Potential Biological Functions of Cytochrome P450 Reductase-dependent Enzymes in Small Intestine

NOVEL LINK TO EXPRESSION OF MAJOR HISTOCOMpatibility COMPLEX CLASS II GENEs

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Background: P450 reductase (POR)-dependent enzymes metabolize numerous endogenous compounds.

Results: Enterocytes of intestinal Por knock-out mice show up-regulation of genes important for immunity and increased geranylgeranyl pyrophosphate levels.

Conclusion: Enterocyte POR-dependent enzymes modulate intestinal expression of major histocompatibility complex class II genes, possibly through intermediates in cholesterol biosynthesis.

Significance: This appears to be the first evidence for a link between POR-dependent enzymes and intestinal immunity.

NADPH-cytochrome P450 reductase (POR) is essential for the functioning of microsomal cytochrome P450 (P450) monooxygenases and heme oxygenases. The biological roles of the POR-dependent enzymes in the intestine have not been defined, despite the wealth of knowledge on the biochemical properties of the various oxygenases. In this study, cDNA microarray analysis revealed significant changes in gene expression in enterocytes isolated from the small intestine of intestinal epithelium-specific Por knock-out (named IE-Cpr-null) mice compared with that observed in wild-type (WT) littermates. Gene ontology analyses revealed significant changes in terms related to P450s, transporters, cholesterol biosynthesis, and, unexpectedly, antigen presentation/processing. The genomic changes were confirmed at either mRNA or protein level for selected genes, including those of the major histocompatibility complex class II (MHC II). Cholesterol biosynthetic activity was greatly reduced in the enterocytes of the IE-Cpr-null mice, as evidenced by the accumulation of the lanosterol metabolite, 24-dihydroxlanosterol. However, no differences in either circulating or enterocyte cholesterol levels were observed between IE-Cpr-null and WT mice. Interestingly, the levels of the cholesterol precursor farnesyl pyrophosphate and its derivative geranylgeranyl pyrophosphate were also increased in the enterocytes of the IE-Cpr-null mice. Furthermore, the expression of STAT1 (signal transducer and activator of transcription 1), a downstream target of geranylgeranyl pyrophosphate signaling, was enhanced. STAT1 is an activator of CIITA, the class II transactivator for MHC II expression; CIITA expression was concomitantly increased in IE-Cpr-null mice. Overall, these findings provide a novel and mechanistic link between POR-dependent enzymes and the expression of MHC II genes in the small intestine.

NADPH-cytochrome P450 reductase (CPR2 or POR (P450 oxidoreductase) is a microsomal flavoprotein that serves as an electron donor for multiple enzymes, including microsomal cytochrome P450s (P450 or CYP) (1), heme oxygenases (HOS) (2), and squalene epoxidase (SQLE) (3). POR-dependent enzymes play important roles in the homeostasis of many endogenous compounds, such as bile acids, cholesterol, heme, steroids, and fatty acids. Furthermore, POR-dependent enzymes are essential for fetal development (4). In humans, mutations in the POR gene result in disordered steroidogenesis and the Antley-Bixler syndrome (5).

To circumvent the embryonic lethality of germ line Por knock-out mice, mouse models employing conditional Por gene knock-out have been developed and used for the investigation of the organ-specific functions of POR-dependent enzymes. For example, two liver-specific Por knock-out mouse models have been developed, and, despite the induction of numerous hepatic P450s, both exhibited impaired drug metabolism, decreased serum cholesterol, and enlarged and fatty livers (6, 7). A whole-body Cpr-low mouse was also developed, in which POR levels are reduced by >70% in all tissues examined (8). This latter model was found to have partial embryonic lethality, altered steroid hormone homeostasis, and infertility in females, in addition to impairments in drug metabolism and decreases in serum cholesterol. In both the liver-Cpr-null and the Cpr-low mice, extensive changes in global gene expression were observed in the livers, which revealed the importance of

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This article contains supplemental Tables 1–4.

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2 The abbreviations used are: CPR or POR, NADPH-cytochrome P450 reductase; P450, cytochrome P450; 24-DHL, 24-dehydroxlanosterol; 24-DHL-TMS, trimethylsilyl derivative of 24-DHL; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GO, gene ontology; HO, heme oxygenase; IE, intestinal epithelium; SI, small intestine; SQLE, squalene epoxidase; LXR, liver X receptor.
Genomic and Biochemical Analysis of IE-Cpr-null Enterocytes

hepatic POR-dependent enzymes in the homeostasis of fatty acids and other lipid metabolites, as well as in the regulation of various metabolic enzymes and transporters (9, 10).

