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DIFFERENTIAL GENE EXPRESSION IN MOUSE LIVER ASSOCIATED WITH THE HEPATOPROTECTIVE EFFECT OF CLOFIBRATE[1]

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Abstract

Pretreatment of mice with the peroxisome proliferator clofibrate (CFB) protects against acetaminophen (APAP)-induced hepatotoxicity. Previous studies have shown that activation of the nuclear peroxisome proliferator activated receptor α (PPARα) is required for this effect. The present study utilizes gene expression profile analysis to identify potential pathways contributing to PPARα-mediated hepatoprotection. Gene expression profiles were compared between wild type and PPARα-null mice pretreated with vehicle or CFB (500mg/kg, i.p., daily for 10 days) and then challenged with APAP (400mg/kg, p.o.). Total hepatic RNA was isolated 4 h after APAP treatment and hybridized to Affymetrix Mouse Genome MGU74v2.0 GeneChips. Gene expression analysis was performed utilizing GeneSpring ® software. Our analysis identified 53 genes of interest including vanin-1, cell-cycle regulators, lipid-metabolizing enzymes, and aldehyde dehydrogenase 2, an acetaminophen binding protein. Vanin-1 could be important for CFB-mediated hepatoprotection because this protein is involved in the synthesis of cysteamine and cystamine. These are potent antioxidants capable of ameliorating APAP toxicity in rodents and humans. HPLC-ESI/MS/MS analysis of liver extracts indicates that enhanced vanin-1 gene expression results in elevated cystamine levels, which could be mechanistically associated with CFB-mediated hepatoprotection.

Keywords

peroxisome proliferators; clofibrate; acetaminophen; vanin-1; hepatoprotection; cysteamine; cystamine; pantothenic acid; pantetheine

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Introduction

Peroxisome proliferators mediate their actions in the liver through binding and activating the peroxisome proliferator activated receptors (PPARs). Of the three identified PPAR isoforms, the α receptor (PPARα) is primarily expressed in liver and is responsible for induction of enzymes involved in uptake, metabolism, and β-oxidation of fatty acids. Clofibrate (CFB) and other peroxisome proliferators are known to protect the liver against the toxic actions of chemicals like acetaminophen (APAP) (Nicholls-Grzemski *et al.*, 1992;Manautou *et al.*, 1994). The precise mechanism of this hepatoprotective effect is not known.

Overexposure to APAP results in fulminant centrilobular hepatic necrosis, which can extend to the periportal regions of the liver lobule (Mitchell *et al.*, 1973a). This drug is primarily metabolized by conjugation with sulfate and glucuronic acid, with a small percentage of the dose undergoing bioactivation by cytochrome P450 enzymes to the reactive intermediate N-acetyl*p*-benzoquinoneimine (NAPQI) (Mitchell *et al.*, 1973a). At non-toxic doses, NAPQI is eliminated from the liver after conjugation with reduced glutathione (GSH) (Mitchell *et al.*, 1973). However, with toxic doses, the main conjugation pathways for APAP become saturated, resulting in increased formation of NAPQI. Consequently, detoxification of NAPQI is compromised when existing stores of GSH are depleted and NAPQI then binds to cellular macromolecules, initiating cell death pathways (Jollow *et al.*, 1973). Administration of CFB does not alterAPAP bioactivation or conjugative pathways in mouse liver, and although GSH levels are increased following repeated CFB treatment, this is not the mechanism of resistance (Manautou *et al.*, 1994;Nicholls-Grzemski *et al.*, 2000b). Furthermore, overall biliary and urinary elimination of APAP metabolites is not changed by CFB, thus ruling out changes in hepatobiliary disposition as an explanation for the hepatoprotective effect of CFB (Manautou *et al.*, 1996;Faig *et al.*, 2000).

APAP toxicity is associated with increased production of reactive oxygen species and is often accompanied by lipid peroxidation (Wendel *et al.*, 1979;Gerson *et al.*, 1985). Therefore, the role of other detoxification and antioxidant enzymes has also been investigated. Changes in the activity of enzymes such as glutathione-s-transferase pi and catalase have been excluded as contributors to CFB-mediated hepatoprotection (Manautou *et al.*, 1994;Chen *et al.*, 2002). Previous studies from our laboratory demonstrated that mice lacking the PPAR α nuclear receptor are not protected by CFB treatment from APAP toxicity (Chen *et al.*, 2000). This suggests that the gene(s) contributing to the hepatoprotective effect of CFB are controlled by PPAR αeither directly or indirectly.

To further investigate the mechanism of CFB-mediated hepatoprotection, we identified differentially expressed genes between wild type and PPAR α-null mice treated with vehicle or CFB (500mg/kg, i.p.), daily for 10 days, followed by a toxic challenge of APAP (400mg/kg, p.o.). Total hepatic RNA was isolated 4 h after APAP treatment, labeled, and hybridized to Affymetrix Murine Genome Array MGU74 v2.0 GeneChips. Gene expression analysis was performed utilizing GeneSpring ® software to determine differentially expressed genes (fold change≥1.5, $p<0.05$). The first comparison was between CFB and vehicle (VEH) pretreated wild type mice, challenged with APAP (WT_CFB_APAP – WT_VEH_APAP). The second comparison was between wild type and PPARα-null mice pretreated with CFB and also challenged with APAP (WT_CFB_APAP – KO_CFB_APAP). Differentially expressed genes that were common to both comparisons were further investigated for their role in CFB-mediated hepatoprotection. Genes linked to peroxisome proliferation, immune function, proteosomal degradation, and transcriptional regulation were most significantly modulated. Of interest, vanin-1 was induced 7-fold in CFB pretreated wild type mice compared to vehicle (WT_CFB_APAP – WT_VEH_APAP) and 52-fold in CFB pretreated wild type mice compared to PPARα-null mice receiving CFB (WT_CFB_APAP – KO_CFB_APAP). All

groups of mice in these comparisons received APAP. Vanin-1 may be of importance to CFB-mediated hepatoprotection because it is responsible for cysteamine synthesis, a potent antioxidant capable of ameliorating APAP toxicity in rodents and humans.

Methods

Chemicals

All chemicals used were purchased from Sigma (St. Louis, MO) and were of reagent grade or better.

Animals

Wild type (+/+) and PPARα-null (−/ −) male sv/129 background mice aged 10–12 weeks were provided by Frank Gonzalez (National Institutes of Health, Bethesda, MD). Mice were housed in community cages with free access to water and feed (rodent diet No. 5001, PMI Feeds, St. Louis, MO) and maintained a 12-h dark/light cycle. Groups of mice (n = 3–4) received daily dosing of CFB (500mg/kg) or corn oil vehicle (5ml/kg), i.p. for 10 days. Mice were challenged with APAP (400mg/kg, p.o.) or 50% propylene glycol vehicle (5ml/kg). This dosing paradigm results in peroxisome proliferation and protection from APAP in wild type, but not PPAR α-null mice (Chen *et al*., 2000). Livers were removed at 4 hr after APAP dosing, snap frozen in liquid nitrogen, and stored at −80°C until assayed. The University of Connecticut Institutional Animal Care and Use Committee (IACUC) approved all experimental animal protocols.

