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The Disruption of Hepatic Cytochrome P450 Reductase Alters Mouse Lipid Metabolism

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To elucidate the role of hepatic cytochrome P450 oxidoreductase (POR) in lipid metabolism, we characterized perturbations in lipid homeostasis in a mouse model deficient in liver POR. Using an integrative approach in which transcriptomics, lipidomics, and various bioinformatic algorithms were employed, a disruption in liver lipid mobilization, oxidation, and electron transport functions were identified. Analyzing the promoters of genes in these biological processes identified common binding motifs for nuclear receptors sensitive to lipid status, while Srebp-1c binding sites were only identified in genes involved in lipid metabolism. POR-null mice had drastic increases in hepatic lipid content (diacylglycerols, triacylglycerols, phosphatidylcholine, and cholesterol esters) and a specific enrichment in *n-7* and *n-9* monounsaturated fatty acids (FAs). It was found that while transporters involved in peroxisomal FA oxidation were induced, mitochondrial oxidation appeared to be more tightly controlled, supporting the increase in monounsaturated FAs. Genes coding for hepatic transporters were differentially expressed, where lipid uptake was induced and efflux repressed, indicating that in the absence of hepatic POR the liver serves as a lipid reservoir. Furthermore, while significant changes in intestinal gene expression were found in POR-deficient mice, only minor changes to plasma and intestinal lipid content were observed. Thus, while liver POR plays an important role regulating gene expression and lipid metabolism locally, the hepatic deficiency of this enzyme reverberates throughout the biological system and produces a coordinated response to the low levels of circulating cholesterol and bile.

Keywords: Oxidoreductase • lipidomics • systems biology • promoter analysis • microarray • knock-out

Introduction

The characterization of animal models in which liver NADPH-cytochrome P450 reductase (POR) is functionally inactivated has clearly demonstrated the critical role this enzyme has on metabolism.[1,2] POR is classically regarded as a microsomal flavoprotein that donates electrons to many enzymes, including the cytochrome P450 monooxygenases, heme oxygenase, squalene epoxidase, and cytochrome b_5.[2,3] Mice with a conditional liver POR deletion were characterized by a disruption of the P450 system and a ~90% reduction in bile acid production. Unexpectedly, POR-null mice were also found to have perturbations in lipid metabolism. While reductions in bile were not unexpected, since a dysfunctional POR prevents the conversion of cholesterol to bile acids by *Cyp7a1* and *Cyp8b1* enzymes,[4,5] the impressive increase in liver lipid levels and the reduction in circulating cholesterol were intriguing. Indeed, the apparent liver-specific accumulation of lipid in POR-null mice may suggest possible perturbations in pathways controlling lipid mobilization and metabolism. Two groups previously examined hepatic gene expression in mice with diminished POR activity, and changes in transcript abundance for genes involved in bile acid and lipid metabolism, as well as members of the P450 family, were reported;[3,6] however, to date, only gene expression changes in the liver have been examined.

Lipids not only have a structural role within a cell, but also serve as signaling molecules that are capable of affecting gene expression and protein abundance in both an endocrine and exocrine fashion.[7,8] As such, this diverse class of metabolites has the ability to directly and indirectly modulate biological functions simultaneously in both the liver and peripheral

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tissues. The use of an integrative approach to assess whether extrahepatic tissues respond to the localized perturbations in liver lipid homeostasis provides an alternate approach to elucidate POR protein function by identifying previously unrecognized pathways within the biological system that are sensitive to this enzyme.

Precedence for an integrative approach with this mouse model in particular has been previously demonstrated by revealing that the murine response to the polyphenol quercetin is shared by the liver and jejunum when hepatic POR function is compromised.[9] While no significant changes in the abundance of quercetin metabolites were identified in POR-deficient mice, those biological processes that are normally dependent on both a functional hepatic POR and are sensitive to quercetin were shifted to regions of the small intestine when liver POR was disrupted. These results reinforced the so-called resiliency of biological systems and their capacity to preserve the full gamut of physiological functions even when challenged with tissue-specific perturbations.[10]

To date, the role of hepatic POR on the regulation of lipid metabolism has not been fully elucidated, and the response of peripheral tissues to a dysfunctional liver POR remain totally unexplored. The present manuscript addresses these points by employing a systems biology approach to both further characterize the underlying mechanisms responsible for the accumulation of lipid in the liver and determine whether the intestine is capable of 'sensing' this accumulation. Gene expression profiling and, more specifically, pathway and promoter analyses permitted those genetic elements that mediate lipid metabolism to be identified in POR-deficient mice. The additional integration of quantitative lipid profiling indicated that, while liver gene expression and lipid metabolism are both highly responsive to a deficiency in liver POR, the gut and plasma are less sensitive to the severe perturbations in hepatic lipid metabolism.

