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Effect of pregnane X receptor (PXR) prototype agonists on chemoprotective and drug metabolizing enzymes in mice

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The effects of known PXR inducers; spironolactone [SPL, (i.p.)], pregnenolone-16 alpha-carbonitrile [PCN, (i.g.)] and dexamethasone (i.p.) on hepatic drug metabolizing enzymes in the male CF1 mouse were examined 24 h after 3 daily doses (50, 100, or 200 mg/kg) in corn oil vehicle. All three compounds produced dose-dependent elevations in cytochrome P450 [CYP3A], glutathione S-transferase [GST] and NAD(P)H quinone oxidoreductase [NQO] activities. Only elevations in CYP3A produced after dexamethasone were statistically significant. An elevation in microsomal epoxide hydrolase [mEH, Ephx1] activity was seen after almost all treatments but was erratic with dose. UDP-glucuronosyltransferase and thioredoxin reductase activities were not increased by any agent. Dexamethasone elevated Cyp1a1/2 mRNA at the low dose but reduced the mRNA transcript and activity of the enzyme at the mid and high doses. The mRNA responses of Ephx1 and Nqo1 showed a close parallel to each other with no increases after dexamethasone or SPL treatment, and elevations at the mid dose of PCN. With the exception of dexamethasone at the high dose, elevations in Gst mRNAs were seen with all doses of the three agents. When a large number of hepatic enzymes are examined, the responses to dexamethasone, SPL and PCN are far from identical, and any dose dependency is agent specific. Induction of enzymes seems more complicated to be controlled by one orphan receptor. This study not only filled the void about the murine PXR-induction profile but also will help in the course of drug development research with respect to extrapolation to human risk assessment.

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1. Introduction

The pregnane X receptor (PXR) binds drugs and xenobiotics affecting the expression of numerous genes involved in the elimination of endobiotics and xenobiotics. Spironolactone (SPL), pregnenolone-16 alpha-carbonitrile (PCN), and dexamethasone are prototypic activators of the PXR in rodents (Johnson and Klaassen, 2002; Buckley and Klaassen, 2009; Martin et al., 2010). Protective enzymes often considered include microsomal epoxide hydrolase (mEH), thioredoxin reductase (TR), and quinone oxidoreductase (NQO), as well as UDP-glucuronosyltransferases (UDPGTs), and glutathione transferases (GSTs). Almost all are involved in maintaining cellular components in their appropriate redox status.

Cytochrome P450 isoform 3A (CYP3A), UDPGT, and GST are known target genes for PXR agonists in human (Duret et al., 2006). In mice, PCN, dexamethasone and SPL were reported to increase Cyp3a mRNA in liver (Maglich et al., 2002; Xu et al., 2005), while, dexamethasone alone increased CYP3A activity (Pirmohamed et al., 1992; Xu et al., 2005). PCN had no effect on the transcript level of Cyp1a1 (Maglich et al., 2002). SPL, dexamethasone, and PCN increased Ugt1a1, Ugt1a6, and Ugt1a9 mRNA levels in mouse liver (Buckley and Klaassen, 2009). In another study, PCN did not affect Ugt1a6 or Ugt2b5 in mice (Maglich et al., 2002; Chen et al., 2003) nor the p-nitrophenol activity (Viollon-Abadie et al., 1999; Chen et al., 2003). Dexamethasone increased Gstm transcripts and protein in murine thymoma cell lines (Briehl et al., 1995), but diminished GST expression and activity in mouse liver (Kim et al., 1998). PCN elevated GST activity and mRNA levels in murine liver (Hammock and Ota, 1983; Gong et al., 2006). Dexamethasone decreased mEH gene expression and activity (Kim et al., 1998), while PCN elevated the enzyme activity (Hammock and Ota, 1983). It is obvious that there is no general consensus or clear understanding of the PXR control of drug metabolizing enzymes in mice, although there is a need for a model better than rats in PXR induction profile, a profile that is very different from a human's (Zhang et al., 1999). In reviewing the literature, it was surprising to discover the paucity of information available on the induction and constitutive expression of murine drug-metabolizing enzymes by PXR agonists. Out of almost a thousand hits on PXR on PubMed.gov, very few were concerned with mice. This lack of information on PXR control of drug metabolizing enzymes especially phase II enzymes may be attributed to the fact that one of the first enzymes linked to this receptor was cytochrome P4503A (CYP3A), a

