sonication buffer (1% sodium dodecyl sulfate[SDS], 10 mM EDTA, 50 mM Tris-HCl (pH8.1), 0.5 mM PMSF, and 100 ng of leupeptin and aprotinin per ml), and incubated on ice for 10 min. Samples were sonicated with an Ultrasonics sonicator (Branson Sonifier 250, Branson, Danbury, CT) at the highest power for six 30-s pulses on ice to an average length of 300 to 600 bp and then microcentrifuged at 16,000 g for 10 min. The supernatant was collected and diluted in ChIP

dilution buffer (0.01% SDS, 1% Triton X-100, 2mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) followed by immunoclearing with 1 µg sheared salmon sperm DNA, 5 µg of bovine serum albumin and protein A/G plus Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA) slurry for 2 hours at 4°C. Precleared chromatin was incubated with 5 µg of rabbit anti-hLXRα polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; sc-13068) or no antibody and rotated at 4°C for 12 hours. After immunoprecipitation, 50 µl protein A/G plus Sepharose and 1 µg of salmon sperm DNA were added, and the incubation was continued for another 1 hour. Precipitates were washed sequentially for 10 min each in low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2m M EDTA, 20 mM Tris-HCl, pH8.1, 500 mM NaCl), and LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH8.1). Precipitates were then washed three times with TE buffer and extracted three times with 1% SDS, 0.1 M NaHCO₃. Elutes were pooled and heated at least 6 hours to reverse the formaldehyde cross-linking. DNA fragments were purified with a GENECLAN Turbo Kit (Q-Biogene, Carlsbad, CA) and analyzed by PCR. The hSULT2A1 gene LXR responsive region was amplified with a primer set at -354 (5'-ATGCACGATTGCAGGATTATTAG-3'); and -55 (5'-GCATGTCACATGTTTGTG-3') as described by Echchgadda *et al.* (Echchgadda *et al.*, 2007b). For PCR, 2 µl from 30 µl DNA extraction and 35 cycles of amplification were used.

Statistical analysis

One-way ANOVA followed by the Dunnett's test was performed to evaluate statistical significance with the difference between means of control and treated Hep G2 cells. Data presented in the figures are means ± SD (standard deviation) of the data collected separately from at least three independent experiments. In all cases, $p < 0.05$ was considered significant, $p < 0.01$ was considered very significant.

Results

hLXRα agonist T0901317 induction of hSULT2A1 expression in Hep G2 cells

Our published results indicated that GEN induced hSULT2A1 in Hep G2 cells (Chen *et al.*, 2008). In this work, we investigated the roles of hLXRα in the induction of hSULT2A1 by GEN in Hep G2 cells. The synthetic agonist of hLXRα, T0901317, significantly induced hSULT2A1 protein expression in Hep G2 cells (Figure 1). This induction is also concentration-dependent. Results showed in Figure 1 suggest that human LXRα may mediate hSULT2A1 transcription in Hep G2 cells.

hLXRα and hRXRα transactivation of genistein induction of hSULT2A1 in Hep G2 cells

It is known that most nuclear receptors, including hLXRs, transactivate gene expression via forming heterodimers with RXRα. Hep G2 cells were co-transfected with hLXRα (1 µg) and hRXRα (1 µg) expression vectors for 6 hours, and then treated with 5.0 µM GEN for 48 hours (Chen *et al.*, 2007b; Chen *et al.*, 2008). The induction of hSULT2A1 protein was evaluated by Western blot. Results showed in Figure 2 suggest that co-transfection of hLXRα and hRXRα expression vectors into Hep G2 cells significantly increased hSULT2A1 induction activity by GEN. The overexpression of hLXRα and hRXRα genes activated GEN induction of hSULT2A1 protein. These results suggest that hLXRα and hRXRα are involved in the GEN induction of hSULT2A1 expression in Hep G2 cells. This supports the hypothesis that hLXRα forms heterodimer with hRXRα and transactivates hSULT2A1 expression induced by GEN.

To further explore the molecular mechanism of hSULT2A1 regulation, the 1.5-Kb fragment of hSULT2A1 (-1463 to +48) was generated by PCR with primer pairs using genomic DNA

extracted from Hep G2 cells. Our sequencing result of the PCR product was the same as the published sequence (**L36191** and **U13065**) (Otterness DM, 1995). The generated promoter sequence was inserted into pGL-3 basic vector (Promega) at the *MluI* and *XhoI* sites, and the reporter vector was used for transfection into Hep G2 cells. Reporter gene assay was used to evaluate the roles of hLXR α in the GEN induction of hSULT2A1 promoter-regulated reporter gene expression in Hep G2 cells.