An intestinal epithelium (IE)-specific Por knock-out mouse (named IE-Cpr-null), in which the Por gene is specifically deleted in the enterocytes, was recently obtained (11). The IE-Cpr-null mice do not display any obvious abnormalities in growth, development, or reproduction, and their intestines appear to have normal structure. However, targeted gene expression analysis showed compensatory increases in the expression of several P450 enzymes in the small intestine (SI) (11). Further pharmacological studies revealed deficiencies of the IE-Cpr-null mice in the first-pass metabolism of oral drugs and dietary contaminants (11–13). Given the known ability of various POR-dependent enzymes to metabolize endogenous compounds, the metabolic disturbances detected in the livers of the liver-Cpr-null mice, and the unique chemical environment of the intestinal enterocytes as the portal of entry for lipid molecules entering from either the diet or the bile, we hypothesize that loss of the enterocyte POR expression will impact the homeostasis of endogenous compounds and the expression of genes that have critical biological functions in the SI. Conceivably, the nature of the biochemical consequences of the Por deletion in the intestine may dictate potential functional deficits in the responses to various environmental challenges, including pathogenic infection, in the IE-Cpr-null mice and potentially in people with POR mutations that cause reduced POR expression.

In the present study, we performed comparative analyses of global gene expression in enterocytes from wild-type (WT) and IE-Cpr-null mice using the Affymetrix Mouse Expression Set 430A 2.0 GeneChip arrays. Groups of genes exhibiting differing expression levels between the IE-Cpr-null and WT mice were identified. Subsequent pathway analysis, conducted using Gene Map Annotator and Pathway Profiler (available from the GeneMAPP Web site), led to the identification of “antigen presentation/processing” among the gene ontology (GO) terms that contained the most significantly changed gene expression. A detailed analysis of gene expression changes related to antigen processing and presentation led us to propose a mechanistic scheme to explain the unexpected increase in genes related to this pathway in the intestine. Additional studies of the levels of relevant endogenous metabolites (including cholesterol, 24-dihydroxycholesterol (24-DHC), farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), heme, and bilirubin) and signaling proteins (including STAT1 and C/EBPα (class II major histocompatibility complex transactivator)) provided evidence in support of a novel link between POR-dependent enzymes and expression of the major histocompatibility complex class II (MHC II) genes, which are important for intestinal immunity.

**EXPERIMENTAL PROCEDURES**

**Animals**—Adult (2.5–4.0-month-old) male IE-Cpr-null mice and WT littersmates were used in all experiments. Protocols for breeding and genotyping were as reported previously (11). Animals were maintained at 22 °C with a 12-h on, 12-h off light cycle and were allowed free access to water and a standard laboratory diet. Animal use protocols were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center.

**RNA Preparation and Microarray Hybridization**—Tissues from IE-Cpr-null and WT mice (8 for each group) were collected for RNA preparation. SI was processed immediately after dissection for enterocyte isolation and RNA preparation, essentially as described (14), except that, following removal of luminal content, the SI was placed in PBS, pH 7.2, containing 1.5 mM EDTA, 3 units/ml heparin, and 0.5 mM dithiothreitol for 10 min, in order to loosen the mucosa before the epithelial cells were collected by scraping. The harvested cells were placed directly into TRIZol (Invitrogen). Total RNA was prepared from enterocytes of individual mice, using TRIZol; the samples were further purified using RNAeasy minicolumns (Qiagen, Valencia, CA). The integrity and purity of the RNA preparations were determined using a RNA 6000 Nano Assay kit on a model 2100 Bioanalyzer (Agilent, Santa Clara, CA). The average of RNA integrity number (RIN) values was 8.2 ± 0.5.

**Mouse Expression Set 430A 2.0 GeneChip arrays** (Affymetrix, Santa Clara, CA) were used for microarray analyses. Each array contained 22,690 probe sets, representing ~14,870 distinct genes. Each probe set consisted of 11 pairs of 25-mer oligonucleotides. Four RNA samples, each prepared by pooling equal amounts of enterocyte RNA from two mice of the same genotype, were analyzed for each group (WT or IE-Cpr-null). Synthesis of biotinylated antisense RNA, array hybridization, staining, washing, and collection of expression data were performed within the Microarray Core Facility of the Wadsworth Center, as described previously (9).

**Microarray Gene Expression Data Analysis**—The experimental data sets were normalized using the Guanine Cytosine Robust Multichip Analysis (for fold change analysis) or Microarray Analysis Suite 5.0 (for absent/present calls) programs of the Genespring 10.0 software package (Agilent). The data for WT mice were used as the base line. Probe sets with raw intensity below the 20th percentile in all eight samples were eliminated, leaving 18,443 probe sets for further analysis. Analysis for significance was performed using the unpaired t test in Genespring 10.0. The ratios of averaged values for each group were used to calculate fold change between two groups. Genes with significantly changed expression were tabulated, along with gene symbol, gene name, transcript identification number, and fold change values, and the data were further examined for reproducibility among multiple probe sets for a given gene, where available. Two programs, MAPPFinder (15) and GenMAPP 2.0 (16), were used to group genes having significantly changed expression according to the GO hierarchy at the level of biological processes, cellular components, and molecular functions as described previously (9).

