



Published in final edited form as:

*Toxicol Sci.* 2006 December ; 94(2): 379–387.

## Identification of Genes Controlled by the Pregnane X Receptor by Microarray Analysis of mRNAs from Pregnenolone 16 $\alpha$ -carbonitrile Treated Rats

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### Abstract

Mammalian liver contains a Pregnane X Receptor (NR1I2), which binds drugs and other xenobiotics, and stimulates (or suppresses) expression of numerous genes involved in the metabolic elimination of foreign compounds and some toxic endogenous substances. In the present study we used microarray analysis to identify genes whose expression in rat liver was significantly altered by pregnenolone 16 $\alpha$ -carbonitrile (PCN) treatment. PCN is a synthetic steroid that induces cytochrome P4503A expression and is hepatoprotective by increasing resistance to subsequent stressful insults. Significant induction was seen for 138 genes while expression of 82 genes was significantly repressed. We found induction of genes known to be induced by PCN, such as enzymes involved in drug metabolism and transport. In addition, many genes were differentially expressed whose functions concerned intracellular metabolism, transport of essential small molecules, cell cycle, and redox balance. Our results support the idea that the domain of PXR controlled gene networks may be even more extensive than currently thought and may extend to functions apart from xenobiotic metabolism.

### Keywords

pregnenolone 16 $\alpha$ -carbonitrile; pregnane X receptor; microarray; gene expression profiling

### INTRODUCTION

It has been known for decades that animals treated with pregnenolone-16 $\alpha$ -carbonitrile (PCN), a synthetic steroid, exhibit increased resistance to subsequent stressful insults. PCN appears to protect an animal by eliciting adaptive responses in the liver (and possibly in other tissues) manifested by such physiologic changes as increase in liver size (Garg *et al.*, 1970), hypertrophy of hepatocytes (Japundzic *et al.*, 1974), and increased bile production (Selye, 1971). Biochemical and molecular studies then verified that PCN induces expression of genes that encode mammalian liver enzymes involved in xenobiotic elimination. For example, PCN

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treatment of rats induces the levels of enzymes involved in oxidative metabolism (cytochromes P4503A) (Gonzalez *et al.*, 1993), solubilization by conjugation (DIG- glucuronosyltransferase (Schuetz *et al.*, 1986; Arlotto *et al.*, 1987), glutathione-S-transferase (Maglich *et al.*, 2002), sulfotransferase (Runge-Morris *et al.*, 1999), and small molecule transport (product of the p-glycoprotein gene (P-gp or ABC1) (Maglich *et al.*, 2002) and Na<sup>+</sup>-independent organic anion transporter 2 (Oatp2) (Staudinger *et al.*, 2001a). These observations of multi-gene induction suggested the participation of a receptor-mediated pathway, an idea that has been verified by the discovery of the pregnane X receptor (PXR, NR1I2), a member of the superfamily of nuclear receptor transcription factors. PXR is expressed in liver and several other parenchymal tissues, specifically binds PCN, interacts with a specific DNA response element associated with PCN responsive genes (Goodwin *et al.*, 2002), and accounts for the pharmacologic characteristics of induction of “drug-metabolizing” enzymes in the liver (Dussault and Forman, 2002). Indeed, many investigators accept that PXR functions as a “xenobiotic sensor” (Blumberg *et al.*, 1998). Conversely, a variety of endogenous substances have been suggested as candidate physiological ligands for the PXR, including pregnanes, steroids, and bile acids (Dussault and Forman, 2002; Orans *et al.*, 2005).

The wide variety of stresses which PCN counteracts suggests additional systems, apart from drug metabolism, that are activated in response to PCN treatment. To test this idea, we previously used the technique of differential display to search for mRNA species that were induced or repressed by PCN treatment. We found 76 mRNA species differential expressed in rat liver treated with PCN. One of these was identified as RT1.B(I)Beta, a component of the major histocompatibility class II (MHC) gene family of the immune system (Jimenez *et al.*, 2002). Another mRNA species induced by PCN was identified as IF1, an inhibitor peptide of the ATP synthase/ATPase complex (Jimenez *et al.*, 2000). The finding that genes connected to immune regulation and mitochondrial energy production, respectively, are activated by PCN, suggested that functions unrelated to drug metabolism may be important in the protective adaptation program of the liver. This hypothesis prompted us to further investigate additional genes that fall under the inductive control of PCN/PXR.

In the present study, we used high-density oligonucleotide arrays to obtain a record of rat liver genes whose expression is affected by PCN. This technology can identify each gene, as well as the magnitude to which it is induced or repressed by PCN treatment when compared to a control. Statistical analysis of differential responses allows for identification of significant genes, and software analysis provides pathway information. Our results indicate that the domain of PCN-controlled genes in the liver is broader than previously recognized, and may provide novel insights into the physiological role of the PXR.

