Ectopic expression of MHC class II genes (RT1.B(I) β/α) in rat hepatocytes in vivo and in culture can be elicited by treatment with the pregnane X receptor agonists pregnenolone 16α-carbonitrile and dexamethasone

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Abstract

The synthetic steroid, pregnenolone-16-α-carbonitrile (PCN), has served for decades as a probe for a postulated series of hepatic defenses activated under situations of environmental “stress”. PCN, an antiglucocorticoid, and also such glucocorticoids as dexamethasone (Dex) appear to stimulate hepatic metabolism and elimination of xenobiotics by binding to the nuclear pregnane X receptor (PXR) which then interacts with a distinct DNA response element associated with induction of cytochrome P450 3A genes. To explore the full domain of genes controlled by PCN/PXR, we used differential display to detect rat liver mRNA species selectively induced by PCN or by Dex. Sequence analysis identified one of many PCN induced cDNA fragments as RT1.B(I)β, a member of the major histocompatibility class II (MHC) gene family usually found only in antigen presenting cells. Northern blot analysis of RNA from rat liver or from cultured hepatocytes confirmed that amounts of RT1.B(I)β mRNA and also of its companion gene, RT1.B(I)α mRNA, became readily detectable within 3–6 hours following treatment with PCN or Dex, whereas no induction was observed in spleen RNA. Induction by PCN of RT1.B(I)β immunoreactive protein was localized to the hepatocytes as judged by immunofluorescence. We conclude that ectopic expression of MHC II genes, an unprecedented effect of steroids or drugs, is rapidly evoked by PCN acting on the liver, directly. The concept of a set of genes coordinately controlled to maintain homeostasis in parenchymal
tissues during toxic stress must now be extended to include the immune system. © 2002 Elsevier Science Inc. All rights reserved.

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Introduction

Years ago, the celebrated biologist, Hans Selye, described a set of integrated host defenses activated against hormonal and metabolic effects of environmental stressors to maintain a physiologic steady state. Investigating this postulated “generalized adaptive syndrome,” he treated animals with one of hundreds of various steroid hormone derivatives and then challenged the animals with one of an array of toxic insults. He identified pregnenolone-16α-carbonitrile (PCN) as the most efficacious “catatoxic” steroid, one that protected the animals from toxic agents through non-classical hormone effects [1]. PCN and some glucocorticoids including the synthetic glucocorticoid dexamethasone (Dex), seemed to activate cellular defense mechanisms, including the capacity of the liver to convert potentially toxic xenobiotics into water soluble derivatives that were more readily excretable in bile [1].

In attempting to define the genetic and biological basis for the protective effects of PCN, we found that treatment of rat liver [2] or of primary cultures of adult rat hepatocytes [3] with PCN or glucocorticoids stimulated the expression of a novel form of cytochrome P450 (now called CYP3A23), a member of the supergene family of microsomal hemoproteins that bind drugs and catalyze their oxidation to oxygenated and more polar derivatives [4]. Typically, gene induction by glucocorticoids involves binding of the steroid to the glucocorticoid receptor protein which then interacts with specific DNA “response elements” associated with one or more genes thus activating or inhibiting gene expression, coordinately. However, induction of CYP3A23 by Dex differs from this classical pathway in its dose-response and agonist-antagonist relationships [5] as well as interspecies differences [6,7] for homologous hepatic CYP3A genes. For example, PCN proved actually to be an antiglucocorticoid that blocks induction of glucocorticoid responsive genes by Dex [8] while it synergistically stimulates Dex-mediated induction of CYP3A23 through enhanced transcription [9,10]. Recently, Kliewer and co-workers [11] isolated the pregnane X receptor (PXR), a novel nuclear steroid receptor that binds PCN, Dex, and other relevant agonists and activates gene transcription when bound to the previously identified CYP3A23 DNA response element [10,12].

