0026-895X/06/6905-1554–1563$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 69:1554–1563, 2006

Vol. 69, No. 5
14571/3105381
Printed in U.S.A.

trans-Stilbene Oxide Induces Expression of Genes Involved in Metabolism and Transport in Mouse Liver via CAR and Nrf2 Transcription Factors

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Received June 10, 2005; accepted January 6, 2006

ABSTRACT

trans-Stilbene oxide (TSO) induces drug metabolizing enzymes in rat and mouse liver. TSO is considered a phenobarbital-like compound because it induces Cyp2B mRNA expression in liver. Phenobarbital increases Cyp2B expression in liver via activation of the constitutive androstane receptor (CAR). The purpose of this study was to determine whether TSO induces gene expression in mouse liver via CAR activation. TSO increased CAR nuclear localization in mouse liver, activated the human Cyp2B6 promoter in liver in vivo, and activated a reporter plasmid that contains five nuclear receptor 1 (NR1) binding sites in HepG2 cells. TSO administration increased expression of Cyp2b10, NAD(P)H:quinone oxidoreductase (Nqo1), epoxide hydrolase, heme oxygenase-1, UDP-glucuronosyltransferase (Ugt) 1a6 and 2b5, and multidrug resistance-associated proteins (Mrp) 2 and 3 mRNA in livers from male mice. Cyp2b10 and epoxide hydrolase induction by TSO was decreased in livers from CAR-null mice, compared with wild-type mice, suggesting CAR involvement. In contrast, TSO administration induced Nqo1 and Mrp3 mRNA expression equally in livers from wild-type and CAR-null mice, suggesting that TSO induces expression of some genes through a mechanism independent of CAR. TSO increased nuclear staining of the transcription factor Nrf2 in liver, and activated an antioxidant/electrophile response element luciferase reporter construct that was transfected into HepG2 cells. In summary, in mice, TSO increases Cyp2b10 and epoxide hydrolase expression in mice via CAR, and potentially induces Nqo1 and Mrp3 expression via Nrf2. Moreover, our data demonstrate that a single compound can activate both CAR and Nrf2 transcription factors in liver.

trans-Stilbene oxide (TSO) is a synthetic proestrogen that belongs to the class of compounds called stilbenes. TSO is commonly used to induce liver drug-metabolizing enzymes and is considered to be a phenobarbital-like compound, because, like phenobarbital, it markedly increases *Cyp2B1/2* expression in rat liver. In rats, exposure to TSO also results in induction of several phase I and phase II drug-metabolizing enzymes, such as Cyp3A1, epoxide hydrolase (Eh), NAD(P)H:quinone oxidoreductase 1 (Nqo1), glutathione-*S*-transferases (Gsts), UDP-glucuronosyl transferase (Ugt), as well as heme-oxygenase (Ho-1) in liver (Kuo et al., 1981; Williams et al., 1984; Goon and Klaassen, 1992; Oguro et al., 1997; Schilter et al., 2000). It has recently been demonstrated that TSO also increases the expression of the multidrug resistance-associated protein (Mrp) 2 and 3, at both mRNA and protein levels in rat liver (Slitt et al., 2003).

The mechanism by which phenobarbital induces *Cyp2B* gene expression has been well described (for review, see

This work was supported by National Institutes of Health grants ES11239, ES09649, and ES09716.

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Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.014571.

ABBREVIATIONS: TSO, *trans*-stilbene oxide; Gst, glutathione-*S*-transferase; Ugt, UDP-glucuronosyl transferase; Eh, epoxide hydrolase; Ho-1, heme oxygenase-1; Mrp, multidrug resistance-associated protein; CAR, constitutive androstane receptor; RXR, retinoid X receptor; PBREM, phenobarbital response element module; NR1, nuclear receptor 1; Nrf2, nuclear factor E2-related factor 2; ARE/EpRE, antioxidant response element/electrophile response element; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline with 0.2% Triton X-100; FITC, fluorescein isothiocyanate; SV40, simian virus 40; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; RLU, relative light units; bDNA, branched DNA signal amplification.

Sueyoshi and Negishi, 2001) and is known to be dependent on the activation and nuclear translocation of the constitutive androstane receptor (CAR) (Wei et al., 2000). The specific mechanism by which phenobarbital activates CAR is not well understood; however, some evidence indicates that phosphorylation-dependent pathways are involved (Kawamoto et al., 1999; Rencurel et al., 2004). After activation and nuclear translocation, CAR heterodimerizes with retinoid X receptor α (RXRα) and binds to the 51-bp phenobarbital response element module (PBREM), subsequently enhancing transcription of *Cyp2B1* (rat) and *Cyp2b10* (mouse) genes. The PBREM consists of two CAR binding sequences, nuclear receptor (NR) 1 and NR2 binding sites. CAR more effectively binds to and activates the NR1 sequence than the NR2 site. Decreased levels or absence of hepatic CAR, as described for female Wistar Kyoto rats and CAR-null mice, respectively, results in the lack of Cyp2B induction by phenobarbital and TSO (Wei et al., 2000; Yoshinari et al., 2001; Cherrington et al., 2003). Furthermore, phase II drug metabolizing enzymes, such as glutathione *S*-transferase A1 and sulfotransferase 2a1, as well as organic anion transporters, such as organic anion transporting polypeptide 2, Mrp2, and Mrp4 are induced in a CAR-dependent manner (Maglich et al., 2002; Assem et al., 2004). Moreover, studies with CAR-null mice have revealed that Mrp3 is induced in liver in a CAR-independent manner, although it is also inducible by compounds considered to be phenobarbital-like microsomal enzyme inducers (Xiong et al., 2002; Cherrington et al., 2003).

An intriguing observation is that some genes that are induced after phenobarbital administration, such as *Nqo1*, *Ho-1*, *Eh*, *Ugt1a6*, and *Gsta1*, are also induced by compounds such as oltipraz, which regulates gene expression through nuclear factor E2-related factor2 (Nrf2) (Ramos-Gomez et al., 2001). Nrf2 translocates from the cytosol to the nucleus, where it binds with other coactivators to an antioxidant/electrophile response element (ARE/EpRE) within the 5′-flanking region of certain genes, such as Nqo1, and activates gene transcription. This suggests that some compounds that activate CAR may also activate Nrf2. CAR and Nrf2 may share similar mechanisms for activation, or CAR and Nrf2 may bind to similar DNA response elements.

Because both TSO and phenobarbital induce many of the same drug metabolism and transport genes in liver, the goal of this study was to determine whether TSO activates CAR in mouse liver and whether it induces expression of genes for drug metabolism and disposition in a CAR-dependent manner. The aims were to determine: 1) the time course for induction of Cyp2b10, Cyp3a11, Nqo1, Eh, Ho-1, Ugt1a1, Ugt1a6, Ugt2b5, and Mrp1–4 in mouse liver after treatment with TSO, 2) whether TSO treatment results in increased localization of CAR in liver nuclei, 3) whether TSO activates response elements that bind CAR in vitro and in vivo, and 4) whether the basal and inducible expression of Cyp2b10, Nqo1, Eh, Ho-1, Ugt1a1, Ugt1a6, Ugt2b5, Mrp2, Mrp3, and Mrp4 is dependent on CAR, using CAR-null mice.