Materials and Methods

Animals and Diet. Animals were fed *ad libitum* a RM3 (E) 801710 Soya-free powdered diet (Special Diets Services, Essex, U.K.) for a period of 2 weeks (providing 11.50% energy from oil, 26.93% energy from protein, and 61.57% energy from carbohydrate). For the precise diet composition, please contact info@sdsdiets.com. CXR Biosciences reared 65 male cytochrome P450 reductase null (KO) mice and an equivalent number of wild-type (WT) C57BL/6 mice, all aged between 6 and 8 weeks; however, only a subset of these animals (the control mice) were used in the present study (the remaining animals were used for a previously reported nutritional study[9]). Both WT and KO mice were adapted to the RM3 diet over a period of 15 days, in which they had *ad libitum* access to this pellet diet. Prior to sacrifice on day 16, animals were fasted for 20 h. While difficult to assess food consumption in animals housed in groups, subgroups of 5 mice per genotype were individually caged (and used eventually for lipidomic analyses) to assess food consumption on days 5, 10, and 15. No significant differences were found between WT and KO mice food consumption (WT, 5.9 ± 0.47 g/day; KO, 6.2 ± 0.93 g/day, $p = 0.48$). The remaining animals were housed 3 per cage, where both temperature and relative humidity were maintained within a range of 19−23 °C and 40−70%, respectively. Twelve-hour periods of light were cycled with 12-h periods of darkness.

Sample Collection and Analysis. Mice were anaesthetized with sodium pentobarbital (70 mg/kg, ip) according to procedures approved by University of Dundee Ethical Review Committee, and all animal work was carried out in accordance with the Animal (Scientific Procedures) Act. Plasma and tissues (liver, jejunum, ileum, and colon) were obtained as previously described.[9] In brief, the small intestine (below the stomach to above the caecum) was removed from mice and divided into three equal sections, corresponding to the duodenum, jejunum, and ileum. The colon was harvested from below the caecum and above the rectum. All gut sections were scraped to isolate epithelial cells, which were subsequently used for both transcriptomic and lipidomic work.

RNA was extracted from the various tissue samples ($n = 9$/tissue/genotype) and pooled into 3 groups of 3 RNAs, as previously described.[9] The protocols detailing RNA preparation for microarray analysis, including cRNA preparation, hybridization, and scanning, are described in Mansourian et al.[11] and are in compliance with MIAME standards.

Samples destined for lipid analysis were extracted, flash-frozen in liquid N_2 ($n = 5$/tissue/genotype), and analyzed by Lipomics Technologies, Inc. (Sacramento, CA) as previously described.[12] In brief, lipids were extracted from tissues in the presence of authentic internal standards by the method of Folch et al.[13] using chloroform:methanol (2:1 v/v). Individual lipid classes within the extract were separated by preparative thin layer chromatography as described by Watkins et al.[14] Isolated lipid classes were trans-esterified in 3 N methanolic HCl in a sealed vial under a nitrogen atmosphere at 100 °C for 45 min. The resulting fatty acid methyl esters were extracted with hexane containing 0.05% butylated hydroxytoluene and prepared for gas chromatography by sealing the hexane extracts under nitrogen. Fatty acid methyl esters were separated and quantified by capillary gas chromatography using a gas chromatograph (Hewlett-Packard model 6890, Wilmington, DE) equipped with a 30 m DB 225MS capillary column (J&W Scientific, Folsom, CA) and a flame ionization detector as described previously.[14]

Gene Expression Data Analysis. RNA was hybridized to the Murine 430A GeneChip (Affymetrix, Santa Clara, CA), which consists of 14 000 full-length, well-characterized genes. The complete data set is publicly available in the NCBI Omnibus through the following accession number: GSE4262. Two complementary approaches were used to analyze the raw data: (A) The Global Error Assessment (GEA) model was applied to the raw GeneChip data for the selection of differentially regulated genes, and then pathway analysis was performed with Ingenuity Pathways, Inc. software (www.Ingenuity.com). Only genes differing in expression between WT and KO with a p-value ≤ 0.001 were used for this analysis. To summarize, the GEA approach takes advantages of several inherent characteristics of microarrays that result in an increased statistical power for the data analyst. When examining genomic data sets, most genes are found to be stably expressed across all conditions. Therefore, rather than treating each gene on the microarray as a unique and unrelated element, neighboring genes are binned into groups of 200 based on similar intensity signals, and the mean squared error is calculated for each bin. The binning of genes greatly increases the statistical power of the GEA approach and yields results that are statistically significant and highly concordant with alternate gene expression validation techniques. For a detailed description and general protocol of the GEA model, please see ref 11. (B) The second method used Genomatix's ChipInspector (version 0.91) and BiblioSpherePE (version 5.13) (www.genomatix.de/).[15]

ChipInspector identifies statistically significant changes (with a false-discovery rate, FDR, ideally set at 0%) based on single probes that can be uniquely mapped to gene transcripts. The final list of differentially regulated transcripts used for subsequent analyses is selected by a user-defined threshold based on the number of significant probes for a given transcript. In this analysis, the threshold was set to ≥3 unique probes with a FDR of 0%. This method reduces the influence of gene transcript annotation errors on the subsequent interpretation of data. The list of significantly changed transcripts was uploaded into BiblioSpherePE. This program builds gene−gene networks based on literature analysis and enables the application of various filter criteria, pathway annotation, GeneOntology (GO) classification, and diverse MESH terms.[16] For the present analysis, the filters used for GO and MESH were "Biological Process" and "Disease", respectively. The over representation of input genes within any filter category is calculated as a *Z*-score based on the expected number of genes within the filter category (GO) or the expected number of co-citations with a MESH filter category. Common promoter models were searched in those categories that were significantly over represented in the BiblioSpherePE analysis using Genomatix's Gene2Promoter for promoter sequence extraction and FrameWorker program.[17]

