Hepatocellular and Hepatic Peroxisomal Alterations in Mice with a Disrupted Peroxisomal Fatty Acyl-coenzyme A Oxidase Gene*

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Peroxisomal genetic disorders, such as Zellweger syndrome, are characterized by defects in one or more enzymes involved in the peroxisomal \(\beta\)-oxidation of very long chain fatty acids and are associated with defective peroxisomal biogenesis. The biologic role of peroxisomal \(\beta\)-oxidation system, which consists of three enzymes: fatty acyl-CoA oxidase (ACOX), enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD), and thiolase, has been examined in mice by disrupting ACOX gene, which encodes the first and rate-limiting enzyme of this system. Homozygous (ACOX \(-/-\)) mice lacked the expression of ACOX protein and accumulate very long chain fatty acids in blood. However, these homozygous mice are viable, but growth-retarded and infertile. During the first 3–4 months of age, the livers of ACOX \(-/-\) mice reveal severe microvesicular fatty metamorphosis of hepatocytes. In such steatotic cells, peroxisome assembly is markedly defective; as a result, they contain few or no peroxisomes. Few hepatocytes in 1–3-month-old ACOX \(-/-\) mice contain numerous peroxisomes, and these peroxisome-rich hepatocytes show no fatty change. At this stage, the basal mRNA levels of HD, thiolase, and other peroxisome proliferator-induced target genes were elevated in ACOX \(-/-\) mouse liver, but these mice, when treated with a peroxisome proliferator, showed no increases in the number of hepatic peroxisomes and in the mRNAs levels of these target genes. Between 4 and 5 months of age, severe steatosis resulted in scattered cell death, steatohepatitis, formation of lipogranulomas, and focal hepatocellular regeneration. In 6–7-month-old animals, the newly emerging hepatocytes, which progressively replaced steatotic cells, revealed spontaneous peroxisome proliferation. These livers showed marked increases in the mRNA levels of the remaining two genes of the \(\beta\)-oxidation system, suggesting that ACOX gene disruption leads to increased endogenous ligand-mediated transcription levels. These observations demonstrate links among peroxisomal \(\beta\)-oxidation, development of severe microvesicular fatty liver, peroxisome assembly, cell death, and cell proliferation in liver.

Peroxisomes are single membrane-bound organelles that are present in virtually all eukaryotic cells. These organelles were initially characterized by the presence therein of \(H_2O_2\)-generating flavin oxidases together with the \(H_2O_2\)-degrading catalase (1). At present >50 proteins have been identified in peroxisomes, and more than half of these participate in lipid metabolism (2). In addition to their role in lipid metabolism, these organelles are required for other specific functions such as \(H_2O_2\)-based respiration, synthesis of bile acids and plasmalogens (membrane phospholipids), and degradation of uric acid (1, 3). Peroxisomal proteins are synthesized on cytoplasmic polysomes and imported into (or targeted to) the organelle post-translationally (4). It is postulated that peroxisomes grow by the import of newly synthesized proteins and give rise to new peroxisomes by division of preexisting organelles (5).

In animal cells, mitochondria as well as peroxisomes oxidize fatty acids via \(\beta\)-oxidation (2). Although the physiological significance of this division of labor is not entirely clear, it appears that long chain and very long chain fatty acids are almost exclusively processed by the peroxisomal \(\beta\)-oxidation system, whereas fatty acids up to 18 carbons in length are oxidized by the mitochondria (2). The peroxisomal \(\beta\)-oxidation system consists of three enzymes, namely \(H_2O_2\)-generating fatty acyl-CoA oxidase (ACOX),1 enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (HD), and 3-ketoacyl-CoA thiolase (6, 7). The importance of peroxisomal \(\beta\)-oxidation of fatty acids in mammalian metabolism is accentuated by the discovery of inherited diseases associated with defects in peroxisome biogenesis and fatty acid \(\beta\)-oxidation in humans (8). Disorders with defective peroxisome assembly such as the Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum’s disease are characterized by an impairment of peroxisomal \(\beta\)-oxidation pathway with a decreased number or an absence of morphologically distinguishable peroxisomes in liver and in other tissues (8–10). Since peroxisomes are the principal sites of oxidation of long and very long chain fatty acids, an impairment of this \(\beta\)-oxidation pathway manifests in elevated plasma and tissue levels of very long chain fatty acids, and disturbances in the biosynthesis of bile acids and plasmalogens (10, 11). In these invariably fatal disorders, the packaging of proteins into peroxisomes is defective; as a result, the newly synthesized peroxisomal enzymes, in particular catalase and enzymes of the \(\beta\)-oxidations system, remain diluted in cytosol and possibly rapidly degraded, resulting in reduced or missing peroxisomal functions (4). As a result, morphologically recog-

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1 The abbreviations used are: ACOX, peroxisomal fatty acyl-CoA oxidase; HD, peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; PPAR, peroxisome proliferator-activated receptor(s); kb, kilobase pair(s); ES, embryonic stem.
nizable peroxisomes are not found in cells of Zellweger syndrome patients, but these cells indeed contain peroxisomal membrane ghosts in which integral 70- and 22-kDa peroxisomal membrane proteins (PMP70, PMP22) are demonstrable by immunomorphological approaches (12–14). These empty membrane ghosts cannot be delineated by immunocytochemical methods aimed at localizing peroxisomal matrix proteins such as catalase and the β-oxidation system enzymes. The defect in Zellweger syndrome cells is most likely the generalized failure to import proteins to the peroxisomal matrix, since these cells do assemble peroxisomal membranes (4). This failure is attributable to defects in receptor(s)/import machinery responsible for garnering proteins with different types of the peroxisomal targeting sequences (4). Other peroxisomal disorders characterized by an absence of a single enzyme of the peroxisomal β-oxidation system, such as the absence of ACOX in pseudo-neonatal adrenoleukodystrophy, appear to display morphologically recognizable peroxisomes but not ghosts in their cells (10, 15).

In addition to the interest in peroxisomes generated by the genetic disorders involving disturbances in peroxisomal β-oxidation, these organelles have also attracted considerable attention as a result of the discovery that sustained increases in peroxisome population, and in the activity of peroxisomal β-oxidation system in liver induced by peroxisome proliferators, lead to the development of hepatocellular carcinomas in rats and mice (16, 17). Peroxisome proliferators are a structurally diverse group of compounds of industrial, pharmaceutical, and agricultural value; they include certain phthalate-ester plasticizers, industrial solvents, herbicides, hypolipidemic drugs, and leukotriene D4 inhibitors (2, 18, 19). When administered to rodents and nonrodents including primates, these agents cause profound proliferation of peroxisomes in hepatic parenchymal cells, as well as marked increases in the activities of the three enzymes involved in peroxisomal β-oxidation of fatty acids (20). The increased activities of these enzymes are related to the rapid and coordinated transcriptional activation of the nuclear genes encoding these enzymes by a receptor-mediated mechanism (20). These receptors, designated as peroxisome proliferator-activated receptors (PPARα, β, and γ), belong to the nuclear receptor superfamily and act as ligand-inducible transcriptional regulators transducing the pleiotropic effects of peroxisome proliferators (21–23). PPAR activates responsive genes by forming a heterodimer with retinoid X receptor; the PPAR-retinoid X receptor heterodimer binds to the cognate peroxisome proliferator response element, which is identified as a direct repeat motif of hexamer half-sites, TGACCT, spaced over a 12-day selection period as described by Mansour et al. (27).