Materials and Methods

Materials. *trans*-Stilbene oxide was purchased from Aldrich (Milwaukee, WI). 5α-Androstan-3α-ol was purchased from Steraloids, Inc. (Newport, RI). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The mouse CAR expression plasmid and $(NR1)_5$-tk-luciferase plasmid were generously donated by Dr. Masa Negishi (National Institute of Environmental Health Sciences, Research Triangle Park, NC). The ARE/EpRE luciferase plasmid was generously donated by Dr. Timothy Dalton (University of Cincinnati, OH). HepG2 cells, Eagle's minimum essential medium, and fetal bovine serum were purchased from American Type Culture Collection (Manassas, VA).

Treatment of Animals. Adult male C57BL/6 mice weighing approximately 22 to 25 g were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were fed Harlan Teklad Rodent Diet Type W (Harlan Laboratories, Madison, WI) ad libitum. Mice were housed in a temperature-, light-, and humidity-controlled environment in cages with hardwood chips. Mice (n = 5/group) were treated with TSO in corn oil twice daily (200 mg/kg, 5 ml/kg i.p.). Livers were removed at 3 and 12 h after TSO administration as well as after 4 days of twice daily TSO administration, immediately frozen in liquid nitrogen, and stored at −80°C. Male C57BL/6 or CAR-null mice (Wei et al., 2000) (n = 3–4) were treated with TSO in corn oil twice daily (200 mg/kg, 5 ml/kg i.p.) for 4 days. For both mouse studies, the control mice received the same volume of corn oil vehicle as the treated animals. All mouse studies were conducted according to Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

Oligonucleotide Probe Sets for bDNA Analysis. Mouse Cyp2b10, Nqo1, Ho-1, Ugt1a1, Ugt1a6, Ugt2b5, and Mrp1–3 probes were used as described previously (Chen et al., 2003; Cherrington et al., 2003; Maher et al., 2005; Aleksunes et al., 2006). The probe set for mouse Eh is described in Table 1. These target sequences were analyzed by ProbeDesigner Software Version 1.0 (Bayer Corp., Emeryville, CA). All oligonucleotide probes were designed with a melting temperature of approximately 63°C. Probes developed in ProbeDesigner were submitted to the National Center for Biotechnology Information (NCBI) for nucleotide comparison by the basic linear alignment search tool (BLASTn) to ensure minimal cross-reactivity with other known mouse sequences and expressed sequence tags.

Branched DNA Assay. Oligonucleotide probes were diluted in lysis buffer supplied in the QuantiGene HV signal amplification kit (Panomics, Fremont, CA). All reagents for analysis (i.e., lysis buffer, capture hybridization buffer, amplifier/label probe buffer, and substrate solution) were supplied in the QuantiGene HV signal amplification kit. Total RNA (1 μg/μl, 10 μl) was added to each well of a 96-well plate containing 50 μl of capture hybridization buffer and 50 μl of a diluted probe set. Total RNA was allowed to hybridize to each probe set overnight at 53°C. Subsequent hybridization steps were carried out according to the manufacturer's protocol, and luminescence was measured with a Quantiplex 320 bDNA luminometer interfaced with Quantiplex data management software version 5.02 (Bayer Corp., Diagnostics Div., Tarrytown, NY).

Western Analysis of Nqo1, Mrp2–4 Expression in Mouse Liver. Western analysis was carried out according to the method reported by Aleksunes et al. (2005). Livers were homogenized in ST buffer (250 mM sucrose and 10 mM Tris-HCl, pH 7.4) containing 50 μg/ml aprotinin and centrifuged at 105,000g for 60 min at 4°C. The resulting supernatant contained the cytosolic fraction and the pellet contained the membrane fraction. Protein concentration was determined by the method of Bradford using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA). Proteins (50 μg of protein/lane) were electrophoretically resolved using polyacrylamide gels (8–10% resolving, 4% stacking) and transblotted overnight at 4°C onto PVDF-Plus membrane (Micron Separations, Westboro, MA). Immunochemical detection of Nqo1 protein (~30 kDa) was performed using purified anti-Nqo1 (Novus Biologicals, Littleton, CO). In brief, blots were blocked with 5% nonfat dry milk in TBS with 0.1% Tween for 2 h and incubated for 1 h with the primary antibody diluted in blocking buffer (1:1000). A peroxidase-labeled secondary antibody (Novus Biologicals) was diluted (1:10,000) in blocking buffer and incubated with blots for 1 h. Immunochemical

detection of Mrp2 (~190–200 kDa), Mrp3 (~180–190 kDa), and Mrp4 (~160–170 kDa) protein was performed using M_2III-5, M_3II-2, and M_4I-10 antibodies, respectively. Anti-Mrp antibodies were generously provided by Dr. George Scheffer (VU Medical Center, Amsterdam, The Netherlands). Membranes were blocked with 1% nonfat dry milk in PBS-Tween for 1 h and incubated for 1 h with the primary antibody diluted in blocking buffer (1:2000 for M_3II-2 and M_4I-10; 1:600 for M_2III-5). A species-appropriate peroxidase-labeled secondary antibody (Sigma Chemical Co., St. Louis, MO) was diluted (1:2000) in blocking buffer and incubated with blots for 1 h. Membranes were stripped and reprobed with a dilution of 1:2500 anti-β-actin rabbit polyclonal antibody (Abcam, Cambridge, MA) to confirm equal protein loading.

Protein-antibody complexes were detected using an ECL chemiluminescence kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and exposed to Fuji Medical X-ray film (Fisher Scientific, Springfield, NJ). The intensity of the protein bands was quantified using Quantity One Software (Bio-Rad Laboratories).

Immunohistochemical Staining. Sections of liver were frozen in liquid nitrogen and stored at −80°C until use. Cryosections (4–5 μm) were obtained using a Leica Jung Frigocut 2800N microtome, thaw-mounted onto Superfrost slides (Fisher Scientific, Pittsburgh, PA) and stored at −70°C in a bag with desiccant until use. All antibody solutions were filtered through 0.22-μm low protein binding Durapore (polyvinylidene difluoride) membrane syringe-driven filter units (Millipore Corp., Bedford, MA). For CAR and Nrf2 detection, sections were air-dried at room temperature for 30 min and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min. Sections were rinsed three times for five min with PBS and blocked at room temperature for 30 min with 5% goat serum/PBS with 0.2% Triton X-100 (PBS-T). The sections were then incubated with anti-CAR (M-150) or anti-Nrf2 (H-300) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), diluted 1:50 in 5% donkey serum/PBS-T, overnight at room temperature. Sections were washed three times for 10 min with PBS-T and incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-labeled secondary antibody to rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:200, and rhodamine-labeled phalloidin (Invitrogen, Carlsbad, CA) diluted 1:200 in 5% goat serum/PBS-T. Slides were washed in PBS three times for 10 min each and then rinsed twice in distilled deionized water. The sections were air dried and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Sections were visualized and analyzed using a Zeiss LSM 510 confocal microscope with LSM software ver. 2.8 (Carl Zeiss Inc., Thornwood, NY).

