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Constitutive active/androstane receptor, peroxisome proliferator-activated receptor α, and cytotoxicity are involved in oxadiazon-induced liver tumor development in mice

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ABSTRACT

Oxadiazon (OX) is a protoporphyrinogen oxidase-inhibiting herbicide that induces porphyria and liver tumors in rodents. Although porphyria is generally considered to be a risk factor for liver tumor development, the mechanisms through which OX mediates tumor development are unclear. Therefore, in this study, we investigated the mechanisms of tumor development by focusing on constitutive active/androstane receptor (CAR), which is essential for the development of tumors in response to several chemicals. After 1, 4, or 13 weeks of dietary treatment with 1000 ppm OX, hepatic *Cyp2b10* expression was induced in wild-type (WT) mice. However, this effect was blocked in CAR-knockout (CARKO) mice. Hepatic *Cyp4a10* expression, indicative of peroxisome proliferator-activated receptor α (PPARα) activation, and cytotoxic changes in hepatocytes were also observed in both groups of mice. After initiation by diethylnitrosamine, 26-week treatment with OX resulted in an increase in proliferative lesions, including foci and adenomas, in both genotypes, and the incidence and multiplicity of proliferative lesions in CARKO mice were higher than those in control mice but lower than those in WT mice. These results suggested that CAR, PPARα activation, and cytotoxicity were involved in the development of liver tumors. Moreover, porphyrin was not apparently involved in OX-induced tumor development.

1. Introduction

There are eight enzymes in the heme biosynthetic pathway; defects in any of these enzymes can lead to some form of porphyria. One of these enzymes, protoporphyrinogen oxidase (PROTOX), oxidizes protoporphyrinogen to protoporphyrin IX (PPIX) in the mitochondria. Variegate porphyria (VP) is a rare genetic metabolic disorder in humans that is characterized by deficient PROTOX function. PROTOX deficiency leads to translocation of protoporphyrinogen from the mitochondria to the cytoplasm in the liver. Protoporphyrinogen is then autoxidized and accumulates in the form of PPIX in the cytoplasm. PPIX is finally excreted in the bile and stool (Singal and Anderson, 2013). In patients with porphyria, including VP, chronic liver abnormalities, particularly mild elevation of serum transaminases, are common and increase the risk for development of hepatocellular carcinoma (Sardh et al., 2013; Singal and Anderson, 2013).

Oxadiazon (OX), an N-phenyl heterocyclic compound, is a photobleaching herbicide that acts by inhibiting PROTOX in plants (Dayan et al., 1997). Accumulation of PPIX following PROTOX inhibition induce photo-oxygenation in the presence of light. OX also induces porphyrin accumulation by inhibiting PROTOX activity in

Abbreviations: AhR, aryl hydrocarbon receptor; ALT, alanine transferase; CAR, constitutive active/androstane receptor; CARKO, CAR knockout; DEN, diethylnitrosamine; MOA, mode of action; OX, oxadiazon; PB, phenobarbital; PCNA, proliferating cell nuclear antigen; PPARα, peroxisome proliferator-activated receptor alpha; PPIX, protoporphyrin IX; PROTOX, protoporphyrinogen oxidase; PXR, pregnane X receptor; VP, variegate porphyria; WT, wild type.

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the rodent liver (Krijt et al., 1993; Matringe et al., 1989). The induction of porphyria has been shown to be associated with rodent hepatocellular carcinoma (Smith et al., 1993). Furthermore, porphyria-mediated tumor development is induced through cytotoxicity, which is thought to be relevant for evaluating human cancer risk (Holsapple et al., 2006). In a previous study, OX was shown to induce cytotoxicity and subsequent liver tumors in combined long-term toxicity/carcinogenicity studies in both rats and mice (Pesticide evaluation report; available in Japanese at http://www.fsc.go.jp/fsciis/evaluationDocument/show/kya20080111001).

Peroxisome proliferator-activated receptor α (PPARα) agonists induce hepatocellular hypertrophy and liver tumors in rodents (Boobis et al., 2006; Klaunig et al., 2003). OX has been reported to activate PPRAα (Richert et al., 1996), and the carcinogenicity of OX in rodents is thought to be mediated by PPARα. Analysis of the mode of action (MOA) in rodents revealed that PPARα agonist-induced liver tumors are not relevant for human risk assessment because activation of PPARα does not lead to increased hepatocellular proliferation in humans or primary human hepatocytes (Lake, 2009).

In addition to PPARα, constitutive androstane/active receptor (CAR) is one of the primary nuclear receptors involved in rodent liver carcinogenicity. Yamamoto et al. provided clear evidence that CAR mediated liver tumor induction by phenobarbital (PB) (Yamamoto et al., 2004). Moreover, our recent studies using CAR-knockout (CARKO) mice suggested that CAR is a key player in hepatocarcinogenicity induced by types of chemicals other than PB, such as piperonyl butoxide and trirazole fungicides (Sakamoto et al., 2013; Tamura et al., 2015). Although no direct evidence has supported a relationship between OX and CAR, the PROTOX inhibitor oxyfluorfen has been reported to activate CAR (Stagg et al., 2012). This result suggested that CAR may also be involved in OX-induced liver tumor development in rodents. In a recent study examining the relevance of CAR-mediated hepatocarcinogenesis in humans using chimeric mice, PB-induced rodent liver tumors developed through CAR were not associated with the risk of liver tumor development in humans (Elcombe et al., 2014). However, several reports showed contradictory results (Braeuning, 2014a; Braeuning et al., 2014b; Tokita et al., 2006). Therefore, risk assessment is necessary to determine whether a test substance is involved in CAR-mediated hepatocarcinogenesis in rodents.

