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Ortho-aminoazotoluene activates mouse constitutive androstane receptor (mCAR) and increases expression of mCAR target genes

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ABSTRACT

2′-3-dimethyl-4-aminoazobenzene (ortho-aminoazotoluene, OAT) is an azo dye and a rodent carcinogen that has been evaluated by the International Agency for Research on Cancer (IARC) as a possible (class 2B) human carcinogen. Its mechanism of action remains unclear. We examined the role of the xenobiotic receptor Constitutive Androstane Receptor (CAR, NR1I3) as a mediator of the effects of OAT. We found that OAT increases mouse CAR (mCAR) transactivation in a dose-dependent manner. This effect is specific because another closely related azo dye, 3′-methyl-4-dimethyl-aminoazobenzene (3′MeDAB), did not activate mCAR. Real-time Q-PCR analysis in wild-type C57BL/6 mice revealed that OAT induces the hepatic mRNA expression of the following CAR target genes: *Cyp2b10*, *Cyp2c29*, *Cyp3a11*, *Ugt1a1*, *Mrp4*, *Mrp2* and *c-Myc*. CAR-null ($Car^{-/-}$) mice showed no increased expression of these genes following OAT treatment, demonstrating that CAR is required for their OAT dependent induction. The OAT-induced CAR-dependent increase of *Cyp2b10* and *c-Myc* expression was confirmed by Western blotting. Immunohistochemistry analysis of wild-type and $Car^{-/-}$ livers showed that OAT did not acutely induce hepatocyte proliferation, but at much later time points showed an unexpected CAR-dependent proliferative response. These studies demonstrate that mCAR is an OAT xenosensor, and indicate that at least some of the biological effects of this compound are mediated by this nuclear receptor.

Introduction

Ortho-Aminoazotoluene (OAT, 2′-3-dimethyl-4-aminoazobenzene, 2-amino-5-azotoluene; also known as Solvent yellow 3, Garnet G base and Fast Garnet GB base) is an anthropogenic compound used for coloring oils, fats, and waxes (National Toxicology Program, 2002), as well as for pharmaceutical purposes. It is also used as a synthetic intermediate in the production of other dyes (HSDB) (2001). In 1994 estimates, the world production of azo dyes was around 0.5 million tons (Stolz, 2001; Pandey et al., 2007). OAT and other azo dyes can be released to the environment in wastewater and other emissions during the manufacturing process. Historically, OAT was the first model of experimental chemical carcinogenesis as demonstrated by Yoshida in the 1930's (Cook, 1947). Since that time, a large amount of data has confirmed the carcinogenic activity of azo dyes. Specifically, OAT is known as a mouse carcinogen (Kaledin et al., 1978; Kaledin et al., 1985), and has been evaluated by the International Agency for Research on Cancer (IARC) as a possible (class 2B) human carcinogen ((IARC) (1975); National Toxicology Program, 2002). Despite more than a half a century of studies on OAT induced carcinogenesis, its mechanism of action is still unknown.

Research interest in azo dyes has focused on significant species- and strain-specific differences in response to the carcinogenic activity of these compounds (Merkulova et al., 2005). This allows OAT to be used as a tool to study the mechanisms of genetic predisposition to the tumor development. OAT is associated with a high incidence of liver tumors in a number of mouse strains (CBA, SWR, DBA/2, A/He, and DD) but has little effect in other strains (AKR and CC57Br) and in rats (Kaledin and Zakharova, 1984; Kaledin et al., 1990; Zacharova et al., 2003). On the other hand, the structural analog of OAT (the isomeric DAB) 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB), which is hepatocarcinogenic to rats, rarely causes tumors in mice

Abbreviations: OAT, *Ortho*-Aminoazotoluene 2′-3-dimethyl-4-aminoazobenzene; 3′-MeDAB, 3′-methyl-4-dimethylaminoazobenzene; CAR, Constitutive Androstane Receptor; CYP450s, Cytochromes P450; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene; BrdU, bromodeoxyuridine.

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(Merkulova et al., 2005). Although the mechanisms of hepatocarcinogenicity of these compounds remain unclear, it is known that they can activate drug metabolism, including both phase I xenobiotic-metabolizing enzymes (CYP450s) and phase II enzymes (such as UGTs and SULTs) and transporters (Mikhailova et al., 2005). Xenobiotic-inducible expression of hepatic biotransformation enzymes and transporters is regulated by the xenobiotic-responsive transcription factors Aryl-hydrocarbon receptor (Ah-R), Pregnane X Receptor (PXR), Constitutive Androstane Receptor (CAR), and others (Xu et al., 2005; Stanley et al., 2006; Pascussi et al., 2008).

We have previously shown that one of these receptors, CAR, is activated strongly by OAT and much more weakly by 3′-MeDAB in mice (Pakharukova et al., 2007). Thus, CAR DNA-binding activity is induced 3-fold in nuclear extracts from the mouse liver after OAT administration and far less so after 3′-MeDAB administration. CAR (NR1I3) is known as a 'xenobiotic sensor', and is a member of the nuclear hormone receptor superfamily (Choi et al., 1997). CAR activity can be modulated by a broad array of structurally diverse compounds (Chang and Waxman, 2006). Our previous results also demonstrated that OAT competed with the known ligand androstenol in a CAR binding assay using cytosol isolated from mouse liver cells. Based on these observations, we hypothesized that OAT could activate mouse CAR and, that at least some of its effects would be mediated by this nuclear receptor.