In Vivo Luciferase Assay Plasmids. The human *CYP2B6* promoter luciferase reporter construct was obtained from Dr. Richard Kim (Vanderbilt University School of Medicine, Nashville, TN). The *CYP2B6* promoter-reporter construct contains a 1.7-kilobase fragment that maintains the core promoter (+39/−364) and the distal enhancer region (−1461/−2013). Male C57BL/6 mice (20–25 g) were administered 1 μg of naked plasmid DNA in sterile saline by a rapid (5-s) tail vein injection in a volume equal to 10% of body weight. Twenty-four hours later, animals were anesthetized with a mixture of ketamine (72 mg/kg), acepromazine (6 mg/kg), and xylazine (6 mg/kg). Luciferin was administered to mice (70 μl of a 50 mg/ml stock solution, i.p.) 5 min before imaging. A VersArray 1300B camera from Roper Scientific (Tucson, AZ), thermoelectrically cooled to −100°C, was used to image the mice. A light-tight imaging chamber was used for all images. Images were acquired in gray-scale, and pseudocolor maps were created with the WinView 32 program (Roper Scientific). Color maps were superimposed over the light image of the mouse using Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA). Light images were acquired with lights mounted inside the box, with an exposure time of approximately 20 ms, using a fast setting for the analog-digital converter. Bioluminescent images were acquired with interior lights turned off, an exposure of 10 min, and with a slow setting on the analog-digital converter. All images were taken with an aperture of f/1.2. This image was considered time 0. Each animal (n = 3) was administered a single dose of either TSO (200 mg/kg i.p., 5 ml/kg) or corn oil (5 ml/kg). Images were taken at 24 and 48 h after TSO administration.

Transient Transfection Assays. Cultured HepG2 cells were transiently transfected with a mouse CAR expression plasmid (mCAR/PCR3) and a $(NR1)_5$-tk-luciferase construct according to a previously published method (Kawamoto et al., 2000). In brief, HepG2 cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum according to the protocol provided by the American Type Culture Collection. In 24-well plates, the $(NR1)_5$-tk-luciferase plasmid (0.1 μg) was cotransfected with pRL-SV40 (0.1 μg; Promega Corp., Madison, WI) into HepG2 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), with or without a mouse CAR expression plasmid (0.2 μg). Approximately 36 h after transfection, cells were cultured in the presence of TSO (25–100 μM) with or without 5α-androstan-3α-ol. To assess TSO activation of the ARE/EpRE, HepG2 cells were transiently transfected with 0.1 μg

TABLE 1

Oligonucleotide probes generated for analysis mouse microsomal epoxide hydrolase (mEH) expression by bDNA signal amplification

Gene *mEH*; GenBank accession number NM_010145. Function refers to the utility of the oligonucleotide probe in the bDNA assay.

Function	Probe Sequence
CE	cacggtattctgacttggtccaTTTTTctcttggaaagaaagt
CE	cttccagggagaacttcctctcTTTTTctcttggaaagaaagt
CE	tccttgtagaagcgctgggaTTTTTctcttggaaagaaagt
CE	tcagaagggaaggctgaatagcTTTTTctcttggaaagaaagt
CE	ttgggctcttcgaaggcagTTTTTctcttggaaagaaagt
LE	agtcattcagagcacagcccaTTTTTaggcataggacccgtgtct
LE	cagccaggcccacaggagTTTTTaggcataggacccgtgtct
LE	caggcctccatcctccagttTTTTTaggcataggacccgtgtct
LE	cagtagatcatgatgttagtcagcagatTTTTTaggcataggacccgtgtct
LE	ggagacaatggttcctgtcgtcTTTTTaggcataggacccgtgtct
LE	gacaccctggcccaagtttTTTTTaggcataggacccgtgtct
LE	tcccctcatgtctatggaccatTTTTTaggcataggacccgtgtct
LE	gggtacttgaccttcacccacttTTTTTaggcataggacccgtgtct
LE	ccatgtaggaataggagatgagtttgTTTTTaggcataggacccgtgtct
LE	ccagggacacgaacttgcgTTTTTaggcataggacccgtgtct
LE	gcgtcatcactgcagctcagTTTTTaggcataggacccgtgtct
BL	ggtggagaacttctctaagatgtagg
BL	cagtgggcacaaagaccttca
BL	ttctggggcatgcaggatc
BL	caaagtggcccccacgtt
BL	gatgtcctgggccagaagc

CE, capture extender; LE, label extender; BL, blocker probe.

of an ARE/EpRE luciferase reporter construct (Dieter et al., 2001) and 0.1 μg of pRL-SV40 (0.1 μg). After exposure to TSO for 24 h, the media was removed and cells were washed with PBS. The PBS was aspirated from the cells, and 100 μl of passive lysis buffer was added to each well. Luciferase activity was determined by the Dual-Glo Luciferase Assay (Promega Corp., Madison, WI).

Statistics. Statistical differences between vehicle- and TSO-treated groups at each time point (3 h, 12 h, and 4 days) were determined by a Student's *t* test. Statistical differences between WT and CAR-null, vehicle- and TSO-treated groups were determined by analysis of log-transformed data using a two-way ANOVA followed by a Duncan's multiple range post hoc test. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and TSO-treated groups, number signs (#) represent a statistical difference ($p < 0.05$) between WT and CAR-null control groups, and daggers (†) represent a statistical difference ($p < 0.05$) between WT and CAR-null mice treated with TSO.

Results

TSO Induction of Metabolism and Transport Genes in Livers from Male C57BL/6 Mice. Previous studies report that genes for drug metabolism and transport in rat and mouse liver are induced after exposure to TSO (Kuo et al., 1981; Thabrew and Emerole, 1983; Williams et al., 1984; Gregus et al., 1989; Goon and Klaassen, 1992; Oguro et al., 1997; Schilter et al., 2000). For induction of liver microsomal enzymes, TSO is usually administered once or twice a day for several days (Gregus et al., 1990; Schilter et al., 2000). In the present study, livers were collected at 3 and 12 h after a single dose of TSO and after 4 days of daily TSO administration. Previous studies have demonstrated that TSO induces Cyp2B1/2 mRNA and activity in rat liver (Schilter et al., 2000) and glutathione *S*-transferase activity in mouse liver (Meijer et al., 1984; Gregus et al., 1989). Figure 1 shows the mRNA levels of Cyp2b10 and Cyp3a11 in mouse liver at various times after administration of the TSO induction regimen. TSO induced Cyp2b10 mRNA expression in liver approximately 17-fold after 12 h and after 4 days of TSO administration. Phenobarbital and phenobarbital-like compounds often induce Cyp2b10 and Cyp3a11, although Cyp3a11 induction is more often associated with pregnane X receptor activation (Joannard et al., 2000; Hoen et al., 2001). In this study, TSO administration did not increase Cyp3a11 mRNA levels in mouse liver at any of the times examined. Cyp1a1 and Cyp4a14 mRNA levels in liver were also not increased at any time points after TSO treatment (data not shown).

Previous studies demonstrated that TSO increases the expression and/or activity of other liver metabolism enzymes, such as Nqo1, Eh, and Ho-1, in rat liver (Kuo et al., 1981; Williams et al., 1984; Oguro et al., 1997). Figure 2 illustrates that TSO administration also increases the mRNA levels of Nqo1, Eh, and Ho-1 in male mouse liver. Nqo1 and Eh mRNA levels were increased approximately 500% and 50% after 4 days of treatment with TSO, respectively, but were not increased at 3 or 12 h after TSO. By contrast, Ho-1 mRNA levels in liver increased 450% 3 h after TSO, but were not increased at 12 h or 4 days after TSO administration.

UDP-glucuronosyltransferases (Ugts) are phase-II enzymes that catalyze the conjugation of a glucuronic acid moiety to numerous endogenous and exogenous substrates.