## MATERIALS AND METHODS

### Experimental animals and treatments

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, animals (five treated, five controls) received a single intraperitoneal dose (100 mg/kg) of PCN suspended in corn oil or vehicle and were sacrificed 8 h later. Livers were isolated and total RNA was extracted as described below. Animals were maintained and research conducted in accordance with guidelines set forth by the Animal Care and Research Committee (University of Colorado). PCN was a gift from The Upjohn Company (Kalamazoo, MI).

### Isolation and quality check of total RNA

Liver sections were excised, immediately homogenized in lysis buffer, and total RNA isolated by using the RNeasy Total RNA Isolation Kit (Qiagen Inc., Valencia, California). The integrity

of the RNA samples was examined by the Agilent 2100 Bioanalyzer. Northern blot analysis of total RNA from each animal was performed to confirm PCN-induced expression of CYP3A. As expected, treatment of rats with 100 mg/kg of PCN for 8 h induced the expression of CYP3A mRNA in all 5 animals (Fig. 1).

### Preparation of RNA for hybridization to microarrays

Total RNA (1–5 µg) was converted to double-stranded cDNA (ds-cDNA) by using the Superscript Choice System (Life Technologies Inc., Gaithersburg, MD). An oligo-dT primer containing a T7 RNA polymerase promoter (Proligo Primers & Probes, Boulder, Colorado) was utilized. The ds-cDNA was purified and recovered by using GeneChip sample cleanup module (Affymetrix Inc, Santa Clara, California). In vitro transcription was performed to generate biotin-labeled cRNA. In vitro transcription was performed using a RNA Transcript Labeling Kit (Enzo, Farmingdale, New York) and ds-cDNA template was transcribed in the presence of a mixture of biotin-labeled ribonucleotides. Biotin-labeled cRNA was purified using GeneChip sample cleanup module (Affymetrix) or an RNeasy affinity column (Qiagen). To ensure optimal hybridization to the oligonucleotide array, the cRNA was fragmented. Fragmentation is performed such that the cRNA fragments are between 50–200 bases in length by incubating the cRNA at 94 C for 35 min in a fragmentation buffer. The sample was then added to a hybridization solution containing 100 mM MES, 1 M NaCl, and 20 mM EDTA in the presence of 0.01% Tween 20. The final concentration of the fragmented cRNA was 0.05 µg/µL.

### GeneChip processing

The Affymetrix GeneChip® Rat Expression Set 230 was used to determine the changes in mRNA expression in response to PCN. The microarray set incorporates data from over 30,200 transcripts and variants, including 28,000 well substantiated rat genes. Set 230A chip contains ESTs (Expressed Sequence Tags) plus full-length sequences while Set 230B contains only ESTs. Hybridization was performed by incubating 200 µL of the sample with the Affymetrix GeneChip® arrays. Hybridizations were performed at 45 C for 16 h using a GeneChip® Hybridization Oven 640 (Affymetrix). After hybridization, the hybridization solutions were removed and the arrays were washed and stained with Streptavidin-phycoerythrin using a GeneChip® Fluidics Station 450 (Affymetrix). Arrays were read at a resolution of 2.5 to 3 microns using the GeneChip Scanner 3000 (Affymetrix).

### Data analysis and statistical and informatic analysis

Detailed protocols for data analysis of Affymetrix microarrays and extensive documentation of the sensitivity and quantitative aspects of the method are described in the documentation of the Affymetrix Microarray Analysis Suite version 5.0 (MAS 5.0, Affymetrix). Briefly, each gene is represented by the use of ~11 perfectly matched (PM) and mismatched (MM) control probes. The MM probes act as specificity controls that allow the direct subtraction of both background and cross-hybridization signals. Raw fluorescence data are processed into normalized expression values. Raw data from array scans were averaged across all gene probes on each array and a scaling factor was applied to bring the average intensity for all probes on the array to 500. This allows any sample to be normalized for comparison to any other comparable sample, i.e. control vs. PCN treatment. Rat 230 type A and B chips were combined, resulting in 31,142 gene fragments. These expression values output by MAS 5.0 were further processed by the method described below to produce a list of 220 genes that are differentially expressed with high confidence.

We applied two preprocessing filters to the output of MAS 5.0 to remove meaningless genes; a variance filter and an absolute call filter. The median filter removes genes without at least moderate variance; genes whose expression does not vary at all seem unlikely to be involved

in these phenotypes. In most circumstances, including this one, it is reasonable to assume that only a modest proportion of genes changed expression levels, and therefore the gene with the median variance is a reasonable model of null variation; i.e., the variation due to other factors. We calculate the variance  $s^2$  for each gene; the null hypothesis is that these variances represent random and Normally distributed noise. We can then compute the statistic  $W = (N-1)s^2/\text{median}(s^2)$ , where  $N$  is the number of observations of the gene, which is approximately chi-square distributed with  $N-1$  degree of freedom (Hogg and Craig, 1978). We calculate a p value for rejecting the null hypothesis that the gene did not vary, and perform the False Discovery Rate (FDR) multiple testing correction (Benjamini and Hochberg, 1995), setting the FDR to be 0.10. The genes that pass through this filter have significantly greater variation than the median variation gene, with at most 10% of that list including genes having true variation less than or equal to median variation. The second filter uses Affymetrix's mRNA detection call to exclude all genes with an Absolute Call of "Absent" in all experiments. These pre-processing steps screen out genes with low total variance, and low mRNA levels; the remainder, 9117 genes, is then analyzed for differences related to experimental condition.