To delineate the importance of disturbances in peroxisomal fatty acid β-oxidation in the pathogenesis of human disorders associated with peroxisomal biogenesis, and to investigate the role of sustained enhancement of this enzyme system in peroxisome proliferator-induced liver tumor development, it is necessary to develop animal models with a null mutation of one or all of the peroxisomal β-oxidation system genes. We have used homologous recombination in mouse strain 129/Sv-derived embryonic stem (ES) cells to generate a deletion in ACOX gene, which is the first and rate-limiting enzyme of the peroxisomal β-oxidation system. Homozygous ACOX mutant (−/−) mice are viable but growth-retarded, develop severe microvascular fatty metamorphosis, and contain few or no peroxisomes in a majority of liver parenchymal cells, which also fail to show the typical hepatic peroxisome proliferation in response to peroxisome proliferators. By 5 months of age, severe steatosis results in focal hepatocyte death, lipogranulomatous reaction, and hepatocellular proliferation. These newly emerging hepatocytes exhibit spontaneous peroxisome proliferation. These changes have been consistently observed in the ACOX −/− mice through at least five subsequent generations, affirming that the phenotypes are linked to the ACOX gene disruption.

**EXPERIMENTAL PROCEDURES**

**Cloning of the Mouse ACOX Gene and Construction of the Targeting Vector**—We screened a mouse genomic library with full-length rat ACOX cDNA (31) as probe and isolated a phage clone (mACOX10a) containing a 14.3-kb DNA segment that included exons 3–14 of the mouse ACOX gene as demonstrated by restriction mapping. Southern hybridization and partial sequencing. We then used oligonucleotide primer sense primer ACOX (5′-AGCGAGCGACGCCGGTAGG-3′) and anti sense primer ACOX (5′-TCAGGGGCTACTGAAG-3′) from exon 8 of this mouse ACOX gene to screen a P1 bacteriophage library made from strain 129/Sv mouse genomic DNA (Genome Systems, St. Louis, MO). Clones 358 and 359, which contained over 50 kb of inserts, were obtained. Since information on full-length mouse ACOX cDNA was not available, it was necessary to use different fragments of rat and human ACOX cDNAs (31–33) to characterize the 129/Sv mouse ACOX genomic clones. A genomic subclone containing exons 3–14 of ACOX was isolated and its restriction map determined (Fig. 1A, W1 Locus). Nucleotide sequences of exons and exon-intron junctions were determined. The polymorphic selection sequence was employed for subsequent recombination into mouse ACOX gene. To construct the targeting plasmid, we used the 7.2-kb pPNT vector, which contains PGK-Neo and HSV-TK cassettes separated and flanked by a number of unique cloning sites (34). We first isolated a 2.8-kb BamHI fragment containing exons 10–13 from ACOX genomic subclone (Hin 12) and ligated into the unique BamHI cloning site in pPNT vector between the PGK-Neo and HSV-TK cassettes, to generate a 10-kb plasmid, pPNT-mACOX1. Subsequently, the 6-kb HindIII-SstI fragment containing exons 3-11 to 7 was isolated from the Hin 12 ACOX genomic subclone and inserted into the XhoI site of pPNT-mACOX1, upstream of the PGK-Neo cassette through blunt end ligation. This procedure yielded the 16-kb targeting vector, pPNT-mACOX2 (Fig. 1) The targeting vector, pPNT-mACOX2, was made by replacing the 1.3-kb SstI-HindIII fragment containing exons 8 and 9 with a 1.7-kb PGK-Neo cassette. In essence, a 6-kb ACOX fragment containing exons 3-II to 7 was used as the 5′ arm, while a 3-kb fragment containing exons 10–13 served as the 3′ arm of the vector flanked externally by the 2.7-kb HSV-TK cassette. Both PGK-Neo and HSV-TK genes are in the same transcriptional orientation as the ACOX gene (Fig. 1A).

**Detecting Homologous Recombination in ES Cells**—ES cell line BK4 cells (a subclone of E14TG2a), derived from strain 129Ola mice (35), were cultured on primary embryonic fibroblast feeder cells in Dulbecco’s modified Eagle’s medium-H (Life Technologies, Inc.) supplemented with 15% fetal bovine serum (Life Technologies, Inc.), 100 μg/mL β-mercaptoethanol, and 2 mM L-glutamine. The construct was linearized at the unique NotI site and introduced into ES cells by electroporation (36). Three experiments were performed, each using 2 × 10⁷ cells and 10 μg of targeting plasmid DNA. Electroporated cells were plated (4 × 10⁵ cells/10-cm plate) and colonies doubly resistant to G418 (200 μg/ml) and ganciclovir (2 μM; a gift from Syntex, Palo Alto, CA) were selected, over a 12-day selection period as described by Mansour et al. (37).
Resistant ES cell colonies were individually picked and expanded. An aliquot of each colony was frozen, and the remainder was used to prepare genomic DNA. For Southern blot analysis, ES cell DNA was digested with XbaI or SstI, electrophoresed through 0.8% agarose gels, and transferred to nitrocellulose. Blots were hybridized with a[9-p]labeled 5B200 probe. For immunoperoxidase localization of peroxisomal proteins, tissue sections were fixed for 4 h by immersion in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4, 4°C). After rinsing in 0.1 M sodium phosphate, pH 7.4, 0.15 M NaCl, 0.1 M lysine for 3 h, tissues were dehydrated in graded series of cold ethanol and embedded in Lowicryl K4M at −20°C. Ultrathin sections were stained with each antibody by the protein A-gold technique. The polyclonal antibodies used in these studies were raised in rabbits against rat catalase, rat ACOX, rat HD, rat urate oxidase, and rat PMP 70 as described elsewhere (42).

RESULTS

Isolation of Mouse Genomic Clones—Using the full-length rat ACOX cDNA as probe, we isolated a plaque with a mouse genomic DNA insert corresponding to exons 3–14 of rat ACOX gene from a BALB/c strain mouse library (Clontech). This ACOX genomic clone was mapped and partially sequenced. Comparison of the exonic sequences (data not shown) indicates strong similarity between mouse, rat, and human ACOX (31–33). Since the use of isogenic DNA appears to enhance the targeting efficiency in the embryonic stem (ES) cells, we then obtained by PCR screening two genomic clones from strain 129/Sv mouse genomic DNA in P1 bacteriophage library (Genome Systems Inc.). One of these clones was characterized, and a subclone corresponding to exons 3–14 of 129/Sv mouse ACOX was obtained. As in the case with its rat counterpart (31), the coding sequences of these exons 3–14 of mouse ACOX are spread over a genomic distance of more than 10 kb (Fig. 1A).

