Trans-stilbene oxide induces expression of genes involved in metabolism and transport in mouse liver via CAR and Nrf2 transcription factors

A L Slitt¹, N J Cherrington², M Z Dieter¹, L M Aleksunes³, G L Scheffer⁴, W Huang⁵, D D Moore⁵, and C D Klaassen¹

¹Department of Pharmacology, Toxicology, and Therapeutics, University of Kansas Medical Center (A.L.S, M.Z.D., C.D.K), Kansas City, KS 66160-7417, ²Department of Pharmacology and Toxicology, University of Arizona, Tucson, AZ 85721-0207 (N.J.C), ³Department of Pharmaceutical Sciences, University of Connecticut, CT 06268 (L.M.A), ⁴Department of Pathology, VU Medical Center, 1081 HV Amsterdam, The Netherlands (G.L.S) and ⁵Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, 77030 (W.H. and D.D.M)
Running title page

Running title: Constitutive Androstane Receptor and trans-stilbene oxide

Corresponding Author:

Curtis Klaassen, Ph.D.

Department of Pharmacology, Toxicology, & Therapeutics

University of Kansas Medical Center

3901 Rainbow Boulevard

Kansas City, KS 66160-7417

Phone: (913) 588-7714

Fax: (913) 588-7501

E-mail: cklaasse@kumc.edu

Text pages: 36

Tables: 2

Figs: 9

References: 34

Abstract: 252

Introduction: 656

Discussion: 1213

Abbreviations: branched DNA signal amplification (bDNA), Constitutive Androstane receptor (CAR), cytochrome P450 (Cyp), Epoxide Hydrolase (Eh), Heme Oxygenase-1 (Ho-1), multidrug resistance-associated protein (Mrp), NAD(P)H:quinone oxidoreductase (Nqo1), Nuclear Factor-E2-related factor2 (Nrf2), trans-stilbene oxide (TSO), UDP-glucuronosyltransferase (Ugt)
Abstract

*Trans*-stilbene oxide (TSO) induces drug metabolizing enzymes in rat and mouse liver. TSO is considered to be 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 protein (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, as compared to 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.
Introduction

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 Cytochrome P450 (Cyp) 2B1/2 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).

Recently it has been demonstrated that TSO also increases the expression of the xenobiotic transporters, Multidrug resistance-associated protein (Mrp) 2 and 3, at both mRNA and protein levels in rat liver (Slitt et al., 2003). Therefore, TSO administration coordinately induces mRNA expression of efflux transporters along with phase-I and -II drug-metabolism enzymes.

The mechanism by which phenobarbital induces Cyp2B gene expression has been well described (for review see: [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
alpha (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 2 CAR binding sequences, Nuclear Receptor 1 (NR1) and Nuclear Receptor 2 (NR2). 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 with organic anion transporters, such as Organic anion transporting polypeptide (Oatp) 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
MOLPHARM/2005/014571

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, 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 which 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)2-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 media, and fetal bovine serum were purchased from American Type Culture Collection (Manassas, VA).

**Treatment of Animals.** Adult male C57BL/6 mice weighing approximately 22-25g 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, *ip*). Livers were removed at 3 and 12 hrs 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, *ip*) for four 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 previously described (Aleksunes et al., 2005a; Cherrington et al., 2003; Chen et al., 2003; Maher et al., 2005). 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 Tm of approximately 63°C. Probes developed in ProbeDesigner was 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 ESTs.

Branched DNA Assay. Oligonucleotide probes were diluted in lysis buffer supplied in the QuantiGene® HV signal amplification kit (Genospectra, 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.).
Western analysis of Nqo1, Mrp2-4 expression in mouse liver. Western analysis was carried out according to Aleksunes et al., 2005b. Livers were homogenized in ST buffer (250 mM sucrose, 10 mM Tris–HCl, pH 7.4) containing 50 µg/ml aprotinin and centrifuged at 105,000 x g 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 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 (ab2346, Novus Biological, Littleton, CO). Briefly, blots were blocked with 5% nonfat dry milk in TBS with 0.1% Tween (TBS-T) for 2 hrs and incubated for 1 hr with the primary antibody diluted in blocking buffer (1:1000). A peroxidase-labeled secondary antibody (NB-710H, Novus Biological, Littleton, CO) was diluted (1:10,000) in blocking buffer and incubated with blots for 1 hr. Immunochemical detection of Mrp2 (~190-200 kDa), Mrp3 (~180-190 kDa), and Mrp4 (~160-170 kDa) protein was performed using M2III-5, M3II-2, and M4I-10 antibodies, respectively. Anti-Mrp antibodies were generously provided by George Scheffer, VU Medical Center, Amsterdam, The Netherlands. Membranes were blocked with 1% nonfat dry milk in phenobarbitalS-Tween for 1 hr and incubated for 1 hr with the primary antibody diluted in blocking buffer (1:2000 for M3II-2 and M4I-10, 1:600 for M2III-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 hr.
Membranes were stripped and reprobed with a dilution of 1:2,500 anti-β-actin rabbit polyclonal Ab (Abcam, Cambridge, MA) to confirm equal protein loading.