The D'Agostino normality test was applied to each chip individually, and all had p values of less than 0.001, indicating normal distributions. We then performed a two-tailed t-test. The false discovery rate procedure is again used to correct for multiple testing, producing a final list of 220 differentially expressed genes at FDR of 0.07. Selection of a lower FDR results in no differentially expressed genes, and in the context of 220 genes, an FDR setting of 0.07 is conservative. The result can be interpreted using the formula:  $\text{FDR} = \text{expected number of false positive}/\text{total number of positives}$ , where total number of positives is 220 and FDR is 0.07, for an expected number of false positives equal to 15 of the 220 genes. Of these, 138 genes were induced and 82 repressed. The entire dataset is available at NCBI GEO (<http://www.ncbi.nlm.nih.gov/geo/>) with accession number GSE4959.

The Affymetrix probe set identifiers for these genes were then mapped to GenBank accession numbers. The Gene Ontology database (<http://www.ncbi.nlm.nih.gov/entrez>) was used to identify known functional information regarding the differentially expressed genes, as presented in Tables 1 and 2 under the heading "Group." The genes for which the annotation was unclear such as ESTs and those of weak identity were excluded from the table. The majority of annotated differentially expressed genes identified were ESTs with unknown functions, a finding most likely the result of an inadequate knowledge of the rat genome and transcriptome.

### Quantitative real-time RT-PCR

Total RNA was treated with DNase I prior to analysis. Real time RT-PCR was performed using the ABI Prism 7700 sequence detector (Perkin Elmer Corp./Applied Biosystems) with the following primers: CYP3A1 (also referred to as CYP3A23) (accession no. X96721) forward primer, 5'-CTCACACTGGAAACCTGGGTC-3', reverse primer, 5'-CGGGTCCCAAATCCGTAGA-3', and TaqMan probe, 5'-TCCTGGCAGTCGTCCTGGTGCTC-3', giving a product of 65 bp; Myd118/GADD45 $\beta$  (accession no. NM\_001008321) forward primer, 5'-GTGACTGAATCATGACCCTGGA-3', reverse primer, 5'-ACCGCCTGCATCTTCTGAAC-3', and TaqMan probe, 5'-CTGGTGGCGAGCGACAACGC-3', giving a product of 67 bp; claudin 1 (accession no. NM\_031699) forward primer 5'-TTGCAGCTTCTGGGTTTCATC-3', reverse primer, 5'-AGGGCAGTGCTGACGATAGAG-3', and TaqMan probe, 5'-TGGCTTCGCTGGGATGGATCG-3', giving a product of 65 bp. Each real time RT-PCR reaction was performed in duplicate and normalized to the 18S ribosomal RNA levels in the same sample. Since 18S ribosomal RNA is abundant when compared to the target, the RNA is diluted approximately 100 fold for 18S quantitation and the dilution factor taken in to account

when calculating the quantities of 18S. The statistical analyses were performed using GraphPad Prism (version 4.0, GraphPad Software, San Diego, CA).

## RESULTS

The profile of hepatic gene expression in rats exposed to PCN was analyzed using the Affymetrix GeneChip® Rat Expression Set 230. Recognizing that each of the large number of genes being tested is effectively a separate hypothesis about contribution to phenotype we applied multiple testing corrections to ensure meaningful results. The array technology we used allows for a comprehensive display of the primary genes affected by PCN, without the need for any prediction concerning which genes to look for. We also recognized that the use of a single animal could result in variability in the expression profile. To accommodate this, we used RNA from 5 vehicle-treated and 5 PCN-treated animals to screen 10 microarray chips as well as statistical analysis to maximize the accuracy of the array data. We found several hundred genes that demonstrated significant increases or decreases in the expression of mRNAs in PCN-treated liver. Standard statistical programs were applied using receiver/operator characteristics to maximize specificity of changes in gene expression and minimize false positives. A threshold for induction and repression was implemented to meet these requirements (see Tables 1 and 2).

Significant induction or repression was seen for 220 genes based on the analysis of hybridization to ten microarray chips. We found induction of 138 genes, including those known to be induced by PCN, such as cytochrome P4503A, aminolevulinic acid synthase (the rate limiting enzyme for heme biosynthesis), UDP glucuronosyltransferase, glutathione S-transferase, and drug-transporting proteins such as Abcb1a. In addition, 82 genes were significantly repressed, including cytochrome P4502F1, for which ethoxycoumarin is a traditional substrate. Many of the genes identified involve functions unrelated to drug metabolism, including various aspects of intracellular metabolism and transport of ions, proteins, and other small molecules. These functions encompass various organelles including the mitochondria, golgi, and plasma membrane. Of the 220 annotated differentially expressed genes, 82 have known process functions (Tables 1 and 2). We found the majority of genes altered by PCN were involved in binding (*e.g.* protein, nucleotide, nucleic acid, calcium ion), metabolism (*e.g.* amino acid, phospholipid, fatty acid), and transport (*e.g.* bile acid, electron).