**Determination of Cholesterol Levels in Plasma and Enterocytes**—The levels of total cholesterol in plasma and enterocytes were determined using a cholesterol assay kit (including esterase for hydrolysis; Cayman, Ann Arbor, MI) according to the manufacturer’s instructions. For enterocytes, cholesterol was extracted prior to analysis, based on the method of Folch et al. (17), with modifications. Briefly, enterocytes were isolated as described above for RNA preparation.
HP-5ms 30 m, 0.25-mm (0.25-μm particle size) column (Phenomenex, Torrance, CA). The chromatographic conditions were those described by Tong et al. (19). FPP and GGPP were monitored by detection of fluorescence at 528 nm, elicited by excitation at 335 nm. The amounts of FPP and GGPP were determined using authentic standards (Sigma-Aldrich).

**Generation of Heme and Bilirubin Contents in Plasma and Enterocytes**—Heme content was measured using a QuantiChrom heme assay kit (BioAssay Systems). Plasma and enterocyte homogenates, prepared as described by Mingone et al. (20), were used in the assay at a concentration of ~5 mg of protein/ml. For bilirubin determination, PBS-washed enterocytes (~400–500 mg wet weight) were homogenized in 0.5 ml of deionized water containing 1 mg of butylated hydroxytoluene, and bilirubin was extracted as described (21). Mesobilirubin was used as an internal standard. All steps were performed under dim light. Bilirubin analysis was carried out using an Agilent 1100 HPLC system with a diode array detector (set to monitor 405 nm) and a Vydac C8 column (250 mm × 4.6-mm inner diameter, 5 μm) (Discovery Sciences, Deerfield, IL), using a solvent system consisting of solvents A (10 mM ammonium acetate) and B (methanol/100 mM ammonium acetate, 90:10, v/v). A 20-min linear gradient from 60% B to 80% B was applied at a flow rate of 0.5 ml/min for sample elution; the column was maintained at 40 °C. Authentic bilirubin (Sigma) was used as the standard.

**Real-time RNA-PCR**—Total RNA was isolated from enterocytes with TRIzol as described under “RNA Preparation and Microarray Hybridization.” Real-time RNA-PCRs were performed according to a general protocol described previously (22), with minor modifications. A full list of the primers used and the optimal annealing temperatures is included in supplemental Table 1. All primers were used at 0.1 μM, except for GAPDH, of which primers were used at 0.4 μM. At the end of the PCR cycles, melting curve analysis was performed to assess the purity of the PCR products. The levels of target gene mRNAs in various total RNA preparations were normalized by the level of GAPDH mRNA in a given sample. All reactions were performed in duplicates. Negative control reactions (no template) were routinely included. Identities of PCR products were confirmed by electrophoretic analysis on agarose gels.

**Immunoblot Analyses**—The basic procedures used for immunoblot analysis were as described (22). The following were used as the primary antibodies: a rat monoclonal antibody to the polymorphic determinant shared by multiple mouse MHC II alloantigens (BD Biosciences, San Jose, CA), a rabbit monoclonal antibody to human STAT1 (42H3, Cell Signaling Technology, Danvers, MA), a rabbit polyclonal antibody to human phospho-Ser-727-STAT1 (Cell Signaling Technology),
a rabbit monoclonal antibody to human β-actin (13E5, Cell Signaling Technology), and a goat polyclonal antibody to rabbit GAPDH (GenScript, Piscataway, NJ). Enterocytes were isolated using the same protocol as described above for RNA preparation, except for immunoblot analysis of STAT1 expression, in which the enterocytes were isolated using an elution method described by Ware et al. (23). Whole-cell lysates were prepared using radiolabile precipitation assay buffer (Thermo Fisher Scientific) according to the manufacturer’s instructions. Protein concentrations were determined by using the bicinchoninic acid method (Fisher) with bovine serum albumin as the standard. For immunoblot quantitation, a model GS-710 calibrated imaging densitometer (Bio-Rad) was used.

RESULTS

Microarray Analysis—By using relatively conservative criteria in fold change (≥1.75 or ≤-0.57) and a p value of ≤0.01, we identified a number of mouse SI genes that were differentially expressed in the enterocytes of the IE-Cpr-null mice, compared with their expression levels in WT mice (34 up-regulated and 17 down-regulated) (Table 1). Genes showing significant differences in expression changes were grouped into functional categories based on GenMAPP, UniProt (24), and additional literature searches. The categories included biotransformation, lipid metabolism, transporters, growth factors, and antigen processing and presentation. Notably, numerous other genes in these and other categories were also found to have altered expression, albeit with smaller fold change or greater p values. For example, although many Cyp genes appeared to be up-regulated in the IE-Cpr-null mice, only Cyp1a1, Cyp1a2, and Cyp51 met the criteria for inclusion in Table 1. In this regard, 27 of the 68 unique mouse P450 genes represented (by 83 probe sets) on the GeneChip were detected in both IE-Cpr-null and WT mice (supplemental Table 2).