The reporter gene assay results demonstrated that overexpression of hLXR α significantly increased GEN induction activity (Figure 3). When RXR α was co-transfected with hLXR α , GEN induction activity was further increased (Figure 3). These results further demonstrated the hypothesis that hLXR α forms heterodimer with hRXR α and mediates the induction of hSULT2A1 by GEN. hSULT2A1 promoter driven luciferase reporter gene assay results agreed well with hSULT2A1 Western blot assay results.

Effects of RNA interference of hLXR α and hLXR β on hSULT2A1 gene expression in Hep G2 cells

To further investigate the roles of hLXR α in hSULT2A1 gene regulation in Hep G2 cells, the RNA interference experiments were carried out. Negative control siRNA (Ambion Inc., ID: 4611) and hLXR α and hLXR β specific siRNAs (Ambion Inc., ID: s19569 and s14684) were used in the interfering experiments. Real-time RT-PCR was used to determine the mRNA copy number of hLXR α and hSULT2A1 in Hep G2 cells. As shown in Figure 4A, the amount of hLXR α mRNA in the control cells is low, when the hLXR α expression vector were transfected, the mRNA copy number of hLXR α dramatically increased ($P<0.01$). When the control and hLXR α transfected cells were co-transfected with hLXR α and hLXR β specific siRNAs, the mRNA copy numbers of hLXR α in the control cells and hLXR α transfected cells decreased about 50% and 80% respectively ($P<0.05$, $P<0.01$). Our real-time RT-PCR results shown in Figure 4B suggest that the expression levels of hSULT2A1 mRNA are closely related to the expression level of hLXR α mRNA. When the mRNA level of hLXRs was knocked down by the transfection of hLXR α and hLXR β specific siRNAs, the hSULT2A1 mRNA expression level were also significantly decreased ($P<0.01$). When the mRNA amount of hLXR α increased through the transfection of hLXR α expression vectors, the mRNA level of hSULT2A1 was also significantly increased ($P<0.01$). These results strongly support the hypothesis that hLXR α was involved in the transcriptional regulation of hSULT2A1.

Chromatin immunoprecipitation (ChIP) assay demonstrated that genistein increased the binding of hLXR α to hSULT2A1 promoter

Human SULT2A1 is relatively well studied for the gene regulation mechanisms compared to other SULTs. Human SULT2A1 promoter region contains two major hormone response element responsible for the binding of various nuclear receptors including IR2 (-187 5'-GCAAGCTCAGTTGACCCCTAAAAT-3' -164) and DR4 (-155 5'-GATAAGTTCATGATTGCTCAACATCTTCA-3' -127) (Chen *et al.*, 2006; Song *et al.*, 2006; Chen *et al.*, 2007b; Echchgadda *et al.*, 2007a; Huang *et al.*, 2011). To demonstrate the recruitment of hLXR α to the hSULT2A1 promoter hormone response elements and to verify the enhancement of GEN for this recruitment in Hep G2 cells, primer set -354 (5'-ATGCACGATTGCAGGATTATTTAG-3'); and -55 (5'-GCATGTCACATGTTTGTG-3') was used for ChIP assay. The primer set used is based on the report by Echchgadda *et al.* (Echchgadda *et al.*, 2007b). In this experiment, Hep G2 cells were transfected with pCMX (control) or hLXR α expression vector for 6 hours, and then cells were treated with DMSO (control) or 5 μ M GEN for 48 hours. Cells were then harvested, and ChIP assay was performed with the use of an anti-hLXR α antibody and the PCR primer set amplifying the anti-hLXR α immunoprecipitated hSULT2A1 chromatin

fragments. The ChIP assay results indicated that overexpression of hLXR α resulted in the recruitment of hLXR α to the hSULT2A1 promoter region (Figure 5). Addition of 5 μ M GEN further increased (activated) the binding of hLXR α to the hSULT2A1 promoter (Figure 5). These results suggest that GEN activates hLXR α binding to the hSULT2A1 promoter to transactivate hSULT2A1 expression.