Protein-antibody complexes were detected using an ECL chemiluminescent kit (Amersham Life Science, Arlington Heights, IL) 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, Hercules, CA).

**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 (PVDF) 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 (phenobarbitalS) for 5 min. Sections were rinsed three times for five min with phenobarbitalS, and blocked at room temperature for 30 min with 5% goat serum/phenobarbitalS with 0.2% Triton X-100 (phenobarbitalS-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/phenobarbitalS-T, overnight at room temperature. Sections were washed three times for 10 min with phenobarbitalS-T and incubated for 1 hr 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 (Molecular Probes, Eugene, OR) diluted 1:200 in 5% goat serum/phenobarbitalS-T. Slides were washed in phenobarbitalS 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 v2.8.

**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-kb fragment which maintains the core promoter (+39/-364) and the distal enhancer region (-1461/-2013), including the phenobarbital-responsive element (unpublished). Male C57BL/6 mice (20-25 g) were administered 1 µg 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 hrs 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 50mg/ml stock solution, ip) 5 min prior to 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 pseudo-color maps were created with the WinView 32 program (Tucson, AZ). Color maps were superimposed over the light image of the mouse, using Adobe Photoshop 6.0. Light images were acquired with lights mounted inside the box, with an exposure time of about 20 msec, using a fast setting for the analog-digital
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 f1.2. This image was considered time 0. Each animal (n=3) was administered a single dose of either TSO (200 mg/kg, ip, 5 ml/kg) or corn oil (5 ml/kg). Images were taken at 24 and 48 hrs following 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). Briefly, HepG2 cells were cultured in MEM supplemented with 10% FBS according to the protocol provided by ATCC. 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 hrs 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 of an ARE/EpRE luciferase reporter construct (Dieter et al., 2001) and 0.1 µg pf pRL-SV40 (0.1 µg). After exposure to TSO for 24 hr, the media was removed and cells were washed with phenobarbitals. The phenobarbitalS 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 hr, 12 hr, 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 crosses (†) represent a statistical difference ($p < 0.05$) between WT and CAR-null mice treated with TSO.
Results

*Induction of metabolism and transport genes in livers from male C57BL/6 mice by TSO.* Previous studies report genes for drug metabolism and transport in rat and mouse liver are induced following 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 hr 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 hr, 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 hr after TSO. By contrast, Ho-1 mRNA levels in liver increased 450% 3 hr after TSO, but were not increased at 12 hrs 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. Some Ugts are induced in rat liver after TSO administration (Seidegard and DePierre, 1982). Figure 3 shows Ugt1a1, 1a6, and 2b5 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 hrs 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 (Figure 4). Mrp1 mRNA expression in liver was not changed after TSO administration. Both Mrp2 and Mrp3 mRNA levels were unchanged at 3 or 12 hr 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 (Mdr) 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 naïve 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 present study, liver sections were immunostained with anti-CAR antibodies to identify CAR localization and determine whether TSO activates CAR prior to increasing gene expression. Figure 6 illustrates CAR localization in liver from vehicle- and TSO-treated mice at 3 hrs. Faint CAR staining was detected in cytosol and nuclei in liver from vehicle-treated mice, whereas, staining of CAR increased in hepatocyte nuclei 3 hr 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 via 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 hr, luciferase
activity was increased in livers from TSO-treated as compared to vehicle-treated mice.
This demonstrates that TSO activates the human Cyp2B6 promoter in liver. At 48 hr,
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 previously reported (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
(DMSO), and the activity was repressed in the presence of 4 µM 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 Figures 1-8 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 utilized to determine whether TSO induced Cyp2b10, Ugt1a1, Ugt1a6, Ugt2b5, Mrp2, Mrp3, Nqo1, Eh, and Ho-1 in liver via a CAR-dependent mechanism. 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 (RLU = 0.96 ± 0.2) than in wild-type mice (14.5 ± 1.5). TSO administration markedly induced Cyp2b10 mRNA expression 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, 1a6, and 2b5 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 (about 50%). TSO did not significantly induce Ugt1a1 mRNA expression at any time after TSO administration. Ugt1a6 expression was about 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 1-4 are members of the Multidrug resistance protein family of ATP Binding Cassette (or ABC) 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 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 due to 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 4-5 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 (RLU = 768 ± 135 vs. 533 ± 39.7, respectively), although this did not reach statistical significance. TSO administration increased Eh mRNA expression 2.2 fold in livers from wild-type mice, whereas TSO only induced Eh mRNA expression 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 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, as compared to liver from vehicle-treated controls. Moreover, TSO activated an ARE/EpRE luciferase reporter construct transiently transfected into HepG2 cells by approximately 2-4 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 (Wei et al., 2000). In mice, it is known that the phenobarbital-induced increase in Cyp2b expression and hepatomegaly is mediated through activation of CAR, a member of the nuclear hormone receptor superfamily. 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, as compared to 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., 1985). Although it is not known how TSO induces gene expression in mouse liver, the data in this study suggests 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. Additionally, Mrp2-4 protein levels in liver were increased after TSO administration, with Mrp4 protein levels being 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 probably does not activate the Aryl hydrocarbon receptor or the Peroxisome Proliferator-Activated Receptor in mice. Interestingly, 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 hr after TSO administration, Cyp2b10 was induced by 12 hr after TSO administration, whereas, Nqo1, Eh, Ugt1a6, Ugt2b5, Mrp2 and 3 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 co-transfected 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 knock-out mice after TSO administration. However, it appears 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 a mechanism besides CAR is involved.