To gain a broader view of the genomic changes in the SI, we used a lower stringency for individual gene expression changes (i.e. ≥25% in fold change and a p value of ≤0.05). The selection of this lowered stringency was based on a published method (15) and on the consideration that accumulation of small changes in a biological pathway might cause observable biological effects. By using this criterion, we identified 153 significantly induced and 247 significantly suppressed genes in the enterocytes of the IE-Cpr-null mice (18). As shown in Fig. 1, 24-DHL, which was readily analyzed as its trimethylsilyl derivative (24-DHL-TMS) by using GC/MS, was not detected in enterocytes from WT mice, but it was abundant in enterocytes of IE-Cpr-null mice. The identity of 24-DHL-TMS was confirmed by comparisons with the results from the analysis of the trimethylsilyl derivative of authentic 24-DHL standard with respect to GC retention times and electron impact mass spectra (Fig. 1). Indistinguishable mass spectra and GC retention times were observed. The mass spectrum of 24-DHL-TMS shows a molecular ion at m/z 500 and a peak at m/z 485 attributed to the loss of a methyl radical (15 Da) from the molecular ion. The base peak in the mass spectrum of 24-DHL-TMS is at m/z 395, which is attributed to the neutral losses of HOSi(CH$_3$)$_3$ (90 Da) and a methyl radical from the molecular ion. The tissue level of 24-DHL was determined by quantitative GC/MS analysis to be 260 pmol/mg enterocytes in the IE-Cpr-null mice (Table 3), a level that is at least 8 times greater than the levels in WT mice (based on a detection limit of 30 pmol/mg). This result confirms that cholesterol synthesis was blocked at CYP51 in the enterocytes of IE-Cpr-null mice.

The loss of hepatic cholesterol synthesis in the liver-Cpr-null mice resulted in drastic decreases in plasma total cholesterol (6, 7). Given the relatively large metabolic capacity of the SI, we questioned whether the loss of enterocyte cholesterol synthesis would impact either circulating or else local tissue levels of cholesterol. As shown in Table 3, the levels of total cholesterol in the enterocytes of IE-Cpr-null and WT mice were not significantly different (both at ~4 nmol/mg enterocytes). Plasma total cholesterol levels were also similar between the mouse strains.
Genomic and Biochemical Analysis of IE-Cpr-null Enterocytes

TABLE 1
Genes that were differentially expressed in enterocytes of WT and IE-Cpr-null mice

| Gene symbol | Ref Seq transcript ID | Fold change | Gene name |
|-------------|-----------------------|-------------|-----------|
| Biotransformation | | | |
| Adh4 | NM_011996 | 0.53 | Alcohol dehydrogenase 4 |
| Akr1c14 | NM_134072 | 0.47 | Aldo-keto reductase family 1, member C14 |
| Cypl1a1 | NM_001136059 | 3.8 | Cytochrome P450 1a1 |
| Cypl2a1 | NM_009993 | 44 | Cytochrome P450 1a2 |
| Cyp51a1 | NM_020010 | 2.4/1.9/1.0 | Cytochrome P450 51 |
| Por | NM_008898 | 0.26 | Cytochrome P450 oxidoreductase |
| Lipid metabolism | | | |
| Acer1 | NM_175731 | 2.4 | Alkaline ceramidase 1 |
| Fdrl1 | NM_010191 | 1.9/1.7 | Farnesyl diphtophate farnesyl transferase 1 |
| Id1 | NM_145360 | 2.0/1.5 | Isopentenyl-diphosphate δ isomerase |
| Lx2 | NM_146406 | 2.0/1.8/1.0* | Lanosterol synthase |
| Mvd | NM_138656 | 2.7/2.4 | Mevalonate (diphospho) decarboxylase |
| Pla2g7 | NM_013737 | 0.24 | Phospholipase A2, group VII |
| Pmvk | NM_026784 | 1.9 | Phosphomevalonate kinase |
| Pppla7 | NM_146251 | 2.4 | Pataxin-like phospholipase domain containing 7 |
| Sdc2 | NM_009128 | 7.1/3.4 | Stearoyl-coenzyme A desaturase 2 |
| Sgk | NM_009270 | 2.5 | Squalene epoxidase |
| Growth factors | | | |
| Btc | NM_007568 | 0.76+/0.47 | Betacellulin, EGF family member |
| Caprin2 | NM_181541 | 0.43 | Caprin family member 2 |
| Erg | NM_007950 | 1.9 | Eripeginulin |
| Nr4 | NM_032002 | 0.41 | Neuregulin 4 |
| Transportsers | | | |
| Mfsc27c/Flvcr2 | NM_145447 | 6.2 | Major facilitator superfamily domain-containing 7C |
| Slc2a2 | NM_018824 | 0.54/0.43 | Solute carrier family 2, member 2 |
| Antigen processing and presentation | | | |
| Ciita | NM_005775 | 4.5/4.4 | Class II transactivator |
| H2-Aa | NM_010378 | 3.0/2.2* | Histocompatibility 2, class II antigen A, α |
| H2-Ab1 | NM_207105 | 2.3/2.1/1.9 | Histocompatibility 2, class II antigen A, β1 |
| H2-Dma | NM_010386 | 2.3 | Histocompatibility 2, class II, locus DMA |
| H2-Dmb1/Dmb2 | NM_010387/NM_010388 | 2.9/3.3 | Histocompatibility 2, class II, locus DMB1/2 |
| H2-Eb1 | NM_010382 | 1.9 | Histocompatibility 2, class II antigen E, β |
| H2-gs10 | NM_00143689 | 1.8 | MHC class I like protein GS10 |
| 0610037M15Rik | NM_903697 | 1.8 | RIKEN cDNA 0610037M15 gene |
| Other | | | |
| 6330442E10Rik | NM_178745 | 0.34 | RIKEN cDNA 6330442E10 gene |
| Acta1 | NM_009606 | 4.1 | Actin, α1, skeletal muscle |
| Acpd1 | NM_133237 | 1.0/0.86*/0.79/0.48 | APC-down-regulated 1 |
| Ghp2 | NM_010260 | 2.2/2.1 | Guanylate nucleotide-binding protein 2 |
| Gm7120 | NM_0013092244 | 1.0/0.49 | Predicted gene 7120 |
| Gphab | NM_172952 | 0.61*/0.47 | Gephyrin |
| Greb1 | NM_015764 | 0.28 | Gene regulated by estrogen in breast cancer protein 1 |
| Hlf | NM_172563 | 0.88*/0.31 | Hepatic leucemia factor |
| Jdp2 | NM_030887 | 2.1 | Jnk dimerization protein 2 |
| Mapk36 | NM_016693 | 2.6 | MAP kinase kinase kinase 6 |
| Mt1 | NM_0013062 | 160/8.0 | Metallothionein 1 |
| Mup1 | NM_00145550 | 400/8.0 | Major urinary protein 1 (and 2/7/8/10/12/17) |
| Pdka4 | NM_013743 | 0.32 | Ppyruvate dehydrogenase kinase isozyme 4 |
| Pfkb3 | NM_133232 | 1.9/1.5* | 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 |
| Prkaa2 | NM_011999 | 1.0/0.57 | Ribosomal protein S6 kinase, polypeptide 2 |
| Sec7d1 | NM_177608 | 1.8/1.2 | SECIS binding protein 2-like |
| Susd2 | NM_001162913 | 1.9 | Sushi domain-containing 2 |
| Tmem184c | NM_145599 | 2.5 | Transmembrane protein 184c |
| Tnfr1 | NM_025655 | 4.2 | Transmembrane and immunoglobulin domain-containing 1 |
| Tubb2b | NM_023716 | 0.54 | Tubulin, β2b |
| Ubld | NM_023137 | 7.5 | Ubiquitin D |

