



Published in final edited form as:

*Drug Metab Dispos.* 2008 August ; 36(8): 1716–1721. doi:10.1124/dmd.108.020867.

## The Nrf2 Activator Oltipraz Also Activates the Constitutive Androstane Receptor

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### Abstract

Oltipraz (OPZ) is a well known inducer of *NAD(P)H:quinone oxidoreductase (NQO1)* along with other enzymes that comprise the nuclear factor E2-related factor 2 (Nrf2) battery of detoxification genes. However, OPZ treatment also induces expression of *CYP2B*, a gene regulated by the constitutive androstane receptor (CAR). Therefore, this study was designed to determine whether OPZ induces gene expression in the mouse liver through activation of CAR in addition to Nrf2. OPZ increased the mRNA expression of both Cyp2b10 and Nqo1 in C57BL/6 mouse livers. As expected, in livers from Nrf2<sup>-/-</sup> mice, OPZ induction of Nqo1 was reduced, indicating Nqo1 induction is dependent on Nrf2 activation, whereas Cyp2b10 induction was unchanged. The robust induction of Cyp2b10 by OPZ in wild-type mice was completely absent in CAR<sup>-/-</sup> mice, revealing a CAR-dependent induction by OPZ. OPZ also induced transcription of the human *CYP2B6* promoter-reporter containing the phenobarbital (PB) responsive element in mouse liver using an in vivo transcription assay. Additionally, OPZ induced in vivo nuclear accumulation of CAR at 3 h but, as with PB, was unable to reverse androstanol repression of mouse CAR constitutive activity in transiently transfected HepG2 cells. In summary, OPZ induces expression of Cyp2b10 and Nqo1 via the activation of CAR and Nrf2, respectively.

The chemopreventive chemical oltipraz (OPZ) (4-methyl-5-(2-pyrazinyl)-1,2-dithiol-3-thione) belongs to a class of compounds known as dithiolethiones. The antitumorigenic activity of OPZ is generally ascribed to its strong induction of enzymes that mediate detoxication processes. Early studies revealed that OPZ increases expression of a number of Phase I and II enzymes, including glutathione-*S*-transferases (GSTs), NAD(P)H:quinone oxidoreductase (NQO1), microsomal epoxide hydrolase, aflatoxin aldehyde reductase, glucuronosyl transferases, as well as enzymes that increase glutathione levels, namely, glutathione reductase and glucose-6-phosphate dehydrogenase (Kensler et al., 1985; Ansher et al., 1986; Davidson et al., 1990; Morel et al., 1993; Primiano et al., 1996).

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Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

Induction of these metabolic enzymes by OPZ has been linked to the transcription factor nuclear factor E2-related factor 2 (Nrf2) and its activation of the antioxidant response element/electrophile response element (ARE/EpRE). Under normal conditions, Nrf2 is sequestered in the cytosol and marked for proteasomal degradation by the protein Kelch-like ECH-associated protein 1 (Keap1). This protein acts as an adapter for Cullin 3-based ubiquitin ligase (E3 ligase), as well as a “receptor” for electrophiles (Cullinan et al., 2004). In the absence of electrophiles/oxidative stress, Nrf2 is ubiquitinated and targeted for degradation by the 26S-proteasome, keeping cellular levels of Nrf2 low and preventing activation of its response element. Electrophilic binding to Keap1 is thought to disrupt the ubiquitination complex and inhibit Nrf2 degradation (Zhang et al., 2004). The increased cellular concentration of unbound Nrf2 in turn leads to ARE/EpRE activation (Zhang, 2006). It has been shown in Keap1 knockout mice that the loss of this Nrf2-Keap1 complex leads to Nrf2 nuclear accumulation and activation (Itoh et al., 2004). Further evidence of the role of Nrf2 in OPZ gene induction of detoxication enzymes was provided through experiments showing that loss of expression of Nrf2 abrogates both the chemoprotective activity of OPZ, as well as the inducibility of many known OPZ-induced genes (Kwak et al., 2001; Ramos-Gomez et al., 2001).

Although early investigation into the activity of OPZ focused on induction of detoxication enzymes like NQO1 and GST, OPZ also alters the expression and activity of certain cytochrome P450 enzymes. OPZ has been shown to induce the expression of CYP1A1, CYP1A2, CYP2B1, CYP2B2, and CYP2E1 (Maheo et al., 1998; Cherrington et al., 2003; Cho and Kim, 2003; Merrell et al., 2008). The induction of at least one of these isoforms, CYP1A1, has been characterized as aryl hydrocarbon receptor-dependent (Le Ferrec et al., 2002), which suggests that OPZ may activate multiple transcription factors.