Lipid Data Analysis. Lipid profiles in the various biological samples were examined using a number of techniques. Heat maps comparing the abundance of lipids were generated by comparing KO versus WT with the Lipomics Surveyor developed by Lipomics Technologies, Inc. (www.lipomics.com/). Lipid clustering in the liver, jejunum, and plasma of WT and KO mice was performed using a Pearson correlation algorithm on lipid data (nmol) with dCHIP software (www.dchip.org). While quantified, the quality of the lipid analysis in the ileum and colon were not suitable for analysis due to a high variability in measurements. Lipomics Technologies have established quality controls to determine 'acceptable' thresholds for the variation of each fatty acid in each lipid class. If the variation of the measured data is above these thresholds (most likely due to a combination of technical and biological variability), then it can be considered too variable for accurate biological interpretation. Lipid nanomol per gram (nmol/g) of tissue and liquid material data can be found in Supplementary Data 1 in Supporting Information.

Results

GEA analysis of hepatic gene expression profiles ($p < 0.001$) identified 1110 genes as differentially expressed in KO mice, where 369 were down-regulated and 741 up-regulated. Subsequent Ingenuity pathway analysis revealed that 'lipid metabolism' and 'drug metabolism' were the most significantly modulated canonical functions affected in mice lacking a functional POR enzyme ($p = 0.0003$ and 0.0008, respectively). A simultaneous analysis with ChipInspector identified 998 transcripts as differentially regulated, with 365 transcripts down-regulated and 633 up-regulated. In perfect concordance with the Ingenuity pathway analysis, a functional analysis using BiblioSpherePE revealed that GO categories 'electron transport' and 'carboxylic acid metabolism' were significantly modulated in KO mice (*Z*-scores of 11.49). Although the functional categories are not labeled identically by the different software, the descriptions for 'lipid metabolism' and 'carboxylic acid metabolism' and for 'drug metabolism' and 'electron transport' are highly similar. Because of this high 'bioinformatic' concordance, the present analysis only focused on genes associated with the processes 'carboxylic acid metabolism' and 'electron transport', with the goal of unraveling the role of hepatic POR on lipid metabolism within the biological system.

We hypothesized that common regulatory mechanisms may underlie the large number of genes differentially expressed in the livers of KO mice. Co-citation analysis and filtering for Gene Ontology (GO) categories within the BiblioSphere software was used to identify smaller subgroups of genes that are likely to be transcriptionally co-regulated. Figure 1 illustrates the GO filtering for the genes from the ChipInspector analysis, plus additional co-citation linked transcription factors (TFs), which belong to the genetic networks underlying 'electron transport' (A) and 'carboxylic acid metabolism' (B) functions. In both cases, the expression levels of nearly all genes associated with either function are increased. Genetic components of 'electron transport' are known transcriptional targets of signal transducer and activator of transcription 5 (*Stat5a*), peroxisome proliferator-activated receptor gamma coactivator-1alpha (*Pgc-1*α), and nuclear receptor 1h3/liver-X receptor alpha (*Lxr*-α)[18−20] (Figure 1A). Similarly, the critical role of PPAR-α in regulating hepatic lipid metabolism is clearly illustrated in 'carboxylic acid metabolism', as is the role of the nuclear receptor 1h4/farnesoid X receptor (*Fxr*)[21,22] (Figure 1B). None of the aforementioned transcription factors are differentially expressed in KO mice. Identifying nuclear receptors as key regulators of genes annotated to either 'electron transport' or 'carboxylic acid metabolism' suggested the possibility of common transcriptional motifs. Modeling frameworks of TF binding sites began by examining all genes present on the GeneChip that are directly connected to PPAR-α in BiblioSphere, then filtered for their association with the "carboxylic acid metabolism" annotation, and finally confirmed they had a promoter binding site for the PPAR family (green connections in Figure 1B). We first identified the maximum number of promoters which shared a common model containing a match to the PPAR family (termed PERO). We obtained a model of PERO_MYT1 (Table 1) within 5 of the 10 promoters identified by the aforementioned criteria. The significance of this model was then evaluated by scanning all annotated mouse promoters and scoring the overlap with the list of differentially regulated genes obtained with ChipInspector. The model matched 2642 promoter sequences, of which 134 of these were in our list of differentially expressed genes found with ChipInspector, resulting in an over representation of 1.8-fold above expected. However, this model was not conserved in the orthologous promoters of rat and human. Assuming that fundamental metabolic control should be conserved across mammals, we implemented an additional criterion to our modeling strategy that considered both rat and human promoters in addition to the mouse, with the goal of reducing the number of false-positive results. While such a criterion can be expected to increase the number of false negatives in our results (by not considering species-specific transcription events, for example), we preferred to err on the side of caution and report results that have only been validated across several species. Since transcript annotation varies between species, we extracted only those promoters which are (a) assigned to orthologous conserved and experimentally verified transcripts and (b) assigned to transcripts coding for a full-length protein. This gave us a set of 12 promoters for 4 mouse genes and their orthologues. The FrameWorker program applies the following additional criteria for model generation: (1) conserved order of transcription factor binding sites (e.g., A-B is not equivalent to B-A), (2) conserved DNA-strand