An interesting observation is that CAR is activated in vitro by high levels of 17-beta 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 anti-estrogenic 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 Antioxidant Response Element/Electrophile Response Element (ARE/EpRE) (Gong et al., 2002). Because Nqo1 is regulated/induced by Nrf2 activation in mouse liver, it is possible that TSO upregulates Nqo1, Ugt2b5, and Mrp3 mRNA expression in mouse liver via activation of Nrf2. It is known that TSO administration depletes glutathione (GSH) levels in liver within 4 hrs 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 glutathione (GSH), it is likely that chronic TSO administration may cause oxidative stress and also activate Nrf2 in addition to CAR. Interestingly, 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 draw to light an 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 induces 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 of: 1) the compounds activate/modify more than one transcription factor/nuclear hormone receptor through receptor through ligand-dependent or ligand-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 bind to each others 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 appears to induce expression of some genes in liver in a CAR-dependent manner, whereas others are induced independent of CAR, and possibly via activation of Nrf2.
References


hepatobiliary and increased hepatovascular transport of acetaminophen-glucuronide after microsomal enzyme induction. Drug Metab Dispos. 18:10-19.


gene expression in hepatocytes and a newly derived human hepatoma cell line.

*J.Biol.Chem.* **280**:4367-73


Footnotes

Send reprint requests to: Curtis D. Klaassen, Ph.D.
Department of Pharmacology, Toxicology, and Therapeutics
University of Kansas Medical Center
3901 Rainbow Boulevard
Kansas City, KS 66160-7417

1Supported by NIH grant ES-11239, ES-09649, ES-09716

2Present address: Department of Pharmacology and Toxicology, University of Arizona,
Tucson, AZ 85721-0207
Legends for figures

Figure 1: Effect of trans-stilbene oxide (TSO) administration on Cytochrome P450 (Cyp) 2b10 and 3a11 mRNA expression in mouse liver. Tissue total RNA was isolated from adult male C57BL/6 mice after 3 hr, 12 hr, or 4 days of TSO administration (200 mg/kg, ip, twice daily) and analyzed by the bDNA signal amplification assay for Cyp2b10 and 3a11 mRNA. The data is presented as mean RLU ± SEM (n=4-5 animals).

Figure 2: Effects of trans-stilbene oxide (TSO) administration on NAD(P)H:quinone oxidoreductase (Nqo1), Epoxide Hydrolase (Eh), and Heme Oxygenase-1 (Ho-1) mRNA expression in mouse liver. Tissue total RNA was isolated from adult male C57BL/6 mice after 3 hr, 12 hr, or 4 days of TSO administration (200 mg/kg, ip, twice daily) and analyzed by the bDNA signal amplification assay for Nqo1, Epoxide hydrolase, and Heme oxygenase-1 mRNA content. The data is presented as mean RLU ± SEM (n=3-5 animals).