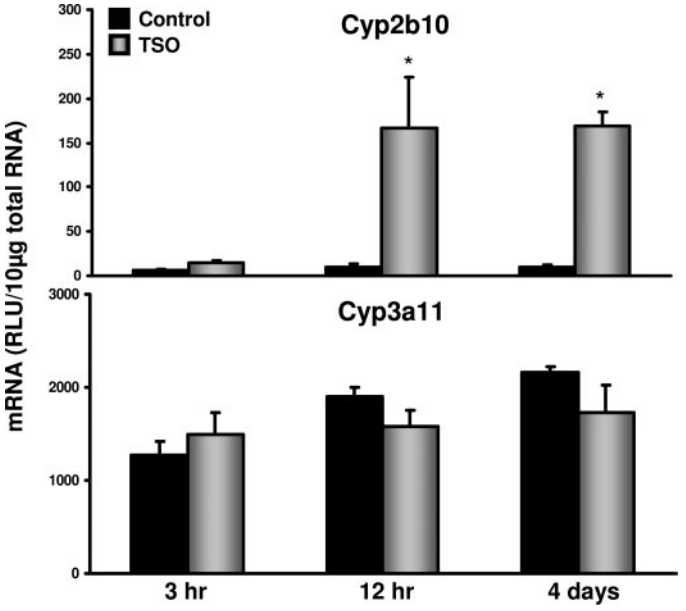


Fig. 1. Effect of TSO administration on Cyp2b10 and Cyp3a11 mRNA expression in mouse liver. Tissue total RNA was isolated from adult male C57BL/6 mice after 3 h, 12 h, or 4 days of TSO administration (200 mg/kg i.p., twice daily) and analyzed by the bDNA signal amplification assay for Cyp2b10 and Cyp3a11 mRNA. The data are presented as mean RLU ± S.E.M. (n = 4–5 animals).

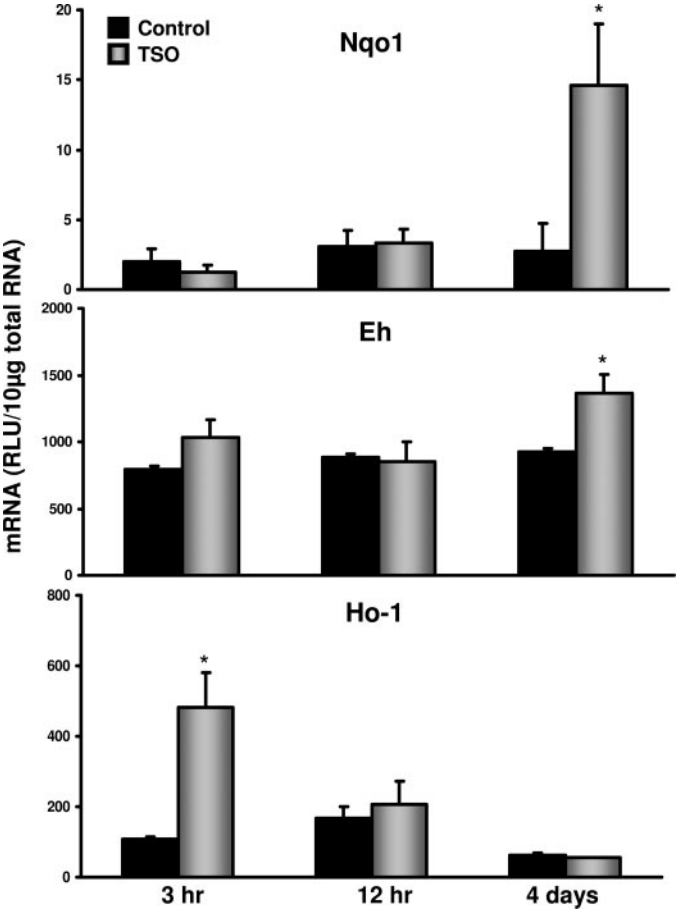


Fig. 2. Effects of TSO administration on Nqo1, Eh, and Ho-1 mRNA expression in mouse liver. Tissue total RNA was isolated from adult male C57BL/6 mice after 3 h, 12 h, or 4 days of TSO administration (200 mg/kg i.p., twice daily) and analyzed by the bDNA signal amplification assay for Nqo1, Eh, and Ho-1 mRNA content. The data are presented as mean RLU ± S.E.M. (n = 3–5 animals).

Some Ugts are induced in rat liver after TSO administration (Seidegard and DePierre, 1982). Figure 3 shows Ugt1a1, Ugt1a6, and Ugt2b5 mRNA levels in mouse liver after TSO administration. Ugt1a6 and Ugt2b5 mRNA levels were slightly increased in mouse liver after 4 days of TSO administration but not increased in liver at 3 or 12 h after TSO treatment. Ugt1a1 mRNA expression was not altered by TSO at any time point.

TSO has been shown to increase Mrp2 and Mrp3 mRNA and protein levels in rat liver (Slitt et al., 2003). Therefore, the effect of TSO on mRNA expression on Mrp1–4 was quantified in mouse liver (Fig. 4). Mrp1 mRNA expression in liver was not changed after TSO administration. Both Mrp2 and Mrp3 mRNA levels were unchanged at 3 or 12 h after TSO, but Mrp2 expression increased by 30% and Mrp3 mRNA expression increased approximately 75% after 4 days of TSO administration. Mrp4 expression was not significantly increased after TSO treatment, although there was a trend for it to increase at 4 days. TSO did not alter the expression of some other transporters in liver, namely organic anion transporting polypeptide 2 (Oatp2), Mrp6, multiple drug resistance protein 1a and 1b (data not shown).

Figure 5 illustrates induction of Nqo1, Mrp2–4 protein levels after 4 days of TSO administration by Western blot. TSO administration increased Nqo1 protein levels in liver cytosol by 140%. In membrane fractions, TSO increased Mrp2 levels 30%, Mrp3 levels by 80%, and Mrp4 levels by 280%.

Nuclear Localization of CAR in Liver after TSO Administration. TSO induced Cyp2b10, which suggested that TSO activates CAR. In naive liver, CAR is localized in the cytosol. Upon treatment with phenobarbital, active CAR translocates to the nucleus, where it heterodimerizes with RXRα, binds to the PBREM, and subsequently activates gene transcription (Swales and Negishi, 2004). Therefore, in the