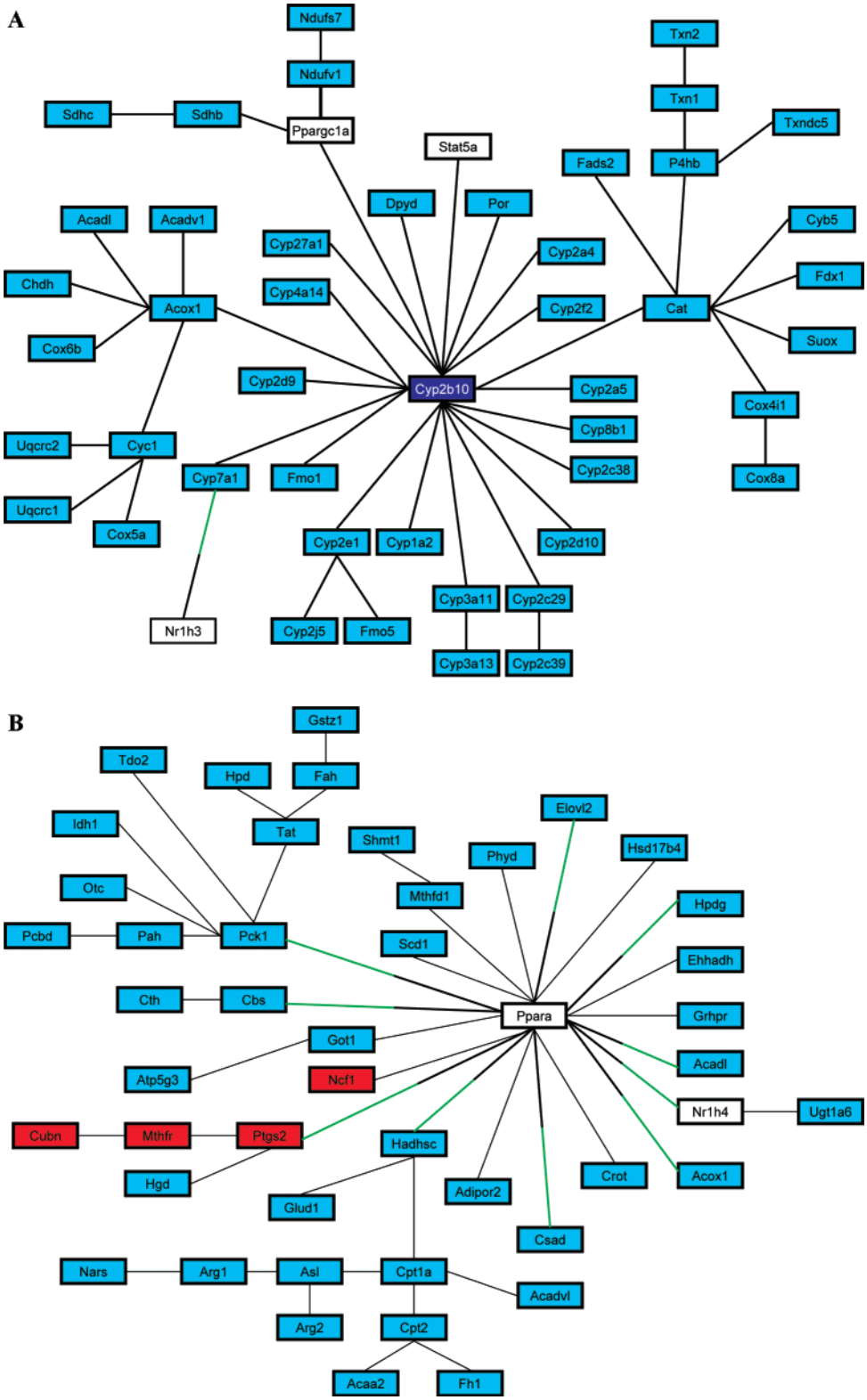

Figure 1. Gene networks for the two hepatic functional annotations most significantly regulated in KO animals. A BiblioSpherePE analysis indicates all genes filtered for (A) GeneOntology category 'electron transport' (comprised of 64 genes) and (B) GeneOntology category 'carboxylic acid metabolism' (comprised of 67 genes). Blue indicates up-regulated genes, red indicates down-regulated genes, and white boxes indicate transcription factors known from literature to be associated with these pathways. Only the shortest paths traversing the networks are shown with *Cyp2b10* as the central node for (A) and *Ppar-*α as the center for (B). A green connection line toward a gene indicates a TF-binding site match for the connected TF in the promoter(s) of that gene that has been experimentally verified. Gene symbols are official mouse NCBI GENE IDs.

orientation (e.g., A−, B+ is not equivalent to A−, B−) and, (3) conserved distance between adjacent binding sites. Using such criteria enables the detection of common models (i.e., composed of 2 or more transcription binding sites) that may be

Table 1. Promoter Models Obtained from Subgroups of Genes Annotated in GO Categories "Carboxylic Acid Metabolism" and "Electron Transport"[a]

model	matches in mouse promoters	matches in analyzed gene-list	over representation in gene-list
Carboxylic Acid Metabolism			
PERO_MYT1[b]	2642	134	1.8
NR2F_HIFF	3751	201	1.9
RXRF_NKXH	6409	305	1.7
NR2F_SP1	1968	120	2.2
EBOX_NKXH	9501	416	1.6
Electron Transport			
FKHD_NR2F	8007	317	1.4
RXRF_LEFF	3192	317	3.6
NR2F_SRFF	3601	174	1.7
RXRF_NR2F	6534	283	1.6
GKLF_TBPF	6762	248	1.3
CAAT_TBPF	5022	191	1.4

