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Alterations in the distribution and orexigenic effects of dexamethasone in CAR-null mice

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Abstract

The constitutive androstane receptor (CAR, NR1I3) has emerged as an important regulator of drug metabolism. CAR responds to a wide spectrum of xenobiotics by inducing expression of cytochrome P450 (CYP) enzymes and a number of other proteins responsible for drug metabolism in the liver. The xenosensor function of CAR overlaps with that of the pregnane X receptor (PXR), another xenobiotic receptor that belongs to the nuclear hormone superfamily. We observed that injection of dexamethasone (Dex), a ligand for the glucocorticoid receptor (GR) and PXR but not CAR, results in an unexpected twofold increase in the stomach weight of CAR-null animals relative to wild-type animals. Here, we show that CAR knockout mice have elevated levels of Dex in the brain, resulting in a more rapid and robust increase in the hypothalamic expression of the GR-responsive target genes encoding neuropeptide Y (NPY) and neuropeptide Y receptor subtype 1 (NPY-R1). As expected, this is accompanied by a higher increase in the food intake of the CAR-null animals. The data described here highlight the complexity of the overlapping functions of CAR and PXR.

Keywords: Drug metabolism; Xenobiotic response; Xenosensor function

1. Introduction

Treatments with drugs or other xenobiotics often result in increased metabolism of the administered drug and altered pharmacokinetics of other coadministered compounds (Fuhr, 2000). The first phase of drug metabolism is primarily mediated by the cytochrome P450 (CYP) enzymes, which catalyze the metabolism of a wide diversity of xenobiotics, including various environmental pollutants, carcinogens, and prescription drugs, as well as endogenous substrates such as steroid hormones (Honkakoski and Negishi, 2000; Xie and Evans, 2001).

The constitutive androstane receptor (CAR), a member of the nuclear receptor superfamily, has emerged as a key player in xenobiotic metabolism (Honkakoski and Negishi, 2000; Wei et al., 2000). CAR is highly expressed in the liver and the small intestine, two key tissues expressing inducible CYP enzymes responsible for steroid and xenobiotic metabolism, and has been shown to mediate the well-studied induction of CYP2B genes by phenobarbital (PB) and 1,4-bis-(2-(3,5,-dichloropyridyloxy)) benzene (TCPOBOP) (Sueyoshi et al., 1999; Tzameli et al., 2000; Wei et al., 2000). Unlike classical nuclear receptors, which are activated by their cognate ligands, CAR has a strong constitutive activity in cell-based reporter assays in the absence of any exogenous ligands (Baes et al., 1994; Choi et al., 1997). This constitutive activity can be inhibited by the inverse agonist ligands androstanol and androstenol (Forman et al., 1998). In untreated livers or primary cultures of hepatocytes, however, CAR is sequestered in the cytoplasm (Kawamoto et al., 1999). Treatment of animals or primary hepatocytes with PB or TCPOBOP results in the translocation of CAR into the nucleus (Kawamoto et al., 1999), where it binds to its cognate DNA response elements as a heterodimer with

Abbreviations: CAR, constitutive androstane receptor; CO, corn oil; CYP, cytochrome P450; Dex, dexamethasone; GR, glucocorticoid receptor; NPY, neuropeptide Y; NPY-R1, neuropeptide Y receptor subtype 1; NPY-R5, neuropeptide receptor subtype 5; PB, phenobarbital; PXR, pregnane X receptor.

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the retinoid X receptor (RXR) and activates the transcription of target genes.

The loss of CAR expression in CAR-null mice results in the complete absence of CYP2B10 induction in response to PB and PB-like inducers in the liver and decreased drug metabolizing capabilities (Wei et al., 2000, 2002). Recently, the list of CAR target genes has been expanded to include genes involved in all phases of xenobiotic metabolism (Maglich et al., 2002), including the CYP3A enzymes (oxidative metabolism phase; Goodwin et al., 2002; Wei et al., 2002), UGT1A enzymes (conjugation phase; Sugatani et al., 2001; Huang et al., 2003), and members of the MRP family of transporters (transport phase; Kast et al., 2002; Xiong et al., 2002; Huang et al., 2003). Many of these genes are also known to be targets for the pregnane X receptor (PXR), another important xenobiotic receptor (Xie and Evans, 2001).