Plasma Alanine Aminotransferase (ALT) Activity

Blood collected at 4 hr afterAPAP was centrifuged at 8,800xg for 5 min. Plasma was then analyzed for ALT activity using Infinity ALT Liquid Stable Reagent (Thermotrace, Melbourne, Australia) as directed by the manufacturer.

Histopathology

A portion of the liver was fixed in 10% phosphate-buffered formalin. Paraffin embedded sections (5μm) were stained with hematoxylin and eosin. Livers were evaluated by light microscopy and graded as previously reported (Bartolone *et al.*, 1989) for the presence and severity of lesions. Grades range from 0–5 (0, no lesions; 1, single or few necrotic cells; 2, 10–25% necrotic cells or mild diffuse degenerative changes; 3, 25–40% necrotic or degenerative cells; 4, 40–50% necrotic or degenerative cells; 5, more than 50% degenerative cells). Histopathology scores greater than 2 indicate significant pathology (Chen *et al.*, 2000).

RNA Isolation

Total tissue RNA was extracted using RNAzol B reagent (Tel-test Inc., Friendswood, TX) according to the manufacturer's protocol. RNA integrity was confirmed by agarose gel electrophoresis through visualization of intact 18S and 28S rRNA.

Probe labeling

Total RNA (5μg) was used for cDNA synthesis. Superscript II (Invitrogen, Carlsbad, CA) was used to reverse-transcribe the RNA in the presence of a T_7-containing oligo dT_{24} primer and second strand generated as recommended by Affymetrix (Santa Clara, CA). Resulting cDNA was purified using GeneChip sample clean-up modules and used as a template for *in vitro* transcription using the GeneChip One-Cycle Labeling Kit (Affymetrix). Generated cRNA was purified using GeneChip sample clean-up modules (Affymetrix). Quantity and purity of the cRNA was determined by absorbance at 260 nm and 260 nm/280 nm absorbance ratio, respectively. The quality of the cRNA was also evaluated on a 2100 BioAnalyzer (Agilent,

Palo Akto, CA). The cRNA was fragmented as recommended by Affymetrix and quality of fragmentation verified by evaluating the sample on a 2100 BioAnalyzer.

Chip hybridization

Hybridization mix was prepared as recommended by Affymetrix. 10μg of labeled, fragmented cRNA was hybridized to individual MGU74v2.0 Murine GeneChips. The arrays were washed, stained, and scanned under low photomultiplier tube setting.

Differential Gene Array Analysis

Gene expression analysis was performed utilizing GeneSpring® software to determine differentially expressed genes (fold change ≥1.5, $p<0.05$). Normalization of gene expression data was processed using MAS 5.0. The primary gene expression comparisons for identifying genes of interest were WT_CFB_APAP – WT-VEH_APAP and WT_CFB_APAP – KO_CFB_APAP. Further comparisons were made to identify genes in control treated groups, which consisted of WT_VEH_VEH – KO_VEH_VEH, WT_CFB_VEH – WT_VEH_VEH, and WT_VEH_APAP – WT_VEH_VEH. Complete listings of these differentially expressed genes are present in Supplemental Table 2 through Supplemental Table 6.

Reverse Transcriptase-PCR Analysis of Hepatic Vanin-1 Expression

Total hepatic RNA (1 μg) was reverse transcribed and PCR amplified using the Promega Access RT-PCR kit. Gene specific mouse primers for vanin-1 were used as previously described (Berruyer *et al.*, 2004). Relative amounts of cDNA synthesized were estimated by amplification of β-actin, using gene-specific primers (Xu *et al.*, 2005). Vanin-1 and β-actin cDNA products were analyzed on agarose gels and quantified by densitometry using a KODAK Image Station 440CF (Eastman Kodak, New Haven, CT).

Reduced and Oxidized Liver Sample Preparation for ESI-LC/MS/MS Analysis

Liver homogenates were generated from 1g of liver in 4.5 ml of a reducing buffer consisting of 20mM dithiothreitol in 50mM ammonium carbonate buffer (pH8.3). Homogenates were incubated for 30 min at 60°C. Homogenates were further diluted (1:1 v/v) in an alkylating buffer to stabilize the free thiol groups. The alkylating buffer contained 200mM iodoacetamide in ammonium carbonate buffer, pH8.3. Oxidized liver homogenates were generated by diluting 1g of liver in 9ml of an alkylating buffer containing 200mM iodoacetamide in ammonium carbonate buffer, pH8.3. Ice cold acetonitrile was added to both oxidized and reduced homogenate samples (1:1 v/v). The tubes were placed at 5°C for 30 min. The samples were centrifuged at 13,000xg rpm for 10 min. Each sample was decanted and mixed in a LC/MS sample vial with 10mM ammonium formate buffer (1:1 v/v) for analysis of cysteamine and pantetheine. Another 10μL of each sample was decanted and mixed with 90μL of 0.1% formic acid for the analysis of pantothenic acid and cystamine.

HPLC-ESI-MS/MS Analysis

Chromatography was conducted with a capillary HPLC system from LC Packings/Dionex (Amsterdam, Netherlands) including an UltiMate quaternary pump and Famos autosampler. A Thermo Electron Corp. (Bellefonte, PA) Aquasil C_{18} reversed-phase column (3μm particle size, 1mm x 150mm) was used for chromatography with a precolumn (1 mm x 10 mm) containing C_{18} reversed-phase resin. For alkylated cysteamine and pantetheine, HPLC mobile phase A consisted of 10mM ammonium formate and B was 10mM ammonium formate in 90% CH_3CN. The separation of alkylated cysteamine was done with a linear gradient starting at 90% A and 10% B to 100% B over 10 min at a flow rate of 50 μL/min. Separation of pantetheine was done by holding 2% B for 1 min then using a linear gradient to 100% B over 9 min. For pantothenic acid analysis, mobile phase A consisted of 0.1% formic acid and B consisted of

10% water, 0.1% formic acid, and 89.9% acetonitrile. Separation was done by holding 2% B for 1 min then using a linear gradient to 100% B over 9 min. A 2 μL sample injection volume was used. Analyte detection and quantification was done by ESI-MS/MS with a Waters Micromass Quattro Ultima triple quadrupole mass spectrometer (Manchester, U.K.). Quantitative analysis was done in positive ionization mode using the SRM transitions $[M+H]^+ \rightarrow [M+H - 17]^+$ and $[M+H]^+ \rightarrow [M+H - 34]^+$ with collision energies of 10 and 18V respectively for alkylated cysteamine. Pantetheine analysis utilized SRM transitions of $[M+H]^+ \rightarrow [M+H - 18]^+$ and $[M+H]^+ \rightarrow [M+H - 130]^+$ with collision energies of 13V for both transitions. The same transitions were used for pantothenic acid with collision energies of 14V. The dwell time for alkylated cysteamine was set at 1 sec and 0.5 sec for pantothenic acid and pantetheine. The capillary voltage was set at 2.92 kV, the cone voltage was set at 36 V, hexapoles 1 and 2 were set at 18.1 and 0 V, respectively. The source and desolvation temperatures were 120 and 350°C. The cone gas flow rate was 99 L/h and the desolvation gas was set at 287 L/h. Argon was used as the collision gas and set at a pressure of 2.55 mTorr. Quantification was made by comparison to a calibration curve prepared by injecting standards at several concentrations.