[a] Model names reflect the names of the Matrix families (Genomatix Matrix library 6.0; family concept (see ref 17). Data indicates the number of occurrences for a given promoter model in the murine genome, the number of matches to genes identified as differentially expressed in KO animals, and their over representation within the data set. Families and relevant TFs represented: NR2F, nuclear receptor subfamily 2 factors, includes HNF4 and ARP1; RXRF, RXR heterodimer binding sites, includes VDR/RXR, LXR, FXR; NKXH−NKX homeodomain factors; EBOX, E-box binding factors, includes SREBP; FKHD, fork head domain, includes the liver enriched HNF3-α, HNF3-β, and HNF3-γ; SRFF, serum response factor family; PERO, peroxisome proliferator-activated receptor; MYT1, MYT1 C2HC zinc finger protein. [b] In contrast to all other models, this model was obtained only from mouse promoters and did not include promoters of rat and human orthologues.

key elements for transcriptional co-regulation. Four simple models, each composed of 2 transcription factors, were found in at least 6 of these 12 gene promoters (Table 1). The set consisted of the promoters of those mouse genes identified to be closely related by the initial PERO_MYT1 model and their orthologues. Models were evaluated by scanning all annotated mouse promoters and comparing them to the ChipInspector gene-list, as described above. These models consisted of matches to transcription factors known to be involved in the regulation of lipid metabolism,[17] such as HNF4, VDR/RXR, LXR, FXR, PPAR, and SREBP. A similar examination of the *Cyp* genes in the 'electron transport' category revealed several of the same transcription factor binding motifs were present (Table 1); however, it is most likely through different binding partners that these transcription factors regulate genes in both categories. In contrast with nuclear receptors, SREBP is not common to both biological processes.

The present study integrated transcriptional and lipidomic data from the liver, plasma, and regions of the intestinal tract. GEA analysis identified 177 differentially expressed genes in the jejunum (89 up-regulated and 88 down-regulated), 324 genes in the ileum (177 up-regulated and 147 down-regulated), and 325 genes in the colon (216 up-regulated and 109 down-regulated) in KO mice. In all gut tissues, Ingenuity pathway analysis (data not shown) revealed that lipid metabolism was the biological function most significantly modulated (p-values between 0.0006 and 3.5×10^{-12}) upon the elimination of liver POR; however, the further examination of significantly regulated pathways revealed that each tissue contributes differently to lipid homeostasis. Genes in liver pathways regulating the metabolism of fatty acids, bile, and arachidonic acid were all significantly induced ($p = 8.9 \times 10^{-5}$, $p = 0.005$, and $p = 0.008$, respectively). In the jejunum, sterol metabolism genes were up-regulated ($p = 2.2 \times 10^{-13}$), while fatty acid metabolism genes were predominantly decreased ($p = 0.005$). In the ileum, genes associated with bile acid metabolism were down-regulated ($p = 0.009$), and in the colon, sterol biosynthesis was generally increased ($p = 0.0002$). The genetic components underlying these functions are detailed in subsequent sections.

Bile Acid Metabolism. Because of a ~90% reduction in bile acids, genes involved in the hepatic bile acid synthesis pathways are up-regulated (*Adhfe1*, *Akr1d1*, *Aldh1a1*, *Aldh1a7*, *Aldh1b1*, *Baat*, *Cyp7a1*, and *Cyp8b1*, between 1.4- and 5.4-fold increase). More specifically, the increased expression of *Cyp7a1* and *Cyp8b1* of 5.4- and 2.3-fold suggests an activation of the classical pathway for bile acid synthesis. In contrast, *Cyp7b1* expression, belonging to the 'alternative' bile acid synthesis pathway, was down-regulated 1.8-fold. Furthermore, both the small heterodimer partner (SHP)-dependent and SHP-independent pathways that control the negative feedback mechanisms regulating bile acid synthesis were down-regulated.[23,24] Indeed, the expression of both *Shp* and hepatocyte nuclear factor 4 α (*Hnf4*α) was decreased 1.8- and 1.4-fold, respectively. This suggests that the classic pathway for bile acid synthesis is preferentially induced following POR inactivation and that, in the absence of circulating bile acids, the negative feedback mechanisms are repressed. In the ileum, two critical players in the enterohepatic circulation of bile acids were up-regulated: farnesoid X-activated receptor (*Fxr*) and ileal bile acid transporter (*Ibat*) (1.5- and 1.6-fold, respectively). Taken together, these findings demonstrate a coherent biological response in both the liver and jejunum to the low levels of bile.