In addition to CYP3A23 [11,13,14] and its human homologue, CYP3A4 [14–17], a growing number of genes under functional control by the PXR have been identified. Many of these genes are involved in elimination of foreign compounds by the liver including digitoxigenin glucuronosyl-transferase [18], liver sulfotransferase [19], the ATP-binding cassette transporter proteins MRP-2 [20] and MDR1 [21], and mouse CYP2B10 [22]. To explore the idea that the PCN/PXR system may extend beyond hepatic drug metabolism to other functions of protection against toxic stress, we prepared rat liver mRNA gene products amplified as cDNAs by the polymerase chain reaction (PCR) and differentially displayed on standard DNA sequencing gels. We found numerous potential gene products induced or repressed by treatment of the animals with PCN or Dex. One of these encodes the mitochondrial ATPase, a protein complex involved in cellular energetics not previously known to be inducible [23]. Presently, we report that another one of these
PCN induced liver mRNAs proved to be RT1.B(I)β, the β chain component of the rat’s major histocompatibility complex class II (MHC II).

**Methods**

**Materials**

Dex and PCN were purchased from Sigma (St. Louis, MO). Other reagents were purchased from the following: The kit for mRNA differential display system was from GenHunter (Brookline, MA), reverse transcriptase (SuperScript II) and RadPrime DNA labeling kit from GIBCO/BRL. 35S-dATP and 32P-dCTP were purchased from Amersham (Arlington Heights, IL). The mouse anti-rat RT1.B(I) monoclonal antibody (OX6) was purchased from Pharmingen (San Diego, CA). Collagenase type I was purchased from Worthington Biochemical (Freehold, NJ) and all other cell culture materials were from Sigma.

**Animals**

Adult female Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 150–200 g were maintained with free access to animal chow and water. After an overnight fast, rats received a single intraperitoneal dose (80 mg/kg) of Dex or PCN suspended in saline or vehicle alone (untreated controls), and at various times thereafter, animals were sacrificed, tissues were excised, and total RNA isolated by the method of Chomczynski and Sacchi [24]. The institutional Animal Care and Use Committee of the University of Colorado HSC approved all procedures.

**mRNA Differential Display**

The protocol for mRNA differential display was as previously described [23]. Briefly, total RNA isolated from livers of female rats treated for 8 hours with Dex or PCN was reverse transcribed using SuperScript II and one of three anchored oligo (dT) primers. The resulting single-stranded cDNA was then amplified in a PCR reaction containing the same oligo (dT) primer and the same arbitrary primer according to the manufacturer’s guidelines (GenHunter). Direct amplification of DNase-treated RNA without prior reverse transcription served as a control for bands arising from contaminating DNA. The PCR-amplified DNA was analyzed in triplicate lanes in a 6% polyacrylamide sequencing gel which was dried and then autoradiographed. Only bands differentially displayed in all three lanes were excised from the dried gels, eluted and reamplified for further characterization by Northern blot analysis.

**Northern Blot Analyses, Cloning and DNA Sequencing**

Northern blot analysis was performed by electrophoresis of total RNA (10 μg/well) isolated from untreated control, Dex-, or PCN-treated animals through a 1% agarose-2.2 M formaldehyde gel, followed by blotting onto a nylon membrane (MSI, Westboro, MA). RNA was cross-linked to the membranes using a UV Crosslinker (Fisher Scientific, Madison, WI) and the membranes hybridized to random-primed cDNA. DNA sequencing was performed by the Sanger dideoxy method [25] on an ABI
Prism 377 Automated Sequencer using the dRhodamine Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). Global alignments of published sequences were made with the NIH Network BLAST program. The cDNA clone pLRβ118, encoding the rat MHC class II RT1.B(I)β [26] and cDNA clone RT1.B(I) alpha 4 [27], encoding the alpha chain of RT1.B(I), were generously provided by Dr. K. Reske, University of Mainz, Germany. The cDNA probe for CYP3A23 has been previously described [28]. The cDNA probes for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Clontech, Palo Alto, CA) or 18S (Ambion) were used to normalize the amount of RNA loaded in each lane. Autoradiographs of Northern blots were quantified by densitometry using a Model GS-670 Imaging Densitometer equipped with Molecular Analyst/Mac version 1.1.1. Image Analysis software (Bio-Rad Laboratories, Hercules, CA). Exposure times used were in the linear range for the film, Kodak XAR-5.