Statistical Analysis

Results are expressed as means ± standard error (SE) for 3 to 4 mice per treatment group. Relative gene expression was compared using a student's t-test or ANOVA and Tukey's post hoc test where appropriate. Differences were considered significant at $p<0.05$. A 1.5-fold change threshold was set for identifying significant changes in gene expression, as previously described (Anderson *et al.*, 2004).

Results

Clustering strategy for differentially expressed genes in CFB-mediated hepatoprotection

To investigate PPARα-dependent pathways linked to the hepatoprotective effect of CFB, we identified differentially expressed genes between wild type (WT) and PPARα-null mice (KO) treated with vehicle (VEH) or CFB (500mg/kg) for 10 days, followed by a toxic challenge ofAPAP. Although the administered dose of APAP (400mg/kg) is considered hepatotoxic (Chen *et al.*, 2000), 4h is not sufficient enough time to detect significant increases in plasma ALT activity (Supplemental Figure 1) or noticeable histopathology under the dosing conditions used in this study (Supplemental Table 1). The 4h time point was selected to evaluate genes modulated by CFB and APAP treatment, rather than those associated with overt pathology at later times after toxicant treatment. RNA was isolated from these livers, labeled, and hybridized to individual Affymetrix MGU74v2.0 murine GeneChips for transcriptional analysis. We selected a 1.5-fold threshold ($p<0.05$) for assessing differentially expressed genes as previously done by Anderson et al. (2004) in another mouse gene array study for peroxisome proliferators. The first differential analysis of genes was between WT_CFB_APAP and WT_VEH_APAP mice. This comparison was performed to identify genes modulated by the combination of CFB and APAP treatments. There were 409 differentially expressed genes in this comparison (Supplemental Table 2).

We previously showed that PPARα-null mice pretreated with CFB are not protected from APAP hepatotoxicity (Chen *et al.*, 2000). Therefore, we compared gene expression profiles between WT_CFB_APAP and KO_CFB_APAP mice. This comparison was made to exclude changes in gene expression resulting from the combination of CFB and APAP treatment independent of PPARα activation. Similar gene sorting restrictions (1.5-fold change threshold and $p<0.05$) resulted in 1026 differentially expressed genes (Supplemental Table 3). Theoretically, gene(s) associated with the hepatoprotective effect of CFB are shared by these two comparisons. We focused our investigation on the 53 genes that were differentially

expressed at the intersection of these comparisons (Figure 1). These 53 genes of interest were assessed for consistency across the biological replicates by heat map analysis (Figure 2).

Further comparisons of differentially expressed genes were made to assess changes among control groups. These comparisons included inherent differences between the WT and KO mice receiving vehicle only (WT_VEH_VEH – KO_VEH_VEH) (Supplemental Table 4), gene changes associated with CFB treatment alone in WT mice (WT_CFB_VEH – WT_VEH_VEH) (Supplemental Table 5), and the effect of APAP on gene expression (WT_VEH_APAP – WT_VEH_VEH) (Supplemental Table 6).

Differentially Regulated Genes

Table 1 shows that the vast majority of down-regulated genes are associated with immune function, including mannose-binding lectin protein c (MBL2), zeta-chain TCR associated protein kinase (ZAP70), and lymphocyte antigen 4 homolog (LY64). Decreased expression of these genes suppresses pathogen recognition, T cell signaling, and B cell proliferation, respectively (Miura *et al.*, 1998;Dean *et al.*, 2005;Yokosuka *et al.*, 2005). Jun B proto-oncogene (JunB) was down-regulated approximately 11-fold. Although JunB has several cellular functions, down-regulation of this gene effectively increases c-Jun regulated cellular proliferation (Shaulian and Karin, 2001). The most significantly down-regulated gene was early growth response 1 (EGR1), which is involved in signal transduction, regulation of cell proliferation, and initiation of apoptosis (reviewed by Adamson and Mercola, 2002). Other genes regulating transcription, such as inhibitor of DNA binding 1 (ID1) and zinc finger protein 68 (ZAP68) were also down-regulated. Another gene of interest is aldehyde dehydrogenase 2 (ALDH2), a mitochondrial enzyme involved in ethanol metabolism and detoxification of aldehydes (Siew *et al.*, 1976). ALDH2 is also a major target for APAP covalent binding (Landin *et al.*, 1996).

The majority of up-regulated genes were associated with peroxisome proliferation and lipid metabolism. These include enoyl-Coenzyme A hydratase (EHHADH), malic enzyme 1 (ME1), and acyl-Coenzyme A oxidase 1 (ACOX1). Two genes linked to proteosomal degradation pathways were also up-regulated approximately 2-fold, proteosomal subunitβ-type 4 (PSMB4) and proteosome subunitα-type 3 (PSMA3). Induction of these genes by peroxisome proliferators has been suggested to contribute to resistance from oxidative stress in the liver by eliminating damaged proteins (Anderson, 2004). Of interest, vanin-1 was one of the most dramatically up-regulated genes in both comparisons of differentially expressed genes. Vanin-1 is a glycosylphosphatidylinositol-anchored enzyme with pantetheinase activity, capable of hydrolyzing pantetheine into pantothenic acid (vitamin B_5) and cysteamine (2-mercapturate) (Figure 3). While vanin-1 is most commonly associated with recycling pantetheine from coenzyme A and acyl carrier proteins in the liver, *de novo* generation of cysteamine by vanin-1 may have a role in hepatoprotection. Cysteamine can exist in a reduced monothiol form or as the dithiol cystamine. Cysteamine and cystamine are potent antioxidants capable of ameliorating APAP toxicity when administered exogenously to rodents or humans (Prescott *et al.*, 1974;Olinescu *et al.*, 1982b;MacDonald *et al.*, 1983;Miller and Jollow, 1986). The higher levels of vanin-1 gene transcript expression were further investigated in wild type and PPAR α-null mice following CFB administration (WT_CFB_APAP, KO_CFB_APAP).