Cholesterol Metabolism. The 1.4-fold increase in HMG-CoA reductase (*Hmgcr*), the rate-limiting enzyme for cholesterol biosynthesis, contributes to the increases in hepatic cholesterol levels described below. Cholesterol biosynthetic pathways are significantly up-regulated in both the jejunum and colon of POR-null mice. Quite remarkably, in the jejunum, all differentially expressed genes associated with cholesterol biosynthesis are induced: *Dhcr7* (1.4-fold), *Lss* (1.4-fold), *Mvk* (1.4-fold), *Mvd* (1.5-fold), *Pmvk* (1.6-fold), *Hmgcr* (1.6-fold), *Idi1* (1.8-fold), *Fdps* (2.1-fold), *Sqle* (2.5-fold), *Fdft1* (2.6-fold), *Cyp51a1* (2.1-fold), and *Srebp2* (1.5-fold). These findings are similarly found in the colon, where cholesterol biosynthetic genes are up-regulated: *Cyp51a1*, *Fdft1*, *Idi1*, and *Lss* were induced 1.4-, 1.8-, 1.9-, and 1.3-fold, respectively. *Lldr* was induced to a lesser extent, but retained significance (1.4-fold). From a transcriptional perspective, multiple tissues are actively trying to synthesize cholesterol. Therefore, one possible explanation for the apparent paradox between low circulating cholesterol levels and the induction of biosynthetic pathways in KO mice is the significant regulation of lipid transport genes, located both within and near the membranes of hepatocytes, and the reduction of bile which consequently reduces cholesterol absorption. Indeed, hepatic plasma membrane transporters involved in lipid uptake, *Cd36* and *Srbi,* were induced 4.3- and 1.3-fold, respectively, while the efflux pump *Abcg8* was decreased 1.4-fold. Furthermore, the increased expression of *Pltp* and *Pctp* of 1.6- and 1.7-fold would lead to increases in the hepatic uptake of phospholipid species. As such, these results suggest the unidirectional transport of lipids into the liver, while reducing lipid export.

Fatty Acid Metabolism. Maintaining fatty acid homeostasis is a complex process in which many pathways in several tissues are in equilibrium; however, the significant accumulation of hepatic lipid suggests that this delicate balance has been disrupted. Pathway analysis revealed that increases in hepatic lipid do not occur because of induced local lipid biosynthesis. This hypothesis is supported by the lack of expression changes

for fatty acid synthetase (*Fas*) and *Srebp1c*, indicating that fatty acid biosynthesis at the mRNA level has not been induced. Rather, the liver is adapting to increases in localized lipid content by inducing both peroxisomal and mitochondrial oxidation pathways. *Abcd1* and *Abcd2*, involved in peroxisomal fatty acid oxidation, are up-regulated 1.3- and 1.6-fold, respectively. Mitochondrial β-oxidation is also induced via the induction of several key genes, such as *Acox1*, *Acsl4*, and *Cpt1a* by 1.2-, 1.6-, and 1.7-fold, respectively; however, the 1.5-fold up-regulation of *Acc2* may counteract mitochondrial oxidation by leading to increases in cellular content of malonyl-CoA, an allosteric inhibitor of CPT1a. This suggests that long-chain polyunsaturated fatty acids (LC-PUFA) are metabolized in peroxisomes, while medium-chain fatty acids are not efficiently processed by the mitochondria. The abundance of medium-chain fatty acids would then serve as ideal substrates for *Scd1* and *Scd2*, both of which are increased 2.2- and 1.5-fold respectively.

Lipidomic Analysis of the Liver, Plasma, and Small Intestine. To assess the relevance of our hypotheses generated by transcriptional profiling, we analyzed the composition of nine principal lipid categories in the liver, of which four were significantly up-regulated ($p < 0.05$) (Figure 2). Quantitative analysis of lipids revealed an increase in DAGs of approximately 150% and a dramatic increase in TAGs of nearly 2000%. To a lesser extent, phosphatidylcholine (PC) and cholesterol ester (CE) abundances were modulated, with overall increases of approximately 21% and 130%, respectively. CEs were found to preferentially incorporate monounsaturated fatty acids (MUFA), as the n-9 and n-7 acyl species (i.e., oleic and palmitoleic acids, respectively) are increased by 636% and 362% in comparison with n-6 (135%) acyl species. Although subtle increases in the MUFA and PUFA components of PC can be seen, the most significant increase was seen with the n-7 fatty acyl species (119%). Furthermore, despite the lack of significant changes in the PE and free fatty acid (FFA) categories as a whole, both the n-9 fatty acids and MUFA subgroup are significantly enriched in these lipid groups. These changes in lipid composition reflect the aforementioned increased expression of hepatic *Scd1* and *Scd2*.

To ascertain the degree by which the dramatic lipid increase in livers of POR-null mice affects extrahepatic tissues, we also examined the lipidome of the plasma and jejunum. While gene expression results suggested significant changes in extrahepatic lipid metabolism, the lipid profiles of both plasma and the jejunum were only minimally changed. Examining the acyl composition revealed that the ~40% decrease in plasma CE was due to significant decreases in CEs enriched with n3- and n6-PUFA species (46% and 45%, respectively) (Figure 2). Changes in CE quantity and composition are supported by the differential gene expression of hepatic cholesterol transporters and peroxisomal fatty acid oxidation genes, as reported above. No other changes in lipid abundance or composition were found in plasma. In the jejunum, only the FFA category was significantly changed. Jejunal MUFA levels were increased by 166%, corresponding to approximately 200% and 100% increase in n9 and n7 acyl species, respectively (Figure 2). The 3.4-fold induction of *Scd2* in jejunal epithelium suggests a molecular mechanism to support these increases in MUFA. No other changes in abundance or composition were found. Clustering the lipid data with a Pearson correlation (Figure 3) further revealed that the inactivation of liver POR does not significantly affect the overall lipid profile of either the jejunum or plasma,