PXR, which is the closest relative to CAR within the nuclear receptor superfamily, has also been implicated in the regulation of a distinct but overlapping set of drug-metabolizing enzymes in response to foreign compounds (Kliewer et al., 1998; Lehmann et al., 1998; Moore et al., 2000; Xie et al., 2000). Although CAR and PXR have been implicated as primary regulators of CYP2B and CYP3A expression, respectively, more detailed studies have revealed extensive overlap in the functions of these receptors (Moore et al., 2000; Xie et al., 2000; Wei et al., 2002). Thus, CAR can activate CYP3A genes, and PXR can induce CYP2B genes. The basis of this functional overlap resides in part in the ability of both CAR and PXR to recognize each other's response elements (Tzameli et al., 2000; Xie et al., 2000; Goodwin et al., 2001). Partial overlap has also been demonstrated at the level of ligand binding (Moore et al., 2000; Wei et al., 2002). Thus, the CAR inducer PB has been shown to act as a ligand for human, but not mouse, PXR (Lehmann et al., 1998). In addition, the pesticide dieldrin and the antifungal agent clotrimazole can activate both CAR and PXR. On the other hand, some ligands are specific for each receptor. For example, the induction of CYP2A4 by chlorpromazine is lost in the CAR-null mice (Wei et al., 2002). The response of CYP2B10 and CYP3A11 to dexamethasone (Dex), a potent orexigen and an agonist for both PXR and glucocorticoid receptor (GR), is lost only in the PXR-null mice (Xie et al., 2000).

In the course of studies on the functional overlap of CAR and PXR, we observed that Dex treatment resulted in an unexpected two- to threefold increase in stomach size in CAR-null mice compared to wild-type animals. Here, we examine the hypothesis that the loss of CAR affects the levels of this orixegenic steroid in the brain. We show that altered Dex pharmacokinetics result in increased expression of neuropeptide Y (NPY) and neuropeptide Y receptor subtype 1 (NPY-R1), known modulators of food intake, in the CAR-null mice and a concomitant increase in food intake.

2. Materials and methods

2.1. Animals and treatment

Mice carrying the CAR mutation have been described elsewhere (Wei et al., 2000). Animals used in all experiments were age-matched (8- to 10-week-old) male mice housed in groups of four or five in plastic microisolator cages at 22 °C with 12:12-h light–dark cycle and had free access to food and water. Mice received injections of Dex (50 mg/kg ip) dissolved in corn oil (CO; Sigma, St. Louis, MO) or an equal volume of CO as control. Twenty-four hours after treatment, the mice were decapitated, and their stomachs were isolated and weighed. At least three mice were used per treatment, and the experiment was repeated three times.

2.2. Northern blot analysis

Two to three mice were used per time point, and the experiment was repeated twice. At different time points, the mice were decapitated, and the total hypothalami were excised between the optic chiasm and the mammillary bodies. The hypothalami were immediately place in liquid nitrogen for later use. Total RNA from pooled hypothalami was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Fifteen micrograms of total RNA was resolved on 1% agarose/2.2 M formaldehyde denaturing gel and transferred to a nylon membrane (Hybond N+, Amersham Biosciences, Piscataway, NJ) by capillary blotting. The probe for NPY, corresponding to position 3-290 of NPY cDNA, was prepared from hypothalamus total RNA using Superscript One-step RT-PCR System (Invitrogen, Carlsbad, CA). PCR primers for NPY were 5′-gctaggaaacacgcgaatgg-3′ and 5′-cacatggaagggtcttcaag-3′. Blots were hybridized using ULTRAHyb solution (Ambion, Austin, TX) with 32P-labeled NPY probe at 42 °C overnight. The blots were then washed twice with 0.1% SDS/ 2X SSC at room temperature followed by one wash with 0.1% SDS/ 0.1X SSC at 55 °C. The blots were subsequently reprobed with a radiolabeled β-actin cDNA (BD Clontech laboratories, Palo Alto, CA). The intensity of signals was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The levels of NPY mRNA were normalized with the levels of β-actin mRNA.