Gene expression of vanin-1

Gene array results show that CFB administration increased vanin-1 gene expression (7-fold) between WT_CFB_APAP and WT_VEH_APAP mice (Figure 4). The differential increase in vanin-1 was even more dramatic (53-fold) between the WT_CFB_APAP and KO_CFB_APAP groups. Greater differential expression of vanin-1 gene between wild type and PPAR α-null

mice pretreated with CFB is due to lower basal levels in null mice. These findings were confirmed by RT-PCR analysis (Figure 5). Hepatic RNA was amplified and analyzed for vanin-1 using gene-specific mouse primers (Pitari *et al.*, 2000). RT-PCR analysis showed a 2.5-fold increase in vanin-1 gene expression between WT_CFB_APAP and WT_VEH_APAP. Similarly, vanin-1 gene expression was increased 22-fold between WT_CFB_APAP and KO_CFB_APAP. No significant changes in β-actin gene expression were detected, confirming equivalent RNA loading.

Intracellular hepatic concentrations of vanin-1 substrate and products

ESI-LC/MS/MS analysis of liver extracts was conducted to determine the intracellular concentrations of pantetheine, pantothenic acid, cysteamine, and cystamine in control and CFB treated WT and null mice without APAP treatment (WT_VEH, WT_CFB, KO_VEH, KO_CFB) (Figure 6). This allowed us to assess changes in hepatic levels of the enzymatic products of vanin-1 by CFB treatment prior to APAP exposure. Hepatic pantetheine levels were elevated in WT_CFB mice compared to vehicles; however, this was not statistically significant. By contrast, pantothenic acid was significantly increased in WT_CFB mice in comparison to WT_VEH (2.4-fold) and KO_CFB mice (3.6-fold). Whereas no significant changes were seen in cysteamine concentrations with any of the treatment groups, the dithiol cystamine was significantly increased (5.3-fold) in WT_CFB mice. Cystamine concentrations did not increase in KO_CFB mice. Since the mechanism by which cysteamine and cystamine elicit protection from APAP is not known, it is unclear how the ratio of cysteamine/cystamine impacts hepatoprotection.

Discussion

The hypolipidemic drug CFB has been used extensively to investigate the mechanisms of hepatoprotection provided by peroxisome proliferators against APAP and other hepatotoxicants. Currently, the precise mechanism has not been fully elucidated. An important clue comes from previous studies demonstrating that mice lacking the PPAR α nuclear receptor are refractory to the protective effects of CFB (Chen *et al.*, 2000), implicating PPARα activation in hepatoprotection. In the present study, we used gene array analysis to identify differentially expressed genes that might contribute to the hepatoprotective effects of CFB. Two comparisons were made: the first one compared differentially expressed genes between CFB and vehicle pretreated wild type mice challenged with APAP (WT_CFB_APAP – WT_VEH_APAP). The second one compared changes in gene expression between wild type and PPAR α-null mice pretreated with CFB and challenged with APAP (WT_CFB_APAP – KO_CFB_APAP). We focused our investigation on the 53 common genes with similar regulation in these comparisons.

The majority of down-regulated genes within this group of common genes were associated with immune function. Given the increasing link between immune function and susceptibility to APAP toxicity (Liu *et al.*, 2004;James *et al.*, 2005), it is tempting to speculate that modulation of multiple genes linked to immune function may contribute to CFB-mediated hepatoprotection. However, primary hepatocytes isolated from mice dosed*in vivo* with CFB are also resistant to APAP toxicity (Nicholls-Grzemski, 2000). Hepatocyte isolation for culturing purposes removes a significant portion of non-parenchymal cells, thus reducing the likelihood that a change in immune function (e.g. cytokines originating from Kupffer cells) is a primary mechanism responsible for CFB-mediated hepatoprotection.

Enhanced cell proliferation is another explanation for hepatoprotection by accelerating tissue repair following chemical injury (reviewed by Mehendale, 2005). This mechanism has been suggested to contribute to CFB-mediated hepatoprotection (reviewed by Mehendale, 2000). The most dramatically down-regulated gene within the set is Egr-1, an important transcription

factor modulating cellular proliferation by controlling mitotic cell progression. However, it is unlikely that down regulation of Egr-1 increases cellular proliferation because mice deficient in Egr-1 have impaired hepatic regeneration (Liao *et al.*, 2004). Down regulation of other genes negatively regulating transcription (MCRS1, ZFP68) and inhibitors of cell growth (JunB) may impact hepatoprotection via hypertrophy and hyperplasia associated with peroxisome proliferation.

Although the events involved in the progression of APAP toxicity are not entirely known, the degree of APAP covalent binding to target hepatic proteins is directly pr oportional to the severity of toxicity (Jollow *et al.*, 1973). Therefore, changes in the expression of APAP binding proteins may modulate toxicity. One such APAP binding protein, ALDH2, was among the down regulated genes identified in our analysis. Although the biological significance of APAP covalent binding to ALDH2 is unknown, adduct formation inhibits the activity of the enzyme (Landin *et al.*, 1996). Presently, it is unclear how altered expression of ALDH2 may impact peroxisome proliferator-mediated hepatoprotection.

A similar study of genes differentially expressed between wild type and PPARα-null mice treated with the peroxisome proliferator Wy14,643 showed significant induction of proteasome maintenance genes (Anderson *et al.*, 2004). The proteasome carries out ubiquitin-dependent and independent proteolysis of damaged and misfolded proteins (Goldberg, 2003). Enhanced proteasomal degradation has been suggested to contribute to peroxisome proliferator-mediated hepatoprotection (Anderson *et al.*, 2004). Two of these proteasomal genes were also significantly up-regulated in the present analysis (PSMA3 and PSMB4). Although enhanced proteosomal activity can be beneficial in removing denatured proteins, one must consider the well-established mechanistic features of the hepatoprotection afforded by CFB. Treatment for 10 days with CFB prevents APAP covalent binding and GSH depletion. This suggests that NAPQI is being neutralized prior to causing GSH depletion and binding to or oxidizing thiols in hepatic proteins. Therefore, it seems that an enhanced removal of damaged or adducted proteins through ubiquination/proteasomal pathways is not critical to the hepatoprotective response resulting from multiple dosing with CFB. It is worth noting that a single dose of CFB provides partial protection against APAP hepatotoxicity. In contrast to the 10 day dosing regimen, a single dose does not alter GSH depletion or protein binding byAPAP. Under this scenario, enhanced proteosomal degradation of modified proteins should play a more prominent role in reducing the severity of APAP toxicity.

Vanin-1 was one of the most significantly up-regulated genes in our analysis. We hypothesized that increased vanin-1 expression translates into elevated hepatic cysteamine and cystamine levels. Interestingly, only dithiol cystamine concentrations were significantly increased in wild type mice pretreated with CFB. Although exogenous administration of cysteamine and cystamine are both hepatoprotective, it is unclear how the redox state of these compounds may affect hepatoprotection. Even though little is known about the equilibrium between cysteamine and cystamine, the present study suggests a dynamic relationship. Given the low mRNA expression of hepatic vanin-1 in KO_CFB mice, correspondingly low levels of hepatic cysteamine would be anticipated. However, PPARα-null mice had hepatic cysteamine levels comparable to wild type, suggesting a greater complexity in hepatic cysteamine regulation.