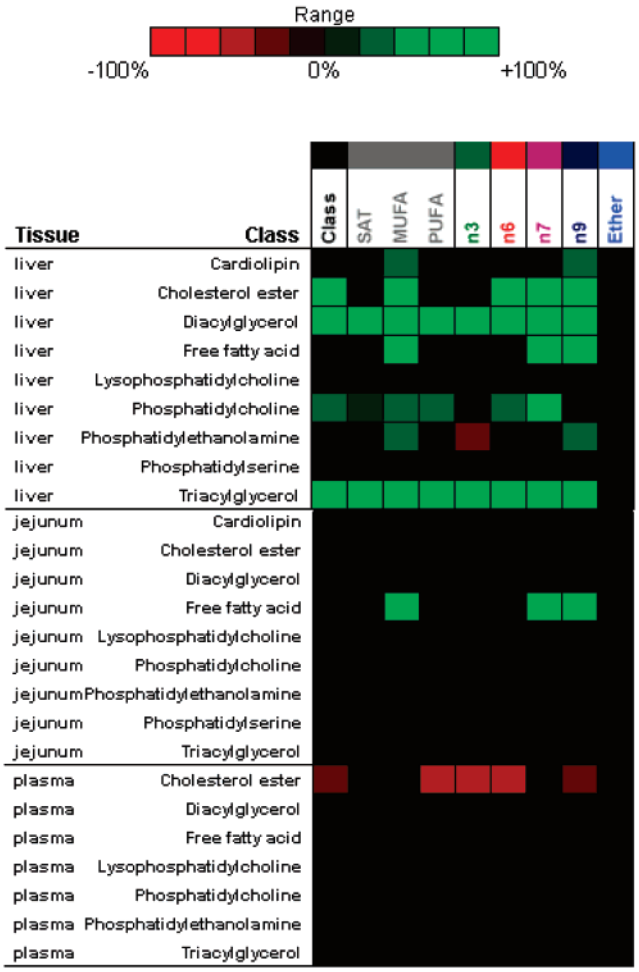

Figure 2. Changes in lipid classes in the liver, jejunum, and plasma of POR-null animals. In the liver cholesterol ester, diacylglycerol, triacylglycerol, and, to a lesser extent, phosphatidylcholine are significantly up-regulated as lipid classes by 130%, 150%, 2000%, and 21%, respectively; however, only DAG and TAG show increases in all fatty acyl species. The composition of cholesterol esters is specifically enriched with MUFA (n7 and n9 fatty acids increased by 362% and 636%, respectively). In the jejunum, no classes are up-regulated following the elimination of hepatic POR. Increases of 166% in jejunal MUFA were found, corresponding to a 200% and 100% increase in n9 and n7 fatty acids, respectively. In the plasma, cholesterol esters were, as a class, significantly reduced by 40%. CE showed specific reductions in PUFA (n3, 46%; n6, 45%) and, to a lesser extent, n9 fatty acids (36%). Green indicates increases, red indicates decreases. Quantitative nmol lipid data can be found in the Supplementary File in Supporting Information.

as these tissues cluster tightly together irrespective of POR functionality.

Discussion

The goal of the present study was to provide insight into lipid metabolism in the liver, circulation, and intestine of mice deficient in liver POR. Because previous studies describing POR-null mice models have neither definitely unraveled the molecular mechanisms leading to perturbations in lipid homeostasis nor assessed the impact of a POR-deficiency outside of the liver, the present study had two principal goals: to elucidate (A) whether the changes observed in gene expression

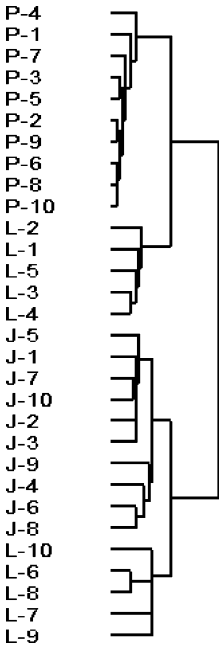

Figure 3. Clustering total lipids in the liver, jejunum, and plasma of wild-type and KO animals with a Pearson correlation algorithm. The disruption of the hepatic POR enzyme has minor effects on the jejunum and plasma lipid profiles, as these tissues cluster together irrespective of POR functionality. P, plasma; L, liver; J, jejunum. Samples 1−5 are WT animals and 6−10 are KO animals.

studies are reflected in the hepatic lipidome, and (B) whether deficiencies in liver POR affect gene expression and lipid metabolism in the small intestine and plasma.

Hepatic gene expression analyses revealed that 'electron transport' and 'carboxylic acid metabolism' were the two most significantly regulated biological processes in KO mice. Changes in the expression of genes associated with electron transport were not unexpected, since the elimination of liver POR systematically disables the entire liver P450 system; however, the induction of all 17 genes identified as differentially expressed and associated with this biological process implies that the system is uniformly responding to, and attempting to compensate for, a deficiency in metabolism. This compensatory notion is further supported by previous findings in which a 5-fold increase in P450 protein was also observed in these mice.[2] This previous finding serves as a valuable independent validation of our microarray study. The search for regulatory promoter elements revealed the presence of nuclear receptor binding motifs common to genes in both biological processes. Indeed, nuclear receptors known to be activated by lipids (such as PPARs, LXR-α, and FXR[25,26]) provide a mechanistic link underlying the increased expression of genes assigned to both biological processes. While these nuclear receptors are recognized regulators of lipid metabolism, the significance of their role in coordinating the biological response to deficiencies in hepatic POR has not been previously appreciated.