Little has been reported previously regarding the strong induction of the *CYP2B* genes by OPZ. The mechanism by which *CYP2B* genes are induced is well documented (Sueyoshi and Negishi, 2001). *CYP2B* inducers activate the constitutive androstane receptor (CAR), which is normally sequestered in the cytoplasm by the cytoplasmic CAR retention protein (Kobayashi et al., 2003). Treatment with CAR activators appears to induce nuclear accumulation through one of two mechanisms: 1) some CAR activators are ligands, such as TCPOBOP in mice and CITCO in humans, and act as direct activators of CAR nuclear translocation; and 2) other CAR activators, such as phenobarbital (PB) and phenytoin, do not appear to be actual ligands and are thought to promote CAR nuclear translocation indirectly (Jackson et al., 2004, 2006; Stanley et al., 2006). In both direct and indirect activation, CAR translocates to the nucleus where it heterodimerizes with the retinoid X receptor (RXR)  $\alpha$ . The CAR/RXR $\alpha$  heterodimer then binds to a PB responsive enhancer module to drive expression of *CYP2B* genes, including *Cyp2b1* in rats, *Cyp2b10* in mice, and *CYP2B6* in humans (Honkakoski and Negishi, 1997; Honkakoski et al., 1998). We have previously shown that OPZ induction of *Cyp2b10* mRNA expression is significantly less in RXR $\alpha$ -deficient mice (Cherrington et al., 2003).

The present study was developed to determine whether, in addition to activating Nrf2, OPZ also activates the nuclear receptor CAR. We report here that OPZ strongly induces *Cyp2b10* in a CAR-dependent manner, that OPZ treatment increases CAR nuclear accumulation, and that OPZ appears to be an indirect activator of CAR. This study shows an uncharacterized mechanism for OPZ-mediated gene induction through activation of the CAR.

## Materials and Methods

### Chemicals

OPZ was a gift of Dr. Ronald Lubet (National Cancer Institute, Bethesda, MD). D-Luciferin was synthesized by Dr. Eugene Mash (University of Arizona, Tuscon, AZ). 5 $\alpha$ -Androstan-3 $\alpha$ -ol (androstanol) was purchased from Steraloids (Newport, RI). PB was purchased from Mallinckrodt, Inc. (Paris, KY). Ketamine HCl and xylazine injectables were purchased from Associated Medical Supply (Scottsdale, AZ). Luciferin was obtained from Molecular Imaging Products Company (Ann Arbor, MI). All the other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO).

### Animals

All the animals were housed and acclimated in a temperature-, light-, and humidity-controlled environment in cages with hardwood chips and were given Harlan Teklad Rodent Diet W (Harlan Laboratories, Madison, WI) and water ad libitum. All the animals were acclimated for at least 1 week before the experiments and were allowed water and standard chow ad libitum. Housing and experimental procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* as determined by the U.S. National Institutes of Health. Male C57BL/6 mice were treated with either corn oil (5 ml/kg), OPZ (150 mg/kg, i.p. in corn oil), or PB (80 mg/kg, i.p. in saline) with an injection volume of 5 ml/kg. The choice of these doses was based on previous work from our laboratory (Cherrington et al., 2002), as well as the preponderance of published literature.

For the dose response, adult male C57BL/6 mice were treated with vehicle or OPZ once daily for 4 days (50, 100, 150, 200, and 250 mg/kg). Male Nrf2<sup>-/-</sup> mice (Aleksunes et al., 2006), CAR<sup>-/-</sup> mice (Wei et al., 2000), or C57BL/6 mice ( $n = 5$  in each group) were treated with either corn oil (5 ml/kg), OPZ (150 mg/kg, p.o. in corn oil), or PB (80 mg/kg, i.p. in saline, 24 h) for 4 days with an injection volume of 5 ml/kg. For mRNA analysis, livers were excised 24 h following the final treatment, snap-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until use. For nuclear extraction, livers were excised 3 h after OPZ treatment, and nuclear protein was isolated as previously described (Sueyoshi et al., 1995) and stored at  $-80^{\circ}\text{C}$  until Western blot analysis of CAR accumulation. To assess the degree of cytosolic contamination in the nuclear extracts, Western blotting was performed against glyceraldehyde-3-phosphate dehydrogenase protein, with minimal cytosolic contamination detected (data not shown).

### RNA Isolation

Total RNA was isolated using RNazol B reagent (Tel-Test Inc., Friendswood, TX) as per the manufacturer's protocol. RNA concentration was determined by UV spectrophotometry, and its integrity was examined by ethidium bromide staining after agarose gel electrophoresis.

### Branched DNA Signal Amplification Assay

Mouse Cyp2b10 and Nqo1 probe sets were used as previously described (Cherrington et al., 2003; Aleksunes et al., 2005). These oligonucleotide probes were diluted in lysis buffer supplied in the Quantigene HV Signal Screen Kit (Panomics, Inc., Fremont, CA). Reagents for analysis were supplied with the kit (i.e., lysis buffer, amplifier/label probe buffer, and substrate solution) or prepared in the laboratory (capture hybridization buffer). Total RNA (1  $\mu\text{g}/\mu\text{l}$ , 10  $\mu\text{l}$ ) was added to each well of a 96-well plate containing 50  $\mu\text{l}$  of capture hybridization buffer and 50  $\mu\text{l}$  of diluted probe set. mRNA was allowed to hybridize to each probe set overnight at  $53^{\circ}\text{C}$ . Subsequent hybridization steps were carried out as per the manufacturer's protocol, and luminescence was measured with a Quantiplex 320 branched

DNA (bDNA) luminometer (Bayer Diagnostics, Tarrytown, NY) interfaced with Quantiplex Data Management Software version 5.02 for analysis of luminescence from 96-well plates.