Both cysteamine and cystamine are potent antioxidants capable of ameliorating APAP toxicity when administered exogenously to rodents or humans (Prescott *et al.*, 1974;Olinescu *et al.*, 1982a;MacDonald *et al.*, 1983;Miller and Jollow, 1986). There are several striking similarities between the protection afforded by cysteamine/cystamine and peroxisome proliferator-mediated hepatoprotection. First, several peroxisome proliferators have been reported to induce vanin-1 gene expression including di(2-ethylhexyl) phthalate (DEHP), Wy-14,643, and fenofibrate (Wong and Gill, 2002;Yamazaki *et al.*, 2002). This observation is important

because multiple peroxisome proliferators protect from APAP hepatotoxicity (Nicholls-Grzemski *et al.*, 1992;Nguyen *et al.*, 1999). In addition to its antioxidant properties, cysteamine decreases covalent binding of APAP to target hepatic proteins and reduces lipid peroxidation (Tredger *et al.*, 1980;Stroo *et al.*, 1985), as also seen with CFB-mediated hepatoprotection (Manautou *et al.*, 1994;Nicholls-Grzemski *et al.*, 2000a). Furthermore, primary hepatocytes isolated from mice treated *in vivo* with CFB are resistant to APAP, even with depletion of GSH by diethyl maleate (Nicholls-Grzemski *et al.*, 2000b). Cysteamine similarly protects mice from APAP toxicity, even when GSH is depleted by butathione sulfoximine pretreatment (Miners *et al.*, 1984). The protection afforded by cysteamine/cystamine is not limited to APAP; it also protects *in vivo* against toxic exposure to carbon tetrachloride and γ-irradiation (Brucer and Mewissen, 1957;Castro *et al.*, 1973). Interestingly, CFB pretreatment also prevents carbon tetrachloride hepatotoxicity *in vivo* (Manautou *et al.*, 1998). Therefore, it seems plausible that peroxisome proliferator-mediated hepatoprotection results from increased vanin-1 expression and elevated hepatic cystamine levels.

The implications of elevated vanin-1 mRNA and cystamine to hepatoprotection by CFB treatment are difficult to assess at the present time. There is only one known antibody that recognizes vanin-1. However, this antibody is not suitable for western blot analysis. Immunohistochemical localization of hepatic vanin-1 is also difficult due to low and diffuse staining (personal communication with P Naquet, Centre d'Immunologie de Marseille-Luminy CNRS-INSERM-Université de la Méditerranée, Marseille, France). There are no known specific depleting agents for cysteamine or cystamine, and inhibitors of vanin-1 are limited to general protein-thiol oxidizing agents that inactivate the enzyme (Ricci *et al.*, 1986). A vanin-1-null mouse lacking pantetheinase activity and liver cysteamine production is available (Pitari *et al.*, 2000). However, the use of these mice to assess the role of vanin-1 in CFB hepatoprotection would be confounded by compensatory responses in these mutants. Vanin-1-null mice have 50% more liver GSH than wild type mice, which makes them resistant to oxidative stress (Berruyer *et al.*, 2004). The higher liver GSH content of vanin-1 mutants is due to the lack of cystamine, which is known to negatively regulates gamma-glutamylcysteine synthetase, the rate limiting enzyme in GSH synthesis (Martin *et al.*, 2004). In the absence of liver cystamine, GSH production increases significantly.

In conclusion, differential gene analysis was used to identify genes involved in CFB-mediated hepatoprotection. Our comparisons yielded 53 genes of interest, including vanin-1. We established that CFB-mediated induction of vanin-1 gene expression is dependent upon PPAR α activation. Induction of vanin-1 results in increased levels of hepatic cystamine, a proven and potent antioxidant capable of protecting from APAP toxicity. Further mechanistic study of cysteamine and cystamine in CFB-mediated hepatoprotection is warranted as additional biochemical tools become available to modulate these potent antioxidants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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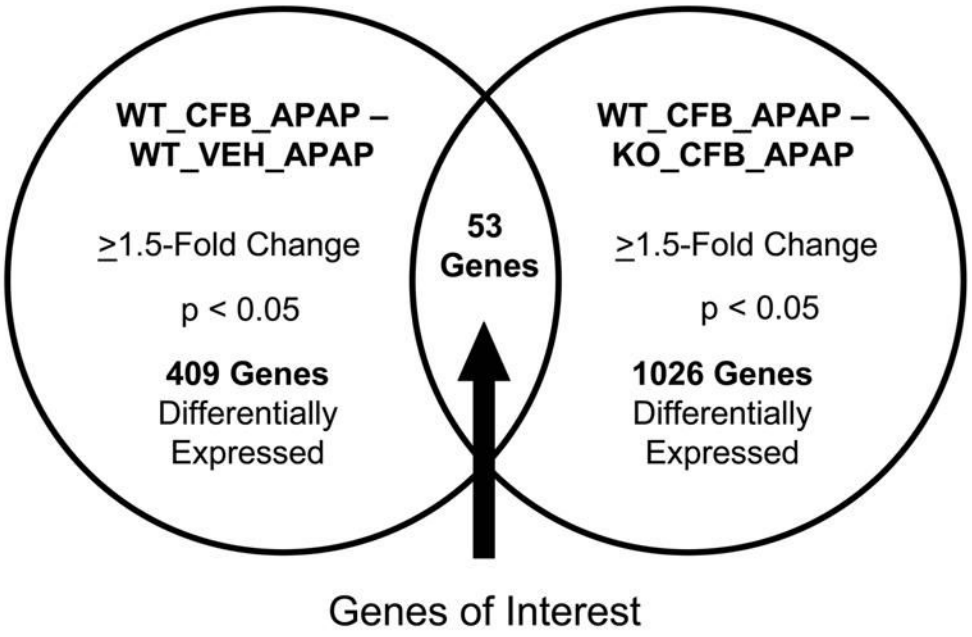

Figure 1.

Clustering analysis of gene(s) associated with PPAR α-mediated hepatoprotection. GeneSpring® analysis identified 409 genes differentially expressed between vehicle and CFB pretreated wild type mice, challenged with APAP (WT_CFB_APAP – WT_VEH_APAP). 1026 genes were differentially expressed between wild type and PPAR α-null mice pretreated with CFB and challenged with APAP (WT_CFB_APAP – KO_CFB_APAP). Common genes differentially expressed in both comparisons were identified as genes of interest. These 53 genes were considered for their possible role in hepatoprotection. Only genes differentially expressed with $p<0.05$ and a fold change ≥1.5-fold were considered significant.