* p < 0.05,

at ~120 ng/ml, or 3.1 mM; data not shown). Thus, de novo cholesterol synthesis in the SI did not noticeably influence cholesterol levels in the plasma or in enterocytes.

Up-regulation of CIITA and MHC II Genes—Genes that are up-regulated in expression in the biological process of antigen processing and presentation in the enterocytes of IE-Cpr-null mice (Tables 1 and 2) included MHC I and II genes (1.8–3.3-fold), the class II transactivator (Ciita, the master regulator of MHC II genes; 4.5-fold), and Cd74 (the MHC II invariant chain, a specialized chaperone; 1.6-fold, p = 0.02, not included in Table 1). These changes were confirmed by RNA-PCR analysis of mRNA expression for Ciita, Cd74, and H2-Aa (as a representative MHC II gene) in enterocytes of IE-Cpr-null and WT mice (Fig. 2); the extents of increase in gene expression determined by RNA-PCR were comparable with or greater than the extents revealed by the microarray data. In addition, the mRNA...
Genomic and Biochemical Analysis of IE-Cpr-null Enterocytes

TABLE 2
Gene ontology terms that showed the most significant gene expression alterations in the enterocytes of IE-Cpr-null mice

Pathway analysis was performed using GenMAPP 2.1, MAP Finder 2.0, and the Min-swstd_20070817.gdb database (available at the GenMAPP Web site). The criteria for identification of genes with significantly increased or decreased expression were as follows: fold change $\geq 25\%$ (or $\leq 0.80$) and $p \leq 0.05$. GO terms are sorted into three types: biological process, molecular function, and cellular component. For each GO term, the number of genes that meet the criteria for a significant increase or decrease was determined (No. changed). This number was compared with the number of genes in the GO term that are measured by the MOE 430A chip (No. of genes measured), for the calculation of the percentage of genes measured in the GO term that meet the criteria for a significant increase or decrease (% changed, in parentheses).

expression of cathepsin E, a gene known to be down-regulated by CIITA (25), also appeared to be decreased in enterocytes of IE-Cpr-null mice, compared with WT mice, as indicated by the microarray data (fold change, 0.21; $p = 0.09$) and by RNA-PCR analysis (by 85%; data not shown).