The exon/intron boundaries of the murine ACOX gene are found to lie at precisely the same locations as those reported for the rat and human ACOX genes (31, 33).

Targeted Disruption of the Mouse ACOX Gene and Generation of ACOX-deficient Mice—To target the ACOX gene in strain 129Sv mouse ES cells (subclone BK4 cells), we constructed a replacement vector by substituting a 1.3-kb Sau3AI segment of cloned 129/Sv mouse genomic DNA containing the ACOX exons 8 and 9, with a 1.7-kb neomycin resistance (PGK-Neo) cassette (Fig. 1A). A 6-kb segment of mouse ACOX gene, composed of exons of 3–7, was used as the 5’ arm, and a 3-kb segment containing exons 10–13 was used as 3’ arm of the vector. A herpes simplex virus-thymidine kinase (HSV-TK) cassette (2.7 kb) was added to the 3’ end of the construct for positive-negative selection (37). The choice of targeting exons 8 and 9 of ACOX gene for disruption was based on the comparison of ACOX sequences of rat, mouse, human, and yeast, which showed particularly high homology between amino acid positions 390–407 and 622–634 corresponding to exons 9 and 13, respectively (data not shown). The vector was designed to replace residues 395–407 encoded within exon 9, since this region shows a high degree of homology to the putative flavin binding site (43). We transfected ES cells with the linearized targeting vector and selected the cells for resistance to G418 and ganciclovir. When DNA samples from 200 double-resistant clones were analyzed by Southern blotting, we found that 15% of these clones possess the correct gene targeting event. We expanded and karyotyped 6 of these clones and found them to be diploid. One of these positive ES clones was utilized for injection into blastocysts from C57BL/6J mice and implanted into pseudopregnant females. This clone yielded founder chimeric mice. All the chimeras obtained revealed a large 129/Sv contribution (>90%), as judged from the coat color. This ES cell line contributed to the germ line transmission of the disrupted allele giving rise to ACOX heterozygotes (ACOX+/−). Mice Homozygous for ACOX Mutant Are Viable but Growth-retarded—Heterozygous F1 mice were phenotypically normal. F1 heterozygotes were intercrossed to produce homozygous (ACOX −/−) mice, which were identified by Southern
wild-type and heterozygous littersmates. During the second week of age, the homozygous mice grew more slowly and their growth retardation continued to manifest up to 24 weeks of age. At the age of 5 weeks, the average body weight of ACOX−/− mice was 9.5 g compared to the average body weight of 19.9 g for wild-type littersmates. Homozygous mice were usually the smallest animals (Fig. 1C), and weighed 40–45% lower than the wild-type littersmates during the first 5 months of age. In animals older than 5 months of age, some weight gain occurred.

**Molecular Characterization of ACOX−/− Mice**—To assess whether the targeted homologous recombination resulted in the ablation of ACOX mRNA, we performed Northern blot analysis on total RNA extracted from livers of these null mice. The 3.8-kb ACOX mRNA is undetectable in the livers of ACOX−/− mice (Fig. 2A, and see below for additional details about Fig. 2A), but it is present in the RNA samples extracted from the livers of wild-type (Fig. 2A) and heterozygous mice (data not shown). To ascertain whether ACOX protein is also missing in these ACOX−/− mice, we conducted immunoblot analysis using a nonspecific polyclonal antibody raised against recombinantly expressed human ACOX protein (32). This antibody

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**Fig. 1. Generation of ACOX-deficient mice.** A, outline of the strategy used to disrupt the ACOX gene. Restriction map and schematic representation of exons 3–14 (solid, numbered boxes) in the mouse ACOX gene (WT Locus). Exons 3–14 were positioned by restriction mapping and nucleotide sequence determination. Targeting Vector, the neomycin resistance gene (PGK-Neo) flanked by 6- and 3-kb ACOX DNA fragments. HSV-TK, the herpes simplex virus thymidine kinase gene. The plasmid was linearized with NotI for electroporation. X denotes homologous recombination. The arrows in the PGK-Neo and HSV-TK cassettes indicate the direction of transcription. The HSV-TK 2-week-old pups generated from crosses of heterozygous mice was di- ergent. The ACOX null mouse (ACOX−/−) and wild-type (+/+) mice maintained on normal diet, or on a diet containing either 0.0125% ciprofibrate (Cip) or 0.125% Wy-14,643 (Wy) for 1 week. The glyoxylated RNA was electrophoresed on a 0.8% agarose gel, blotted onto a nylon membrane, and probed with nine different random-primed 32P-labeled cDNA probes as shown. All cDNA probes used for analysis are from a rat source except PPARα, which is of mouse origin. B, Western blot analysis of extracts prepared from the liver of wild-type (+/+) and ACOX−/− mice on control diet (Con) or on a diet containing either 0.025% ciprofibrate (Cip) or 0.125% Wy-14,643 (Wy). The blots were probed with rabbit polyclonal anti-ACOX serum, anti-HD serum, anti-catalase (CTL) serum, or anti-urate oxidase (UOX) serum. Bands corresponding to the 72- and 51-kDa subunits of ACOX, 78-kDa subunit of catalase, and 35-kDa subunit of urate oxidase are shown. C, ACOX activity in the liver of wild-type (+/+) and ACOX−/− mice on control diet (Con) or a diet containing a peroxisome proliferator ciprofibrate (Cip), Wy-14,643 (Wy) for 1 week. D, ratio (Cp/Ac) of very long chain fatty acids in the plasma of wild-type (+/+) and ACOX−/− mice. Con, control diet; Wy, Wy-14,643 containing diet.
FIG. 3. Absence of ACOX protein and development of hepatic steatosis in ACOX −/− mice. A and B, immunoperoxidase staining for peroxisomal ACOX in liver of wild-type (+/+ ) mouse (A) and ACOX −/− mouse (B) fed a diet containing 0.0125% ciprofibrate, a peroxisome proliferator, for 1 week. Intense cytoplasmic granular staining for ACOX is seen in the liver of wild-type mouse, but is absent in ACOX-deficient mouse. Low magnification, histological appearance of liver (hematoxylin and eosin-stained sections) of wild-type (+/+ ) mouse (C) and 2-month-old ACOX (−/− ) mice (D). Hepatic parenchymal cells of young ACOX-deficient mice reveal severe steatosis involving the entire liver lobule (D). Numerous microvesicular fatty droplets are seen in the cytoplasm (E and F) of 4–5-month-old ACOX −/− mice; liver cell death and lipogranulomas
recognized the 72-kDa and 51-kDa subunits of ACOX in liver from wild-type mice, but not in the liver of ACOX null mice (Fig. 2B). We also assayed ACOX activity in liver using a procedure specific for peroxisomal ACOX (38). No ACOX activity is detectable in the liver of ACOX null mice, but not in the liver of ACOX+/− animals (Fig. 2B). To assess whether the loss of ACOX enzyme results in the accumulation of very long chain fatty acids in the plasma, we determined the C26:0/C22:0 ratio in the plasma of ACOX null mice and compared with the wild-type mice. Very long chain fatty acid levels are markedly affected by the ACOX mutation, resulting in significant increase in the C26:0/C22:0 ratio (Fig. 2D). The mean C26:0 level in the plasma of 2-month-old ACOX null mice was 1.36 μg/ml, as compared to 0.55 μg/ml in the wild type. To further confirm the disruption of ACOX gene in these mice, we examined the inducibility of this enzyme in liver by two structurally diverse peroxisome proliferators, ciprofibrate or Wy-14,643. No increase in hepatic ACOX activity was discerned in ACOX null mice. This was further confirmed by immunoperoxidase staining of liver from wild-type and ACOX null mice fed ciprofibrate; this procedure revealed the characteristic positive staining for ACOX protein in the wild-type mouse (Fig. 3A), and completely negative staining in the ACOX null mouse (Fig. 3B; for additional details, see below). Immunoelectron microscopy confirmed the absence of ACOX protein in the cytosol or within the peroxisome matrix in hepatocytes of ACOX null mice (results not shown). Thus, these data demonstrate convincingly that the targeting event to ablate the ACOX gene was indeed successful. None of the phenotypes were observed in wild-type and heterozygous littermates of similar genetic background, and the ACOX null mice presented these phenotypes through at least five generations over a 1-year period.