2.3. Analysis of food intake

The food intake was measured using metabolic cage systems for rodents (Mini Mitter, Bend, OR). Two mice were used per cage, and the experiment was repeated three times. Animals were placed into metabolic cages for 4 days prior to treatment. On the 5th day, at 9:30 a.m., the animals were treated either with CO or Dex (50 mg/kg). The weight

of the food consumed and the fecal output were measured at the same time the next morning.

2.4. Semiquantitative RT-PCR

The relative levels of NPY-R1 and neuropeptide receptor subtype 5 (NPY-R5) were measured at different time points after injection with Dex (50 mg/kg) using Superscript One-step RT-PCR System (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation. Hypothalamus total RNA (250 ng) was used for each reaction. Reverse transcription was performed in 50 μl total volume for 45 min at 54 °C. This was immediately followed by PCR; 3 min at 93 °C, 34 cycles of 1 min at 93 °C, 45 s at 56 °C, and 45 s at 72 °C. The last cycle was followed by a final extension step, 5 min at 72 °C. PCR primers for NPY-R1 (accession: 6754881) were the following: forward—5′ -tgaactcaactctgctctcc-3′ located between nucleotides 2 and 21; reverse—5′ -cagcctgtgagagtcagatg-3′ located between nucleotides 605 and 624 with amplicon size of 622 bp. The primers for NPY-R5 (accession: AF049329) were the following: forward—5′ -ggaggttaaactttgaagagc-3′ located between nucleotides 3 and 22; reverse—5′ -atgaatcggagggccatgac-3′ located between nucleotides 662 and 682 with amplicon size of 679 bp. And the primers for β-actin were 5′ -gactacctcatgaagatcctg-3′ and 5′ -gaaggaaggctggaaaagtg-3′ . The PCR products were run on 1.2% agarose gel with 0.05 μg/ml ethidium bromide and subsequently visualized under UV light. The intensity of signals was quantified as described above.

2.5. In vivo ^{3}H-dexamethasone distribution study levels

At least three mice per genotype per time point were used for this experiment. The mice received an injection of 50 mg/kg ip Dex, which contained 250 uCi/kg ^{3}H-Dex [(1,2,4,6,7-^{3}H)-Dex, specific activity 1 mCi/ml; Amersham Pharmacia Biotech, Piscataway, NJ]. At the specified time, the mice were decapitated, and 100 μl of trunk blood was isolated and diluted in solvable (Packard, Meriden, CT) for determination of radioactivity in the blood. Liver and brain tissues were isolated and immediately homogenized using solvable (Packard) according to the manufacturer's instructions. All samples were diluted in UltimaGold scintillation counting fluid (Packard), and the radioactivity was determined in a Tricarb β-counter (Beckman, Fullerton, CA).

2.6. Statistical analysis

For measurements of food intake and stomach weight, we used the single-tailed Student t test for unpaired data. For the ^{3}H-Dex levels, the double-tailed Student t test was used. Values of $P<.05$ were accepted as statistically significant.

3. Results

3.1. Determination of stomach weight and food intake in Dex-treated animals

Administration of Dex by intraperitoneal injection resulted in a dramatic increase in stomach weight in CAR-null compared to wild-type animals 24 h later ($P<.003$; Fig. 1a). This increase in stomach weight is due to an increase in stomach content, inasmuch as the weight of the empty stomachs was the same in all groups tested (data not shown). No differences were observed in the histology of the tissues in the various groups.