Hepatocyte Cultures and Drug Treatment

Primary cultures of adult rat hepatocytes were prepared as previously described [29]. Hepatocytes freshly isolated by collagenase perfusion of the livers of untreated rats were incubated in a humidified atmosphere of 95% air/5% CO₂ in a modification of Waymouth MB-752 medium containing 0.1 μM insulin as the only hormone. Hepatocytes were plated onto 60 mm plastic culture dishes coated with Matrigel (approximately 300 μg). Isolated hepatocytes were maintained in the defined culture medium for 72 hours prior to treatment. Inducers were added as 1000-fold stocks in dimethyl sulfoxide. Matrigel was prepared from the Engelbreth-Holm-Swarm tumor provided by Dr. Hynda Kleinman, NIDR, NIH, Bethesda, MD.

Immunofluorescence

Pieces of liver (0.5 mm³) were dissected from control and PCN treated animals, rapidly placed on OCT compound (Miles, Elkhart IN) at 4 °C, frozen in a dry ice acetone bath, and stored at -70 °C. Cryostat sections (4 μm) were prepared and mounted on plus charged slides, dried at 22 °C, and fixed in methanol at -20 °C for 5 minutes just prior to immunolabeling. All incubations for immunolabeling were at 22 °C and as follows: (1) 15 minutes in phosphate-buffered saline (PBS) with 10% fetal calf serum (FCS) and 0.25% glycine; (2) 90 minutes with mouse anti-rat RT1.B(I) monoclonal antibody (OX6) diluted 1:100 with PBS containing 10% FCS; (3) two 10 minute washes with PBS; (4) 30 minutes with goat anti-mouse IgG conjugated with Alexa-488 (Molecular Probes, Eugene OR) diluted 1:800 with PBS containing 10% fetal calf serum; (5) two 10 minute washes with PBS, and finally mounted in MOWIOL (Calbiochem, San Diego, CA). The fluorescence was recorded on Ektachrome 35 mm film with a Nikon Diaphot inverted microscope, the film scanned with a Nikon slide scanner, and the final digital image pasted together and labeled using Adobe Photoshop. In all of these imaging steps, care was taken to apply the same exposure times and digital manipulations to the images of the control and PCN treated tissues.

Results

We previously identified a total of 76 differentially displayed bands of liver RNA from rats treated for either 8 or 24 hours with Dex or PCN as compared to RNA bands from untreated animals [23]. One of
the selected bands displaying PCN inducibility yielded a cDNA fragment of approximately 280 bp (Fig. 1). Northern blot analysis revealed that this cDNA fragment hybridized with an ~1.4 kb transcript from RNA isolated from the liver of a PCN-treated rat. Comparison of the sequence of the isolated cDNA to the NIH GenBank Database identified this clone as RT1.B(I)β, the β chain component of the major MHC class II gene in the rat. Sequence alignment with the 3′ end of the published RT1.B(I)β sequence (GenBank accession no. X56596) shows a 98% sequence identity (Fig. 1).