**Development of Fatty Liver in ACOX−/− Mice**—In ACOX null mice, the liver appeared pale and enlarged. The absolute liver weights of ACOX null mice were higher than those of age-matched controls (data not shown). In 5-month-old ACOX null mice, the liver weight accounted for ~11% of the body weight compared to about 4% in age-matched wild-type animals. Histologic examination of livers of 5–8-week-old ACOX null mice revealed severe microvesicular fatty metamorphosis of hepatocytes (Fig. 3, C and D). Oil red O stain for lipid confirmed the microvesicular steatosis (data not shown). Fatty change affected all hepatocytes irrespective of their distribution within the liver lobule (Fig. 3D). In these hepatocytes the cytoplasm is filled with numerous small lipid droplets, which do not displace the nucleus peripherally (Fig. 3, E and F). In 2–4-month-old mice, the microvesicular fatty metamorphosis in liver was extensive. During this period, focal hepatocyte death was encountered leading to the formation of lipogranulomatous reaction (Fig. 4, E and F). By 5 months of age, focal proliferation of hepatocytes with increased mitotic activity was evident. These newly emerging hepatocytes did not contain lipid droplets, but had abundant, intensely eosinophilic granular cytoplasm (Fig. 4G). In 6–7-month-old ACOX null mice, the number of steatotic hepatocytes decreased, and they were replaced by regenerating cells with granular cytoplasm. These proliferating hepatocytes were present as expanding foci or in later stages as nodular proliferations. The livers of heterozygotes (ACOX +/−) showed mild fatty change (data not shown).

**Peroxisomal Absence or Paucity in Steatotic Liver Cells of ACOX−/− Mice**—Ineffective or impaired peroxisome assembly, a hallmark of peroxisomal genetic disorders such as Zellweger syndrome, can result in a total absence or a marked reduction in the number of peroxisomes in various organs, but the reduction is more pronounced in liver (9). In the Zellweger syndrome liver cells, peroxisome profiles are fewer than those seen in neonatal adrenoleukodystrophy liver cells (11). To gain a deeper understanding of the magnitude of peroxisomal paucity/loss in ACOX null mice, we examined many semithin sections of livers that were processed for the cytochemical localization of peroxisomal catalase by the alkaline 3,3-diaminobenzidine histochemical procedure (41). In the wild-type mice, peroxisomes are randomly distributed in all liver cells and appear as diaminobenzidine-positive dark brown dots due to peroxisomal concentration of catalase (Fig. 4A); the number of these organelles increases dramatically in all liver cells following the administration of a peroxisome proliferator, such as ciprofibrate in the diet (Fig. 4, B and C). Evaluation of semithin sections of liver of 1–2-month-old ACOX null mice, either on a control (Fig. 4, D and E), or a peroxisome proliferator-containing diet (Fig. 4, F and G), revealed a conspicuous absence of recognizable diaminobenzidine positive catalase-containing organelles in a vast majority of hepatocytes that exhibit microvesicular fatty metamorphosis (Fig. 4, D–G). Only an occasional hepatocyte revealed abundant clusters of diaminobenzidine-positive catalase-containing peroxisomes, and such hepatocytes lacked steatosis (Fig. 4, D–G). In 5–7-month-old ACOX null mice, there was a perceptible increase in the number of hepatocytes lacking fatty vacuoles, but showing conspicuous, spontaneous peroxisome proliferation in the absence of an exogenous peroxisome proliferator (Fig. 4, H and I).

To confirm the possible defects in peroxisomal assembly, we undertook extensive ultrastructural survey of livers of ACOX null mice. Peroxisomes are either absent or unrecognizable at the ultrastructural level in a vast majority of liver cells of 4–8-week-old ACOX null mice (Fig. 5A), essentially confirming the light microscopic findings in diaminobenzidine-stained sections (Fig. 4, D and E). Hepatocytes with fatty vacuoles either lacked or contained few peroxisomes (Fig. 5A), whereas in a rare field such as the one illustrated in Fig. 5B, an adjacent hepatocyte devoid of lipid droplets shows numerous peroxisomes. In animals older than 4 months, the number of hepatocytes with spontaneous peroxisome proliferation increased as the numbers of steatotic hepatocytes decreased (Fig. 5, C and D). This marked increase in the number of peroxisomes in hepatocytes of ACOX-deficient mice (Fig. 5, B–D) is similar to (or even more pronounced than) the increases occurring in wild-type mice exposed to peroxisome proliferators (18). When peroxisomes were detected in hepatocytes with numerous lipid droplets, they were usually reduced in number and appeared smaller in size. In such cells, the peroxisomal matrix density was also markedly diminished, and few membrane-bound structures are recognized as peroxisomes only by the presence of one or more urate oxidase-containing cores (data not shown). Immunoelectron microscopy of these peroxisome-rich hepatocytes, as expected, confirmed the absence of ACOX, but these organelles did show the presence of catalase and HD (data not shown).