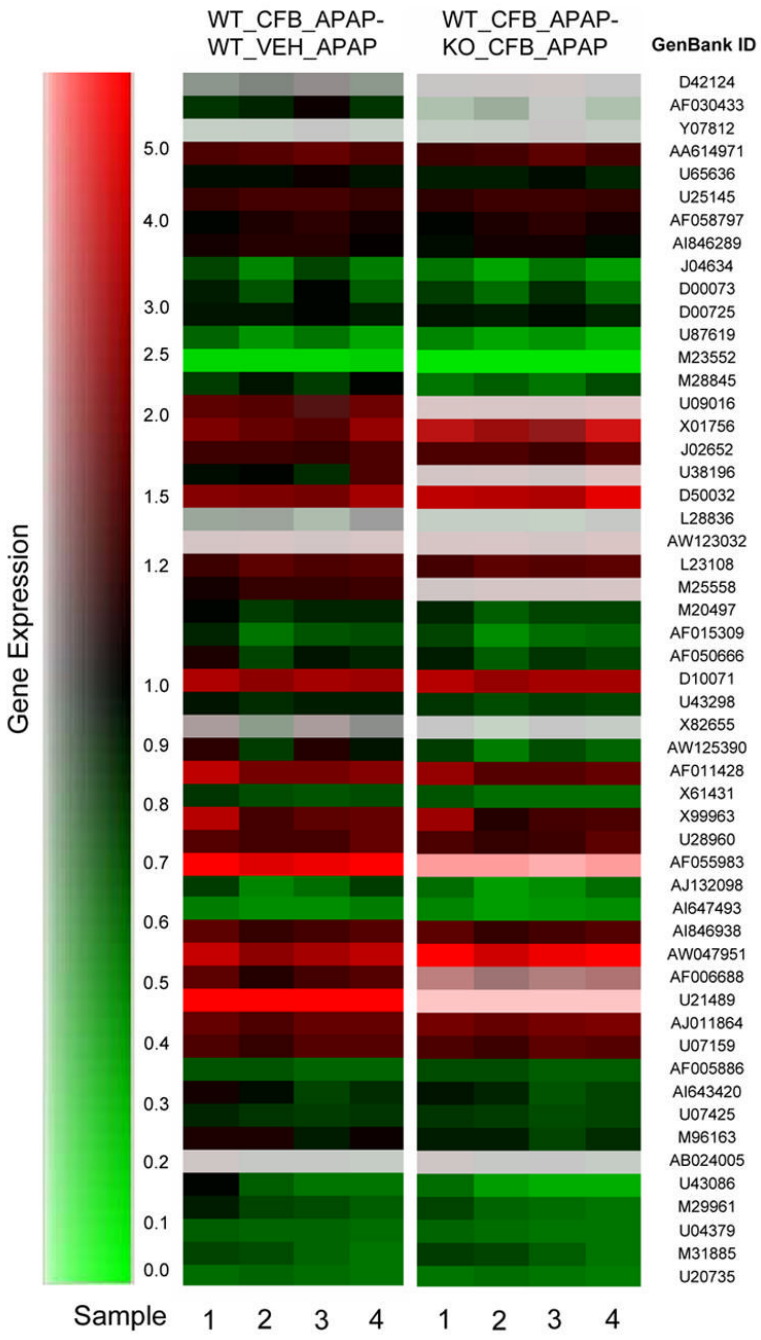

Figure 2.

Heat map representation of normalized, log-transformed relative intensities from the 53 genes identified for their role in PPARα-mediated hepatoprotection. Individual samples from WT_CFB_APAP – WT_VEH_APAP comparisons were normalized to the mean of WT_VEH_APAP (left box), using GeneSpring ® software. Similarly, individual samples from WT_CFB_APAP – KO_CFB_APAP comparisons were normalized to the mean of KO_CFB_APAP (right box).

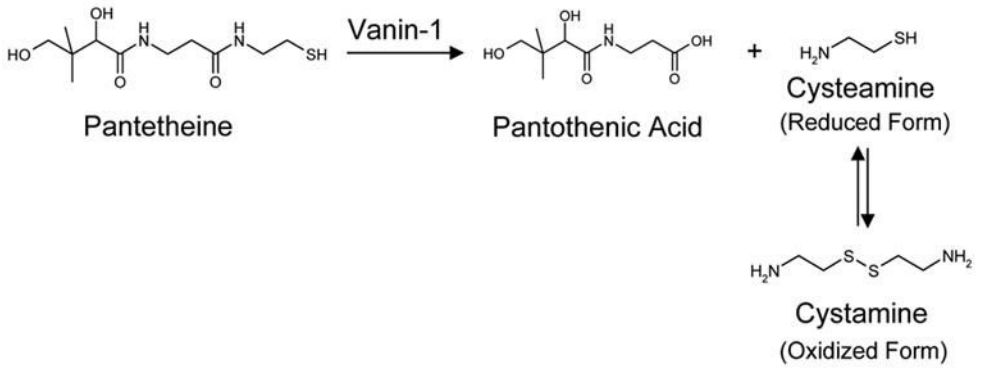

Figure 3.
Schematic of vanin-1 pantetheinase activity. Hydrolysis of pantethenine is catalyzed by vanin-1 forming pantothenic acid and cysteamine. The reduced, monothiol cysteamine is also capable of existing in an oxidized form, the dithiol cystamine.

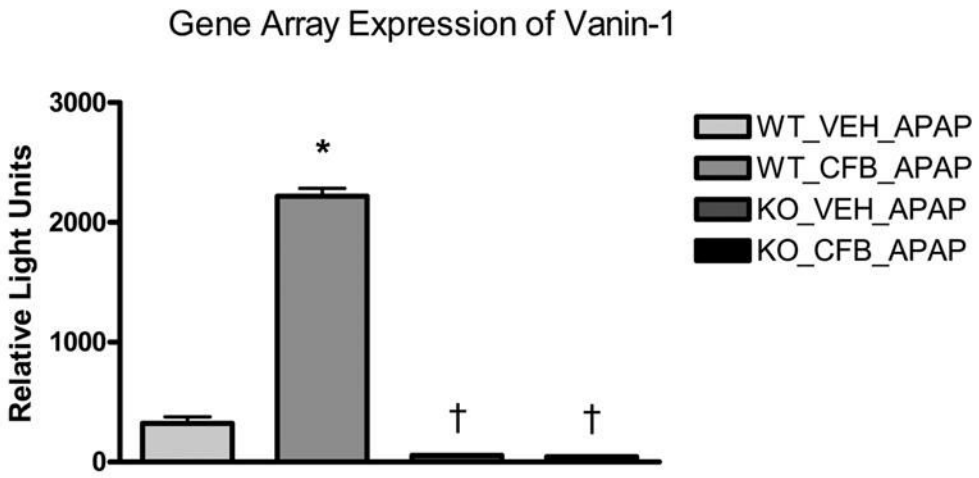

Figure 4.

Vanin-1 gene array expression analysis in vehicle and CFB pretreated wild type mice, challenged with APAP (WT_VEH_APAP, WT_CFB_APAP). Similar analysis was conducted in PPARα-null mice (KO_VEH_APAP, KO_CFB_APAP). RNA isolated from both groups of mice was labeled and hybridized to Affymetrix GeneChips as described under the methods section. Results of vanin-1 gene expression were analyzed by GeneSpring ® software and expressed as mean optical density ± SE for 3 to 4 mice per treatment group. Asterisks (*) denote a statistical difference from control and daggers (†) indicate a statistical difference from wild type ($p<0.05$).