The required cellular machinery for cholesterol biosynthesis is present in nearly all tissues throughout the body; however, the principle locations are the liver and intestine.[27] In response to the low levels of circulating cholesterol, genes associated with sterol biosynthesis in both the liver and various regions of the gut (except ileum) were significantly induced. Despite the highly significant up-regulation of gut biosynthetic pathways, no changes in intestinal cholesterol content were found. In the liver, the rate-limiting enzyme HMGCR was transcriptionally induced, and a 130% increase in cholesterol levels was found. Interestingly, the altered expression of various transporters involved in hepatic cholesterol transport (*Abca1*,[28] *Srbi*,[28] *Abcg8*[29]) suggests a transcriptional regulation of key players in liver sterol mobilization. Quantitative lipid analysis in the plasma revealed that decreased abundance of CEs stems predominantly from a reduction in LC-PUFA enriched CEs. Examining the gene expression data set for mechanistic clues to explain this reduction in LC-PUFA enriched CEs enabled us to hypothesize that this occurs because of changes in oxidation processes. LC-PUFA destined for oxidation are first processed by peroxisomes and subsequently by mitochondria.[30] The present study found increases in peroxisomal transporters (*Abcd1* and *Abcd2*), suggesting an increase in the potential for LC-PUFA oxidation. However, while gene expression data found increases in several genes of the mitochondrial oxidation pathway, the data also suggested that this process may be inhibited by the formation of ACC2-derived malonyl-CoA, which has previously been demonstrated to inhibit lipid oxidation.[31] Indeed, individuals with genetic defects in β-oxidation genes have previously been found to have significant increases in hepatic lipid content.[32,33] Thus, it is highly plausible that the dramatic increase in liver MUFA (specifically in the CE and free fatty acid pools) stems from an inability to metabolize fatty acids in the mitochondria, while the decrease in LC-PUFA occurs because of an increase in peroxisomal oxidation.

The principal lipid classes accumulating in the liver of POR-null mice are DAGs and TAGs. Because no changes in DAGs and TAGs were seen in the plasma, our conclusion that mice deficient in liver POR are unable to efficiently mobilize this increased lipid content is reinforced. As such, deficiencies in liver POR result in the liver serving principally as a lipid reservoir. Although fatty acid synthesis is not induced, it is not down-regulated either, suggesting that it is proceeding normally and would lead to an eventual increase in the local lipid pool if feedback mechanisms are dysfunctional. Furthermore, the increased expression of genes such as *Cd36*, *Pltp*, *Pctp*, and *Fatp4* further suggests that the liver is tending to promote lipid uptake and storage over efflux. Intriguingly, the overall induction of lipid synthetic pathways is reflected in the composition of DAG and TAG pools. Indeed, all fatty acid species (n3, n6, n7, and n9) are increased within these pools, suggesting no particular pathway regulating fatty acid incorporation into DAG and TAG is specifically affected by a dysfunctional POR enzyme.

Lipid analyses in the jejunum and plasma revealed relatively minor changes in the absence of liver POR. Indeed, clustering lipid profiles of mice with and without hepatic POR did not significantly modify the overall lipid content of the jejunum and plasma, as revealed by clustering the quantitative lipid data. In contrast, the profile of the liver was remarkably changed. Whereas in WT animals the plasma reflected liver lipid content, the lipid profile of the liver clustered more tightly with that of the jejunum in KO animals. This presents an intriguing finding suggesting that, when lipid mobilization in the liver is disrupted, the plasma lipid profile can remain relatively resistant to these changes.

Conclusion

Integrating lipid profiling with gene expression analyses has provided a means to further elucidate those molecular mech-

anisms that change in mice deficient in hepatic POR. The concordance between the two analytical platforms (transcriptomic vs lipidomic) in the liver was excellent, and serves as a valid approach for generating novel hypotheses to unravel protein function that cannot be accomplished with as much confidence using either platform individually. Such an approach has further revealed that, while gene expression and lipid metabolism in extrahepatic tissues are sensitive to changes in hepatic POR functionality, the lipidome is only minimally affected. The gene expression changes observed in the gut can most likely be attributed to decreases in luminal bile and lipid content, and are therefore indirectly regulated by a deficiency in hepatic POR. Indeed, both lipid and bile biosynthetic pathways are up-regulated in the jejunum, ileum, and colon. Furthermore, bile acid metabolism is transcriptionally induced by both the ileum and liver in a coordinate manner, but the minor changes in the lipid profiles of gut and plasma suggests that the aberrant accumulation of lipid is confined to the liver. In conclusion, our findings imply that the hepatic lipid accumulation stemming from POR deficiencies has only a minor effect on lipid metabolism in the biological system at large. Taken together, hepatic POR can be considered an enzyme critical for the proper functioning of lipid mobilization and metabolism predominantly within the mouse liver.

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Supporting Information Available: A complete table of all quantitative lipid data (nmol/g of tissue and liquid material) for the liver, plasma, and jejunum can be found in the supporting material. This data set contains quantitative data for 40 different fatty acyl species in each animal, where samples 1−5 are WT animals and 6−10 are KO animals. This material is available free of charge via the Internet at http://pubs.acs.org.

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