**Refractoriness of Steatotic Hepatocytes of ACOX−/− Mice to Peroxisome Proliferator-induced Peroxisome Proliferation**—When wild-type mice are treated with a peroxisome proliferator such as ciprofibrate or Wy-14,643, the number and volume density of peroxisomes increase dramatically in liver parenchymal cells (18). This profound peroxisome proliferative response constitutes the hallmark of the characteristic pleiotropic responses induced by these agents (2, 17, 28). In this study,
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Fig. 4
4–10-week-old ACOX \( ^{-/-} \) mice treated with a peroxisome proliferator failed to show peroxisome proliferative response in liver cells that display microvesicular steatosis (Fig. 4, F and G). Numerous peroxisomes were seen in a few hepatocytes, and such cells were surrounded by hepatocytes devoid of discernible peroxisomes (Fig. 4F). As expected, peroxisome proliferation was seen in all hepatocytes of wild-type mice exposed to peroxisome proliferators (Fig. 4, B and C). Consistent with the nonresponsiveness to the peroxisome proliferative effects is the failure of these chemicals to increase liver weight in ACOX \( ^{-/-} \) mice. Marked hepatomegaly occurred in wild-type mice fed ciprofibrate or Wy-14,643, but not in ACOX \( ^{-/-} \) mice (data not shown).

Other Two Genes of the \( \beta \)-Oxidation System Show Higher Basal Expression in the Liver of ACOX \( ^{-/-} \) Mice—ACOX is the initial and rate-limiting enzyme of the peroxisomal \( \beta \)-oxidation pathway (6, 7); this pathway consists of enzymes that are coordinately induced by peroxisome proliferators (20). In the livers of rats and mice, peroxisome proliferators induce all three genes of the peroxisomal \( \beta \)-oxidation system, namely ACOX, HD, and thiolase, in conjunction with peroxisome proliferation (20). Northern and Western blot analyses of liver of ACOX \( ^{-/-} \) mice treated with peroxisome proliferators further confirmed the ACOX deficiency (Fig. 2, A and B). Since this \( \beta \)-oxidation system consists of three coordinately regulated genes, it appeared necessary to assess whether the inducibility

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**Fig. 4. Peroxisomal mosaicism—peroxisomal paucity, defective peroxisome proliferator-induced peroxisome proliferation, and spontaneous peroxisome proliferation in the liver of ACOX-deficient mice.** Light microscopic appearance of liver as revealed in semithin sections of tissue that was processed for the cytochemical localization of catalase using the alkaline 3′,3′-diaminobenzidine substrate. A, wild-type (+/+) mouse fed control diet shows peroxisomes randomly distributed in hepatocyte cytoplasm, as dark brown dots indicating the presence of diaminobenzidine reaction product (arrows); B and C, when these wild-type mice are treated with a peroxisome proliferator such as ciprofibrate, a marked proliferation of peroxisomes occurs in all hepatocytes (B), as evidenced by numerous brown-stained granules (C). D and E, ACOX \( ^{-/-} \) mice (2 months old), show no catalase containing brown dots (peroxisomes) in a majority of hepatocytes except for a rare liver cell with clusters of peroxisomes appearing as brown granules (arrows in D, and E). F and G, these 2-month-old ACOX mutant mice, male and female, show no peroxisome proliferation in a majority of hepatocytes when fed a diet containing a peroxisome proliferator, such as ciprofibrate. Fat droplets of different sizes are seen in hepatocyte cytoplasm. Arrows point to an occasional hepatocyte with numerous peroxisomes but no steatosis (F and G). The sinusoidal erythrocytes are stained positively (dark brown color) due to the peroxidatic activity of hemoglobin. H and I, focal (H) and nodular proliferations (I) of hepatocytes occur in the livers of ACOX \( ^{-/-} \) mice, 5 months or older, and these hepatocytes reveal spontaneous peroxisome proliferation and are resistant to fatty change.
of remaining two genes of the β-oxidation pathway is affected as a consequence of ACOX gene knock-out. Northern blots revealed that the basal mRNA levels of HD and thiolase are increased appreciably in the liver of 2-month-old ACOX −/− mice when compared to wild-type animals (Fig. 2A). Of interest, but nonetheless consistent with the failure of peroxisome proliferation, is the abrogation of the inducibility of HD and thiolase genes of the peroxisomal β-oxidation system in the livers of ACOX −/− mice treated with peroxisome proliferators (Fig. 2A). As expected, the mRNA levels of all three β-oxidation system genes increased >30-fold in the livers of wild-type mice treated with peroxisome proliferators for 1 week (Fig. 2A). Increases in ACOX and HD proteins are also noted on immunoblotting in the livers of wild-type mice treated with ciprofibrate or Wy-14,643 (Fig. 2B). Ablation of ACOX gene resulted in a failure of induction of HD protein in the liver by peroxisome proliferators (Fig. 2B), which is consistent with the non-inducibility of HD mRNA in these animals (Fig. 2A).

In the livers of 6-month-old ACOX −/− mice, the basal expression of HD and thiolase genes was pronounced as evidenced by the marked increase in the amounts of mRNA on Northern analysis (data not shown). The increases in the HD and thiolase mRNAs in liver in ACOX −/− appeared consistent with the observed spontaneous peroxisome proliferation. This up-regulation of the two downstream genes may be due to compensatory mechanism involving endogenous PPARα agonists or increase in PPARα in regenerating hepatocytes. No further increases in the mRNAs in the livers were noted when these 6-month-old animals were treated with a peroxisome proliferator for 1 week; whether diminished metabolism of endogenous PPARα agonists, such as fatty-acyl CoAs in the ACOX −/− mouse liver, contributes to this phenomenon remains to be examined.

mRNAs of Microsomal Enzymes Involved in Lipid Metabolism Were Also Up-regulated in the Liver of ACOX −/− Mice—Peroxisome proliferators induce microsomal fatty acid ω-hydroxylases along with the fatty acid-metabolizing peroxisomal β-oxidation system. The microsomal fatty acid ω-hydroxylases are a distinct subfamily (CYP4A4 of P450 superfamily of monoxygenases (27, 44, 45). In the rat liver, peroxisome proliferators induce CYP4A1, CYP4A2, and CYP4A3 due to transcriptional enhancement (46, 47). In this study, we found an increase in the basal hepatic mRNA levels of CYP4A1 and CYP4A3 in 4–8-week-old ACOX −/− mice (Fig. 2A). This increase appears similar to the increases in the basal HD and thiolase mRNA levels of the peroxisomal β-oxidation system (Fig. 2A). In these ACOX −/− animals, both ciprofibrate and Wy-14,643 failed to induce CYP4A1 and CYP4A2 mRNA levels in liver; in contrast, these agents caused a 5-fold increase in the mRNA levels of these two genes in the livers of wild-type mice (Fig. 2A). The data on the basal expression of peroxisomal and microsomal genes involved in the metabolism of fatty acids suggest intrinsic up-regulation of these genes in the livers of ACOX −/− mice, presumably by fatty acid overload as fatty acids are known to activate PPAR, thereby increasing the transcription of responsive genes (22, 25, 48).