The expression of CYP3A1 and selected genes identified as being differentially expressed by microarray analysis was validated by quantitative RT-PCR. We chose CYP3A1 for validation because its expression, known to be highly induced by PCN in rat liver (Quattrochi and Guzelian, 2001), was not identified in the array analysis as being differentially expressed, although PCN-stimulated expression was found for rat CYP3A18 (2.74-fold) and transcripts similar to human CYP3A3 (1.50-fold). We found by RT-PCR analysis of RNA from 5 control and 5 PCN-treated animals (the same RNA preparations used for the microarray) and using primers specific to the CYP3A1 sequence that CYP3A1 expression was induced 8.2-fold over control animals (Fig. 2). This discrepancy is most likely due to cross-hybridization of multiple, homologous CYP3A transcripts, some of which are highly expressed constitutively, to the arrayed CYP3A1 target oligonucleotides.

In our study, PCN stimulated the highest level of expression of a gene product not involved in drug metabolism, namely Myd118/GADD45 $\beta$ . There was good agreement between microarray and RT-PCR data for expression of GADD45 $\beta$  10.36-fold vs. 16.77-fold, respectively (Table 1 and Fig. 2). GADD45 $\beta$  is a member of a family of genes that has been shown to be induced by DNA damage and other genotoxic stresses, as well as by terminal differentiation and apoptotic cytokines (Liebermann and Hoffman, 1998). Indeed, a large number of differentially expressed genes were found in the functional category characterized by pathways involved in



cell cycle, growth suppression, apoptosis, cell signaling (Table 1 and 2), providing additional evidence that the activated PXR functions as a sensor of xenobiotic stress. Such findings also evoke important therapeutic considerations. If PCN can bypass immediate early transcription factors to directly stimulate the process of DNA replication and mitosis as has been proposed for the constitutive androstane nuclear receptor (CAR) (Columbano *et al.*, 2005), then clinically relevant PXR agonists such as rifampicin may be useful for stimulating proliferation of transplanted donor livers, provided that drug-drug interactions can be avoided (Costa *et al.*, 2005).

Claudin 1 was also selected for validation because it was identified as a gene that statistically displayed one of the highest levels of suppression, (see Table 2). There was also good agreement between microarray and RT-PCR data for changes in expression of claudin 1: -3.85 versus -5.26, respectively (Table 2 and Fig. 2). Claudins are a family of integral membrane proteins important for tight junction formation (Van Itallie and Anderson, 2004). Gap junctional intercellular communication is essential in the maintenance of tissue homeostasis and for the maintenance of differentiated liver functions; it also contributes to the protection against various cellular stresses (*e.g.* radiation, oxidative, and heat shock). Our findings of substantial suppression of claudin 1 expression in intact rat liver by PCN treatment may provide an explanation for the observation that PCN inhibits gap junctional intercellular communication in rat liver (Kolaja *et al.*, 2000).

## DISCUSSION

Design, analysis, and interpretation of gene expression microarray experiments requires careful attention to statistical and bioinformatic considerations. Uncorrected  $p$  values for each gene over a certain threshold can be deceptive; testing 10,000 genes at a critical value of  $p < 0.05$  means that 500 genes would be expected to appear positive by chance. We used the false discovery rate correction (Benjamini & Hochberg, 1995), a well accepted method in microarray analysis, to create statistically reliable gene lists with a reliable (and controllable) estimate of the number of false positives.

Molecular studies of the effects of PCN on the liver have traditionally focused on the structure, function and inducibility of enzymes involved in xenometabolism. However, recent work has identified specific genes involved in mitochondrial metabolism and immune response. In addition, reports that activation of the SXR (human PXR) appears to elicit expression of genes related to bone homeostasis (Tabb *et al.*, 2003) and that PXR mRNAs (and its splice variants) are present in many human extrahepatic tissues such as brain, bone marrow and heart (Lamba *et al.*, 2004) suggest a broad metabolic role for this receptor and the domain of genes it controls. The present results give further evidence for the multi-system effect of PCN. This steroid, acting specifically through the PXR receptor, simultaneously induces or represses literally hundreds of genes, most of which remain unidentified at this time. This suggests that the adaptive response of the liver requires the involvement of a surprisingly wide group of cellular systems, aside from metabolism and excretion of foreign toxic compounds.

