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Comparison of the hepatic and thyroid gland effects of sodium phenobarbital in wild type and constitutive androstane receptor (CAR) knockout rats and pregnenolone-16α-carbonitrile in wild type and pregnane X receptor (PXR) knockout rats

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ABSTRACT

A number of chemicals produce liver and thyroid gland tumours in rodents by nongenotoxic modes of action (MOAs). In this study the hepatic and thyroid gland effects of the constitutive androstane receptor (CAR) activator sodium phenobarbital (NaPB) were examined in male Sprague-Dawley wild type (WT) rats and in CAR knockout (CAR KO) rats and the effects of the pregnane X receptor (PXR) activator pregnenolone-16α-carbonitrile (PCN) were examined in WT and PXR knockout (PXR KO) rats. Rats were either fed diets containing 0 (control) or 500 ppm NaPB or were dosed with 0 (control) or 100 mg/kg/day PCN orally for 7 days. The treatment of WT rats with NaPB and PCN for 7 days resulted in increased relative liver weight, increased hepatocyte replicative DNA synthesis (RDS) and the induction of cytochrome P450 CYP2B and CYP3A subfamily enzyme, mRNA and protein levels. In marked contrast, the treatment of CAR KO rats with NaPB and PXR KO rats with PCN did not result in any increases in liver weight and induction of CYP2B and CYP3A enzymes. The treatment of CAR KO rats with NaPB had no effect on hepatocyte RDS, while PCN produced only a small increase in hepatocyte RDS in PXR KO rats. Treatment with NaPB had no effect on thyroid gland weight in WT and CAR KO rats, whereas treatment with PCN resulted in an increase in relative thyroid gland weight in WT, but not in PXR KO, rats. Thyroid gland follicular cell RDS was increased by the treatment of WT rats with NaPB and PCN, with NaPB also producing a small increase in thyroid gland follicular cell RDS in CAR KO rats. Overall, the present study with CAR KO rats demonstrates that a functional CAR is required for NaPB-mediated increases in liver weight, stimulation of hepatocyte RDS and induction of hepatic CYP enzymes. The studies with PXR KO rats demonstrate that a functional PXR is required for PCN-mediated increases in liver weight and induction of hepatic CYP enzymes; with induction of hepatocyte RDS also being largely mediated through PXR. The hepatic effects of NaPB in CAR KO rats and of PCN in PXR KO rats are in agreement with those observed in other recent literature studies. These results suggest that CAR KO and PXR KO rats are useful experimental models for liver MOA studies with rodent CAR and PXR activators and may also be useful for thyroid gland MOA studies.

1. Introduction

A number of nongenotoxic chemicals have been shown to produce liver and thyroid gland tumours in rodents (Gold et al., 2001; Huff et al., 1991). Both rodent liver and thyroid gland tumour formation may be associated with induction of hepatic xenobiotic metabolising enzymes. For example, a number of nongenotoxic chemicals, including phenobarbital (PB) and/or its sodium salt (sodium phenobarbital; NaPB), have been shown to produce liver tumours in rats and/or mice (Cohen, 2010; Elcombe et al., 2014; Lake, 2009) by a mode of action (MOA) involving activation of the constitutive androstane receptor (CAR). In an evaluation of the MOA for PB-induced rodent liver tumour formation by Elcombe et al. (2014), the key events were identified as CAR activation, altered gene expression specific to CAR activation,

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increased cell proliferation, the development of altered hepatic foci and finally liver tumour formation. Associative events for this MOA included liver hypertrophy, induction of cytochrome P450 (CYP) enzymes (particularly CYP2B subfamily enzymes) and inhibition of apoptosis.

Some nongenotoxic chemicals, including some CAR activators, have been shown to produce thyroid gland tumours in rodents by a MOA in which circulating thyroid hormone levels are decreased as a result of increased hepatic metabolism and clearance (Capen, 2001; Dellarco et al., 2006; Hill et al., 1998; McClain et al., 1989; Meek et al., 2003). In this MOA, thyroxine (T_4) conjugation is enhanced due to the stimulation of hepatic microsomal UDPglucuronosyltransferase (UGT) enzymes towards T_4 as substrate resulting in increased biliary excretion of T_4, a decrease in serum triiodothyronine (T_3) and/or T_4 levels and a compensatory increase in serum thyroid stimulating hormone (TSH) levels. In the rodent thyroid gland, the increase in TSH levels results in thyroid follicular cell hypertrophy and hyperplasia, with chronic stimulation subsequently producing thyroid follicular cell tumours (Capen, 2001; Curran and DeGroot, 1991; Hill et al., 1998).

In establishing the MOA for rodent liver tumour formation by CAR activators, studies in transgenic mice lacking CAR (i.e. CAR knockout (KO) mice) have been particularly useful. Such studies have demonstrated that PB does not increase liver weight, produce liver hypertrophy, does not induce CYP2B enzymes or replicative DNA synthesis (RDS) and does not promote liver tumours in CAR KO mice (Huang et al., 2005; Scheer et al., 2008; Wei et al., 2000; Yamamoto et al., 2004).

Recently, transgenic rat models lacking either the CAR or the pregnane X receptor (PXR), namely CAR KO and PXR KO rats, have been developed and have become commercially available (Forbes et al., 2017). The objective of this study was to evaluate the usefulness of the CAR KO and PXR KO rat models for liver and thyroid gland MOA studies. Male Sprague-Dawley (i.e. wild type; WT) and CAR KO rats were treated with NaPB and male WT and PXR KO rats were treated with pregnenolone-16α-carbonitrile (PCN). NaPB and PCN were selected as known rodent liver CAR and PXR activators, respectively (Elcombe et al., 2014, Lake et al., 1998; Martignoni et al., 2006; Omiecinski et al., 2011).