### In Vivo Transcription Assay

Male C57BL/6 mice (20–25 g; Charles River Laboratories, Raleigh, NC) were administered 10 µg of naked plasmid DNA (*CYP2B6* promoter luciferase reporter construct) as a rapid (5-s) tail vein injection in sterile saline at a dose volume equal to 10% of body weight. Eighteen hours later, animals were anesthetized with a mixture of ketamine (72 mg/kg) and xylazine (6 mg/kg). Mice were injected i.p. with luciferin (70 µl of a 50-mg/ml stock solution) 5 min before imaging. A VersArray 1300B camera (Princeton Instruments, Trenton, NJ) thermoelectrically cooled to –100°C, with an aperture of *f*1, connected to a light-sealed imaging chamber was used for all the images. Images were acquired in gray scale, and pseudocolor maps were created with the WinView 32 program (Princeton Instruments). Color maps were superimposed over the light image of the mouse using Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA), and these images were considered time 0. Each animal (*n* = 3) was administered a single i.p. dose of either OPZ (150 mg/kg) or corn oil at a dose volume of 5 ml/kg, and imaging was repeated 24 h after dosing.

### Western Blot Analysis

Nuclear extracts from control, PB-, and OPZ-treated C57BL/6 mice were resolved on a SDS-10% polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Blots were incubated 1 h at room temperature in blocking buffer containing 5% nonfat milk in Tris-buffered saline/Tween 20. After 3-h incubation with a rabbit anti-CAR polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), the membrane was incubated 1 h with a goat anti-rabbit IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology). Western blots were visualized using enhanced chemiluminescence advanced reagents (Amersham, Arlington Heights, IL) as per manufacturer's protocol.

### In Vitro Luciferase Assay

Cultured HepG2 cells were transiently transfected with a mouse CAR (mCAR) expression plasmid (mCAR/pCR3.0) (Sueyoshi et al., 1999) and a *CYP2B6*-tk-luciferase construct. The human *CYP2B6* promoter luciferase reporter construct was obtained from Dr. Richard Kim (Vanderbilt University School of Medicine, Nashville, TN). The *CYP2B6* promoter luciferase reporter construct contains a 1.7-kilobase fragment that maintains the core promoter (+39/–364) and the distal enhancer region (–1461/–2013) including the PB responsive element (PBREM) (R. Kim, unpublished data).

Briefly, HepG2 cells were cultured in Eagle's minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) according to the protocol provided by American Type Culture Collection (Manassas, VA). In 24-well plates, the *CYP2B6*-tk-luciferase plasmid (0.1 µg) was cotransfected with pRL-tk transfection control (0.001 µg) (Promega Corp., Madison, WI) into HepG2 cells using Transfectene (Qiagen, Hilden, Germany) with or without a mouse CAR expression plasmid (0.025 µg). Twenty-four hours after transfection, cells were cultured in the presence of dimethyl sulfoxide (DMSO; 0.2%), PB (1 mM), TCPOBOP (250 nM), or OPZ (5, 20, 50, 100 µM) with or without androstanol (10 µM). After 24 h of exposure, the media were removed and cells were lysed with 100 µl of passive lysis buffer. Luciferase activity was determined by the Dual-Glo Luciferase Assay per manufacturer's protocol (Promega Corp.).

## Statistical Analysis

Data are expressed as mean  $\pm$  S.E. For multiple comparisons, an analysis of variance was performed followed by Duncan's multiple range test. The level of significance was set at  $p$  0.05.

## Results

### Induction of Cyp2b10 and Nqo1 Expression in C57BL/6 Mice

Nqo1 mRNA levels were significantly increased by OPZ (22-fold) but were not induced by administration of PB (Fig. 1). However, both PB and OPZ treatment resulted in robust increases in mRNA expression of Cyp2b10 compared with control.

### Dose Response of Induction of Nqo1 and Cyp2b10 to OPZ Treatment

The increase of Nqo1 mRNA levels by OPZ (Fig. 2) reached significance at a dose of 100 mg/kg and continued to increase through the higher doses, reaching an induction of 6-fold by 250 mg/kg. Although Cyp2b10 induction by OPZ did not reach significance until 200 mg/kg, the induction was more profound, with 47- and 74-fold increases at 200 and 250 mg/kg.

### Induction of Cyp2b10 and Nqo1 Expression in Nrf2- and CAR-Deficient Mice

To determine the involvement of Nrf2 and CAR in the induction of Cyp2b10 and Nqo1, Nrf2- and CAR-null mice were used. As an indication of Nrf2-mediated induction, treatment of wild-type mice with PB and OPZ resulted in 2.5- and 3.6-fold increases in Nqo1 mRNA, respectively (Fig. 3). This induction of Nqo1 was ablated in the Nrf2<sup>-/-</sup> animals, as has been previously noted for OPZ (Kwak et al., 2001). The absence of Nrf2 had no effect on the induction of Cyp2b10 mRNA, as the robust induction by PB and OPZ was maintained in Nrf2<sup>-/-</sup> animals.

Treatment of wild-type mice with PB or OPZ resulted in a greater than 30- and 10-fold increase in Cyp2b10 mRNA, respectively (Fig. 4). This robust induction was completely absent in the CAR<sup>-/-</sup> animals treated with PB and OPZ, as has been previously noted for PB (Wei et al., 2000). In contrast, the induction of Nqo1 mRNA was maintained in CAR<sup>-/-</sup> animals.