To determine whether this unexpected stomach phenotype was due to an increase in food intake, the feeding response of the animals was determined. Dex resulted in a significantly higher increase in food intake in the CAR-null mice over the 24 h after treatment, compared to wild-type animals (29.5% vs. 50.3%, $P<.04$; Fig. 1b). The increase in

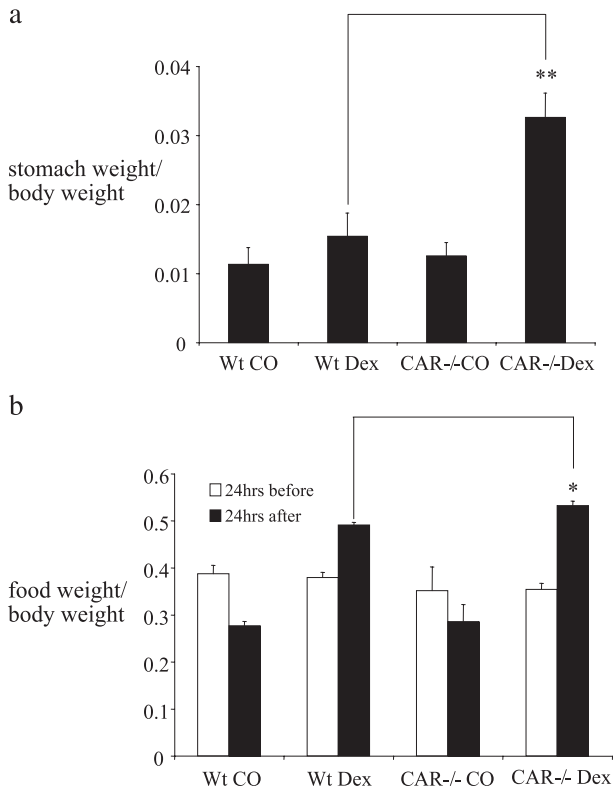

Fig. 1. The effect of Dex injection on (a) stomach weight and (b) food intake. Animals were placed into metabolic cages for 4 days prior to treatment. On the 5th day, animals were weighed and then injected with either CO or Dex (50 mg/kg ip). Exactly 24 h after injection, the animals were decapitated, and their stomach weight and food intake were recorded. (a) Treatment with Dex resulted in a significant increase in the stomach weight of CAR-null animals. (b) Dex injections resulted in a higher increase in food intake in CAR-null animals versus wild-type animals. Each bar represents mean ± S.E.M. of six to nine animals/group. $**P<.01$; $*P<.05$.

food intake was associated with a comparable increase in fecal output after Dex injection in both genotypes (data not shown).

3.2. Determination of the levels of Dex in the brain

Dex acts centrally on the hypothalamus to increase food intake (Cavagnini et al., 2000). To test the hypothesis that the higher stomach weight and increased food intake in CAR-null mice is due to higher CNS levels of Dex, we assessed the levels of the steroid in the brains of CAR-null and wild-type animals at different time points after injection with ^{3}H-Dex. At all time points prior to 24 h, CAR-null mice had higher brain levels of Dex, with the most statistically significant increases at 0.5 and 12 h (Fig. 2a). The CAR-null animals also had significantly higher Dex levels in their blood at 0.5, 3, 6, and 12 h, compared to wild-type animals (Fig. 2b). The levels of Dex in the livers of CAR-null animals were not significantly different from those in the livers of wild-type animals (data not shown).

