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Involvement of constitutive androstane receptor in liver hypertrophy and liver tumor development induced by triazole fungicides

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ABSTRACT

We clarified the involvement of constitutive androstane receptor (CAR) in triazole-induced liver hypertrophy and tumorigenesis using CAR-knockout (CARKO) mice. Seven-week-old male CARKO and wild-type (WT) mice were treated with 200 ppm cyproconazole (Cypro), 1500 ppm tebuconazole (Teb), or 200 ppm fluconazole (Flu) in the diet for 27 weeks after initiation by diethylnitrosamine (DEN). At weeks 4 (without DEN) and 13 (with DEN), WT mice in all treatment groups and CARKO mice in Teb group revealed liver hypertrophy with mainly Cyp2b10 and following Cyp3a11 inductions in the liver. Teb also induced Cyp4a10 in both genotypes. Cypro induced slight and duration-dependent liver hypertrophy in CARKO mice. At week 27, Cypro and Teb significantly increased eosinophilic altered foci and/or adenomas in WT mice. These proliferating lesions were clearly reduced in CARKO mice administered both compounds. The eosinophilic adenomas caused by Flu decreased in CARKO mice. The present study indicates that CAR is the main mediator of liver hypertrophy induced by Cypro and Flu, but not Teb. In contrast, CAR played a crucial role in liver tumor development induced by all three triazoles.

1. Introduction

Liver hypertrophy and liver tumors are major treatment-related events in rodent toxicity or carcinogenicity studies of xenobiotics (Thoolen et al., 2010). Liver hypertrophy can cause increases in liver weights, average sizes of hepatocytes (hepatocellular hypertrophy), and the functional capacity of the liver. Lack of liver hypertrophy and tumors in constitutive androstane receptor (CAR; NR113)-knockout (KO) mice treated with phenobarbital (PB), a prototypical CYP2B inducer, has provided clear evidence that CAR plays an essential role in PB-induced liver hypertrophy and tumor development (Wei et al., 2000; Yamamoto et al., 2004). In contrast, epidemiologic studies of human patients undergoing long-term PB therapy showed no increase in the incidence of hepatic cancer (International Agency for Research on Cancer (IARC), 2001; La Vecchia and Negri, 2014; Lamminpää et al., 2002). Liver hypertrophy had been accepted as a key event of liver tumor development in rodents (Holsapple et al., 2006). However, a recent study showed that CAR activation plays an important role in hepatocarcinogenesis and that hypertrophy is associated with hepatocarcinogenesis (Elcombe et al., 2014). In a flow-chart diagram demonstrating the strategy of the National Toxicology Program for incorporating predictive hepatic transcriptomic datasets, hypertrophy induced by compounds in a 90-day toxicity study was shown to be the primary change associated with the formation of liver tumors. In contrast, at the Third International ESTP Expert Workshop, liver hypertrophy was suggested to have a low predictive value in the identification and elucidation of carcinogenic agents; however, no conclusive evidence was provided (Hall et al., 2012). Our previous study using C3H strain-derived CARKO mice showed that the pathway of CAR-mediated liver hypertrophy is different from that in hepatocarcinogenesis in mice treated with piperonyl butoxide (PBO) or decabromodiphenyl ether, both of which are CYP2B inducers, indicating that liver hypertrophy is not a key event for hepatocarcinogenesis (Sakamoto et al., 2013).

Triazoles, a class of antifungal agents that are widely used as fungicides or pharmaceutical drugs, exert their fungicidal activity by inhibiting 14α-demethylase activity, which is involved in sterol biosynthesis (Ghannoum and Rice, 1999). Various triazoles have been reported to induce liver hypertrophy and tumor development in rodent carcinogenicity studies (INCHEM, 1987, 1992, 1994, 1997, 2001; Regulatory Affairs Department, SDS Biotech K.K., 1997). Three recent studies have indicated that some triazoles induce CAR-mediated liver hypertrophy and/or gene expression in rodents (Currie et al., 2014; Nesnow et al., 2009; Peffer et al., 2007). Aside from these

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studies, there is no clear evidence regarding the involvement of CAR in rodent liver tumorigenesis by long-term triazole treatment.