The mouse Citta gene has three distinct promoters (pI, pIII, and pIV) that are utilized in a cell-specific manner, each producing a unique mRNA, pI and pIII are used exclusively by immune cells, whereas pIV is utilized in multiple cell types (26), including enterocytes (27). The two probe sets for Citta on the GeneChip are homologous to regions shared by mRNAs from all three promoters. However, RNA-PCR (Fig. 2) analysis revealed increased expression from pIV, but not from pI or pIII, in the enterocytes of IE-Cpr-null mice, compared with expression in WT mice. Furthermore, the magnitude of the increase in Citta pIV expression was similar to that of the increase of total Citta transcripts, determined by use of either a general CIITA primer or by the microarray analysis (both 4–5-fold).

Thus, the induction of CIITA occurred in enterocytes, rather than in immune cells that conceivably could have been co-isolated with the enterocytes.

Microarray analysis revealed up-regulation of multiple MHC II genes, including H2-Aa, H2-Ab1, and H2-Eb1, in IE-Cpr-null enterocytes (Table 1). Using an antibody that recognizes both H2-A and H2-E MHC II proteins, we confirmed up-regulation of MHC II protein expression in whole cell lysates from IE-Cpr-null enterocytes (~4-fold, compared with WT mice) (Fig. 3). The two bands detected on immunoblots represent the $\alpha$ and $\beta$ subunits of MHC II proteins (28).

Potential Mechanistic Link between Por Gene Deletion and MHC II Up-regulation.—The expression from CIITA pIV can be induced by IFN-$\gamma$ in multiple cell types (26), including enterocytes (27). Evidence in support of the involvement of IFN-$\gamma$ or its downstream mediators in the up-regulation of CIITA in the IE-Cpr-null mice was obtained in the analysis of the microarray data, which indicated 1.5-fold or greater increases in the expression of several other genes known to be inducible by IFN-.$\gamma$. These included Cd74, guanylate-binding protein 2, MHC I, MHC II, metallothionein 1, and ubiquitin D (Table 1) (29–31). The positive regulatory effects of IFN-$\gamma$ on the expression of these genes are likely to be mediated through activation of STAT1 (29). This contention is supported indirectly by our microarray data showing decreased expression (fold change = $0.50$, $p = 0.02$) of Skp2 (S phase kinase-associated protein 2), which can result from increased STAT1 expression (32), in the enterocytes of IE-Cpr-null mice.

Direct evidence for increased expression of STAT1 protein in IE-Cpr-null enterocytes was obtained by immunoblot analysis, using an anti-STAT1 antibody (Fig. 4), which detected the two bands representing the STAT1 $\alpha$ and $\beta$ isoforms (33). The levels of both STAT1 isoforms were increased (~3.2-fold) in whole-cell lysates from IE-Cpr-null enterocytes, compared with the levels in WT enterocytes. Evidence was also obtained for increased activation of STAT1 via phosphorylation at serine 727, a signaling event that is known to be required for maximal activity of the transcription factor (34). As shown in Fig. 4, the level of phosphorylated STAT1 was increased (~4.2-fold) in enterocytes from IE-Cpr-null mice in comparison with the levels in enterocytes from WT mice.

The observed up-regulation of STAT1 and CIITA in the IE-Cpr-null enterocytes is in contrast with the known inhibitory effects of statins on IFN-$\gamma$-mediated induction of Citta transcription in macrophages and microglia (35, 36). The statins inhibit the production of intermediates in the cholesterol synthetic pathway (including FPP and GGPP) through inhibition of the pivalostream upstream enzyme, HMG-CoA reductase (37). The molecular mechanism responsible for the inhibitory effects of statins on CIITA transcription is thought to involve
decreases in cellular levels of the isoprenoids and consequent reduction in prenylation of Rho family GTPases; the latter event may lead to decreased expression of both STAT1 and CIITA (cf. Ref. 36). We reasoned that, if the same mechanistic link were involved, but in the opposite direction, this could explain the increases in STAT1 and CIITA expression in the IE-Cpr-null enterocytes; the POR loss–related blockage of cholesterol synthesis at CYP51 would result in accumulation of FPP and GGPP. As shown in Table 3, the levels of enterocyte FPP and GGPP were both increased in the IE-Cpr-null mice, compared with the levels in WT mice; the relative extent of increase for FPP (51 fmol/mg, a 24% increase) was lower than that for GGPP (16 fmol/mg; a 70% increase), given the much higher levels of FPP than GGPP in the WT mice.

Heme Metabolism and Homeostasis—HO activity is involved in the breakdown of heme, producing CO, bilirubin, and free iron (2). The absence of POR expression in the IE-Cpr-null enterocytes was accompanied by a −90% decrease in total HO activity in enterocyte microsomal preparations, compared with the activity in WT mice (data not shown), consistent with the known dependence of HO on POR (2, 6). However, no significant difference was found in enterocyte heme levels between IE-Cpr-null and WT mice (Table 3). Moreover, enterocyte levels of bilirubin, a heme metabolite capable of regulating STAT1 and MHC II gene expression (38, 39), were likewise not significantly different between IE-Cpr-null and WT mice (Table 3). These results suggest that enterocyte HO activity does not play an essential role in controlling enterocyte levels of heme or its metabolite, bilirubin. Notably, although the biochemical mechanisms involved in heme export from SI are not fully understood (40), preliminary analysis (data not shown) of the expression of genes potentially related to heme transport revealed compensatory changes that may at least partially explain why the heme content in the IE-Cpr-null enterocytes was not different from that in WT mice.