Detection of Catalase, Urate Oxidase, PMP70, and PPARα in the Livers of ACOX −/− Mice—In the livers of 2-month-old ACOX −/− mice, the mRNA levels of catalase (data not shown) and urate oxidase (Fig. 2A) appeared essentially unaffected when compared to wild-type mice. PMP70 mRNA was detectable in liver of ACOX −/− mice by Northern blotting, but barely so in wild-type ACOX +/+ mice. A slight increase in PMP70 mRNA in liver was observed in ACOX +/+ mice treated with a peroxisome proliferator, but not in ACOX −/− animals. Catalase protein levels in the livers of untreated and peroxisome proliferator-treated ACOX −/− and ACOX +/+ mice did not differ significantly (Fig. 2B). A modest increase in urate oxidase protein content is seen in the liver of peroxisome proliferator-treated wild-type mice, but not in the livers of ACOX −/− mice (Fig. 2B). No appreciable differences in PPARα mRNA levels were observed in the livers of ACOX −/− and ACOX +/+ mice; however, by the immunoperoxidase method, PPARα staining was diminished in 2-month-old ACOX −/− liver when compared to wild-type controls. In the wild-type control mouse liver, PPARα appeared particularly prominent in hepatocytes of the centrilobular region (49), and when these animals were fed ciprofibrate PPAR immunostaining became diffuse (data not shown). In ACOX −/− mouse liver, whether control or peroxisome proliferator-fed, only a few cells with no microvesicular fatty change, also rich in peroxisomes, appeared to stain positive for PPARα (data not shown).

Catalase Is Mostly Cytosolic in Steatotic Hepatocytes of ACOX −/− Mice—In liver cells and cultured fibroblasts of Zellweger syndrome patients, functionally active catalase is present in normal amounts, but is predominantly cytosolic and not peroxisome-bound (12, 50, 51). Since morphologically distinct peroxisomes are largely absent in a majority of hepatocytes of ACOX −/− mice, we studied the cellular and subcellular localization of catalase by immunohistochemical and immunogold procedures, respectively. Immunohistochemically, using antibodies against rat catalase, this enzyme was demonstrable, at the light microscopic level, in all hepatocytes in the livers of untreated and peroxisome proliferator-treated ACOX −/− mice (data not shown). Few hepatocytes stained intensely with this antibody technique, which correlated with the peroxisome-rich cells observed in semithin sections. In peroxisome-defective hepatocytes of ACOX −/− mice, the subcellular localization of catalase, as visualized by the immunogold method, is mostly cytosolic, as compared to the typical peroxisomal matrix localization of this protein when these organelles are properly assembled in some hepatocytes of these ACOX-deficient mice (data not shown).

Peroxisomes Are Present in Kidneys of ACOX −/− Mice—Peroxisomes are seen in the proximal tubular epithelium of kidney of ACOX −/− mice, and as expected these organelles in this null mutant contained no immunocytochemically demonstrable ACOX protein. Catalase and HD proteins were visualized in the renal peroxisomes in ACOX −/− mice (data not shown). The morphological and biochemical characterization of peroxisomes in cells other than those of liver and kidney is necessary to gain a broader perspective of the implications of ACOX null mutation on peroxisome assembly.

ACOX −/− Mice Are Sterile—Both male and female 4-month-old ACOX −/− mice appeared to be sterile. In ACOX −/− mice, testes are smaller compared to wild-type controls. The significant histologic finding is the remarkable reduction in Leydig cell population in the testis of ACOX −/− mice when compared to age matched wild-type animals (data not shown). In addition, seminiferous tubules of ACOX −/− mice revealed some degree of hypospermatogenesis and reduction in spermatid numbers, despite apparent normal distribution of spermatogonia and primary spermatocytes (data not shown). Few multineutated spermatids are found in some seminiferous tubules of ACOX −/− mice. Several round degenerating spermatids and only a few spermatoza are seen in the lumen of epididymis of ACOX −/− mice. These observations suggest that progression through spermatogenesis is affected in ACOX −/− mice. A decrease in the number of Leydig cells, as well as hypocellularity and maturation arrest in seminiferous tubules have been described in patients with adrenoleukodystrophy (52). The ovaries of 3-month-old ACOX −/− mice were smaller,
but appeared histologically unremarkable when compared to age-matched wild-type mice.

**DISCUSSION**

**Fatty Acid β-Oxidation**—In lower eukaryotes such as yeasts and fungi, peroxisomes appear to be the only subcellular site of fatty acid β-oxidation (3). In plants, peroxisomes also participate in the β-oxidation of fatty acids, but whether plant mitochondria possess a functional β-oxidation system remains controversial (8). In animal cells, peroxisomes as well as mitochondria degrade fatty acids via β-oxidation. The mechanism of β-oxidation in both organelles is similar (7). Nevertheless, it is important to note that the enzymes in each system are different gene products and that mitochondrial fatty acid metabolism generates energy, whereas the peroxisomal β-oxidation system produces H$_2$O$_2$ in the first oxidation step, which is lost as heat (2, 3).

Peroxisomal β-oxidation involves the sequential participation of ACOX, HD, and finally 3-ketoacyl-CoA thiolase (6, 7). Although the substrate spectra of mitochondrial and peroxisomal β-oxidation partly overlap, an important distinction is that mitochondria catalyze the β-oxidation of the bulk of short (<C$_6$), medium (C$_6$–C$_{12}$), and long chain (C$_{14}$–C$_{20}$) fatty acids, whereas peroxisomes are involved in the preferential β-oxidative chain shortening of very long chain (>C$_{20}$) fatty acids to long chain fatty acids that can be further oxidized in the mitochondria (2). The physiological significance of the β-oxidation of very long chain fatty acids only in peroxisomes is unclear, but the relevance of this substrate specificity is underscored in some peroxisomal genetic disorders, such as Zellweger syndrome, that are characterized by disturbances in the ability of peroxisomes to β-oxidize fatty acids (53). As a result, excessive accumulation of very long chain fatty acids in these conditions leads to multiple deleterious anatomical and functional defects (10, 15, 54). The hallmark of these genetic conditions involving disturbances in peroxisomal lipid metabolism is defective peroxisome biogenesis (8, 12, 50, 55). To delineate precisely the pathogenesis and the pathological consequences of peroxisomal β-oxidation enzyme system defects, it is essential to have animal models. Accordingly, we have used gene targeting in ES cells to generate mice with a null mutation in the ACOX locus, with the anticipation that such a mutant animal would provide insights into the role of peroxisomal ACOX in the selective metabolism of very long chain fatty acids and thus serve as an animal model for Zellweger-like syndromes (9, 10). In addition, these ACOX mutants would also be useful in evaluating the implications of peroxisome proliferator-induced pleiotropic responses (17).