Figure 3: Effects of trans-stilbene oxide (TSO) administration on UDP-glucuronosyltransferase (Ugt) 1a1, 1a6, and 2b5 mRNA expression in mouse liver. Tissue total RNA was isolated from adult male C57BL/6 mice after 3 hr, 12 hr, or 4 days of TSO administration (200 mg/kg, ip, twice daily) and analyzed by the bDNA signal amplification assay for Ugt1a1, 1a6, and 2B5 mRNA content. The data is presented as mean RLU ± SEM (n=4-5 animals).
Figure 4: Effects of trans-stilbene oxide (TSO) administration on Multidrug resistance-associated protein (Mrp) 2, 3, and 4 mRNA expression in mouse liver. Tissue total RNA was isolated from adult male rats after 3 hr, 12 hr, or 4 days of TSO administration (200 mg/kg, ip, twice daily) and analyzed by the bDNA signal amplification assay for Mrp1-4 mRNA content. The data is presented as mean RLU ± SEM (n=4-5 animals).

Figure 5: Effect of trans-stilbene oxide (TSO) pretreatment on NAD(P)H:quinone oxidoreductase (Nqo1), Multidrug resistance-associated protein (Mrp) 2-4 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, Mrp2-4, or β-actin. (50 µg protein/lane, n=3 mice /treatment).

Figure 6: Immunohistochemical detection of CAR in mouse liver after trans-stilbene oxide (TSO) administration. Livers were isolated from adult male C57BL/6 mice 3 hr after TSO administration (200 mg/kg, ip). 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 = 630x.

Figure 7: Activation of a Cyp2B6 promoter-luciferase reporter construct in mouse liver after vehicle (CON) or trans-stilbene oxide (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 hrs later, animals were anesthetized with ketamine (72 mg/kg), acepromazine (6 mg/kg), and xylazine (6 mg/kg) and 5 min prior to imaging at times 0, 12, and 48 hr, mice were injected with luciferin ip. At time 0, mice were injected with a single dose of corn oil vehicle (5 ml/kg, ip) or TSO (200 mg/kg, ip). Images were collected at 0, 12, and 48 hrs after vehicle or TSO administration.

**Figure 8:** Activation of the (NR1)$_5$-tk-luciferase reporter construct in HepG2 cells after treatment with 1,4-bis [2-3,5-dichloropyridyloxy)]benzene (TCPOBOP) and Trans-stilbene oxide (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 hrs after transfection, the cells were treated with DMSO, TCPOBOP (250 nm), or TSO (10-100 µm) in the presence or absence of 4 µM 5α-androstan-3α-ol (androstenediol). 24 hrs after TCPOBOP and TSO treatment the cell lysates were collected and luciferase activity was determined by the Dual-Glo™ Luciferase Assay. The data is presented as the mean fold activation ± SEM (n=3 wells/treatment).

**Figure 9:** Effect of TSO administration on Nrf2 translocation in mouse liver and Antioxidant Response Element/Electrophile Response Element (ARE/EpRE) activation in HepG2 cells. Left panel: 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 = 630x. Right Panel: HepG2 cells were transiently transfected with an ARE/EpRE luciferase reporter construct (0.1 µg) and pRL-SV40 (0.1 µg) using lipofectamine 2000. 36 hrs after transfection, cells were treated with TSO (25 and 100 µm). 24 hrs later, luciferase activity was determined by the Dual-Glo™ Luciferase Assay. The data is presented as the mean fold activation ± SEM (n=3 wells/treatment).
### Tables

#### TABLE 1: Oligonucleotide probes generated for analysis mouse microsomal epoxide hydrolase (mEH) expression by bDNA signal amplification