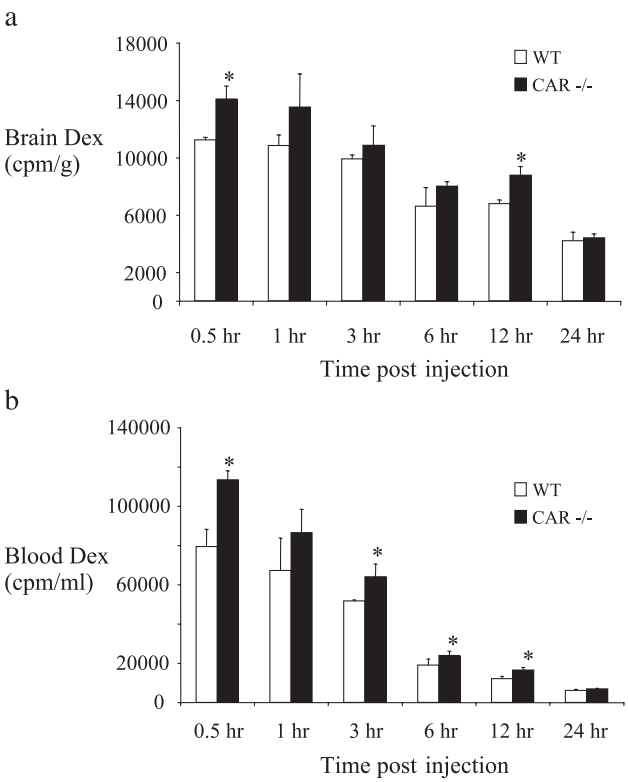

Fig. 2. Dex levels in the brain (a) and blood (b) of wild-type and CAR-null animals at different time points. Animals were treated with a single intraperitoneal injection of Dex (50 mg/kg, 250 μCi/kg). At different times, animals were decapitated, tissues were isolated, and Dex levels were measured by a scintillation counter. Compared to wild-type animals, CAR-null animals had higher Dex levels in their brain as well as their blood. Each bar represents mean ± S.E.M. of three to four animals/group. * $P < .05$.

3.3. Evaluation of NPY and orexigenic NPY receptors levels in the hypothalamus

Dex induces expression of NPY (Misaki et al., 1992) and NPY receptor subtype 1 (NPY-R1) in vitro and in vivo (Weng et al., 1995). Considering the important role of NPY and NPY receptors (NPY-R1 and NPY-R5) in the hypothalamic control of food intake, we assessed the expression of these genes in the hypothalami of wild-type and CAR-null animals after Dex injection. The relative levels of NPY mRNA in wild-type and CAR-null hypothalami are shown in Fig. 3a. In this experiment, Dex injection produced a rapid increase in NPY mRNA in the hypothalami of CAR-null animals that persisted until 6 h after injection. In marked contrast, the increase in NPY levels in the hypothalami of Dex-treated wild-type animals was not evident until 6 h postinjection.

Compared to uninjected control animals, Dex treatment did not produce any changes in the expression of NPY-R5 in the hypothalamus of wild-type or CAR-null animals. However, Dex injection elicited a gradual increase in the expression of NPY-R1 in wild-type animals starting at 6 h postinjection and reaching a maximum at 24 h (Fig. 3b). As observed with NPY, a stronger and more rapid increase in the expression of this receptor was seen in the CAR-null animals, with obvious induction at 1 h after injection and a maximum response at 6 h (Fig. 3b).

4. Discussion

The results described here demonstrate that mice lacking CAR have altered blood and brain distribution of the drug Dex at early time points after intraperitoneal injection. The CAR-null mice exhibit increased sensitivity to the orexigenic effects of Dex, which is likely to be due to the higher brain levels of this drug. The elevated Dex levels in the brain of CAR-null animals led to a higher and more rapid increase in the hypothalamic expression of the potent orexigenic peptide NPY and its receptor, NPY-R1, compared to Dex-treated wild-type animals. This was accompanied by increased food intake of the CAR-null animals, which is consistent with the well-documented ability of NPY and NPY-R1 agonists to increase feeding behavior (Inui, 1999; Cavagnini et al., 2000; Yokosuka et al., 2001).