Discussion

There is more and more evidence in favor of soybean diets. They have been reported to reduce incidence of different cancers, cardiovascular diseases, and osteoporoses. Some of the beneficial effects may come from soy isoflavones including genistein, daidzein, glycitein etc. Isoflavones belong to the family of phytoestrogens. Isoflavones came into science field almost one hundred years ago, although publication on isoflavones only tremendously increased during last decade or so based on PubMed searching results. In recent years, more and more reports demonstrated that isoflavones bind to different kinds of nuclear receptors including those that induce drug metabolizing enzymes such as peroxisome proliferator-activated receptors (PPARs) (Mezei *et al.*, 2003; Scatena *et al.*, 2004; Ricketts *et al.*, 2005), steroid hormone receptors (SHRs) (Jacobs and Lewis, 2002), and estrogen related receptors (ERRs) (Suetsugi *et al.*, 2003; Ricketts *et al.*, 2005). Isoflavones have been reported to induce cytochrome P450s (Mezei *et al.*, 2002; Ferraris *et al.*, 2005), although reports on isoflavones induction of drug metabolizing enzymes are very limited. Reports on gene regulation of SULTs by isoflavones are very limited. Genistein, a natural isoflavone found in soybean products, has been reported to have both chemopreventive and chemotherapeutic potential against estrogen-responsive diseases, including inhibition of tumor cell growth (Miodini, 1999; Setchell, 1999; Mitchell, 2000; Xiang, 2002), lowering of serum cholesterol, and prevention of bone loss in rodents (Kirk, 1998; Nakajima, 2001; Ishimi, 2002; Paik, 2003). Genistein is the most potent estrogenic compound in soy and soy products (Chen, 2007). In humans, genistein plasma or serum levels derived from ingesting soy foods range from 1 μ M to about 5 μ M (Maubach, 2003; Safford, 2003; Allred, 2004; Wiseman, 2004). To obtain relevant data from *in vitro* models, Klein and King (2007) recommended using a genistein concentration of 5 μ M as the upper limit for *in vitro* studies (Klein, 2007). This concentration represents the maximal physiological serum level (free form) of genistein achievable by diet or dietary supplementation.

Phase I and phase II drug metabolizing enzymes are well known to be regulated by endogenous hormones as well as by therapeutic drugs and xenobiotics. We previously reported that isoflavone GEN induces hSULT1A1 and hSULT2A1 in human Hep G2 and Caco-2 cells. The induction was time-dependent and dose-dependent (Chen *et al.*, 2008). However, the molecular mechanisms underlying the GEN induction of hSULT1A1 and hSULT2A1 in human Hep G2 cells remain to be determined.

In this study, Western blot analysis (for endogenous hSULT2A1), hSULT2A1 promoter driven luciferase reporter gene assay, RNA interference experiments, and chromatin immunoprecipitation (ChIP) assay were used to evaluate the roles of hLXR α in GEN induction of hSULT2A1. The typical hLXR agonist, T0901317, significantly induced hSULT2A1 gene expression (Figure 1), suggesting the involvement of hLXR. Results shown in Figure 2 and 3 demonstrate that hRXR α transfection activated GEN induction activity. When hRXR α was co-transfected together with hLXR α , hRXR α and hLXR α synergistically increased GEN induction of hSULT2A1, for both hSULT2A1 Western blot results and the luciferase reporter gene assay results. These results support the hypothesis that hLXR α transactivates the induction of hSULT2A1 by GEN via forming hLXR α /hRXR α heterodimer in Hep G2 cells. GEN induced hSULT2A1, which suggests that GEN may function as an agonist of hLXR α . The results of RNA interference experiments (Figure

4) further support our working hypothesis that the expression of hSULT2A1 was closely related to the expression level of hLXR α . When hLXR α expression vector were transfected into Hep G2 cells, both hLXR α and hSULT2A1 expression levels were significantly increased, which suggests that hLXR α can mediate the up-regulation of hSULT2A1. When the hLXR expression was knocked down by hLXR specific siRNAs, the mRNA level of hSULT2A1 was also significantly decreased. RNA interference experimental results agreed with endogenous induction of hSULT2A1 results and luciferase reporter gene assay results. The chromatin immunoprecipitation (ChIP) assay results indicated that overexpression of hLXR α resulted in the recruitment of hLXR α to the hSULT2A1 promoter (Figure 5). In addition, the treatment with GEN further increased the recruitment (Figure 5), suggesting that GEN may act as an agonist of hLXR α and transactivate hSULT2A1. This result is consistent with previous reports which showed that LXR/RXR heterodimers bind to DR4 (Chawla, 2001). Our results also agree with recent reports that LXRs agonist T0901317 induced SULT2A1 expression in human hepatocytes and LNCaP cells (Uppal *et al.*, 2007; Lee *et al.*, 2008).

Over expression of LXR increased hSULT2A1 protein expression (Western blot of hSULT2A1, Figure 2) and increased luciferase protein expression driven by hSULT2A1 promoter (luciferase activity, figure 3). The effect of the over expression of LXR on these protein expressions is similar. However, the effect of the over expression of LXR on hSULT2A1 mRNA level is much higher (figure 4). This is in agreement with our past experiences; the effect of an inducer on mRNA expression is usually much higher than the effect on the protein expression. The increased level of mRNA may not completely convert to protein expression. Some other factors may limit the expression of the protein from increased mRNA level.