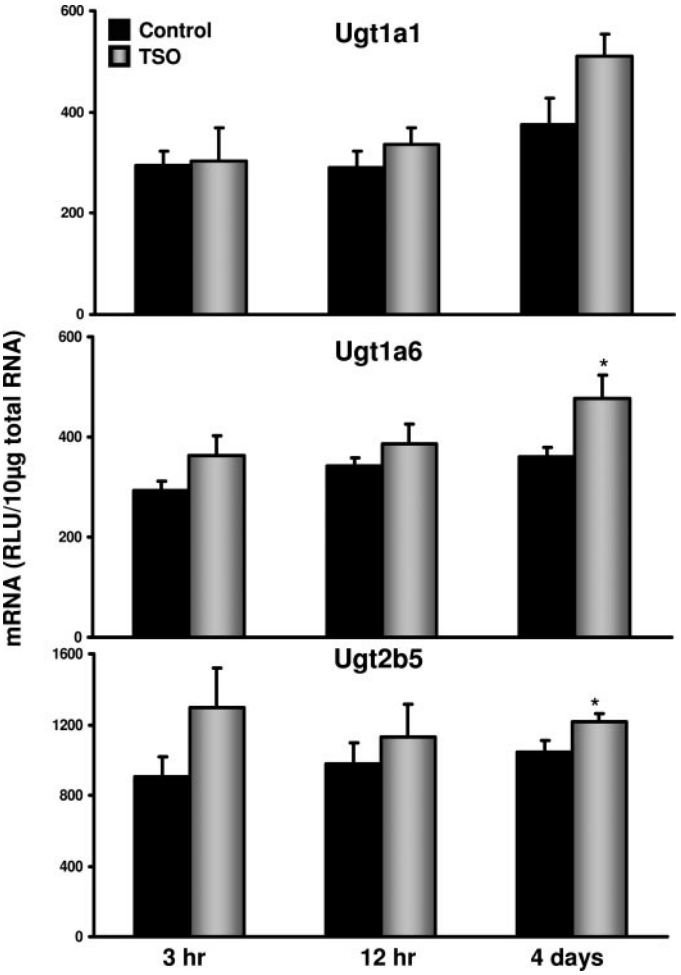


Fig. 3. Effects of TSO administration on Ugt1a1, Ugt1a6, and Ugt2b5 mRNA expression in mouse liver. Tissue total RNA was isolated from adult male C57BL/6 mice after 3 h, 12 h, or 4 days of TSO administration (200 mg/kg i.p., twice daily) and analyzed by the bDNA signal amplification assay for Ugt1a1, Ugt1a6, and Ugt2b5 mRNA content. The data are presented as mean RLU ± S.E.M. (n = 4–5 animals).

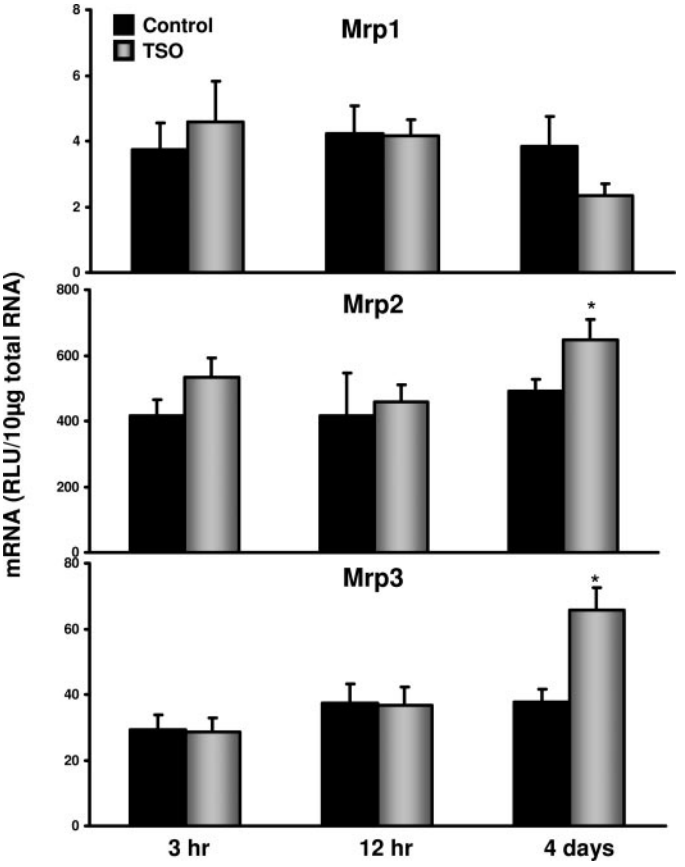


Fig. 4. Effects of TSO administration on Mrp2, Mrp3, and Mrp4 mRNA expression in mouse liver. Tissue total RNA was isolated from adult male rats after 3 h, 12 h, or 4 days of TSO administration (200 mg/kg i.p., twice daily) and analyzed by the bDNA signal amplification assay for Mrp1, Mrp2, Mrp3, and Mrp4 mRNA content. The data are presented as mean RLU ± S.E.M. (n = 4–5 animals).

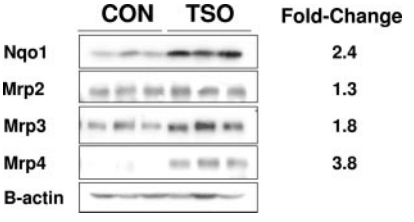


Fig. 5. Effect of TSO pretreatment on NAD(P)H:quinone oxidoreductase (Nqo1), Mrp1, Mrp2, Mrp3, and Mrp4 protein levels in mouse liver. Western blots of cytosol (Nqo1) or crude membrane fractions (Mrp2–4) isolated from livers of male C57BL/6 mice treated with vehicle (CON) or TSO for 4 days were stained with antibodies that detect mouse Nqo1, Mrp2, Mrp3, Mrp4, or β-actin (50 μg of protein/lane, n = 3 mice/treatment).

present study, liver sections were immunostained with anti-CAR antibodies to identify CAR localization and to determine whether TSO activates CAR before increasing gene expression. Figure 6 illustrates CAR localization in liver from vehicle- and TSO-treated mice at 3 h. Faint CAR staining was detected in cytosol and nuclei in liver from vehicle-treated mice, whereas staining of CAR increased in hepatocyte nuclei 3 h after TSO administration.

TSO Activation of CAR in Vitro and in Vivo. Staining of CAR increased in nuclei in hepatocytes after TSO administration, suggesting that TSO increases gene expression by inducing translocation of CAR into the nucleus and activating phenobarbital responsive elements. Therefore, in vivo and in vitro activation assays were employed to determine whether TSO administration activates a promoter construct that contains a PBREM, and whether TSO activates the NR1 CAR binding site contained in the PBREM. Figure 7 illustrates in vivo activation of the human Cyp2B6 promoter transfected into livers from mice administered vehicle or TSO. By 24 h, luciferase activity was increased in livers from TSO-treated compared with vehicle-treated mice. This demonstrates that TSO activates the human Cyp2B6 promoter in liver. At 48 h, the luciferase activity returned to control values.

Figure 8 illustrates in vitro TSO activation of a luciferase construct containing five copies of the NR1/DR4 CAR binding element $(NR1)_5$ with or without mouse CAR. In cells not transfected with the mouse CAR-expression plasmid, there was no activation of the $(NR1)_5$ reporter construct after TSO treatment. As reported previously (Kawamoto et al., 2000), transfection of HepG2 cells with mouse CAR results in activation of the $(NR1)_5$ reporter construct in HepG2 cells cultured in the presence of dimethyl sulfoxide, and the activity was repressed in the presence of 4 mM 5α-androstan-3α-ol (androstenol). As expected, the potent CAR agonist 1,4-bis[2-(3,5- dichloropyridyloxy)]benzene (TCPOBOP), activated CAR and the $(NR1)_5$ reporter construct in the presence of 5α-androstan-3α-ol. TSO also abrogated the 5α-androstan-3α-ol induced-repression of mouse CAR, and activated the $(NR1)_5$ reporter construct in HepG2 cells in a concentration-dependent manner. TSO did not activate pGL3 vector (data not shown).

TSO Induction of Metabolism and Transport Genes in Livers from Wild-Type and CAR-Null Mice. Data from Figs. 1 to 8 strongly suggest that TSO activates CAR and CAR-responsive elements. Phenobarbital induction of Cyp2b10 in liver is robust in mice that express CAR but is diminished in livers from CAR-null mice (Wei et al., 2000). Therefore, in Table 2, CAR-null mice were used to determine whether TSO induced *Cyp2b10*, *Ugt1a1*, *Ugt1a6*, *Ugt2b5*, *Mrp2*, *Mrp3*, *Nqo1*, *Eh*, and *Ho-1* in liver via a CAR-dependent mechanism gene expression. Because most genes were induced after 4 days of TSO administration, this time point was chosen to examine induction in wild-type and CAR-null mice.