### Real-Time in Vivo Activation of the Human CYP2B6 Promoter in Mice

The ability of OPZ to activate transcription of the human *CYP2B6* promoter containing the PBREM was determined by transfecting a human *CYP2B6* promoter-reporter luciferase reporter via rapid i.v. hydrodynamic injection via the tail vein and a real-time in vivo imaging assay before and after dosing. OPZ treatment resulted in robust activation of the human *CYP2B6* promoter as seen in Fig. 5.

### Nuclear Accumulation of CAR in PB- and OPZ-Treated Mice

To determine the ability of OPZ to induce nuclear accumulation of CAR, C57BL/6 mice were treated with corn oil (5 ml/kg), PB (80 mg/kg), and OPZ (150 mg/kg). Western blot analysis of nuclear extracts shows an increase in CAR nuclear accumulation in both PB- and OPZ-treated animals (Fig. 6).

### Ability of OPZ to Overcome Androstanol Inhibition of CAR

Cotransfection of HepG2 cells with a mouse *CAR* expression construct and *CYP2B6* reporter gene containing the PBREM resulted in a 5-fold increase in luciferase expression (Fig. 7). Known mCAR activity modulators and ligands androstanol and TCPOBOP were

used to further investigate the mechanisms of CAR activation. As expected, treatment with the mCAR inhibitor androstanol repressed transcriptional activation of the *CYP2B6* reporter. In contrast, cotreatment with the direct CAR activator TCPOBOP was sufficient to overcome this inhibition. Neither PB nor OPZ was able to overcome androstanol repression of CAR at the doses examined.

## Discussion

OPZ was recognized as a microsomal enzyme inducer over 25 years ago, with apparent 4- to 6-fold increases in the activity of liver GSTs in mice treated with the drug (Ansher et al., 1983). The induction of GST and other cytoprotective enzymes has been termed the “antioxidant response” (Jaiswal, 1994) and is central to the proposed mechanism of action of OPZ in cancer prevention. The antioxidant response is an important mechanism for cellular defense and is mediated by the Nrf2/ARE pathway, resulting in the induction of several chemoprotective genes, including *NQO1*, *HO-1*, and *GSTA1* and *GSTA2* (Kensler et al., 1985). It is important to note that the induction of NQO1 by OPZ has been shown to be dependent on Nrf2 activation (Ramos-Gomez et al., 2001). Our current data showing the loss of Nqo1 induction in Nrf2<sup>-/-</sup> mice support that finding.

Although the induction of these genes has been shown to be ARE/EpRE-dependent, OPZ is also capable of inducing other genes in an Nrf2-independent manner (Le Ferrec et al., 2002; Ebert et al., 2005). For example, *CYP2B* is induced by OPZ yet is considered a hallmark gene of CAR activation. The mechanism of induction of *CYP2B* isoforms has been well characterized, and currently no ARE/EpRE consensus sequences have been identified in their 5′-flanking regions. Consistent with these facts, our data show that OPZ activates gene transcription through activation of multiple and distinct transcription factors, including Nrf2 and CAR.

To analyze the role of both CAR and Nrf2 in OPZ gene induction, CAR- or Nrf2-deficient mice were treated with OPZ or the known CAR activator PB. Our data indicate that Nrf2 and CAR are required in the regulation of gene induction by OPZ. As expected, the loss of Nrf2 resulted in loss of Nqo1 induction, whereas induction of Cyp2b10 mRNA was unaffected. These results both confirm a role for Nrf2 in Nqo1 regulation and suggest an Nrf2-independent mechanism for the induction of Cyp2b10 mRNA by OPZ. The complete abrogation of Cyp2b10 mRNA induction in CAR-null mice treated with OPZ strongly implicates CAR as the regulatory mechanism. These data agree with our previously published data, which revealed that loss of the obligatory CAR-binding partner RXR $\alpha$  abrogates OPZ induction of Cyp2B (Cherrington et al., 2003).

Two additional lines of evidence in this study characterize OPZ as a CAR activator. First, OPZ treatment results in transcriptional activation of a human *CYP2B6* promoter construct in transiently transgenic mice. The promoter region of this construct contains the PBREM, the well characterized CAR DNA-binding sequence. The evident induction of this reporter gene in OPZ-treated mice compared with corn oil controls shows the ability of OPZ to induce CAR-driven genes in vivo. Second, OPZ induces CAR nuclear accumulation in hepatocytes of OPZ-treated mice. Because the main component of CAR activation appears to be nuclear translocation (Kobayashi et al., 2003), these findings firmly establish OPZ as a CAR activator. Interestingly, the OPZ dose used in these in vivo studies did not cause maximal induction of the CAR target gene *Cyp2b10* (Fig. 2). This indicates that CAR may have not been fully activated at that dose. The increase in CAR activity by OPZ treatment seen even at this lower dose helps to support OPZ as a CAR activator.