Cyproconazole (Cypro), tebuconazole (Teb), and fluconazole (Flu), classified as fungicides (Cypro and Teb) or antifungal medicines (Flu), induce liver hypertrophy and/or liver tumors in rodents (INCHEM, 1994; Regulatory Affairs Department, SDS Biotech K.K., 1997; Sun et al., 2006). We recently conducted a short-term study in which CARKO mice were treated with Cypro, Teb, or Flu for 7 days (Tamura et al., 2013). Our results indicated that these 3 triazoles produced CAR-mediated effects on liver hypertrophy in a dose-dependent manner; interestingly, the proportion of involvement varied for each compound. However, the study did not clarify the involvement of CAR in liver hypertrophy after long-term treatment or in the development of liver tumors induced by these triazoles.

The present study was conducted to investigate the involvement of CAR in the subacute effects of Cypro, Teb, and Flu on liver hypertrophy and liver tumor development induced by these 3 triazoles using CARKO mice and to elucidate the mode of action (MOA) of tumor promotion by these triazoles. The initiation and promotion rodent model was used for the determination of liver tumor development.

2. Materials and methods

2.1. Chemicals

Cypro (CAS No. 94361-06-5, purity 96.7%), Teb (CAS No. 107534-96-3, purity 97.3%), and Flu (CAS No. 86386-73-4, purity > 99.5%) were obtained from LKT Laboratories, Inc. (St. Paul, MN, USA). Diethylnitrosamine (DEN, CAS No. 55-18-5, purity 99.9%) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

2.2. Animals

Male CARKO mice (Kodama et al., 2004; Yamamoto et al., 2004), in the C3H/HeNCrl background, were provided courtesy of Dr. M. Negishi (NIEHS) 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 animal experimental protocols were approved by the Animal Care and Utilization Committee of NIHS and followed NIHS guidelines for the care and use of laboratory animals.

2.3. Treatments

Six-week-old male WT and CARKO mice were randomly allocated to 4-, 13-, or 27-week treatment groups by body weight. To clarify the effects of triazoles on liver tumor development, mice from both genotypes in the 13- and 27-week groups were administered a single intraperitoneal injection of 90 mg/kg DEN as a liver tumor initiator at 6 weeks of age as previously described (Diwan et al., 1986; Sakamoto et al., 2013). The initiation-promotion model for carcinogenicity in the liver is accepted as a useful method for detection of the promotion stage for hepatocarcinogenicity of chemicals (Klaunig, 2013). All treated mice (4-, 13-, and 27-week groups) were given 200 ppm Cypro, 1500 ppm Teb, or 200 ppm Flu in the basal diet (CRF-1, Oriental Yeast, Co. Ltd, Tokyo, Japan) beginning at 7 weeks of age. The selected doses of Cypro and Teb were chosen based on doses shown to induce liver hypertrophy and tumorigenesis in previous studies in mice (INCHEM, 1994; Peffer et al., 2007; Regulatory Affairs Department, SDS Biotech K.K., 1997). In a 21-month carcinogenicity study of Teb in mice (INCHEM, 1994), liver tumors were not induced in mice given less than 1500 ppm Teb. Although the dose that induced hepatotoxicity was considered to be higher than the maximum tolerated dose (MTD) for carcinogenicity in risk assessment of chemicals, we chose this dose to investigate CAR involvement in liver tumor development by Teb. Since Flu did not induce liver tumors at up to 10 mg/kg by gavage in a Diflucan (registered trademark of Pfizer, Inc., NY, USA) carcinogenicity study (Diflucan patient information sheet; available at http://www.pfizer.com/pfizer/download/uspi_diflucan.pdf), we set 200 ppm as the concentration of Flu equal to approximately 25 mg/kg/day by gavage, based on the dose shown to induce *Cyp2b10* gene expression and liver hypertrophy (Sun et al., 2006). WT and CARKO mice in the control groups were fed a basal diet for 4, 13 or 27 weeks ad libitum. For each genotype, 5 or 6 mice per group were allocated to the 4- or 13-week treatment groups, and 22–25 mice per group were allocated to the 27-week treatment group. The exact numbers of mice examined at each observation are described in Table 2.

2.4. Observation of mice

During the treatment period, clinical signs were evaluated for all mice at least once a day. Body weight and food intake were measured once a week up to week 13 and once every 2 weeks thereafter. Mice found dead or euthanized when moribund were necropsied to determine their cause of death. After 4, 13, or 27 weeks of treatment, blood from all mice was withdrawn from the heart under deep gas inhalation 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 hepatotoxicity (SRL, Inc., Tokyo, Japan).