Table 2 illustrates Cyp2b10 mRNA expression in livers from wild-type and CAR-null mice after 4 days of TSO administration. The constitutive expression of Cyp2b10 mRNA was lower in livers of CAR-null than in wild-type mice (0.96 ± 0.2 RLU versus 14.5 ± 1.5 RLU, respectively). TSO administration markedly induced Cyp2b10 mRNA expression by 30-fold in livers from wild-type mice, and this induction was significantly abated to only 8-fold in livers from CAR-null mice.

Ugt1a1, Ugt1a6, and Ugt2b5 expression in livers from wild-type and CAR-null mice was also examined after TSO administration (Table 2). Ugt1a1 expression was lower in livers from CAR-null mice than in wild-type mice (approximately 50%). TSO did not significantly induce Ugt1a1 mRNA expression at any time after TSO administration. Ugt1a6 expression was approximately 50% lower in livers from CAR-null mice compared with wild-type mice. Ugt1a6 mRNA expression was not induced in livers from wild-type or CAR-null mice after TSO. Basal Ugt2b5 mRNA expression in liver was not significantly different between wild-type and CAR-null mice. In livers from wild-type mice, TSO increased Ugt2b5 mRNA expression by 60%, whereas in livers from CAR-null mice, it increased Ugt2b5 mRNA levels 40%.

Mrp 1 to 4 are members of the multidrug resistance protein family of ATP binding cassette transporters. Table 2 illustrates the effect of TSO on induction of Mrp2, -3, and -4 in livers from wild-type and CAR-null mice. The constitutive mRNA expression of Mrp2 in liver was slightly lower in livers from CAR-null mice than from wild-type mice. Mrp2 was not significantly induced in liver by TSO treatment in livers of

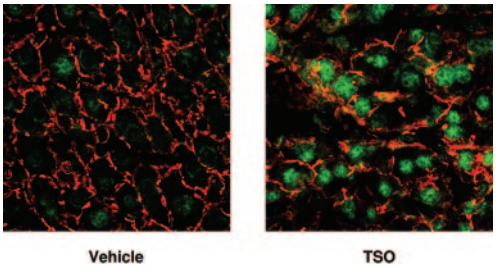


Fig. 6. Immunohistochemical detection of CAR in mouse liver after TSO administration. Livers were isolated from adult male C57BL/6 mice 3 h after TSO administration (200 mg/kg i.p.). Cryosections were incubated with anti-CAR primary antibodies followed by incubation with FITC-conjugated secondary antibodies (green) and rhodamine-labeled phalloidin that binds to actin (red). Magnification, 630×.

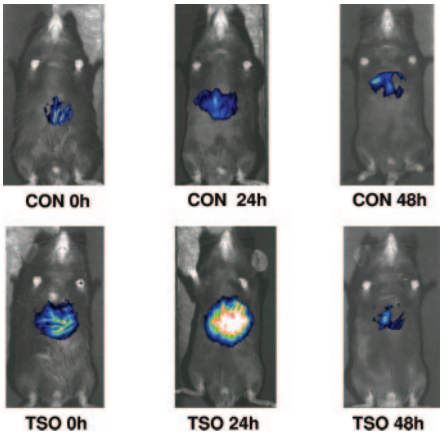


Fig. 7. Activation of a *Cyp2B6* promoter-luciferase reporter construct in mouse liver after vehicle (CON) or TSO administration. Male C57BL/6 mice were injected with 1 μg of naked plasmid DNA in a rapid (5-s) tail vein injection in sterile saline in a volume equal to 10% of body weight. Twenty four h later, animals were anesthetized with ketamine (72 mg/kg), acepromazine (6 mg/kg), and xylazine (6 mg/kg) and 5 min before imaging at times 0, 12, and 48 h, mice were i.p. injected with luciferin. At time 0, mice were injected with a single dose of corn oil vehicle (5 ml/kg i.p.) or TSO (200 mg/kg i.p.). Images were collected at 0, 12, and 48 h after vehicle or TSO administration.

the wild-type mice but was induced in livers from CAR-null mice. The constitutive expression of Mrp3 did not differ in livers from wild-type and CAR-null mice. However, after TSO administration, Mrp3 mRNA was induced approximately 2-fold in livers from both wild-type and CAR-null mice. The basal expression of Mrp4 tended to be lower in livers from CAR-null mice, but this was not statistically significant. TSO administration also tended to increase Mrp4 in livers from both wild-type and CAR-null mice, but this observation was also not statistically significant (possibly because of the small number of mice used).

Table 2 shows Nqo1, Eh, and Ho-1 mRNA expression in livers from wild-type and CAR-null mice after 4 days of TSO administration. Nqo1 mRNA expression in liver did not differ between wild-type and CAR-null mice. TSO induced Nqo1 mRNA expression by 4- to 5-fold in livers from both wild-type and CAR-null mice. Eh expression tended to be lower in livers from wild-type mice than in livers from CAR-null mice (768 ± 135 RLU versus 533 ± 39.7 RLU, respectively), although this did not reach statistical significance. TSO administration increased Eh mRNA expression by 2.2-fold in livers from wild-type mice, whereas TSO induced Eh mRNA expression by only 1.7-fold in livers from CAR-null mice. Ho-1 mRNA expression in livers from CAR-null mice was approximately 40% of that detected in livers from wild-type mice. Consistent with data from Table 1, Ho-1 expression was not induced in livers from wild-type or CAR-null mice after 4 days of TSO administration.

Induction of Nqo1 in CAR-null mice suggests that TSO may activate gene expression through a mechanism besides CAR. Nqo1 induction after treatment with antioxidants and chemicals that cause oxidative stress is mediated through activation of the antioxidant response element/electrophile response element (ARE/EpRE). Therefore, the observed