Gene profiling studies confirm that PCN induces or represses expression of a large number of mRNA species. For example, transgenic mice expressing a constitutively active variant of the PXR indicate 168 ESTs are differentially expressed (about half induced) as compared to wild-type mice (Rosenfeld *et al.*, 2003). The authors emphasize that the induced gene products appear to function in activation, solubilization, or elimination of xenobiotics. Still, their results do identify gene products whose functions are unrelated to drug metabolism such as those involved in cell cycle kinetics and growth factors. It is important to note that few of the identified sequences affected by PCN treatment of the rat in the present study appear as differentially expressed genes in these transgenic mice. Another study of wild-type mice

focusing on 38 genes selected for their involvement in liver xenobiotic metabolism and elimination found that PCN induced expression 1.5 fold or greater of 16 genes analyzed by real time quantitative PCR analysis (suppression of mRNA was not examined) in the liver, whereas only 4 genes were differentially expressed in PCN-treated PXR-null mice (Maglich *et al.*, 2002). Finally, examination of the effects of rifampin on mRNA expression in primary cultures of human hepatocytes (an inducer of the human PXR) found that 32 genes were induced as analyzed by either cDNA-based or oligonucleotide-based microarrays (Rae *et al.*, 2001). Although left without comment by the authors, 1 of the induced genes is not involved in drug metabolism or transport (a DNA mismatch repair protein, PMS1). This finding could be important given that PCN is an anticarcinogen (Argus *et al.*, 1978).

Because we sampled the liver at 8 h of exposure, we believe that most of the observed effects are likely to be primary changes effected by PCN, rather than secondary consequences of primary stimulated genes. However, further analysis is required to demonstrate the direct effect of PXR on the transcriptional activation of individual genes. Furthermore, we recognize that statistical significance of PCN altered gene expression (as demonstrated here) does not equate necessarily to the biological significance. Even modest changes can be important if the gene product limits the rate of a metabolic pathway, whereas in other functional contexts large changes may be biologically inconsequential. There is little evidence that in the rat, PCN interacts with CAR or other liver X-receptors, which may have overlapping functions in regulation of drug metabolism. Moreover, our previous studies of the MHCII gene by PCN in the liver indicated that induction could be distinguished from inductive effects of glucocorticoids (dexamethasone), which are also known to interact with the PXR, by comparing the 6 and 18 h treatments (Jimenez *et al.*, 2002) (verification of this hypothesis will require isolation of each gene and detailed mechanistic studies of its response to the PCN/PXR activation). While transgenic mice have the advantage of avoiding pharmacologic non-specificities, the observed differentially expressed genes could reflect secondary responses to the longstanding activation of PXR, beginning with prenatal development. Such models also are unable to simulate the acute activation of the adaptive response of the liver and unable to recognize effects of induction and deinduction that accompany challenges by endogenous or exogenous inducers. Such differences in timing, approach, and in species may account, in part, for the lack of extensive correspondence between PCN-mediated differential expression of genes. Indeed, for reasons that are not clear at this time, our array results failed to return positive signals for MHCII as was expected from our studies of PCN induction of this gene in rat liver and in primary cultures of rat hepatocytes (Jimenez *et al.*, 2002), even though treatment conditions (*e.g.* dose, time) were identical. IF1 inhibitor peptide, previously identified as being differentially expressed in rat liver (Jimenez *et al.*, 2000) was also not identified by the microarray analysis, but this was most likely due to differences in its time-dependent expression. Accordingly, a combined approach of genetic models, pharmacologic challenges, and cell culture studies offers the best prospect of elucidating the complex mechanisms that undoubtedly underlie the highly conserved PXR pathways.

Given the conclusive evidence that PXR mediates both induction and repression of genes involved in complex metabolic pathways of not only xenobiotics but also endogenous substrates such as lipids (Edwards *et al.*, 2002) and bilirubin (Roy-Chowdhury *et al.*, 2003), it might be expected that targeted disruption of the PXR gene would produce animals which, if viable at all, would exhibit significant metabolic disturbances. However, PXR-null mice appear normal and exhibit few, if any, manifestations of altered metabolism (Xie *et al.*, 2000); Staudinger *et al.*, 2001b). These findings suggest that PXR may function, in part, as a standby receptor to elicit a unique set of responses under conditions of stress. Many forms of stress raise levels of glucocorticoids, which in high concentration can directly activate the PXR. Such stress need not represent xenobiotic challenges. Considering the variety of systems induced by PCN and that PXR is activated by glucocorticoids (dexamethasone), it is likely that the role of

PXR is to trigger the liver to adapt to stresses that affect high levels of endogenous glucocorticoids. Use of array technology for screening large numbers of genes to detect changes stimulated by PCN treatment represents, at best, an approach termed hypothesis generation rather than hypothesis testing. Genes of potential interest, unexpectedly discovered by this approach to be regulated by PCN treatment, as for example our findings of GADD45 $\beta$  and claudin 1, must be verified and evaluated for significance by detailed follow-up studies. For instance, while GADD45 $\beta$  up-regulation in livers of rats exposed to PCN conforms to Selye's thesis that PCN affords protection from toxic agents (i.e., GADD45 $\beta$  protects hepatocytes from apoptosis), suppression of claudin 1, a cell-cell adhesion molecule, is counterintuitive, since intercellular junctions contribute to the protection against various toxic stresses. Given our findings that PXR may stimulate expression of many genes outside the drug metabolizing system, we continue to pursue our hypothesis that PXR may serve as the stress receptor. Indeed, our initial results may simply be the molecular representations of the physiologic findings 50 years ago by Hans Selye who predicted that "catatoxic" steroids such as PCN would be found to activate a system of multiple cellular and hormonal changes (as opposed to effects restricted to a single specific tissue such as the liver) in maintaining body homeostasis in an individual exposed to "environmental stress".