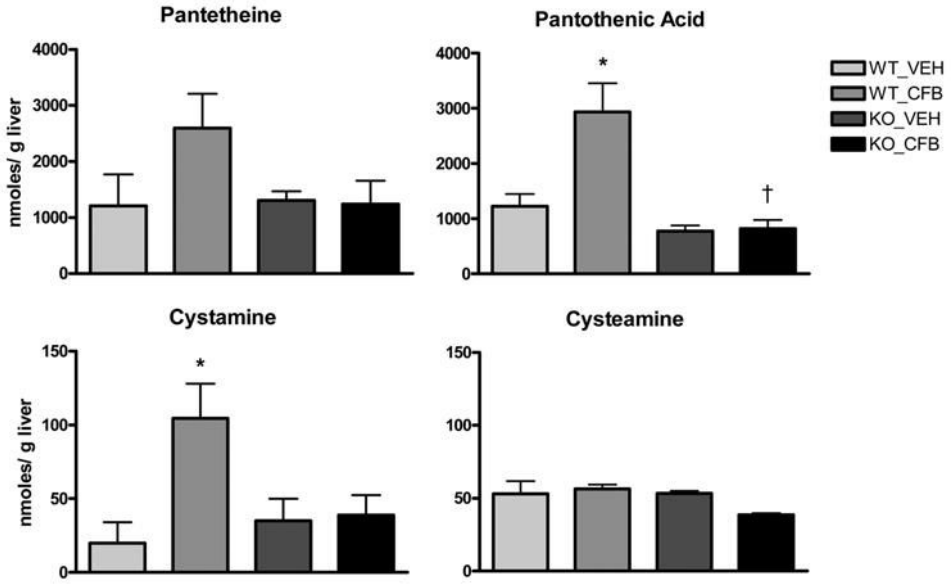

Figure 5.

Confirmation of vanin-1 gene array expression by RT-PCR analysis in vehicle and CFB pretreated wild type mice, challenged with APAP (WT_VEH_APAP, WT_CFB_APAP). Similar analysis was conducted in PPAR α-null mice (KO_VEH_APAP, KO_CFB_APAP). RNA from individual samples was amplified and analyzed for vanin-1 gene expression using gene specific mouse primers. The optical density (O.D.) of bands was quantified with a Kodak® Digital Science Image Station and expressed as mean O.D. x mm ^{2} ± SE for 3 to 4 mice per treatment group. Asterisks (*) denote a statistical difference from vehicle and daggers (†) indicate a statistical difference from wild type ($p<0.05$).

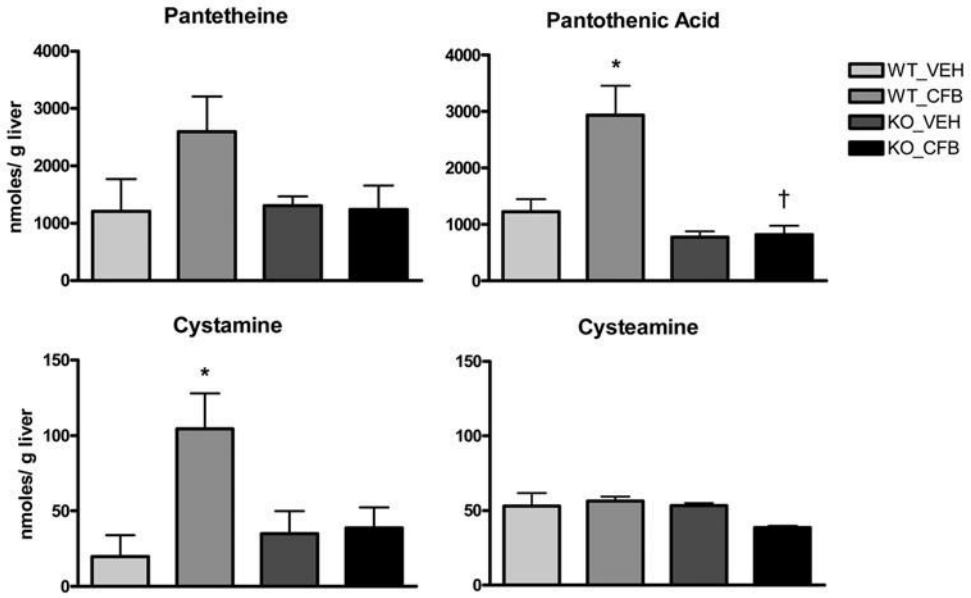

Figure 6.

ESI-LC/MS/MS analysis of mouse liver for pantetheine, pantothenic acid, cystamine, and cysteamine following CFB administration. Liver extracts were generated and analyzed as described in the methods section from wild type (WT) andPPARα-null (KO) mice treated with vehicle (VEH) or CFB. These mice did not receive an APAP challenge. Results are expressed as nmoles/g of liver ± SE for 3 to 4 mice per treatment group. Statistical differences ($p<0.05$) from vehicle and wild type are marked by asterisks (*) and daggers (†), respectively.

Table 1

nes of interest investigated for their role in PPAR α-mediated protection.