In vivo, CAR can be activated either directly by ligand binding (as in the case of TCPOBOP) or indirectly through the activation of currently unknown pathways (as with PB) (Stanley et al., 2006). In cultured cells lines, however, transfected CAR freely translocates to the nucleus without exposure to CAR activators (Kobayashi et al., 2003). This is apparent in our study by the increase in *CYP2B6*-promoter driven activity of the luciferase reporter gene on the addition of mCAR. The treatment of these cells with the inverse agonist androstanol repressed the activity of mCAR and the transcriptional activation of the luciferase reporter gene. Androstanol is a CAR ligand, and binding results in a conformational change in CAR that inhibits the recruitment of the transcriptional machinery (Wright et al., 2007). The ability of TCPOBOP to overcome this inhibition has been previously shown and results from the direct interaction of TCPOBOP with CAR, which both displaces androstanol and promotes the recruitment of the transcriptional machinery (Sueyoshi et al., 1999; Tzamelis et al., 2000). In the current study, TCPOBOP treatment, but not PB or OPZ treatment, was able to reverse the inhibitory effects of androstanol. Although OPZ cotreatment resulted in a slight increase in luciferase activity, this increase never reached significance ( $p > 0.05$ ). Although these findings suggest that OPZ acts similarly to PB to indirectly activate CAR, further investigation of any OPZ-CAR interactions is necessary to definitively rule out OPZ as a CAR ligand.

The mechanism by which PB indirectly activates CAR remains to be elucidated, although the involvement of a number of kinases and phosphatases has been proposed. The involvement of the extracellular signal-regulated kinase (ERK) in the repression of CAR nuclear translocation is of particular interest. Previous work has shown that epidermal growth factor is able to attenuate CAR-mediated induction of *CYP2B* by PB treatment (Bauer et al., 2004). Inhibitors of mitogen-activated protein kinase kinase 1/2 (a kinase downstream of epidermal growth factor signaling) have been shown to potentiate the induction *CYP2B* by PB (Joannard et al., 2006). Most recently, ERK1/2 de-phosphorylation (deactivation) was shown to be associated with CAR nuclear translocation (Koike et al., 2007). Our findings that OPZ activates CAR may provide additional support to the importance of ERK1/2 in the regulation of CAR. OPZ has been shown to inhibit the constitutive phosphorylation (activation) of ERK1/2 and has been shown to inhibit the activity of mitogen-activated protein kinase kinase 1 (Kang et al., 2003). Whether this inhibition of ERK signaling by OPZ is responsible for the activation of CAR is unclear and under investigation.

The data presented in this manuscript show that OPZ modulates gene expression through CAR activation, in addition to Nrf2. These results add to previous findings on the dual activation of Nrf2 and CAR. Trans-stilbene oxide and diallyl sulfide have both been identified as activators of both CAR and Nrf2 (Slitt et al., 2006; Fisher et al., 2007). The addition of yet another structurally diverse compound to this group raises the possibility of cross-talk between the two factors, although further studies are needed to determine the extent of any connections.

## Acknowledgments

This work was supported by National Institutes of Health Grants ES011646 and ES006694 (to N.J.C.), ES09716 and ES09649 (to C.D.K.), and DK46546 (to D.D.M.).

## ABBREVIATIONS

<b>OPZ</b>	oltipraz
<b>GST</b>	glutathione-S-transferase

<b>NQO1, NAD(P)H</b>	quinone oxidoreductase
<b>Nrf2</b>	nuclear factor E2-related factor 2
<b>ARE/EpRE</b>	antioxidant response element/electrophile response element
<b>Keap1</b>	Kelch-like ECH-associated protein 1
<b>CAR</b>	constitutive androstane receptor
<b>TCPOBOP</b>	1,4-bis-[2-(3, 5-dichloropyridyloxy)] benzene
<b>CITCO</b>	6-(4-chlorophenyl)imidazo[2,1- <i>b</i> ][1,3]thiazole-5-carbalde-hyde <i>O</i> -(3,4-dichlorobenzyl)oxime
<b>PB</b>	phenobarbital
<b>RXR</b>	retinoid X receptor
<b>bdNA</b>	branched DNA
<b>mCAR</b>	mouse constitutive androstane receptor
<b>PBREM</b>	phenobarbital responsive element
<b>DMSO</b>	dimethyl sulfoxide
<b>ERK</b>	extracellular signal-regulated kinase