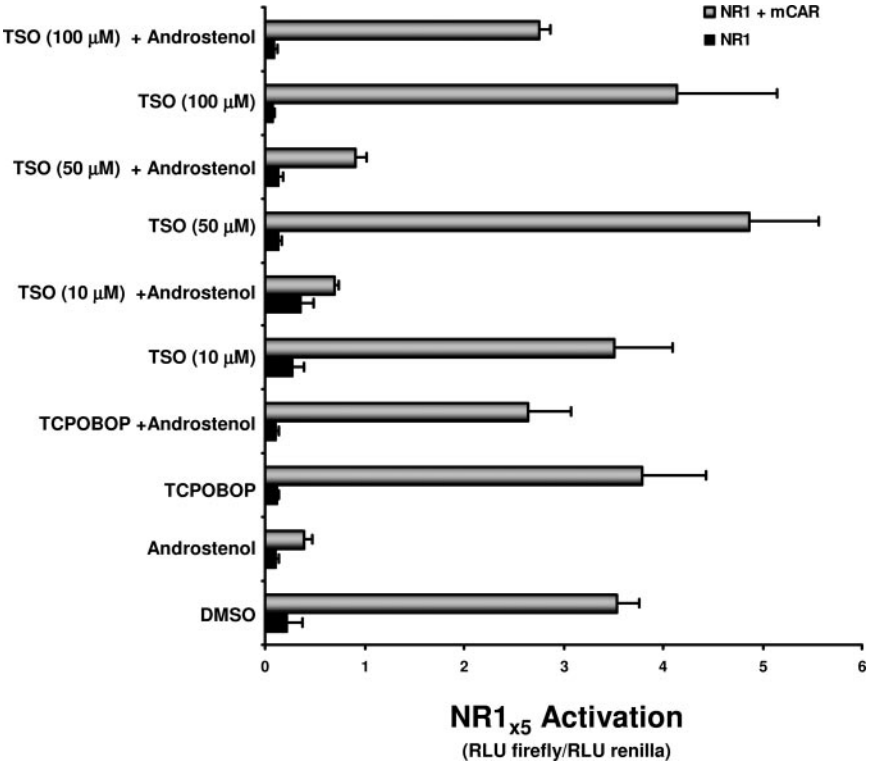


Fig. 8. Activation of the $(NR1)_5$-tk-luciferase reporter construct in HepG2 cells after treatment with TCPOBOP and TSO. HepG2 cells were transiently transfected with the $(NR1)_5$-tk-luciferase plasmid (0.1 μg) and pRL-SV40 (0.1 μg) into HepG2 cells, with or without a mouse CAR expression plasmid (0.2 μg). 36 h after transfection, the cells were treated with dimethyl sulfoxide, TCPOBOP (250 nm), or TSO (10–100 μm) in the presence or absence of 4 μM 5α-androstan-3α-ol (androstenol). Twenty-four hours after TCPOBOP and TSO treatment, the cell lysates were collected and luciferase activity was determined by the Dual-Glo luciferase assay. The data are presented as the mean -fold activation ± S.E.M. (n = 3 wells/treatment).

TABLE 2

Effect of TSO administration on mRNA expression in livers from wild-type and CAR-null mice

Total RNA was isolated from livers of wild-type and CAR-null mice after 4 days of TSO administration (200 mg/kg, i.p.) and analyzed by the bDNA signal amplification assay for Cyp2b10, Nqo1, Eh, Ho-1, Ugt1a1, Ugt1a6, Ugt2b5, Mrp2, Mrp3, and Mrp4 mRNA content. The data is presented as mean RLU ± SEM. (n = 3–4 animals).

	Wild-Type		CAR-Null	
	Vehicle	TSO	Vehicle	TSO
Cyp2b10	14.5 ± 1.0	426.5 ± 8.0*	1.6 ± 0.2#	54.6 ± 1.3*†
Nqo1	10.4 ± 2.1	53.0 ± 10.4*	9.1 ± 0.9	39.5 ± 6.1*
Eh	767.8 ± 135.1	1701.3 ± 172.0*	533 ± 39.7	888.6 ± 97.6*†
Ho-1	66.4 ± 6.6	62.2 ± 9.3	27 ± 5.5#	23.9 ± 6.4#
Ugt1a1	166.5 ± 20.0	187.2 ± 10.6	58.1 ± 11.8#	61.1 ± 3.0#
Ugt1a6	143.3 ± 33.8	175.2 ± 3.5	68.3 ± 10.9#	84.7 ± 4.0#
Ugt2b5	920.7 ± 195.6	1479.7 ± 97.3*	642.9 ± 75.0	921.5 ± 84.1*†
Mrp2	514.2 ± 122.6	588.6 ± 38.5	311.2 ± 18.6#	439.1 ± 43.7*
Mrp3	149.0 ± 34.5	339.4 ± 43.7*	93.1 ± 10.0	170.0 ± 15.2*
Mrp4	3.7 ± 0.7	5.8 ± 0.7	2.4 ± 0.2	4.2 ± 0.9

* Statistical difference ($P < 0.05$) between control and TSO-treated groups.
# Statistical difference ($P < 0.05$) between WT and CAR-null control groups.
† Statistical difference ($P < 0.05$) between WT and CAR-null mice treated with TSO.

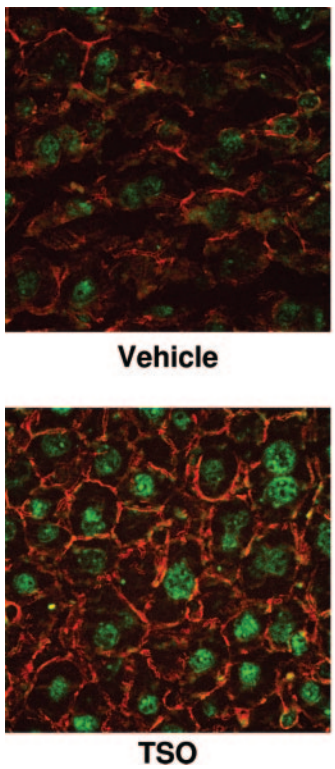
CAR-independent induction of some genes after TSO administration could be mediated through activation of Nrf2. The data illustrated in Fig. 9 support this hypothesis. Nrf2 staining in nuclei was increased in liver after 4 days of TSO administration, compared with liver from vehicle-treated controls. Moreover, TSO activated an ARE/EpRE luciferase reporter construct transiently transfected into HepG2 cells by approximately 2- to 4 -fold, in a concentration-dependent manner.

Discussion

For many years, it has been known that treatment with phenobarbital causes induction of Cyp2b activity in liver of mice and rats. In mice, the phenobarbital-induced increase in Cyp2b expression and hepatomegaly is known to be mediated through activation of CAR, a member of the nuclear hormone receptor superfamily (Wei et al., 2000). Furthermore, studies using CAR-null mice have shown that CAR expression is necessary for induction of Cyp2b10 in liver, as well as other hepatic genes for biotransformation (Maglich et al., 2002). In Wistar-Kyoto rats, CAR expression in liver is also associated with inducibility of Cyp2b by phenobarbital treatment (Yoshinari et al., 2001). Studies demonstrated that the decreased phenobarbital induction of Cyp2B1 in female Wistar-Kyoto rats, compared with male Wistar-Kyoto rats, correlates with decreased CAR protein levels and CAR binding to the PBREM in liver.

Like phenobarbital, TSO administration also induces Cyp2B1/2 in rat liver. TSO is considered a "phenobarbital-like" compound because it induces Cyp2B1/2 expression as well as Cyp3A1, Eh, Gst, and Nqo1 expression in liver (Pickett and Lu, 1981; Williams et al., 1984; Slawson et al., 1996; Schilter et al., 2000). Few studies have addressed TSO induction in mice. In mice, TSO administration increases Gst activity in liver (Gregus et al., 1989). Although it is not known how TSO induces gene expression in mouse liver, the data in this study suggest that TSO increases expression of some genes in mouse liver in a CAR-dependent manner. In this study, TSO increased Cyp2b10 expression in liver from C57BL/6 mice at 12 h and 4 days. In addition, TSO administration caused a robust induction of Cyp2b10 mRNA expression in livers from wild-type mice but not in livers from CAR-null mice. These data suggest that TSO activates CAR in liver, which is consistent with previously published data that document the lack of phenobarbital induction of Cyp2b10 induction in CAR-null mice (Wei et al., 2000).