Fig. 1. mRNA differential display gel of amplified cDNAs from rat liver RNA. RNA was isolated from control (C), Dex (D) or PCN (P) treated female rats (8 hour treatment) and was processed for differential display. A cDNA band (subsequently identified as MHC class II RT1.B(I)β) was evident only in lanes containing RNA from PCN treated rats. When excised and amplified by PCR it gave an apparent length of 278 bp when submitted to electrophoresis on a 1% agarose gel (see lower insert). Northern blot hybridization of rat liver RNA using the excised PCR cDNA product as a probe confirmed reactivity with an mRNA species in the liver RNA of a PCN treated rat (see upper insert). The DNA sequence for the isolated cDNA fragment from the differential display corresponds to the RT1.B(I)β gene. Italicized sequence represents the 202 bases from the 3′ untranslated region of the isolated cDNA fragment identical to the RT1.B(I)β reported cDNA sequence (GenBank accession no. X56596). The remaining 76 bases complete the 3′ sequence to the polyA+ tail.
To further confirm the identity of the isolated cDNA fragment as RT1.B(I)β, we probed Northern blots of liver RNA from PCN treated animals with either the differential display cDNA fragment (DD probe) or a nearly full-length cDNA clone encoding RT1.B(I)β (pLRβ118 probe), and found a single, common band induced in the liver by PCN treatment (Fig. 2). The presence of other, smaller, apparently non-induced bands visible only on blots that were hybridized with the pLRβ118 probe (Fig. 2) likely reflect the cross-reactivity of the RT1.B(I)β cDNA probe to other class II genes including RT1.D(I) (Dr. K. Reske, personal communication). Reanalysis of the blot revealed also induction by PCN of an mRNA band that hybridized with a cloned cDNA of RT1.B(I)α [27], the companion subchain for the complete heterodimer RT1.B(I) molecule (Fig. 2). Because treatment of rats with PCN failed to induce the expression of RT1.B(I)β mRNA in the spleen, an organ laden with lymphocytes and other immune cells (Fig. 2), it seemed unlikely that steroid induced expression of RT1.B(I)β mRNA in the liver reflects an effect on resident hepatic immune cells.

Examining the temporality of gene expression in rat liver, we found, as has been reported previously [9], that induction of CYP3A mRNAs was detectable within 3 hours (approximately 3 fold) after a single injection of Dex or PCN and increased thereafter with Dex being a more efficacious inducer at both 18 and 30 hours (approximately 30 fold) when compared to PCN (approximately 8 fold) (Fig. 3). In contrast, the amount of RT1.B(I)β mRNA was induced by PCN (but not by Dex) at 3 hours (> 70 fold), 6 h (30 fold), and 8 hours (18 fold) (not shown) and declined thereafter while Dex induced RT1.B(I)β mRNA did not become detectable until 18 hours (>20 fold) (Fig. 3). From these experimental results, we concluded that both PCN and Dex coinduce CYP3A and RT1.B(I)β and α mRNAs in rat liver in vivo.

It was important to exclude the possibility that PCN or Dex induced hepatic RT1.B(I)β mRNA, indirectly, by causing the systemic release of endogenous mediators (such as INFγ) [30] which...
subsequently target the hepatocyte. Accordingly, we exposed primary, nonproliferating cultures of adult rat hepatocytes to a defined medium containing PCN or Dex, and found that the RT1.B(I)\(h\) mRNA became detectable (>20 fold) within 3–6 hours (Fig. 4). The induction of RT1.B(I)\(h\) mRNA was sustained for 48 hours in the presence of PCN but diminished and disappeared in Dex treated cultures (Fig. 4). As we have observed many times previously, the same cultures responded to PCN or Dex with an induction of CYP3A23 mRNA within 6 hours that progressively increased thereafter (>90 fold at 48 hours) (Fig. 4). These results indicate that PCN and Dex rapidly induce RT1.B(I)\(h\) mRNA in the hepatocyte, directly, because these cultures contain no serum and only a small fraction of non-parenchymal cells (1-2%) (e.g. Kupffer cells) [31].

The observed differences in the induction of MHC II mRNA seen in primary cultures as compared to in vivo (Fig. 3) suggest that other factors not in culture are involved in modulating the responses seen with each inducer.

A final set of experiments verified that induction of RT1.B(I)\(h\) mRNA is accompanied by the appearance of RT1.B(I)\(h\) protein. Immunofluorescence of MHC II in sections of liver labeled with the OX6 antibody showed staining localized to the hepatocytes throughout the lobule in PCN treated

Fig. 3. Time course of expression of rat liver RT1.B(I)\(h\) mRNA. RNA isolated at the indicated times from control (C) or from Dex (D) or PCN (P) treated female rats was analyzed on Northern blots (10 \(\mu\)g/lane) hybridized with the differential display-isolated cDNA as the probe for RT1.B(I)\(h\). Then, the blot was stripped and was reprobed with a CYP3A cDNA probe. Finally, the same membrane was reprobed with a GAPDH cDNA probe to evaluate equal loading (for example lane C, 3 h was underloaded).