DISCUSSION

Numerous changes in gene expression were observed in the enterocytes of IE-Cpr-null mice in comparison with expression levels in WT mice. However, in contrast to the obvious pathological changes seen in the liver of the liver-Cpr-null mice (enlarged, fatty liver, with necrotic lesions) (6, 7), the changes in gene expression in IE-Cpr-null mice were not accompanied by gross cellular and anatomical changes in the intestine (11). An important reason for the tissue differences between liver and intestine in their response to POR loss may be the fact that the intestinal enterocytes have a short life span, ~3 days in mice (41); thus, a phenotype (such as lipidosis) that takes considerable time to develop may not be observable. Additionally, some of the POR loss–related metabolic deficiencies in the enterocytes are extracted ion chromatograms (XIC) of m/z 395 from the analysis of 5 μg of 24-DHL standard, derivatized to form 24-DHL-TMS (A); a derivatized lipid extract from WT mice (B); and a derivatized lipid extract from IE-Cpr-null mice (C). The mass spectrum for the 24-DHL-TMS peak at 40.7 min (D) from the analysis of the lipid extract from IE-Cpr-null enterocytes is identical to the mass spectrum for the peak at 40.6 min (E) for the 24-DHL-TMS standard. A peak corresponding to 24-DHL-TMS was not detected in the derivatized extract from WT mice (B).
Enterocytes were isolated from male, age-matched (2–4-month-old) WT and IE-Cpr-null mice. The values presented are means ± S.D. (n ≥ 3).

| Strain     | Cholesterol (nmol/mg tissue) | 24-Dihydrolanosterol (pmol/mg tissue) | FPP (fmol/mg tissue) | GGPP (fmol/mg tissue) | Heme (nmol/mg protein) | Bilirubin (fmol/mg tissue) |
|------------|------------------------------|---------------------------------------|----------------------|-----------------------|------------------------|--------------------------|
| WT         | 4.4 ± 0.6                    | 209 ± 28                              | 23 ± 5               | 3.3 ± 0.5             | 31.7 ± 18.3            |
| IE-Cpr-null| 4.0 ± 0.3                    | 260 ± 18                              | 39 ± 6               | 3.6 ± 0.3             | 29.3 ± 5.2            |

* ND, not detected; detection limit was ~30 pmol/mg tissue.

p < 0.01, compared with WT value.

p < 0.001, compared with WT value.

FIGURE 2. Differential expression of genes related to antigen presentation and processing in the enterocytes of WT and IE-Cpr-null mice. RNA samples prepared from enterocytes of 2.5–3-month-old male mice (n = 3) were used for quantitative RNA-PCR analysis. The levels of various target transcripts were normalized to the level of GAPDH mRNA in the same RNA sample. Relative levels of each transcript in the two mouse strains were determined, and the results are shown in arbitrary units obtained by setting the GAPDH-normalized values for the WT samples to 1. The values represent mean ± S.D. (error bars). *, p = 0.05; **, p = 0.01. Data represent typical results from two experiments.

FIGURE 3. Immunoblot analysis of MHC II protein expression in enterocytes of WT and IE-Cpr-null mice. A, mice were fasted overnight, and whole-cell lysates (30 µg/lane) were prepared from enterocytes of three individual, 3-month-old male WT or IE-Cpr-null mice and analyzed on immunoblot with an anti-MHC II antibody. B, results from densitometry analysis. The two bands were combined for determination. Data represent typical results (normalized to GAPDH) from three experiments. *, p < 0.05. Error bars, S.D.