The alteration in the levels of Dex in the brain of the CAR-null animals is presumably a reflection of the higher blood levels of this drug. The basis for this increase is unclear, but one possibility is that the loss of CAR leads to a change in the rate of metabolism of Dex in the liver—the primary site of drug metabolism. CYP3A enzymes are involved in the metabolism of more than 60% of pharmaceutical drugs, including Dex (Cholerton et al., 1992). The induction of these enzymes by Dex is intact in the CAR-null animals, and the loss of CAR does not have a large effect on the somewhat variable basal levels of CYP3A

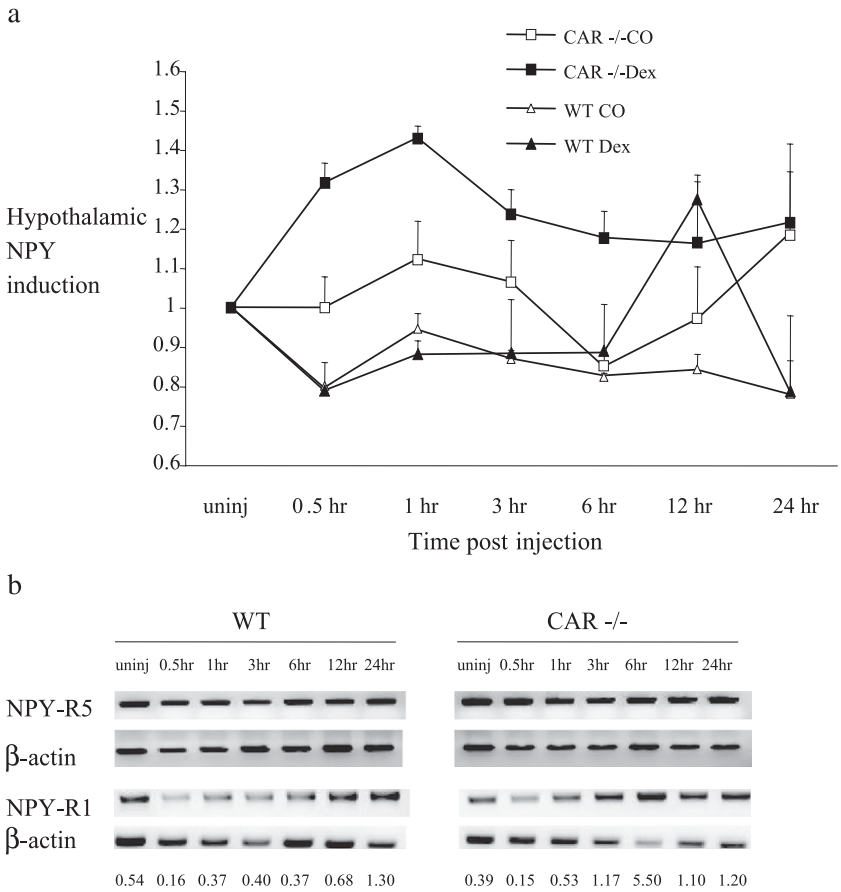

Fig. 3. Expression of NPY (a) and NPY-R5 and NPY-R1 (b) in the hypothalamus after treatment with Dex. Animals were treated with a single intraperitoneal injection of either CO or Dex (50 mg/kg). At different times, animals were decapitated, and hypothalamus total RNA was prepared. RNA was then analyzed by Northern blotting for NPY and semiquantitative RT-PCR for NPY receptors. Dex treatment produced a more rapid and more robust increase in the expression of NPY and NPY-R1 in the CAR-null versus wild-type animals. For NPY expression, points represent means ± S.E.M. of four to six animals/group. Fold induction is relative to NPY levels in the animals that received no injection (uninj). Numbers represent the ratio of NPY-R1 band intensity to the respective β-actin band intensity. * $P < .05$.