The ACOX Mutant Phenotype—We generated a mouse model with a complete lack of the peroxisomal ACOX (pseudoneonatal adrenoleukodystrophy or peroxisomal acyl-CoA oxidase deficiency disease) to study the molecular and cellular implications of the loss of ACOX activity, particularly with respect to hepatic peroxisomal alterations. Inactivation of the >25-kb ACOX gene, which undergoes alternative splicing (31, 33), requires a careful design of the targeting vector to ensure that an ACOX null mutant can be generated. Since two different nucleotide sequences for exon 3 are detected in rat, mouse, and human (exons 3-I and 3-II; see Refs. 31 and 33), we chose to delete exon 9, which has a highly conserved region (amino acid residues 395–407 of rat, mouse, and human enzyme) suggestive of a flavin-binding site (91, 43). We generated a complete deficiency for ACOX at the mRNA and protein levels in all tissues studied. Consistently, all tissues of the homozygous mutant mice are completely devoid of ACOX activity. As expected, the loss of peroxisomal ACOX activity in the homozygous mutant mice resulted in increased levels of very long chain fatty acids in blood. An increase in plasma hexacosanoic acid (C$_{26:0}$) levels and a decrease in the levels of decosanoic acid (C$_{22:0}$) fatty acid resulted in abnormal C$_{26:0}$/C$_{22:0}$ ratio, reminiscent of the changes observed in Zellweger syndrome and pseudoneonatal adrenoleukodystrophy patients (15, 51). In patients with Zellweger syndrome, adrenoleukodystrophy, and pseudoneonatal adrenoleukodystrophy, neurological abnormalities, although variable, that are typically present are related to the defect in very long chain fatty acid metabolism (10). In the present study, no appreciable neurological manifestations were detected in the ACOX null mutant mice by 6 months of age. It should be emphasized that clinical manifestations of acyl-CoA deficiency in children are milder than those of Zellweger syndrome (51). This may be attributable, in part, to the accumulation predominantly of very long chain fatty acids in fatty acyl-CoA oxidase deficiency, whereas both very long chain fatty acid and bile acid abnormalities coexist in Zellweger syndrome patients and also in patients with peroxisomal HD or thiolase deficiency (51). The absence of bile acid intermediates di- and trihydroxycoprostanic acids in peroxisomal ACOX deficiency is most likely due to the presence in human liver peroxisomes of two acyl-CoA oxidasases, namely the ACOX, which oxidizes the CoA esters of very long chain fatty acids, and the branched-chain acyl-CoA oxidase (trihydroxycoprostanoyl-CoA oxidase), which participates in the oxidation of esters of 2-methyl-branched chain fatty acids and of the bile acid intermediates (2). Accordingly, the loss of ACOX will affect the metabolism of very long chain fatty acids without interfering with the bile acid metabolism, whereas the absence of any one or both of the downstream enzymes of the peroxisomal β-oxidation system (HD or thiolase) will interfere with the bile acid metabolism. Nonetheless, whether neurological dysfunctions develop in older ACOX-deficient mice as a delayed consequence of persistent elevation of these toxic fatty acids remains to be seen.

We have detected no obvious embryonic or neonatal lethality associated with homozygosity for this ACOX mutation. Mice homozygous for the ACOX null mutation show no obvious external phenotype at birth. However, by 2 weeks of age, the growth retardation was obvious, and continued to manifest during the first 5 months of age of these animals. ACOX-deficient mice, less than 5 months of age, were 40–45% underweight compared with age-matched controls. Some of the older animals appeared to gain weight. The potential cause of decreased body weight in these mutant mice remains speculative at this point. It may be related to malnutrition resulting from decreased food intake and or malabsorption. Additionally, severe microvesicular steatosis of liver developing in these younger ACOX-deficient mice can represent a cause as well as a consequence of this malnutrition.

Both male and female homozygous ACOX /−/− mice were sterile when tested at 6–20 weeks of age. Histology of testes revealed absence of Leydig cells and severe reductions in spermatids and mature sperm. Absence of fully differentiated Leydig cells has been noted in patients with X-linked adrenoleukodystrophy (52), a common peroxisomal disorder characterized by the absence of very long chain fatty acyl-CoA synthase (54). This enzyme converts very long chain fatty acids into acyl-CoA esters for oxidation by the peroxisomal ACOX (2). In essence, in conditions that lack either acyl-CoA synthase or ACOX, there is accumulation of very long chain fatty acids. The similarity in the testicular phenotype in X-linked adrenoleukodystrophy patients and in the ACOX /−/− mouse may be somehow related to this metabolic derangement. Mutant and wild-type female reproductive tissues were indistinguishable histologically. Whether male and female infertile ACOX /−/− mice are sterile remains to be determined.
mice eventually become fertile with further aging remains to be ascertained.

**Fatty Liver in ACOX Mutants**—As indicated above, ACOX-deficient mice exhibit marked hepato- megaly with steatosis. The liver changes include diffuse microvesicular steatosis and a depletion of glycogen. Excessive accumulation of lipid in hepatocytes, occurring in certain disease states and a variety of experimental conditions, visually presents, at the light microscopic level, either as microvesicular type (numerous small lipid droplets in the cytoplasm, which do not displace the nucleus to the periphery), or as macrovesicular type (usually a single huge vacuole pushing the nucleus to a side, so-called signet-ring, appearance of the cell). While macro- and microvesicular fatty changes may be present in the same liver, and either form can manifest following any type of injury, it is generally held that microvesicular form of steatosis is seen in conditions where there is continued subtoxic insult (chemical, anoxic, or hypoxic) to the liver (56). Although an increase in liver uptake of lipids cannot be ruled out in these ACOX null mutant mice, it is reasonable to assume that the microvesicular steatosis in these animals is due to decreased fatty acid oxidation resulting from ACOX deficiency. In addition, the resulting excessive levels of very long chain fatty acids potentially inhibit protein synthesis and can cause damage to subcellular organelles, including mitochondria. Free fatty acids are highly cytotoxic and act as potent detergents damaging cellular membranes (57, 58). It is well known that decreased rate of mitochondrial fatty acid β-oxidation, resulting from exposure to CCl₄, white phosphorus, ethionine, or choline deficiency, or in other conditions such as alcoholic liver damage, causes fatty liver (56). We now demonstrate for the first time that decreased or absence of peroxisomal fatty acid β-oxidation also produces steatosis. Thus, an increase in fatty acid levels can lead to steatosis and that sustained elevations of these very long chain fatty acids in ACOX null mutant mice would predispose to subsequent steatonecrosis of hepatocytes. We have demonstrated the presence of lipogranulomas in the steatotic livers of ACOX /−− mice; these lipogranulomas predominate in livers with hepatocellular death due to long-standing steatosis (59). Inflammation is encountered in fatty livers in association with lipogranulomas, as fat droplets become extracellular following rupture of overly distended hepatocytes. Lipogranulomas consist of aggregates of lymphocytes, foamy macrophages, and an occasional eosinophil. Steatonecrosis appears to serve as a stimulus for hepatocellular regeneration in 4–5-month-old ACOX null mutant mice. The regenerating hepatocytes appear as focal proliferations and exhibit resistance to steatosis. Whether these focal hepatocellular proliferations progress into neoplastic nodules remains to be seen. It is possible that as these animals age, the entire liver may be replaced by these new hepatocytes that are resistant to lipid accumulation. This could predispose to accumulation of very long chain fatty acids in nonhepatic locations and may lead to delayed damage to nervous system. This ACOX null mutant mouse can serve as a model to delineate the natural history of nonalcoholic fatty liver. Nonalcoholic fatty liver is widely believed to be a benign condition with little or no risk of disease progression (57). It is possible that the fatty liver in this ACOX /−− mouse can demonstrate progression to cirrhosis with age as a result of fatty acid-mediated hepatotoxicity.