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subfamily of phase I enzymes that are involved in the metabolism of almost two-thirds of drugs that undergo oxidation (Zhang et al., 1999).

Since PXR affects many genes of drug-metabolizing enzymes, extrapolation of results to human in experiments using mice would be inappropriate unless we clearly understand the similarities/differences in responses of these animals to human. To fill the void and to better understand which enzymes are controlled through the PXR in mice, a comprehensive examination was undertaken of the effects of PXR agonists on the mRNAs and activities of a range of drug-metabolizing enzymes in murine liver.

2. Materials and methods

2.1. Chemicals

Dexamethasone, spironolactone, pregnenolone-16 alpha-carbonitrile and all other reagents were purchased from Sigma (St. Louis, MO) except where indicated in the specified methods.

2.2. Animal treatment and maintenance

Adult male CF-1 albino mice (3 month-old, 25–35 g) were obtained from Charles River Laboratories and were maintained in a humidity (50 ± 5%) and temperature (20–25 °C) controlled environment on a 12-hour light/dark cycle with free access to standard rodent pellet food and tap water. The mice were housed in clear cages, 5–6 animals per cage. The pregnenolone-16 alpha-carbonitrile (PCN) was administered in corn oil by gavage (intragastric, i.g.), while dexamethasone and spironolactone (SPL) were administered in corn oil by intraperitoneal injection (i.p.). The three agents were administered at 50, 100, and 200 mg/kg, daily, for 3 consecutive days.

All animal procedures were conducted in concordance with NIH guidelines for the humane care of laboratory animals.

2.3. Biological sample preparation

Animals were sacrificed 24 h after the final dose, a blood sample was collected for immediate serum preparation, and the livers were quickly perfused in situ (via the hepatic portal vein) with normal ice cold saline. A 100 mg sample of liver was removed, homogenized in 2 ml of TRIzol solution (Invitrogen; Carlsbad, CA) and frozen at −80 °C for later RNA isolation. The gall bladder was then carefully dissected away, and the remaining liver homogenized in 0.25 M sucrose (20% w/v) and subjected to 3-stage differential centrifugation (9000 × g for 15 min, 19,000 × g for 15 min, and 105,000 × g for 60 min) to prepare the cytosolic and microsomal fractions. Protein content of both fractions was determined by the method of Lowry et al. (1951) using Folin-Ciocalteu's phenol reagent (Sigma; St Louis, MO) and the fractions were stored at −80 °C until assayed for enzyme activity.