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank ID</th>
<th>Function</th>
<th>Probe Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEH</td>
<td>NM_010145</td>
<td>CE</td>
<td>cacgggtattcttgacttgccatTTTTTctcttgaaaggaagt</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CE</td>
<td>cttccaggagaactctctctctTTTTTctcttgaaaggaagt</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CE</td>
<td>tctttggagaagcgctgggaTTTTTctcttgaaaggaagt</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CE</td>
<td>tccaaggggaagctgaatagcTTTTTctcttgaaaggaagt</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CE</td>
<td>ttgggctctcgaaggcagTTTTTctcttgaaaggaagt</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LE</td>
<td>agttcactacagacacagcccaTTTTTtaggcatagaccctgtct</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LE</td>
<td>caggcagccacagaggtTTTTTtaggcatagaccctgtct</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LE</td>
<td>caggccttcactctccagttTTTTTtaggcatagaccctgtct</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LE</td>
<td>cagttagatatcatgtgtttagtcagcagaaTTTTTtaggcatagaccctgtct</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LE</td>
<td>ggagacactgtctgtctgctTTTTTtaggcatagaccctgtct</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LE</td>
<td>gacaccctggcccaagtTTTTTtaggcatagaccctgtct</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LE</td>
<td>tccccctagctgtctctgtaccaTTTTTtaggcatagaccctgtct</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LE</td>
<td>ggtactacgttagcttccacccactctTTTTTtaggcatagaccctgtct</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LE</td>
<td>cacttaggaagatagagatgatTTTTTtaggcatagaccctgtct</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LE</td>
<td>caggcagacagaaactctggcTTTTTtaggcatagaccctgtct</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LE</td>
<td>gcgtcatcacagctagctagTTTTTtaggcatagaccctgtct</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BL</td>
<td>ggtgggaacactcttcaagatgtagg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BL</td>
<td>cagtggccaccaagacccctca</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BL</td>
<td>tctggggcatcaggatc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BL</td>
<td>caaagltgccccccacgt</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BL</td>
<td>gatgtctgcccagagacg</td>
</tr>
</tbody>
</table>

*Function refers to the utility of the oligonucleotide probe in the bDNA assay (CE, capture extender; LE, label extender; BL, blocker probe).*
TABLE 2: Effect of TSO administration on mRNA expression in livers from wild-type and CAR-null mice

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

Total RNA was isolated from livers of wild-type and CAR-null mice after 4 days of TSO administration (200 mg/kg, ip) and analyzed by the bDNA signal amplification assay for Cytochrome P450 2b10 (Cyp2b10), NAD(P)H:quinone oxidoreductase (Nqo1), Epoxide Hydrolase (Eh), Heme oxygenase-1 (Ho-1), UDP-glucuronosyltransferase (Ugt) 1a1, 1a6, 2b5, Multidrug resistance-associated proteins (Mrp) 2, 3, and 4 mRNA content. The data is presented as mean RLU ± SEM. (n=3-4 animals). 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 crosses (†) represent a statistical difference (p < 0.05) between WT and CAR-null mice treated with TSO.
FIGURE 1

The figure shows the mRNA expression levels of Cyp2b10 and Cyp3a11 in response to TSO treatment over time. The y-axis represents mRNA (RLU/10µg total RNA), and the x-axis represents time points: 3 hr, 12 hr, and 4 days. The figure compares the mRNA expression levels between Control and TSO treatments. The Cyp2b10 expression is significantly higher in the TSO group compared to the Control group at the 3 hr and 4 days time points, as indicated by the asterisks. The Cyp3a11 expression levels are also shown, but without significant differences indicated between the groups.
FIGURE 2

- **Nqo1**: The graph shows a significant increase in mRNA levels for Nqo1 over time with TSO treatment compared to the control group. The * symbol indicates statistical significance.

- **Eh**: Similar to Nqo1, there is a noticeable increase in mRNA levels for Eh with TSO treatment over time. The * symbol denotes statistical significance.

- **Ho-1**: The mRNA levels for Ho-1 show a more modest increase with TSO treatment compared to controls, and the * symbol indicates statistical significance.

The x-axis represents time points (3 hr, 12 hr, 4 days), while the y-axis represents mRNA (RLU/10µg total RNA).
FIGURE 3

**Ugt1a1**

**Ugt1a6**

**Ugt2b5**

mRNA (RLU/10µg total RNA)

Control

TSO

3 hr 12 hr 4 days

This article has not been copyedited and formatted. The final version may differ from this version.
FIGURE 4
<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>TSO</th>
<th>Fold-Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nqo1</td>
<td></td>
<td></td>
<td>2.4</td>
</tr>
<tr>
<td>Mrp2</td>
<td></td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>Mrp3</td>
<td></td>
<td></td>
<td>1.8</td>
</tr>
<tr>
<td>Mrp4</td>
<td></td>
<td></td>
<td>3.8</td>
</tr>
<tr>
<td>B-actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5
FIGURE 6
FIGURE 7
Figure 8

NR1x5 Activation

(RLU firefly/RLU renilla)
FIGURE 9

ARE/EpRE Activation

\[ \frac{\text{RLU}_{\text{Firefly}}}{\text{RLU}_{\text{Renilla}}} \]

![Vehicle](image1)

![TSO](image2)

**TSO (µm)**

<table>
<thead>
<tr>
<th>0</th>
<th>25</th>
<th>100</th>
</tr>
</thead>
</table>

* *