Fig. 4. Expression of RT1.B(I)\(h\) mRNA in primary cultures of rat hepatocytes following treatment with Dex or PCN. Isolated hepatocytes were maintained in a defined culture medium for 72 hours and then were incubated for the indicated times with medium containing 10 \(\mu\)M Dex or 10 \(\mu\)M PCN. RNA harvested from the cultures, was processed by gel electrophoresis (10 \(\mu\)g/ lane) and was analyzed on Northern blots hybridized with the isolated differential display cDNA fragment, or with a CYP3A cDNA probe. The same membrane was reprobed with a GAPDH cDNA probe to adjust for RNA loading.
Fig. 5. Immunofluorescence micrograph of RT1.B(I)β localization in PCN treated rat liver. Cryostat sections of control spleen (A), control liver (B and E) and PCN treated liver (C and F) were labeled with mouse anti-rat RT1.B(I) monoclonal antibody (OX6) and subsequently with fluorescently conjugated goat anti-mouse IgG. Panels B and C are low magnification micrographs illustrating the overall labeling pattern for RT1.B(I)β. Unaffected by PCN treatment, heavily labeled cells are observed particularly in the periportal region (pp) and dispersed throughout the tissue. However, after PCN treatment (C) a lighter staining, in a web like pattern, is evident throughout the section that is not present in the control tissue or in tissue labeled with pre-immune serum (D). This is shown at a higher magnification in F as indicated by the arrow heads. The stars in E and F indicate the voids of the hepatocyte nuclei. The bar in A, B, and C is 20 μm and in D, E, and F is 10 μm.
animals (faint, uniform green signals appear on the hepatocyte cell surface; Fig. 5), while only diffuse background fluorescence was apparent in control animals. In addition, sections of liver from untreated control and PCN treated animals contained bright punctate staining associated with the portal tracts, although these signals were not affected by PCN treatment. Specificity of the antibody was demonstrated by lack of reactivity in tissues stained with pre-immune serum (Fig. 5D).

Discussion

Historically, it has been assumed that the protective effects of PCN against “stressful” toxic insults largely involve stimulation by the steroid of multiple components of the liver’s “drug metabolizing system” [32], of its biliary excretion process [33], and of its production of essential membrane components such as cholesterol [34]. Our present findings expand the impact of PCN beyond xenobiotic elimination by the liver, and thus, occasion a reappraisal of this concept. We found that PCN and Dex activate expression of RT1.B(I)β in rat liver and in primary cultures of rat hepatocytes, cells that do not customarily express MHC class II genes. Indeed, these pivotal mediators of the immune response are generally restricted to professional antigen presenting cells with the exception that IFN-γ can induce MHC II genes in many tissues over a 24–48 hour period [35]. Rapid, ectopic activation of these immune responsive genes in the liver by a drug is unprecedented to our knowledge. The function of MHC II induction in the liver is unknown at present, but need not necessarily involve the immune system. Recently, MHC-I has been shown to play an unexpected pivotal role in organizing interneuronal connections in the developing brain [36]. PCN, presumably acting through its newly identified receptor, PXR [11], may activate a much broader and more fundamental set of disparate genes to maintain homeostasis than was previously accepted [21,23] Differential display (see Fig. 1) affords the opportunity to catalog additional induced or inhibited gene products that may be unexpected members of this postulated set of PCN/PXR controlled genes.