expression, at least by Northern blot analysis (Wei et al., 2002). This is consistent with the fact that in untreated animals, CAR is sequestered in the cytoplasm of wild-type hepatocytes and thus should not contribute to basal CYP3A expression. Recently, however, GR has been implicated in the induction of CAR mRNA and protein expression (Pascussi et al., 2003). Moreover, Dex was shown to increase both basal and PB-mediated nuclear translocation of CAR in human hepatocytes (Pascussi et al., 2000). Thus, although Dex is not an agonist ligand for CAR, it is possible that CAR activation in response to the relatively high doses used in these studies results in a relatively rapid alteration in Dex metabolism in the wild-type mice that is lost in the CAR-null mice. One prediction of this hypothesis is that decreased Dex metabolism in the livers of PXR knockout mice should also result in a similar stomach phenotype. Indeed, treatment of PXR-null animals with the same dose of Dex did result in the enlarged stomach phenotype seen in the CAR-null animals (data not shown). Moreover, treatment of wild-type animals with a higher dose of Dex (100 mg/kg) resulted in the same phenotype. Pretreatment of these animals with the potent CAR activator TCPOBOP, which dramatically induces CYP3A expression in a CAR-dependent manner in the liver, abolished the effect of Dex. This observation is in accordance with the idea that the induction of CYP3A prior to Dex injection leads to an accelerated metabolism of this drug and hence blunts its effects on food intake.

An alternative explanation for the increased brain levels of Dex is a defect in the blood brain barrier (BBB) in the CAR-null mice, particularly inasmuch as Dex treatment of mdr1-null mice results in higher levels in the brain (Schinkel et al., 1995). However, we have found that neither loss of CAR function or CAR activation produces any change in mdr1 expression at the BBB. Moreover, although Dex is known to induce the expression of mdr1 in the liver and intestine through PXR, the latter is not expressed at the BBB (Demeule et al., 1999; Yokogawa et al., 2002), and Dex does not alter mdr1 expression in rat brain endothelial cells (Regina et al., 1999).

The higher levels of NPY and NPY-R1 expression in the Dex-treated CAR-null animals might explain the higher

increase in their food intake compared to wild-type animals. This is consistent with the observation that the increase in the mRNA levels of NPY-R1, but not NPY-R5, results in amplified sensitivity of animals to the orexigenic effects of NPY (Kalra et al., 1998; Yokosuka et al., 2001). The higher increase in food intake seen in the CAR-null animals may partially explain the bigger stomachs that these animals have after treatment with Dex. Inasmuch as these animals did not show a higher increase in their fecal output compared to wild-type animals, it is also possible that a slower rate of gastric emptying may play a role in this phenotype as well. Several reports have suggested that central application of NPY produces a reduction in gastric motility and secretions (Humphreys et al., 1992; Matsuda et al., 1993; Kaga et al., 2001). A scenario can be envisaged in which a higher more rapid increase in the levels of NPY in the brain of CAR-null animals induces an increase in food intake as well as a slower rate of gastric emptying. A combination of these effects might account for the observed stomach phenotype.

The results described here highlight the complexity of the overlap in the functions of the xenosensors, CAR and PXR. The modulation in the distribution of the drug Dex, a known ligand for GR and PXR, in the CAR-null animals is intriguing. Inasmuch as Dex is widely administered for different conditions, the determination of the factors that lead to its altered distribution is of clinical importance. For example, Dex and phenytoin, an anticonvulsant agent, are frequently administered concurrently to prevent consequences of central nervous system pathology. Dex and phenytoin are both ligands for PXR, and the latter is also a CAR activator. While each agent would be expected to increase drug metabolism on its own, the combination of both leads to further increases in this process and, therefore, decreased effective concentrations in these patients (Jubiz et al., 1970; Recuenco et al., 1995). Whether the administration of a CAR inhibitor would lead to higher effective levels of phenytoin in the brain, thus less aggressive dosing of this drug, awaits further investigation.

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