Our data suggest that cholesterol from de novo synthesis in the enterocytes only contributes to a small degree to the total intracellular cholesterol pool. The enterocytes readily obtain cholesterol from extracellular sources in addition to synthesis within the enterocytes; cholesterol in LDL from plasma, cholesterol absorption from the diet, and cholesterol delivered in bile from the liver are all potential sources of enterocyte cholesterol (reviewed in Refs. 45 and 46). Although both endogenously synthesized and absorbed cholesterol can be found in multiple cellular compartments, it has been proposed that, in the enterocytes, absorbed cholesterol is primarily converted to cholesterol esters for transport to the liver in chylomicrons, whereas locally synthesized cholesterol is primarily used for metabolism (47). These same authors suggested that the enterocytes respond differently to increases in cholesterol synthesis versus cholesterol absorption. In this regard, our gene expression data strongly support the concept that enterocytes respond to specific changes in a specific pool of cholesterol (i.e., decreased cholesterol synthesis) despite the absence of changes in total cholesterol levels.
Notably, we cannot rule out the possibility that the IE-Cpr
null enterocytes also compensated for the loss of cholesterol
synthesis through changes in cholesterol uptake or export. 
These changes might also explain, in part, our observation that
the expression of LDL receptor (based on RNA-PCR data (not shown)), a brush border protein
critical for cholesterol absorption in enterocytes (48). In addi-
tion, cholesterol efflux via ABCA1 (ATP-binding cassette A1) 
to HDL plays a key role in reverse cholesterol transport (49).
The genes encoding major cholesterol efflux transporters
(Abca1 and Abcg5/g8) as well as several others encoding enzymes
and transporters involved in cholesterol metabolism and dispo-
sition are regulated by the liver X receptor (LXR), an
oxysterol activated nuclear receptor (50). The loss of POR/P450
activity could potentially lead to decreased levels of oxysterol
(9) and thus decreased activation of LXR. However, our cDNA
microarray data did not show decreases in the expression of
Abca1, Abcg5, Abcg8, or any other LXR target genes, in either SI
or liver of the IE-Cpr-null mice, which suggests that a compen-
satory response, if present, was not initiated via a reduced activ-
ation of LXR. Nonetheless, further studies to directly measure
the enterocytes of IE-Cpr-null mice. This mechanism is based on our present observation of the up-regulation of STAT1/PSTAT1, CIITA pIV, and the MHC IIs in the IE-Cpr-null enterocytes and on previously reported evidence (26, 36, 54, 55), including the involvement of IFN-γ, the effects of statins, and the finding that a decrease in protein prenylation of Rho GTPases by GGPP leads to decreased activation of STAT1 and CIITA (36). However, the increased activation of STAT1 in the enterocytes of IE-Cpr-null mice was unlikely to be mediated by IFN-γ, given the fact that we did not observe an increased level of IFN-γ in the serum or any signs of intestinal inflammation upon histological examination of the IE-Cpr-null mice (data not shown).

Although the available data support a link between GGPP accumulation and increased CIITA and MHC II expression in the IE-Cpr-null enterocytes, it remains possible that other mechanisms also contribute. Of particular relevance are HOs, which also depend on POR for function (2, 6). HO1 was reported to play a role in regulating STAT1 and MHC II expression; studies with dendritic cells show that silencing or inhibition of HO1 results in up-regulation of MHC II expression through induction of CIITA and increased phosphorylation of STAT1 (38). Conversely, bilirubin, a product of heme metabolism, was shown to suppress MHC II expression by reducing expression of CIITA mRNA and reducing STAT1 phosphorylation in endothelial cells (39). Nonetheless, in the enterocytes of IE-Cpr-null mice, the loss of HO activity (data not shown), as a result of the POR loss, was not accompanied by a significant decrease in bilirubin levels. This latter result is explainable by the presence of an alternative source of enterocyte bilirubin, derived from the liver via enterohepatic recirculation (56). Therefore, it is unlikely that the loss of HO activity contributed to the observed increase in MHC II genes in the enterocytes of IE-Cpr-null mice.

In summary, we have explored the potential biological functions of POR-dependent enzymes in the SI through genomic and biochemical analyses of the enterocytes of the IE-Cpr-null mouse. Our findings revealed a novel mechanistic link between POR-dependent enzymes in cholesterol synthesis and the expression of MHC II genes in the enterocytes. This finding, which defines a new physiological/pathological role of intestinal POR/P450 enzymes in modulating the expression of regulators of intestinal immunity, may have important clinical significance. POR is a direct target of inhibition by various drugs and other xenobiotic compounds (e.g. cyclophosphamide (57), ellipticine (58), and cadmium (59)). Furthermore, numerous genetic polymorphisms of the human POR gene that affect either POR expression or POR activity have been identified (60–63). It is conceivable that a decrease in POR activity in the human intestine, either as a result of chemical inhibition or because of POR genetic variations, would also lead to significant increases in the expression of the MHC II genes. Alterations in antigen processing and presentation in the intestine can potentially alter immune responses to antigens. Studies in mice have shown that enterocytes release exosomes that, through MHC II molecules, present antigens to cells of the immune system, resulting in either tolerance (64) or the stimulation of immune responses (65). Furthermore, MHC II expression has been implicated as a factor involved in disease states of the intestine, such as celiac disease (66) and inflammatory bowel disease (67). Additionally, an increase in protein prenylation, resulting from decreases of POR activity and consequent increases of levels of GGPP and FPP, may lead to increased activation of Ras, an oncoprotein, which requires isoprenylation for activation (37, 68) and has been implicated in the development of a large fraction of cancers of the intestine (69, 70). Therefore, any drug that targets cholesterol synthetic enzymes below the branch point of isoprenoid synthesis or has a potential to inhibit POR itself should be monitored for its potential to elicit changes in intestinal antigen processing and presentation or to enhance tumorigenesis in the intestine. This is especially true for drugs that are given orally (as most drugs are) because these would be exposed directly to the intestine.

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17786 JOURNAL OF BIOLOGICAL CHEMISTRY
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