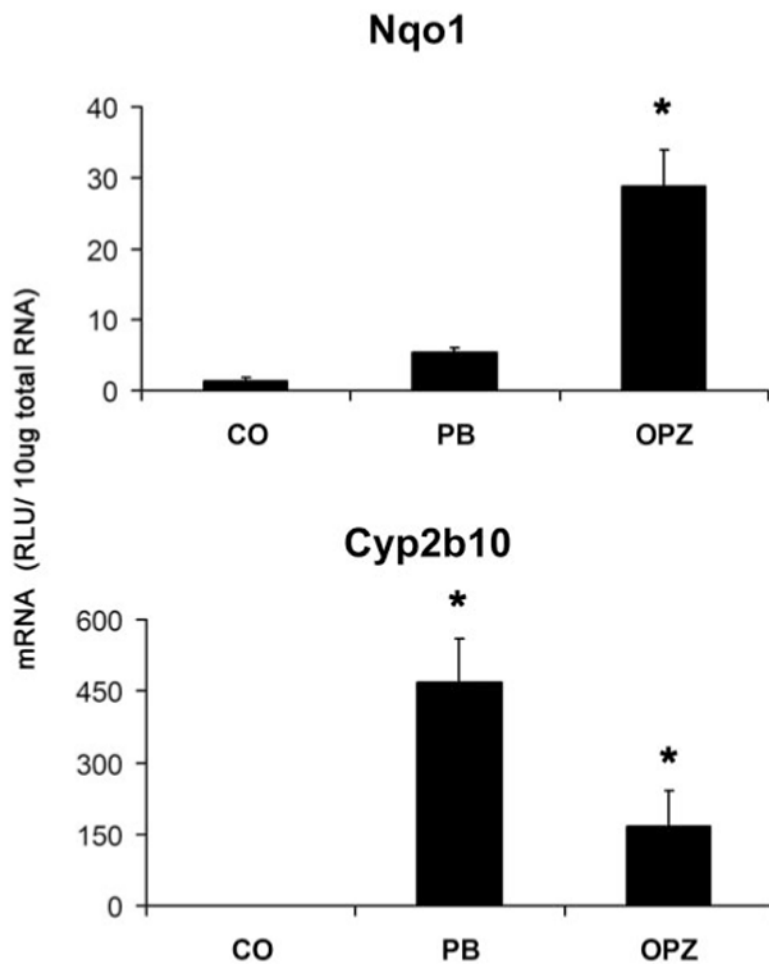
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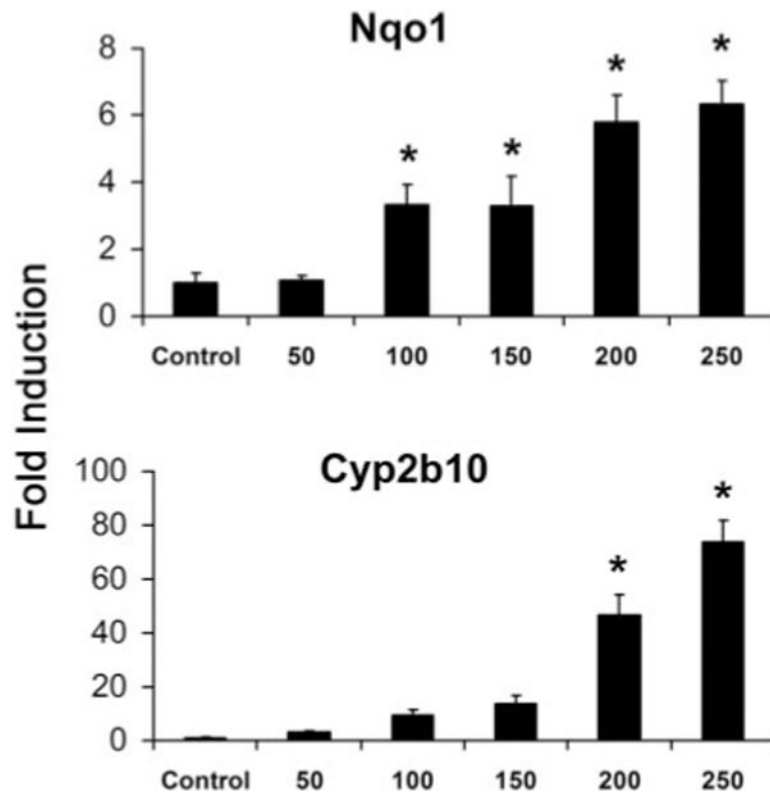


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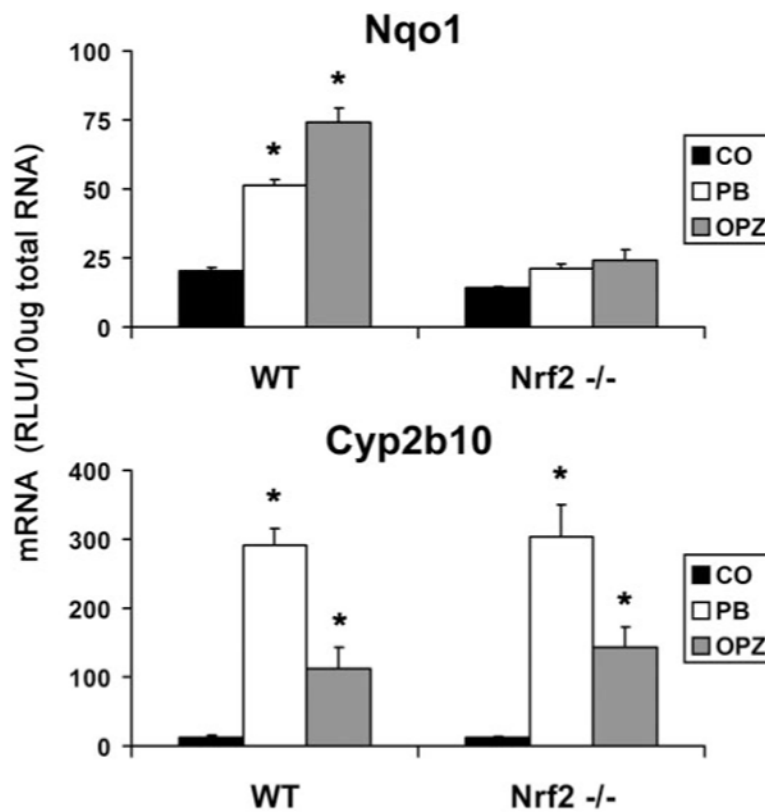
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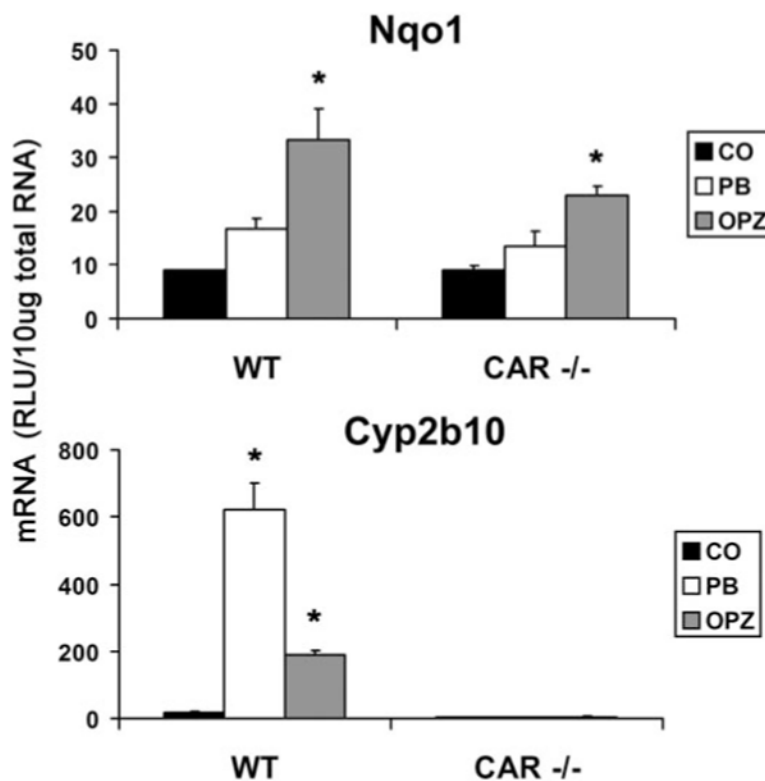
**Fig. 1.** Nqo1 and Cyp2b10 mRNA induction. C57BL/6 mice were treated with corn oil, PB (80 mg/kg), or OPZ (150 mg/kg). Total RNA was isolated, and mRNA levels were analyzed by the bDNA signal amplification assay. mRNA levels are expressed as mean relative light units (RLU)/10  $\mu$ g total RNA  $\pm$  S.E.M. ( $n = 3$ ); \*, significantly different from vehicle ( $p < 0.05$ ).