### Acknowledgements

This work was supported by National Institutes of Health Grant ES05744 (PSG) and AA13524 (LH). Microarray analysis was performed by the University of Colorado at Denver and Health Sciences Center Microarray Core Laboratory. Quantitative RT-PCR was performed by the University of Colorado Cancer Center PCR Core Facility.

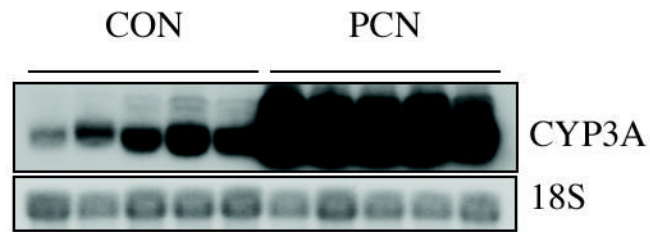
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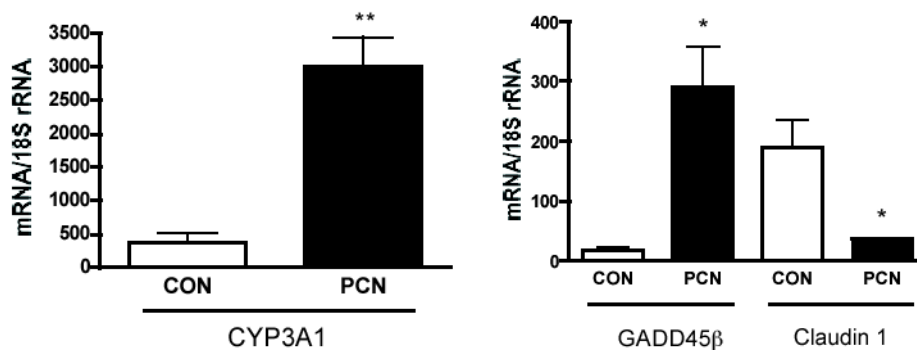


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**Figure 1.** CYP3A expression from control and PCN-treated animals. Total RNA was extracted from the livers of rats treated with vehicle solvent or PCN as described in “Materials and Methods.” Total liver RNA was analyzed by northern blotting for expression of CYP3A using a cDNA probe that recognizes all CYP3A isoforms. The blot was subsequently probed with a cDNA encoding 18S to verify equal loading.



**Figure 2.**

Validation by TaqMan quantitative RT-PCR of selected genes that were altered in the livers of PCN-treated animals. Expression of CYP3A1, GADD45β, and Claudin 1 was determined by quantitative RT-PCR. Data are presented as the mean ± standard deviation of the level of specific gene mRNA expression per 18S ribosomal mRNA as described in “Materials and Methods.” Quantitative RT-PCR was performed on RNA from 5 control and 5 PCN-treated animals (CYP3A1, left graph) and 3 control and 3 PCN-treated animals (GADD45β and Claudin 1, right graph) (the same RNA preparations used for the microarray) and using primers specific to the CYP3A1 sequence (also referred to as CYP3A23, accession no. X96721), GADD45β sequence (NM\_001008321), and Claudin 1 sequence (NM\_031699). P < 0.05\* and P < 0.001\*\* compared to control animals with unpaired t test.