lated Genes of Interest in CFB-mediated Hepatoprotection Abbreviation	GenBank Designation	Affymetrix Probeset Identification	WT_CFB_APAP - WT_VEH_APAPFold Change	WT_CFB_APAP - KO_CFB_APAPFold Change	Gene Description	Gene Function
GR1	M28845	98579_at	−13.18	−19.89	Early growth response 1	Transcriptional Regulation/ Cellular Proliferation
NB	U20735	102362_i_at	−9.48	−11.58	Jun B proto-oncogene	Cell Growth and Differentiation
RS1	AF015309	98563_f_at	−4.56	−4.80	Microspherule protein 1	Transcriptional Repression
K2	M96163	92310_at	−4.46	−4.87	Polo-like kinase 2 (Drosophila)	Cell Cycle Regulation
RPR	AW123032	97491_at	−4.40	−3.20	Heterogeneous nuclear ribonucleoprotein R	Developmental
IT4	U43086	93956_at	−3.39	−4.19	Interferon-induced protein with tetratricopeptide repeats 4	Immune Function
D1	M31885	100050_at	−3.05	−2.49	Inhibitor of DNA binding 1, dominant negative helix-loop-helix	Transcriptional Regulation
PUD1	AI846938	95057_at	−2.93	−2.70	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	Acute phase response/ protein degradation
INA3K	D00725	92583_at	−2.79	−2.46	Serine (or cysteine) proteinase inhibitor, clade A, member 3K	Acute phase protein
P70	U04379	93661_at	−2.89	−3.39	Zeta-chain (TCR) associated protein kinase 70kDa	Immune Function
P68	AB024005	95521_s_at	−2.64	−1.98	Zinc finger protein 68	Transcription Repression
AG3	AI643420	96167_at	−2.60	−2.58	BCL2-associated athanogene 3	Apoptosis
LD1	AF050666	95566_at	−2.56	−1.90	Glycosylphosphatidylinositol specific phospholipase D1	Lipid Metabolism
DH2	AI647493	96057_at	−2.54	−2.33	Aldehyde dehydrogenase 2 family (mitochondrial)	Lipid Metabolism / Aldehyde Detoxification
AL	Y07812	99089_at	−2.49	−3.19	Mal, T-cell differentiation protein	Membrane transport/ Apoptosis
HB	U25145	101738_at	−2.47	−2.58	Luteinizing hormone beta polypeptide	Reproduction
TM3	AW125390	160253_at	−2.43	−3.10	Interferon induced transmembrane protein 3 (1-8U)	Developmental
Y64	J04634	98000_at	−2.43	−2.59	Lymphocyte antigen 64 homolog, radioprotective 105kDa	Immune System
AFK	D42124	102919_at	−2.28	−2.45	v-Maf musculoaponeurotic fibrosarcoma oncogene homolog K	Negatively Regulates Antioxidants
PCS	M23552	104072_at	−2.14	−3.01	Amyloid P component, serum	Acute Phase Protein
BL2	U09016	97427_at	−1.97	−2.59	Mannose-binding lectin (protein C) 2, soluble (opsonic defect)	Immune System
3B	D10071	97435_at	−1.97	−1.72	Coagulation factor XIII, B polypeptide	Clotting
TR	D00073	95350_at	−1.95	−2.19	Transthyretin (prealbumin, amyloidosis type I)	Albumin Production

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lated Genes of Interest in CFB-mediated Hepatoprotection

Abbreviation	GenBank Designation	Affymetrix Probeset Identification	WT_CFB_APAP - WT_VEH_APAPFold Change	WT_CFB_APAP - KO_CFB_APAPFold Change	Gene Description	Gene Function
KK1	AF030433	99881_at	−1.93	−2.43	Dickkopf homolog 1 (Xenopus laevis)	Development
AIL	AA614971	98988_at	−1.79	−1.95	Molecule possessing ankyrin repeats induced by lipopolysaccharide (MAIL), homolog of mouse	Immune System
PIND1	U07425	100966_at	−1.79	−1.64	Serine (or cysteine) proteinase inhibitor, clade D (heparin	Acute phase protein
PIB	U87619	93388_s_at	−1.73	−1.60	Spi-B transcription factor (Spi-1/PU.1 related)	Immune System
PEP	M29961	102373_at	−1.71	−1.99	Glutamyl aminopeptidase (aminopeptidase A)	Immune System
HB	X99963	101030_at	−1.63	−1.55	Ras homolog gene family, member B	Actin cytoskeleton
RNB2	X82655	97010_at	−1.53	−1.87	Cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal)	Neurologic Signaling
05L	AF011428	93445_at	−1.51	−2.07	CD5 antigen-like (scavenger receptor cysteine rich family)	Immune System/ Transcriptional Regulation

ed Genes of Interest in CFB-mediated Hepatoprotection

oreviation	GenBank Designation	Affymetrix Probeset Identification	WT_CFB_APAP - WT_VEH_APAPFold Change	WT_CFB_APAP - KO_CFB_APAPFold Change	Gene Description	Gene Function
MB3	U43298	92759_at	37.80	34.62	Laminin, beta 3	Cellular Adhestion
ADH	AJ011864	97316_at	18.83	46.89	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A	Peroxisome Proliferation and Lipid Metabolism
NN1	AJ132098	104165_at	6.79	54.09	Vanin-1	Immune System/ Lipid Metabolism/ Oxidative Stress
D36	L23108	93332_at	5.87	7.79	CD36 antigen (collagen type I receptor, thrombospondin	Cell Adhesion/ Lipid Metabolism
TP	U28960	100927_at	4.65	2.93	Phospholipid transfer protein	Peroxisome Proliferation/ Lipid Metabolism
PD1	M25558	161753_f_at	4.50	1.63	Glycerol-3-phosphate dehydrogenase 1 (soluble)	Peroxisome Proliferation/ Lipid Metabolism
OX1	AF006688	101515_at	3.37	7.73	Acyl-Coenzyme A oxidase 1, palmitoyl	Peroxisome Proliferation/ Lipid Metabolism
BI	X61431	97248_at	3.03	2.70	Diazepam binding inhibitor (GABA receptor modulator, acyl- Coenzyme A binding protein)	Peroxisome Proliferation/ Lipid Metabolism
K1D	AI846289	97264_r_at	2.95	5.81	Casein kinase 1, delta	Microtubule Formation
CS	X01756	98132_at	2.04	2.31	Cytochrome c, somatic	Oxidative Phosphorylation/ Apoptosis
CD3	L28836	93045_at	2.25	4.42	ATP-binding cassette, sub-family D (ALD), member 3	Peroxisome Proliferation/ Lipid Metabolism
E1	J02652	101082_at	1.95	4.63	Malic enzyme 1, NADP(+)-dependent, cytosolic	Oxidative Phosphorylation/ Peroxisome Proliferation
DLN2	D50032	93882_f_at	1.90	3.80	Trans-golgi network protein 2	Membrane Trafficking
ADL	U21489	95425_at	1.81	2.02	Acyl-Coenzyme A dehydrogenase, long chain	Peroxisome Proliferation/ Lipid Metabolism
G1IP	AW047951	94473_at	1.78	1.89	Pituitary tumor-transforming 1 interacting protein	Cell Cycle Regulation
CNI	AF005886	94819_f_at	1.76	2.00	Cyclin I	Cell Cycle Regulation

lated Genes of Interest in CFB-mediated Hepatoprotection

Abbreviation	GenBank Designation	Affymetrix Probeset Identification	WT_CFB_APAP - WT_VEH_APAPFold Change	WT_CFB_APAP - KO_CFB_APAPFold Change	Gene Description	Gene Function
BP4	**M20497**	100567_at	1.75	4.07	Fatty acid binding protein 4, adipocyte	Peroxisome Proliferation/ Lipid Metabolism
MB4	**U65636**	98557_f_at	1.69	1.83	Proteasome (prosome, macropain) subunit, beta type, 4	Ubiquitination/ Proteosomal Degradation
PP1	**U38196**	97803_at	1.68	2.96	Membrane protein, palmitoylated 1, 55kDa	Tumor Suppression
HAB	**AF058797**	98053_at	1.63	1.50	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, beta polypeptide	Signal Transduction
ADM	**U07159**	92581_at	1.61	2.33	Acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	Peroxisome Proliferation/ Lipid Metabolism
MA3	**AF055983**	92544_f_at	1.60	1.53	Proteasome (prosome, macropain) subunit, alpha type, 3	Ubiquitination/ Proteosomal Degradation

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