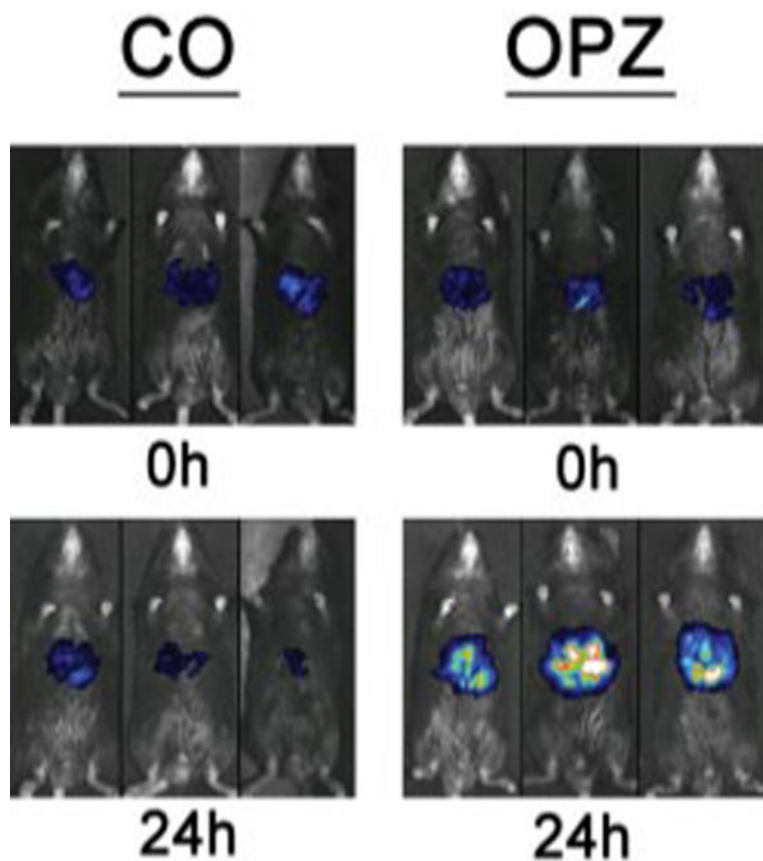
**Fig. 2.** Dose response of induction of Nqo1 and Cyp2b10 to OPZ treatment. Total RNA was isolated from adult male C57BL/6 mice after treatment of OPZ (50, 100, 150, 200, 250 mg/kg, i.p., once daily, 4 days). The mRNA levels of Cyp2b10 and Nqo1 were determined by the bDNA signal amplification assay. The data are presented as mean -fold induction  $\pm$  S.E.M. ( $n = 5-6$ ); \*, significantly different from vehicle ( $p < 0.05$ ).