The data in this study also show that TSO administration at various times increases mRNA levels of Nqo1, Eh, Ho-1, Ugt1a6, Ugt2b5, Mrp2, and Mrp3 in livers of male C57BL/6 mice. In addition, Mrp2 and Mrp3 protein levels in liver were increased after TSO administration, and Mrp4 protein levels were increased by 280%. TSO did not alter the expression of Cyp1a1, Cyp3a11, or Cyp4a14, organic anion transporting polypeptide 2, Mdr1a, 1b, 2, Mrp1, 5, 6, or CAR, or Nrf2 (data not shown) in liver of male C57BL/6 mice. Because TSO did not increase Cyp1a1 or Cyp4a2/3 mRNA levels in liver, TSO



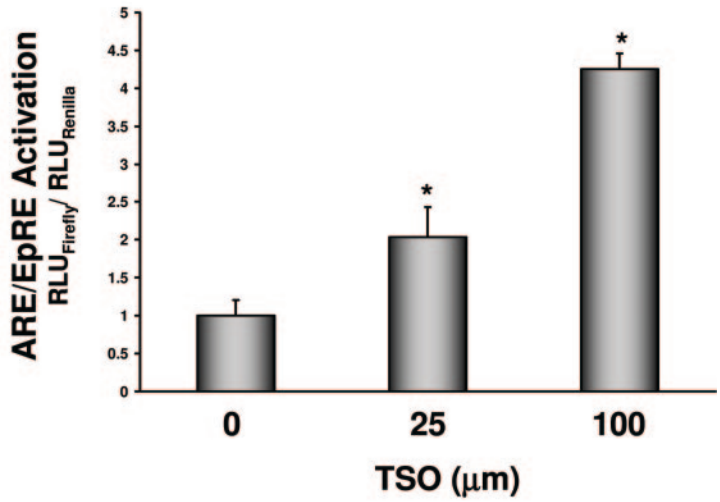


Fig. 9. Effect of TSO administration on Nrf2 translocation in mouse liver and ARE/EpRE activation in HepG2 cells. Left, representative micrograph of Nrf2 localization in liver collected from male C57BL/6 mice after TSO administration (200 mg/kg, twice daily) for 4 days. Cryosections were incubated with anti-Nrf2 antibody followed by incubation with FITC-conjugated secondary antibodies (green) and rhodamine-labeled phalloidin that binds actin (red). Magnification, 630×. Right, HepG2 cells were transiently transfected with an ARE/EpRE luciferase reporter construct (0.1 μg) and pRL-SV40 (0.1 μg) using Lipofectamine 2000. Thirty-six hours after transfection, cells were treated with TSO (25 and 100 μm). Twenty-four hours later, luciferase activity was determined by the Dual-Glo luciferase assay. The data are presented as the mean -fold activation ± S.E.M. (n = 3 wells/treatment).

probably does not activate the aryl hydrocarbon receptor or the peroxisome proliferator-activated receptor in mice. It is noteworthy that not all of the genes induced in C57BL/6 liver were induced at the same time after TSO administration. Ho-1 was induced by 3 h after TSO administration and Cyp2b10 by 12 h, whereas Nqo1, Eh, Ugt1a6, Ugt2b5, Mrp2, and Mrp3 mRNA levels in liver were increased after 4 days of TSO administration. This differential induction between genes in liver suggests that TSO may increase gene expression in liver through more than one mechanism.

The most apparent mechanism by which TSO induces expression of these genes in liver is through activation of CAR. The data presented in this study demonstrate that TSO activates CAR and CAR-responsive DNA binding sequences in vivo and in vitro, and illustrate that TSO: 1) increases nuclear localization of CAR, 2) activates a sequence of the human Cyp2B6 promoter in vivo, which contains a PBREM, 3) activates the NR1/DR-4 CAR binding site in HepG2 cells cotransfected with a mouse CAR expression plasmid, and 4) induces Cyp2b10 and Eh (partially) via a CAR-dependent manner. To date, no studies have defined mechanism(s) by which TSO induces gene expression in liver. These data agree with previously published data by Cherrington et al. (2003) that document an abolished induction of Cyp2b10 in livers from RXRα liver-specific knockout mice after TSO administration. However, it seems that TSO induction of Nqo1, Ugt2b5, and Mrp3 occur via a CAR-independent manner. Furthermore, the observation that induction of Cyp2b10 and Mrp3 is partially attenuated, but not completely ablated, in CAR-null mice indicates that a mechanism besides CAR is involved.

An interesting observation is that CAR is activated in vitro by high levels of 17-β estradiol and estrone, and repressed by progesterone and androgens (Kawamoto et al., 2000). Furthermore, compounds that are considered to be endocrine disrupters, such as methoxychlor, can activate CAR (Blizard et al., 2001). Stilbenes are a class of compounds with estrogenic or antiestrogenic properties, and rat liver microsomes can metabolize TSO to form estrogenic metabolites (Sugihara et al., 2000). The present data demonstrating that TSO antagonizes androstenol-induced repression in vitro suggests that TSO may bind CAR directly and is thus consistent with the results with estradiol and other estrogenic agents.

TSO induced Nqo1, Ugt2b5, and Mrp3 mRNA expression equally in liver from wild-type and CAR-null mice, which suggests that TSO also activates a CAR-independent pathway for induction of gene expression. The regulation of basal and inducible expression of the mouse Nqo1 gene is well characterized as being regulated by Nrf2 binding to an ARE/EpRE (Gong et al., 2002). Because Nqo1 is regulated/induced by Nrf2 activation in mouse liver, it is possible that TSO up-regulates Nqo1, Ugt2b5, and Mrp3 mRNA expression in mouse liver via activation of Nrf2. It is known that TSO administration depletes GSH levels in liver within 4 h after administration, and this is postulated to result in oxidative stress (Oguro et al., 1997; Sasaki et al., 2002). Because the ARE/EpRE is responsive to compounds, such as diethyl maleate, that deplete cellular GSH, it is likely that long-term TSO administration may cause oxidative stress and also activate Nrf2 in addition to CAR. The present data illustrate that TSO administration increases nuclear staining of Nrf2 in mouse hepatocytes and increases activity of an ARE/EpRE reporter construct.

These data enlighten the interesting observation that some genes in liver that are induced by phenobarbital and phenobarbital-like compounds may be induced through Nrf2. As mentioned above, phenobarbital induces not only Cyp2b10 in mouse liver but also Nqo1, Ho-1, and Eh—all genes regulated by Nrf2. However, it is not known whether phenobarbital activates the ARE/EpRE. Furthermore, it was recently demonstrated that ARE/EpRE activators, such as oltipraz and ethoxyquin, increase expression of Cyp2B1/2 and Cyp2b10 in rat and mouse liver, respectively, suggesting that these compounds activate CAR in addition to Nrf2 (Cherrington et al., 2003). Cross-talk between the Nrf2 and CAR activation pathways could occur with TSO and oltipraz because: 1) the compounds activate/modify more than one transcription factor/nuclear hormone receptor through ligand-dependent or -independent mechanisms (i.e., phosphorylation status), 2) the target genes may contain CAR- and Nrf2-responsive elements in their 5′-flanking region that would be activated by both CAR and Nrf2, 3) CAR and Nrf2 could each bind to the other's cognate response elements, and 4) the compounds increase metabolism, which in turn, may cause oxidative stress subsequently activating Nrf2.

In summary, TSO increases the mRNA levels of several genes that encode phase I and phase II drug metabolizing enzymes and drug transporters in mouse liver. Moreover, TSO seems to induce expression of some genes in liver in a CAR-dependent manner, whereas others are induced independently of CAR and possibly via activation of Nrf2.

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