In the present study, we aimed to clarify the mechanisms through which OX induces tumor development and to evaluate its relevance in humans. To this end, we investigated the relationships among cytotoxicity and activation of PPARα and CAR. Moreover, we also used CARKO mice to detect CAR activation by OX.

2. Materials and methods

2.1. Chemicals

OX (CAS No. 19666-30-9, purity 99.8%) and diethylnitrosamine (DEN; CAS No. 55-18-5, purity 99.9%) were obtained from WAKO Pure Chemicals Industries, Ltd. (Osaka, Japan) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively

2.2. Animals

Male CARKO mice (Kodama et al., 2004; Yamamoto et al., 2004) in the C3H/HeNCrl background were provided by courtesy of Dr. Masahiko Negishi (National Institute of Environmental Health Sciences) and bred at the National Institute of Health Sciences (NIHS). C3H/HeNCrlCrlj mice were purchased at 5 weeks of age (Charles River Laboratories Japan Inc., Kanagawa, Japan) and served as wild-type (WT) mice. All experimental animal protocols were approved by the Animal Care and Utilization Committee of NIHS and were carried out following NIHS guidelines for the care and use of laboratory animals. All experimenters treating genetically modified animals were educated by the Gene Recombination Experiment Safety Committee of NIHS.

2.3. Study design

Experiment 1 (Exp. 1) was performed to investigate the time-dependent effects of OX in the liver and the role of CAR on this effect during short-term OX treatment. Six-week-old male WT and CARKO mice were allocated to the 1-, 4-, or 13-week treatment group (5 animals/group) according to body weight. All treated mice were given 1000 ppm OX in the basal diet (CRF-1; Oriental Yeast, Co. Ltd, Tokyo, Japan) from 6 weeks of age. The time points of the experiment were based on our previous study (Maeda et al., 2015). The dose 1000 ppm was chosen as the hepatocarcinogenic dose of OX based on liver tumor induction in mice; this does of OX significantly increased the incidence of hepatocellular adenoma and carcinoma in combined long-term toxicity/carcinogenicity studies in mice (Pesticide evaluation report; available in Japanese at http://www.fsc.go.jp/fsciis/evaluationDocument/show/kya20080111001). In control groups, WT and CARKO mice were fed a basal diet ad libitum in the same manner as in the treated groups for 1, 4, or 13 weeks. For plasma PPIX measurement, 6-week-old male WT and CARKO mice (5 animals/group) were administered 1000 ppm OX in the basal diet for 4 weeks, and blood samples were obtained

Experiment 2 (Exp. 2) was performed to clarify the dose–response relationship between hepatocellular injury and PPIX accumulation in the liver. Six-week-old male WT and CARKO mice (5 animals/group) were treated with 0 (control), 300, 1000, or 3000 ppm OX for 4 weeks. The dose of OX used in Exp. 1 (1000 ppm) was selected as an intermediate dose, and 300 ppm and 3000 ppm were selected with a common ratio of approximately 3 in order to examine differences in liver damage.

Experiment 3 (Exp. 3) was performed to clarify the effects of OX on liver tumor development. All WT and CARKO mice (50 animals/genotype) were administered a single intraperitoneal injection of 90 mg/kg DEN as a liver tumor initiator at 6 weeks of age, as previously described (Sakamoto et al., 2013). The initiation-promotion model for carcinogenicity in the liver is accepted as a useful method for detecting the stage at which chemicals promote hepatocarcinogenicity (Diwan et al., 1986). Two weeks after DEN initiation, 25 mice per genotype were given 1000 ppm OX for 26 weeks in the basal diet.

In Exps. 1, 2, and 3, clinical signs were monitored for all mice at least once per day during the treatment period. Body weight and food intake were measured once per week. After the final treatment, blood from all mice was withdrawn from the vena cava under isoflurane anesthesia, and all animals were then euthanized by exsanguination for necropsy. Serum samples were stored at −30 °C until use. Serum alanine transferase (ALT) levels were measured to examine cytotoxicity (SRL, Inc., Tokyo, Japan). The blood samples from additional animals in Exp. 1 were used only for plasma PPIX measurement

2.4. Histopathology

In Exps. 1 and 2, the livers were weighed, and the left and median lobes were fixed in 10% neutral-buffered formaldehyde solution, routinely processed, and stained with hematoxylin-eosin (H&E), Schmorl's reaction, Prussian blue, or Periodic acid Schiff (PAS) for histopathological examination. Remaining livers were frozen in OCT (Sakura Finetek, Tokyo, Japan), and a part of each tissue was sectioned for Oil red O staining.