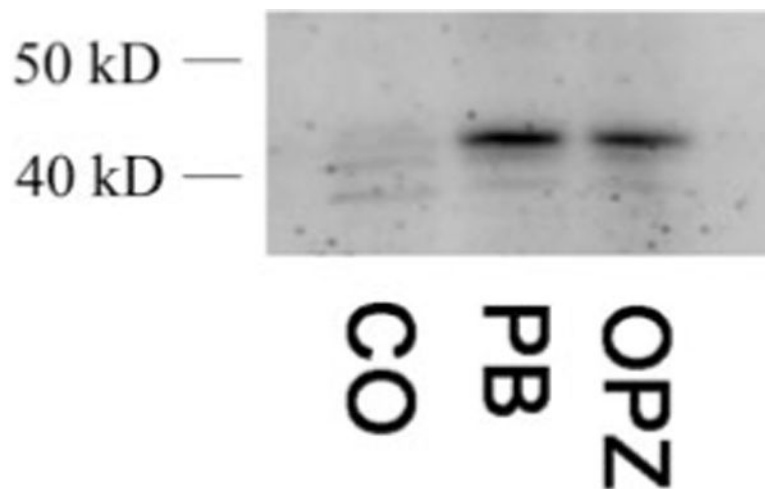
**Fig. 3.** Induction of Cyp2b10 and Nqo1 mRNA in wild-type and Nrf2-null mice. Total RNA was isolated from wild-type and Nrf2<sup>-/-</sup> mice treated with OPZ (150 mg/kg) and PB (80 mg/kg) and analyzed by the bDNA signal amplification assay. mRNA levels are expressed as mean relative light units (RLU)/10  $\mu$ g total RNA  $\pm$  S.E.M. ( $n = 5$ ); \*, significantly different from vehicle ( $p < 0.05$ ).



**Fig. 4.** Induction of Cyp2b10 and Nqo1 mRNA in wild-type and CAR-null mice. Total RNA was isolated from wild-type and CAR<sup>-/-</sup> mice treated with OPZ (150 mg/kg) and PB (80 mg/kg) and analyzed by the bDNA signal amplification assay. mRNA levels are expressed as mean relative light units (RLU)/10  $\mu$ g total RNA  $\pm$  S.E.M. ( $n = 5$ ); \*, significantly different from vehicle ( $p < 0.05$ ).

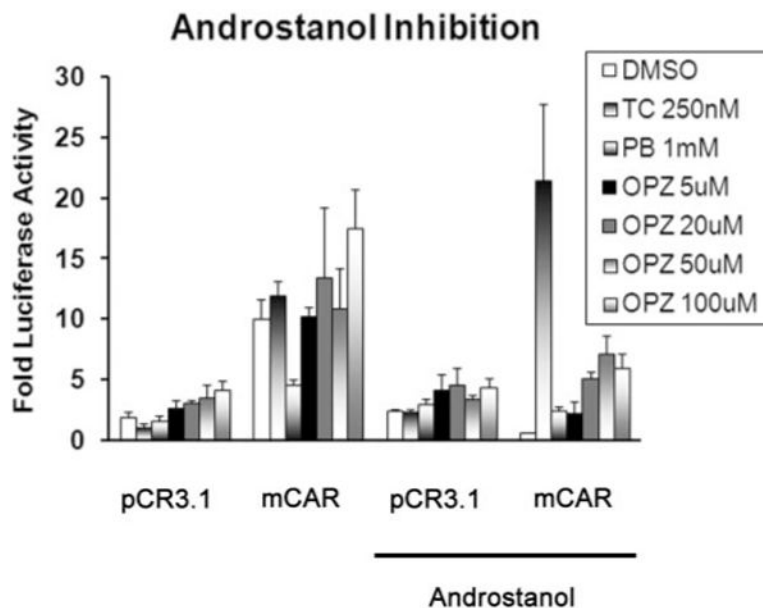


**Fig. 5.** In vivo activation of the human *CYP2B6* promoter-luciferase reporter construct in mouse liver after vehicle or OPZ administration. Male C57Bl/6 mice were injected with 10  $\mu$ g of naked plasmid DNA in a rapid (5-s) tail vein injection in sterile saline in a volume equal to 10% of body weight. Following a 16-h recovery period, animals were anesthetized with a solution of ketamine (72 mg/kg) and xylazine (6 mg/kg) and injected with luciferin i.p. At time 0, mice were injected with corn oil vehicle (5 ml/kg, i.p.) or OPZ (150 mg/kg). Images were collected at 0 and 24 h following vehicle or OPZ administration.



**Fig. 6.** CAR nuclear accumulation. C57Bl/6 mice were treated with corn oil, PB (80 mg/kg), or OPZ (150 mg/kg). Nuclear extracts were immunoblotted to detect the presence of CAR.



**Fig. 7.**

Activation of the *CYP2B6*-tk-luciferase reporter construct in HepG2 cells after treatment with TCPOBOP, PB, and OPZ. HepG2 cells were transiently transfected with the *CYP2B6*-tk-luciferase plasmid (0.1  $\mu$ g) and pRL-TK (0.001  $\mu$ g) into HepG2 cells, with or without a mouse *CAR* expression plasmid (0.25  $\mu$ g). Twenty-four hours after transfection, the cells were treated with DMSO, TCPOBOP (TC, 250 nm), PB (1 mM), or OPZ (5, 20, 50, 100  $\mu$ m) in the presence or absence of 10  $\mu$ M 5 $\alpha$ -androstan-3 $\alpha$ -ol. Twenty-four hours after treatment the cell lysates were collected, and luciferase activity was determined by the Dual-Glo Luciferase Assay (Promega Corp.). The data are presented as the mean -fold activation + S.E.M. ( $n = 3$  wells/treatment); \*, significantly different from DMSO control ( $p < 0.05$ ).