RT1.B(I)β and α are members of the MHC class II membrane receptors (glycoprotein alpha and beta chain heterodimers) involved in presentation of foreign antigens to T-helper lymphocytes. The rat MHC encodes three isotypic class II α and β heterodimers referred to as RT1.B, RT1.D and RT1.H [reviewed in [30]. Unlike the ubiquitously expressed MHC class I molecules, MHC class II genes are expressed constitutively only in such antigen presenting cells as B-lymphocytes, thymic epithelium, dendritic cells, and macrophage cell types [30,37]. MHC class II molecules can be found in many cell types including epithelial cells in inflammatory states under the influence of INFγ, TNF–α, or IL4 [30], in such states as rat pyloric mucosal cell infiltrate following treatment with the carcinogen, MNNG [38], or in salivary glands of rats treated with mercury chloride [39]. However, the rapid appearance of RT1.B(I) α and β (within 3 hours) or any MHC II gene product in steroid treated liver was completely unforeseen. Indeed, corticosteroids have been shown to down regulate MHC class II in B cells and macrophages [40], and we found down regulation of the RT1.B(I)β in the spleen of Dex treated rats (not shown) while in vascular endothelial cells, Dex was reported to slightly stimulate expression of MHC class II [41]. It seems unlikely that resident liver macrophages (Kupffer cells) account for the results presented here, because (i) PCN and Dex induction of RT1.B(I)β was reproduced in primary cultures of hepatocytes isolated from nonparenchymal cells (Fig. 4), (ii) because no induction of RT1.B(I)β in the spleen (Fig. 2) was observed, and (iii) because immunofluorescence localized the PCN induced RT1.B(I) immunoreactive protein to the hepatocytes across the lobule (Fig. 5). Moreover, others have reported that Dex
treatment of cultured macrophages inhibits expression of MHC II molecules [42]. However, we did 
observe detectable RT1Bβ mRNA in some control animals and noninduced immunoreactive protein in 
the portal tracts, likely the contribution of some inflammatory cells. Further studies are required to 
determine if our finding of RT1.B(I)β as a possible accessory liver function is related to the suggested 
involvement of the MHC with the development of diabetes [43], hypertension [44], liver disease [45] or 
liver cancer [46]. If the liver lacks expression of costimulator molecules such as B7, as has been 
demonstrated for interferon-stimulated renal epithelial cells, then class II expression in the liver could 
involve a role for hepatocytes in immune surveillance or in down-regulation of autoimmune responses 
[47].

PCN serves as a convenient pharmacologic probe for its receptor, PXR [11], because PCN does not 
activate (or may actually inhibit) the glucocorticoid receptor [8] while Dex binds to both receptors. 
Therefore, it is believed that PCN distinguishes PXR mediated events. The mechanism involves binding 
of the liganded PXR receptor to one of several forms of a direct repeat (TGAAC1n3TGAAC1) or an 
everted repeat (TGAAC1n6AGGTCA) in the DNA associated with CYP3A, and presumably, with other 
PCN responsive genes in several species [7]. PXR is abundantly expressed in the liver, stomach, and 
intestine [11]. Although responsiveness of gene expression to PCN per se is a phenomenon largely 
restricted to rodents, an homologous human receptor has recently been identified [14–17]. This receptor 
binds the antibiotic, rifampicin, but not PCN. This finding corresponds to the observation that the 
CYP3A4 gene, the predominant form of human liver cytochrome P450, contains a DNA response 
element (an everted repeat) similar to that for its rat homologue, CYP3A23 [7], and is inducible in 
human liver and in cultured human hepatocytes by rifampicin [48]. It has been suggested that this 
evolutionarily conserved receptor and responsive genes in its domain may function as a “steroid 
hormone sensing system” [16]. We presently do not know whether the induction of MHC II by PCN or 
Dex works through activation of the PXR. The different time course of induction observed in these 
studies suggests that Dex and PCN could be acting separately by mechanisms involving different 
receptors or through cooperation between the PXR and glucocorticoid receptor. Further mechanistic 
studies are necessary to resolve this issue.

Conclusion

We conclude that direct contact of PCN with the liver rapidly evokes ectopic expression of RT1.B β1 
mRNA, an unprecedented effect of steroids or drugs. We believe the present identification of immune 
response genes under the control of PCN in parenchymal tissues, especially if this can be confirmed in 
humans, suggests that this system serves a more central biological role. We are encouraged to pursue 
Selye’s prediction that “catatonic” steroids would be found to activate a system of physiologically 
relevant, multiple cellular and hormonal changes that maintain homeostasis to an individual exposed to 
environmental stress.

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