2.4. Enzyme activity

Serum alanine aminotransferase (sALT) activity was determined according to Wroblewski and LaDue (1956), using a coupled reaction by monitoring the serum dependent change in absorbance of NADH oxidation at 340 nm in the presence of optimized concentrations of L-alanine, α-ketoglutarate and purified lactic dehydrogenase enzyme. Cytosolic quinone oxidoreductase (NQO) activity was determined from the 3,3 -methylene-bis-(4-hydroxycoumarin)-inhibited rate of reduction of 2,6-dichlorophenolindophenol by NADH, monitoring absorbance changes at 600 nm (Benson et al., 1980). Cytosolic glutathione S-transferase (GST) activities were determined from the change in absorbance at 340 nm resulted from conjugation of glutathione with 1chloro-2,4-dinitrobenzene [CDNB] (Habig and Jakoby, 1981). Cytosolic thioredoxin reductase (TR) activity was determined using the aurothioglucose-sensitive rate of reduction of 5,5 dithio-bis-(2-nitrobenzoic acid) by NADPH monitored at 412 nm (modified from the method of Hill et al., 1997). Microsomal UDP-glucuronosyltransferase (UDPGT) activity was determined with 4-nitrophenol as the substrate (Franklin and Finkle, 1986), and microsomal epoxide hydrolase activity (mEH) was determined with cis-stilbene oxide as the substrate (Hammock et al., 1985). 7-methoxyresorufin (Sigma-Aldrich®, St. Louis, MO) O-demethylase activity, determined from the rate of fluorescence increase due to the formation of the resorufin (Ex 544 nm, Em 612 nm) was utilized as a monitor of CYP1A1/2 (Burke et al., 1994). Microsomal CYP3A activity was determined from the 6β-hydroxylation of testosterone (Sigma, St. Louis, MO) where the testosterone metabolite was separated by HPLC and quantified from its absorbance at 236 nm (Guengerich et al., 1986). Any elevation in the activities of the cytosolic or microsomal enzymes in the current study does not distinguish between an activation of existing enzyme and an increase arising from increased amounts of enzyme protein.

2.5. mRNA determination

Hepatic mRNA levels were determined by Northern blotting of 20 μg of total RNA isolated by TRIzol extraction. Gel electrophoresis, nucleic acid transfer to membranes and ^{32}P probe labeling, washing stringency, and development conditions were all performed as described previously (El-Sayed et al., 2006a). The sequences and homologies of cDNA probes for the alpha (a), mu (m), and pi (p) glutathione S-transferase (Gst), UDP-glucuronosyltransferase (Ugt-1a1, -1a6, -1a9, -2b5), NAD(P)H-quinone oxidoreductase (Nqo1), thioredoxin reductase (Txnrd1), and microsomal epoxide hydrolase (Ephx1) mRNAs are described in detail elsewhere (El-Sayed et al., 2006a, b). For Cyp1a1/2, the probe spanned the region 1511–2250 of rat CYP1A1 (X00469) which has 92 and 87% homology with mouse Cyp1a1 (BC125444) and Cyp1a2 (BC018298), respectively. The probes used for Cyp1a1 and Cyp1a2 were more than 93% similar and hence reported as Cyp1a1/2. All mRNA bands were normalized to the same-sample cyclophilin mRNA band to control for gel loading and transfer variations. Results are expressed as fold change from the proper corn oil-control animals.

2.6. Statistical analysis of data

Results are expressed as the mean ± S.E.M. Treated group size was 4–6 animals. Statistical analyses were performed using ANOVA, followed by Fisher's protected least significant difference multiple range test. The data were compared against those treated with corn oil which in turn were not statistically different from naïve animals. PCN data were compared to those of corn oil administered intragastrically, and the dexamethasone and SPL data were compared to data of corn oil injected intraperitoneally. Differences were considered significant at P values of <0.05.

3. Results

None of the treatments caused acute hepatotoxicity, since serum ALT was not elevated by any treatment (Table 1). No significant difference in body weight was recorded (data not shown). During the dosing period, clinical signs of toxicity such as hair loss, bleeding, shortness of breath or reduced food consumption were not reported either. All three treatments caused modest elevations in CYP3A activity but only increases reported after dexamethasone treatment at mid and high doses achieved statistical significance (Table 2). Insignificant reductions were seen in Cyp1a1/2 transcripts and again only dexamethasone treatment caused a significant increase in the enzyme transcript at 50 mg/kg and a significant reduction at the highest dose (Table 2). A reduction was also seen after the mid dose of dexamethasone but it did not achieve statistical significance ($P\sim0.06$). These changes in cyp1a1/2 were reflected in enzyme activity, where dexamethasone treatment at both the mid and high doses evoked reductions in CYP1A1/2 (MROD) activities. PCN and SPL at the three

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