2.5. Pathological and immunohistochemical examinations of the liver

At weeks 4 and 13, the livers were weighed and fixed in 10% neutral buffered formaldehyde solution. Livers were routinely processed and stained with hematoxylin-eosin (H&E) for histopathological examination. The severity and distribution of hepatocellular hypertrophy were classified using the following criteria: mild or moderate (more severe than mild) only at the centrilobular area, and marked when severe hepatocellular hypertrophy was observed at the centrilobular to midzonal area.

At week 4, serial sections of H&E-stained samples were incubated with anti-CYP2B antibodies (Enzo Life Sciences Inc., Farmingdale, NY, USA) at 4 °C overnight. The sections were then reacted with secondary antibodies conjugated to peroxidase-labeled dextran polymers (Histofine Simple Stain mouse MAX PO, Nichirei, Tokyo, Japan) and visualized by 3-3′-diaminobenzidine reactions (Sigma Chemical Co., St. Louis, MO, USA) as previously described (Tamura et al., 2013).

Hepatocyte proliferating activity at weeks 4 and 13 was examined with anti-PCNA antibodies (Dako, Japan) using serial sections for CYP2B immunohistochemistry. Sections were processed in the same manner as for CYP2B immunohistochemical stain. Proliferating activity of the hepatocytes was evaluated using the site and intensity combined score (Tamura et al., 2013). Briefly, 5 fields of view containing a whole lobe were randomly selected from each animal. Next, the total number of PCNA-positive hepatocytes in 5 fields of view was counted at 10× magnification of the objective lens under a light microscope. PCNA-positive cells were classified into four grades: 0, 1, 2, or 3 corresponding to the total number of positive cells (<10, 10–25, 26–50, or >50, respectively).

In the 27-week treatment group, the livers were cut into 12 pieces (6 pieces each from the median and left lobes) to count the number of altered hepatocellular foci and adenomas. The classification of liver proliferating lesions, i.e., altered focus and adenoma, was in accordance with the criteria of the International Harmonization of Nomenclature and Diagnostic Criteria of Lesions in Rats and Mice (Thoolen et al., 2010). Each focus and adenoma was subclassified into 3 types: eosinophilic, basophilic, and others (mixed and clear types), based on their morphological patterns observed by staining with H&E, because PB treatment mainly induces eosinophilic foci (Yamamoto et al., 2004). The incidence (the number of mice bearing each type) and multiplicity (the number of each type per animal) of proliferative lesions were calculated.

2.6. Real-time reverse transcription polymerase chain reaction (RT-PCR)

At autopsy in the 4-week treatment group, the liver tissue from the left lobe was immediately frozen at –80 °C until use. Total RNA of the liver was extracted using ISOGEN (Nippon Gene Co. Ltd., Tokyo, Japan). After reverse transcription using 1 μg of total RNA with a High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), real-time PCR (7900HT Fast Real-time PCR System, Applied Biosystems) was performed using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) following the manufacturer's protocols. The primer-probe sets used in the present study were for the following genes: *Cyp1a2* (Mm00487224_m1), *Cyp2b10* (Mm01972453_s1), *Cyp3a11* (Mm00731567_m1), *Cyp4a10* (Mm01188913_g1), and *P450 reductase* (Mm00435876_m1). The expression level of each gene was calculated by the relative standard curve method and normalized to endogenous *GAPDH* (TaqMan Rodent GAPDH Control Reagent, Applied Biosystems). The mRNA expression levels of *Gadd45beta* and *Mdm2* were analyzed according to previously published methods (Ozawa et al., 2011).

2.7. Statistical analyses

The values of the treated groups were compared with those of the control group for the same genotype, and the values in CARKO mice were compared with the corresponding group of WT mice. The values for body weight (comparison between treated and control groups), absolute and relative liver weights, mRNA expression levels, and multiplicity of proliferative lesions were analyzed by the F-test to evaluate the homogeneity of variance. Student's or Welch's t-tests were then performed for homogenous or heterogeneous variances, respectively. Statistical analysis for incidences of proliferative lesions was performed by Fisher's test. Results for these tests were considered to be significantly different at $p < 0.05$ and $p < 0.01$, respectively.

3. Results

3.1. Clinical signs, food consumption, and body and liver weights

No treatment-related clinical signs were detected throughout the treatment period. Final body weights at weeks 4, 13, and 27 are shown