**Table 1**  
Genes significantly up-regulated by PCN

<b>Fold Change</b>	<b>Gene/Description</b>	<b>Gene Symbol</b>	<b>GenBank Accession No.</b>	<b>Group</b>
10.36	Growth arrest and DNA-damage-inducible 45 beta	Gadd45β	NM_001008321	Binding-protein
5.92	Moderate similarity to p53-associated protein (H. sapiens)	MDM2	CAA78055	Binding-protein
4.28	Cytochrome P45026, retinoic acid	Cyp26	NM_130408	Metabolism
4.01	Moderate similarity to suppression of tumorigenicity 5	St5	NM_005418	Transport-electron Signal transduction Tumor suppression
3.67	Aminolevulinic acid synthase 1	Alas1	NM_024484	Metabolism- heme biosynthesis
3.07	P450 (cytochrome) oxidoreductase	Por	NM_031576	Transport-electron
2.74	Moderate similarity to Ras association, domain family1 (H. sapiens)	RALGDS/AF6	NM_007182	Signal transduction Tumor suppression
2.74	Cytochrome P450, 3a18	Cyp3a18	NM_145782	Metabolism
2.55	ATP-binding cassette, sub-family C (CFTR/ MRP), member 3	Abcc3	NM_080581	Transport- electron Binding-ATP
2.45	Moderate similarity to HUMAN sorting nexin 8	SNX8	NM_013321	Transport
2.40	Similar to B-cell leukemia/lymphoma 3 (predicted)	Bcl3 (predicted)	XM_214856	Intracellular signaling Transport- protein Cell cycle
2.35	Moderate similarity to yeast Sec31p homolog (H. sapiens)	SEC31p	NM_199530	Unknown
2.28	Strong similarity to HUMAN calcium-binding mitochondrial carrier protein	SLC25A12	NM_003705	Binding-calcium ion Transport
2.24	Similar to retinol dehydrogenase 10 (all-trans) [ <i>Mus musculus</i> ]	Rdh10	NM_133832	Oxidoreductase activity
2.08	ATP-binding cassette, sub-family B (MDR/ TAP), member 1A	Abcb1a	NM_133401	Binding-ATP Transport
2.03	GTP cyclohydrolase 1	Gch	NM_024356	Hydrolase activity
1.90	Lactamase, beta 2	Lactb2	NM_00102427	Unknown
1.85	Cyclin D1	Ccnd1	NM_171992	Binding-protein Cell cycle
1.84	Strong similarity to HUMAN ARL-6 interacting protein-2	ARL6	NM_032146	Hydrolase activity Binding-protein
1.83	BCL2/adenovirus E1B 19 kDa-interacting protein 3-like	Bnip3	NM_053420	Apoptosis
1.79	Liver UDP-glucuronosyltransferase, phenobarbital-inducible form	Ugt2b3	NM_153314	Metabolism- glucuronosyl transferase
1.78	Strong similarity to mitogen inducible gene mig-2 (H. sapiens)	PLEKHC1	Z24725	Binding-protein
1.78	Protein phosphatase 2a, catalytic subunit, beta isoform	Ppp2cb	NM_017040	Protein phosphatase
1.74	DnaJ homolog, subfamily b, member 9	Dnajb9	NM_012699	Protein binding-heat shock
1.71	Mannosyl (alpha-1,6-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase	Mgat2	NM_053604	Transferase activity
1.69	PDZ domain containing 1	Pdzk2	NM_133226	Binding-Protein Intracellular signalling
1.69	Tumor necrosis factor receptor superfamily, member 1a	Tnfrsf1a	NM_013091	Receptor activity Intracellular signalling
1.67	Glutathione S-transferase, mu 1	Gstm1	NM_017014	Metabolism-glutathione
1.67	Purinergic receptor P2Y, G-protein coupled 2	P2ry2	NM_017255	Binding-nucleotide
1.66	BCL2/adenovirus E1B 19 kDa-interacting protein 3-like	Bnip3l	NM_053420	Apoptosis
1.63	Phospholipase A2, activating protein	Plaa	NM_053866	Metabolism-phospholipid
1.62	TERF1 (TRF1)-interacting nuclear factor 2	Tinf2	NM_001006962	Binding-protein
1.60	ATP-binding cassette, sub-family C (CFTR/ MRP), member 2	Abcc2	NM_012833	Binding-ATP Transport
1.58	RAB2, member RAS oncogene family	Rab2	NM_031718	Binding-nucleotide Protein transport
1.55	Cytochrome P450, subfamily 3A, polypeptide 3	Cyp3a3	NM_013105	Metabolism Transport-electron
1.55	Heat shock 22kDa protein 8	Hspb8	NM_053612	Protein binding-heat shock
1.53	Valosin-containing protein (p97)/p47 complex-interacting protein p135	Ycip135	NM_176857	Binding-protein
1.52	Proteasome (prosome, macropain) 26S subunit, non-ATPase 4	Psm4	NM_031331	Transport-fluid
1.49	Moderate similarity to mitochondrial ribosomal protein L11 (H.sapiens)	MRPL11	NM_016050	Structural constituent of ribosome