2. Materials and methods

2.1. Materials

7-Benzyloxyquinoline and 7-hydroxyquinoline were purchased from Cypex (Dundee, UK). NaPB (purity ≥ 99%), 5-bromo-2′-deoxyuridine (BrdU), and other CYP substrates and metabolites were obtained from Sigma-Aldrich (Poole, Dorset, UK), whereas PCN (purity 99%) was obtained from Enzo Life Sciences Inc. (Farmingdale, NY, USA). For Western immunoblotting, an anti-rat CYP1A antibody was obtained from Corning (Corning B.V., Amsterdam, The Netherlands), whereas anti-rat CYP2B, CYP3A and CYP4A antibodies were obtained from the Biomedical Research Institute (University of Dundee, Scotland, UK). Secondary antibodies were horseradish peroxidase (HRP)-linked anti-sheep IgG (Abgene, Blenheim Road, Epsom, Surrey, UK) and anti-rabbit IgG (GE Healthcare, Little Chalfont, Buckinghamshire).

2.2. Animals and treatment

Male Sprague-Dawley WT, CAR KO and PXR KO rats were obtained from Horizon Discovery (Boyertown, PA, USA). Rats were allowed free access to water and powdered or pelleted RM1 laboratory animal diet (Special Diets Services, Witham, Essex, UK) and were housed singly in accommodation with a 12:12 h light:dark cycle. Temperature and relative humidity were maintained between 19 and 23 °C and 40 and 70%, respectively. Rats were allowed to acclimatize to these conditions for at least 5 days before use. All animal procedures were performed under a UK Home Office license and all animal studies were approved by the Ethical Review Committee of the University of Dundee (Dundee, Scotland, UK). Male Sprague-Dawley WT, CAR KO and PXR KO rats (14 weeks old at study start) were implanted subcutaneously with osmotic pumps (Alzet model number 2ML1, Charles River UK Ltd., Margate, Kent, UK) containing 15 mg/ml BrdU in phosphate buffered saline pH 7.4. Rats were then either fed powdered diets containing 0 (control) or 500 ppm (500 mg/kg) NaPB or were dosed with 0 (control) or 100 mg/kg/day PCN orally for 7 days. For PCN, control animals received corresponding quantities (10 ml/kg body weight) of the corn oil vehicle. Male rather than female rats were used in this study, as male animals are more often used for rodent liver CAR activator MOA studies. At the end of the treatment period rats were killed by exposure to carbon dioxide gas and blood taken for plasma analysis. The liver of each rat was removed, weighed and sampled for morphological and biochemical investigations. The thyroid glands and trachea of each animal were removed intact and fixed in 10% neutral buffered formalin (NBF). After at least 24 h of fixation, the thyroid glands were carefully dissected from the trachea, blotted dry and weighed, prior to returning to the fixation medium. The duodenum of each animal was also excised.

2.3. Plasma analysis

Levels of PB in plasma were determined by protein precipitation followed by quantification by high performance liquid chromatography-mass spectrometry-mass spectrometry (LC–MS/MS) employing a Waters 2795 separation module equipped with a Waters Quattro micro mass spectrometer operated in negative ion mode with electrospray ionisation. Plasma PB levels were determined employing a Kinetex SB column (100 × 4.6 mm, 3 µm), with a mobile phase of 80:20 (v/v) methanol/water at a flow rate of 0.5 ml/min, with detection using a transition of 231.46 → 231.27. Levels of PCN in plasma were determined by protein precipitation followed by quantification by LC–MS/MS employing a Waters 2795 separation module equipped with a Waters Quattro micro mass spectrometer operated in positive ion mode with ACPI ionisation, using a transition of 342.30 → 224.23. Plasma PCN levels were determined employing a Luna C18(2) column (150 × 2 mm, 5 µm) at 35 °C employing mobile phases of methanol and 0.1% (v/v) formic acid in water with gradient elution at a flow rate of 0.4 ml/min.

2.4. Hepatic morphological investigations

Two samples of liver from each rat (one from the left lobe and one from the median lobe) were fixed in NBF, dehydrated, embedded in paraffin wax and approximately 5 µm sections were stained with hematoxylin and eosin. Sections were examined by light microscopy and the degree of liver hypertrophy scored as either minimal (grade 1), slight (grade 2), moderate (grade 3) or marked (grade 4). For each group, the liver hypertrophy severity grades for each animal were summed and a group mean severity grade calculated.

2.5. Replicative DNA synthesis (RDS)

Liver, thyroid gland and duodenum (as a methodology positive control) samples were fixed in NBF for approximately 36 h prior to processing into paraffin blocks. BrdU immunocytochemistry was performed as described previously (Ross et al., 2010). Image capture and acquisition were carried out using a Zeiss Imager A1 microscope and Improvision imaging software, Volocity version 4 (Perkin Elmer, Bucks, UK) was used for data analysis. The BrdU labelling index (i.e. percentage of either hepatocyte or thyroid follicular cell nuclei undergoing RDS) for each animal was determined by counting approximately 3000 nuclei in 10 random areas from both the left and median lobes of the liver and at least 3000 nuclei in 20 fields from the thyroid gland.