Multiple nuclear receptors including the PXR, CAR, VDR, FXR, and RXR α were reported to be responsible for induction of SULT2A1 in cultured human cells and *in vivo* in the mouse liver (Song *et al.*, 2001; Sonoda *et al.*, 2002; Echchgadda *et al.*, 2004; Song *et al.*, 2006; Chen *et al.*, 2007a; Echchgadda *et al.*, 2007b; Seo *et al.*, 2007; Uppal *et al.*, 2007). LXR should crosstalk with different nuclear receptors for the regulation of SULT2A1. This crosstalk will make the regulation of SULT2A1 in various tissues different. Nuclear receptors such as AhR (Han *et al.*, 2006) and ER (Adams *et al.*, 2012) also interact with GEN ; however, their roles in SULT2A1 gene regulation is not known.

In summary, our results demonstrate that hLXR α play important roles in the induction of hSULT2A1 by GEN in Hep G2 cells. LXRs play important roles regulating various biological processes. GEN can act as an agonist of LXR. GEN may play important roles in hydroxysteroid regulation and controlling certain metabolic disorders. This may partially explain the important biological functions of phytoestrogens.

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Abbreviations

SULT sulfotransferase

LXR	Liver X receptor
GEN	genistein
DHEA	dehydroepiandrosterone
hSULT2A1	human dehydroepiandrosterone sulfotransferase (DHEA-ST)

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Highlights

- Liver X receptor α mediated genistein induction of hSULT2A1 in Hep G2 cells.
- LXRA and RXRA dimerization further activated this induction.
- Western blot results agreed well with luciferase reporter gene assay results.
- LXRs gene silencing significantly decreased hSULT2A1 expression.
- ChIP analysis suggested that genistein enhances hLXRA binding to the hSULT2A1 promoter.

Figure 1. Dose-dependent T0901317 induction of hSULT2A1 in Hep G2 cells

Hep G2 cells were grown in 10-cm plates and treated with different doses of hLXR agonist T0901317 (0, 0.05, 0.25, 1.0 μM) for 48 hours. Representative Western blot and densitometry analysis shows hSULT2A1 protein expression in Hep G2 cell cytosol. The histograms with standard deviation are average values from three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with control group.

Figure 2. hLXR α and hRXR α activation of hSULT2A1 expression in Hep G2 cells

Hep G2 cells were grown in 10-cm plates and transfected with pCMX, hLXR α and/or hRXR α expression plasmid DNA (1 μ g). After 6 hours of transfection, Hep G2 cells were treated with DMSO or 5.0 μ M GEN for 48 hours. Representative Western blot and densitometry analysis show hSULT2A1 protein expression in Hep G2 cell cytosol. The histograms with standard deviation are average values from three independent experiments. * P <0.05, ** P <0.01 compared with control group.

Figure 3. hLXR α and hRXR α transactivation of hSULT2A1 promoter driven luciferase reporter activity in Hep G2 cells

Hep G2 cells were transfected with luciferase reporter vector regulated by the hSULT2A1 promoter (from -1463 to +48), hLXR α , and/or hRXR α vectors as indicated in the figure. The transfected cells were treated with DMSO, 5 μ M GEN, and/or 1 μ M 9-cis-retinoid acid as indicated. Values represent averages from three independent experiments; each independent transfection was performed in duplicate. * P <0.05, ** P <0.01, **** P <0.001 compared with control group.

Figure 4. Effect of RNA interference of hLXR α and hLXR β on hSULT2A1 mRNA expression

Hep G2 cells were either not transfected (column 1 and 2 from left in A and B) or transfected with hLXR α (column 3 and 4 from left in A and B). hLXR α and hLXR β siRNAs were co-transfected in column 2 and 4 (in A and B). Cells were harvested after 48 hours and total RNA were purified and analyzed for real-time RT-PCR with gene specific primers for hLXR α (A) or hSULT2A1 (B) as described under Methods section. Relative RNA numbers were calculated using standard curve method. Each treatment was analyzed in triplicate and the data shown were average of three independent experiments. * was used when treatment was compared with negative control group. # was used when LXR+siRNA(LXR) group was compared with LXR group. *, $p < 0.05$; **, $p < 0.01$; ***/###, $p < 0.001$.

Figure 5. Recruitment of hLXR α onto the hSULT2A1 gene promoter revealed by ChIP analysis in Hep G2 cells

Hep G2 cells were grown to 95% confluence and then were transfected with LXR α expression plasmid or pCMX vector plasmid. After 6 hours, cells were treated with 5 μ M GEN or 0.1% (V/V) DMSO. After 48 hours incubation, collected cell pellets were used for the isolation of nuclei. ChIP assay was performed as described under Methods section. The hSULT2A1 gene LXR responsive region was amplified with a primer set at -354 (5'-ATGCACGATTGCAGGATTATTAG-3'); and -55 (5'-GCATGTCACATGTTTGTG-3') as described by Echchgadda *et al.* (Echchgadda *et al.*, 2007b).