In the current study, we investigated the effects of OAT and 3′ MeDAB on the activity of mCAR using transient transfections with an appropriate reporter system. Studies in WT ($Car^{+}/^{+}$) and $Car^{-}/^{-}$ mice examined the effect of OAT and 3′-MeDAB on the expression of CAR target genes. In order to determine a possible role of CAR in the development of liver tumors caused by OAT, we explored hepatocyte proliferation at both relatively short times and much longer times after OAT treatment of WT and CAR KO mice. We observed that OAT specifically activated CAR target genes in the short term, but did not induce hepatocyte proliferation. However, OAT did induce CAR-dependent hepatocyte proliferation at much later times. Overall, these studies demonstrate that CAR mediates biological effects of OAT.

Materials and methods

Caution. Azo dyes used in this study are toxic and should be handled with care.

Chemicals. Fast Garnet GBC base (*Ortho*-Aminoazotoluene/OAT), (97% purity) (CAS number 97-56-3) and 1,4-*bis*-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) (CAS number 76150-91-9) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). 3′-Methyl-4-dimethylaminoazobenzene (98%) (CAS number 55-80-1) was purchased from ABCR GmbH&Co.KG (Germany). 5α-Androstan-3α-ol (CAS number 1224-92-6) was purchased from Steraloids Inc. (Newport, RI). Solvents were obtained from Fisher Scientific (Houston, TX) unless otherwise noted.

Transactivation assay. The mouse CAR (mCAR) expression plasmid was described previously in Forman et al., 1998, and the CAR reporter (LXRE-TK-Luc reporter plasmid) was described in Tzameli et al., 2000. All the procedures were carried out according to Hernandez et al., 2007. Briefly, transactivation assays were performed in HepG2 human hepatoma cells (ATCC, Rockville, MD) cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin (Invitrogen), under 5% CO2 at 37 °C. Cells were plated in 24-well plates at 1×10^5 cells per well. Twenty-four hours after plating, cells were transfected with 200 ng of CAR expression vector (pCMX-mCAR) or the pCMX vector alone and cotransfected with 200 ng of LXRE-TK-Luc reporter using "lipofectamine 2000" reagent as per company protocol (GIBCO BRL). 100 ng of pCMX-β-galactosidase (Promega, Madison, WI) was cotransfected to normalize transfection efficiency. Twelve hours following transfection, cells were treated with different concentrations of OAT or 3′-MeDAB in combination with 10 μM androstanol (CAR's inverse agonist which represses its constitutive activity and increases the sensitivity of the assay (Forman et al., 1998)) or vehicle control, and incubated for 12 h. 0.1% DMSO was used as a vehicle for all chemicals and 500 nM TCPOBOP was used as a positive control for CAR activation. Subsequently, cell lysates were assayed for firefly luciferase activities and normalized to β-galactosidase activities using the Dual-Luciferase reporter assay system (Promega, Madison, WI-not promega) according to the manufacturer's instructions. All experiments were done in quadruplicate, and the data is presented as mean ± S.D from two to three separate assays.

Animals. All experiments were done upon approval of the protocol by the Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC). Mice were maintained in a pathogen-free Baylor College of Medicine Transgenic Mice Facility under a constant 12-h light/dark cycle and fed standard rodent chow and water ad libitum. Wild type (WT) C57BL/6 mice (sensitive to hepatic tumor induction with OAT (Zacharova et al., 2003)) and CAR KO ($Car^{-}/^{-}$) mice (Wei et al., 2000) (>10 backcrosses to C57BL/6 background) were used in the experiments.

Animal treatment. For gene expression studies, eight- to ten- week old CAR KO and WT male mice (three per group) were injected with OAT or 3′-MeDAB (225 mg/kg of body weight) intraperitoneally (i.p.). Controls received an equivalent amount of the vehicle (corn oil). TCPOBOP (at a dosage of 3 mg/kg body weight, i.p.) was used for the positive and negative controls in WT and CAR KO mice respectively. Animals were euthanized by cervical dislocation 3 h (for RNA extraction) and 6 h (for protein preparation) after dosing. Livers were removed, diced into several pieces and stored at −80 °C.

For the first set of cell proliferation studies, eight- to ten- week old CAR KO and WT male mice (five per group) were administered by a single i.p. injection of OAT at a dose of 225 mg/kg of body weight. Controls (three mice per group) received an equivalent amount of the vehicle (corn oil). Animals were euthanized by cervical dislocation 3 days and 1 week after dosing. Immediately after death, mice were weighed and the livers were excised and weighed. The liver index was determined by dividing the liver weight by the weight of the mouse. Liver sections were fixed in Histochoice MB Fixative (histology grade, Electron Microscopy Sciences, Hatfield, PA) and processed for immunohistochemistry.

For the second set of cell proliferation studies, thirteen-days old CAR KO and WT male mice (nine per group) were administered by a single i.p. injection of OAT at a dose of 225 mg/kg of body weight. According to previous publications, suckling mice were more sensitive to the hepatocarcinogenic effect of OAT compared to adult animals (Il'nitskaya et al., 2004). Controls (also thirteen-days old CAR KO and WT male mice, three per group) received an equivalent amount of the vehicle (corn oil). 7 months after the administrations, bromodeoxyuridine (BrdU, 50 mg/kg, Sigma Chemical Co., St. Louis, MO), which intercalates into double stranded DNA during replication thus identifying the cells in S-phase, was administered intraperitoneally 2 h before sacrificing the animals. Preparation and processing of the liver sections was as in the first set of cell proliferation studies.

Quantitative real-time polymerase chain reaction (Q-PCR). To analyze messenger RNA (mRNA) levels, total RNA was isolated from homogenized livers using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm. RNA integrity was confirmed by visualization of intact 18S and 28S rRNA under UV light. Then the cDNAs were synthesized using the SuperScript™ III RT (Invitrogen, Carlsbad, CA) in accordance with manufacturer's instructions. Real-time quantitative PCR (RTQ-PCR)