<b>Fold Change</b>	<b>Gene/Description</b>	<b>Gene Symbol</b>	<b>GenBank Accession No.</b>	<b>Group</b>
1.48	Moderate similarity to elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 1 (H.sapiens)	ELOVL1	NM_022821	Fatty acid biosynthesis
1.47	Sjogren syndrome antigen B	Ssb	NM_031119	Binding-RNA
1.46	Eukaryotic translation initiation factor 4 gamma, 1 (predicted)	Eif4g1 (predicted)	XM_213569	Binding-RNA
1.44	t-Complex protein 1	Tcp1	NM_012670	Protein biosynthesis Binding-nucleotide Protein folding
1.44	Lectin, galactose binding, soluble 3	Lgals3	NM_031832	Binding-protein Cell adhesion
1.41	Similar to SAR1a gene homolog mRNA	Sara1	NM_001007739	Binding-nucleotide
1.40	RAB6, member RAS oncogene family	Rab6	XM_574503	ATPase/GTPase activity
1.38	Moderate similarity to Poly(A) polymerase alpha (PAP) (H. sapiens)	PAPOLA	NM_032632	Binding-RNA Transferase activity
1.37	Fibronigen-like protein 1	Fg11	NM_172010	Cell growth
1.33	Strong similarity to small GTP-binding protein; RAB1B, member RAS oncogene family (H.sapiens)	RAB1B	NM_030981	Binding-nucleotide Signal transduction
1.32	Heterogeneous nuclear ribonucleoprotein U	Hnrpu	NM_057139	Binding-nucleotide RNA processing
1.29	Eukaryotic translation initiation factor 2, subunit 1 (alpha )	Eif2s1	NM_019356	Binding-nucleic acid Binding-protein
1.27	DnaJ (Hsp40) homolog, subfamily A, member 2	Dnaja2	NM_032079	Protein biosynthesis Protein binding-heat shock
1.26	Leukocyte cell-derived chemotaxin 2 (predicted)	Lect2 (predicted)	XM_341486	Metalloendopeptidase activity Chemotaxis

**Table 2**

Genes significantly down-regulated by PCN

<b>Fold Change</b>	<b>Gene/Description</b>	<b>Gene Symbol</b>	<b>GenBank Accession No.</b>	<b>Group</b>
-1.19	Non-metastatic cell expressed protein 3 (nucleoside diphosphate kinase)	Mne3	NM_053507	Kinase activity Nucleotide biosynthesis
-1.25	Death-associated protein	Dap	NM_022526	Apoptosis
-1.27	RNA terminal phosphate cyclase domain 1	Rtcd1	NM_001004227	Metabolism-RNA
-1.28	Cytochrome P450 monooxygenase	Cyp2t1	NM_134369	Cytochrome-electron transport
-1.32	Apoptotic peptidase activating factor 1	Apaf1	NM_023979	Apoptosis
-1.35	Low density lipoprotein receptor-related protein 11 (predicted)	Lrp11 (predicted)	XM_238072	Receptor activity Endocytosis
-1.35	Ras homolog gene family, member E	Arhe	NM_001007641	Binding-GTP
-1.39	Dipeptidylpeptidase 4	Dpp4	NM_012789	Catalytic activity aminopeptidase
-1.39	Cytochrome P450, subfamily 2F, polypeptide 1	Cyp2f1	NM_019303	Cytochrome-electron transport
-1.41	Kinesin family member C2	Kifc2	NM_198752	Binding -ATP Microtubule motor activity
-1.41	Acyl-CoA synthetase long-chain family member 4	Acs14	NM_053623	Catalytic activity Metabolism-fatty acid
-1.43	Glutamate dehydrogenase 1	Glud1	NM_012570	Binding-nucleotide Metabolism-amino acid
-1.45	Moderate similarity to B-cell CLL/lymphoma 7A (H.sapiens)	BCL7A	NM_020993	Actin binding
-1.49	Methylmalonyl CoA epimerase (predicted)	Mcee (predicted)	XM_215213	Metabolism- L-methylmalonyl-CoA
-1.59	Carbonic anhydrase 2	Ca2	NM_019291	Carbonic anhydrase activity
-1.61	Liver glycogen phosphorylase	Pygl	NM_022268	Catalytic activity Metabolism-carbohydrate
-1.75	Matrilin 1, cartilage matrix protein	Matn1	NM_001006979	Structure-extracellular
-1.79	Aquaporin 8	Aqp8	NM_019158	Transport-water
-1.79	Aquaporin 9	Aqp9	NM_022960	Transport-amine Canalicular bile acid
-1.92	Rab38, member of RAS oncogene family	Rab38	NM_145774	Binding-nucleotide Transport-protein
-1.92	Arginine vasopressin receptor 1A	Avpr1a	NM_053019	G-protein coupled receptor activity
-2.04	CTL target antigen	Cth	NM_017074	Metabolism-amino acid
-2.04	Strong similarity to secreted modular calcium-binding protein1 (H. sapiens)	SMOC1	NM_001034852	Binding-calcium ion
-2.08	Similar to NOGO-interacting mitochondrial protein (H. sapiens)	RTN4IP1	NM_032730	Oxidoreductase activity
-2.27	Glrx1 glutaredoxin 1 (thioltransferase)	Glrx 1	NM_022278	Transport-electron
-2.38	Moderate similarity to protein vitrin (H. sapiens)	VIT	NP_444506	Unknown
-2.50	Myotubularin related protein 7	Mtmr7 (predicted)	XM_240417	Actin binding Phospholipid dephosphorylation
-3.85	Claudin 1	Cldn1	NM_031699	Binding-protein
-7.14	Low density lipoprotein receptor-related protein 5 (predicted)	Lrp5 (predicted)	XM_215187	Receptor activity