**Peroxisome Mosaicism in the Livers of ACOX −/− Mice**—Aside from the widely distributed storage of lipid in hepatocytes, the most striking observation in the liver morphology of the ACOX-deficient homozygous mouse is the absence or marked reduction in the number of peroxisomes in a majority of hepatocytes. Consequently, in the bulk of the liver parenchyma, catalase is localized in the cytoplasm. In these peroxisome-deficient hepatocytes, peroxisomal membrane ghosts are observed. Few peroxisomes, when recognized in these cells, usually have markedly diminished matrix density with a prominent urate oxidase core. An occasional hepatocyte adjacent to these peroxisome-deficient hepatocytes contains more peroxisomes than those normally found in the hepatocytes of wild-type (ACOX +/+ ) mice. These peroxisome-rich hepatocytes in ACOX −/− mice show no steatosis and account for <1% of liver cell population. In the livers of older animals, there appeared a progressive increase in the number of hepatocytes with spontaneous peroxisome proliferation. The reason for the peroxisomal abundance in these few hepatocytes is not evident, but this may be due to increased levels of endogenous ligand and transcriptional activation of the PPAR-responsive genes. In these cells catalase is localized in the peroxisome matrix. Peroxisomal mosaicism has been recently described in the livers of patients with peroxisomal deficiency disorders (60). In one patient, less than 1% of hepatocytes possessed peroxisomes (60).

The reasons for the mosaicism of hepatocytes pertaining to the distribution of peroxisomes in ACOX −/− mice remain to be elucidated. In this animal model, there is a clear concordance between the paucity of peroxisomes and presence of severe steatosis in hepatocytes. We speculate that excess accumulation of potentially toxic very long chain fatty acids in hepatocytes disrupts the highly permeable peroxisome membranes and interferes with peroxisomal protein import. It is also worth noting that in these ACOX −/− mice, hepatocytes with defective peroxisome assembly exhibit very little PPARα on immunohistochemical staining. On the other hand, hepatocytes with abundant PPARα, located near centrilobular portion of liver lobule (49), have more peroxisomes and less lipid accumulation. Thus it would appear that regenerating hepatocytes, as they become resistant to lipid accumulation, begin to assemble peroxisomes normally in these mutant mice. Additional studies are needed to determine if hepatocytes with numerical increases in peroxisomes have more PPARα and those with few or no peroxisomes (displaying abundant steatosis) have lower levels of this PPARα isoform but higher levels of other PPAR isoforms such as PPARγ (23). It is conceivable that down-regulation of PPARα in liver may lead to up-regulation of PPARγ, which may contribute to hepatic steatosis as PPARγ isoform, has recently been demonstrated to be a potent regulator of adipogenesis (61, 62).

In this context it is pertinent to note that mice lacking PPARα expression as a result of targeted gene disruption did possess near normal complements of peroxisomes in hepatocytes, but such mice failed to respond to the proliferative effects of peroxisome proliferators (63). These observations imply that PPARα isoform, as well as other isoforms such as PPARγ (23), may not be critical in the peroxisome assembly. Furthermore, while PPARα appears essential for peroxisome proliferator-induced pleiotropic effects in mouse liver as evidenced in PPARα null mice, other PPAR isoforms may not be that relevant for peroxisome proliferation (63). Surprisingly, our studies with ACOX −/− mice demonstrate that peroxisomal disruption is severe in contrast to PPARα −/− mice. More surprising, however, is the failure of induction of peroxisome proliferation in the livers by peroxisome proliferators in these ACOX −/− mice, and the abrogation of ciprofibrate- and Wy-14,643-inducible increases in the mRNA levels of the remaining two genes of the β-oxidation system, although the basal levels of their mRNAs were increased in ACOX −/− mouse liver compared to the wild-type control. We propose that the increased basal levels of HD and thiolase gene expression, as well as in the
basal expression of CYP4A1 and CYP4A3 in ACOX mutants, are due to the influx of fatty acids into the liver, as very long chain fatty acids are known to serve as the physiological activators of the PPARα (25, 48). The lack of response of ACOX
−/− to hepatic peroxisome proliferators such as ciprofibrate and Wy-14,643 may be due to the saturation of activation of PPARα because of the influx of fatty acid overload and or reductions in PPARα transcription in steatotic cells. Another possibility, although less likely, is that ACOX, the first and rate-limiting enzyme of the β-oxidation pathway, is required for the desaturation (oxidation reaction) of fatty-acyl CoA into a 2-trans-enoyl-CoA and this substrate may be necessary to trigger the activation of HD gene transcription. Hepatic cell lines generated from ACOX
−/− mice should assist in exploring these possibilities.

Models of Peroxisomal Genetic Disorders—Peroxisomal genetic disorders that exhibit disturbances in lipid metabolism are characterized by multiple anatomical and functional deficits (10). These disorders are subdivided into three categories: A, B, and C (4). In the first two groups, although a single gene is affected in each patient, multiple peroxisomal proteins remain in the cytosol because of defects in import pathways involving proteins with the peroxisomal targeting signals, PTS1 or PTS2 (4). The group A peroxisomal disorders, which includes Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum’s disease, and hyperpieric acidemia, lacks both PTS1 and PTS2 import pathways, and as a consequence morphologically recognizable peroxisomes are absent or greatly reduced in number. The paucity of peroxisomes in this group results in almost generalized loss of peroxisomal functions. In group B, namely in patients with rhizomelic chondrodysplasia punctata, the defect is limited to PTS2 import pathway, and as a result multiple peroxisomal functions are lost due to the inability of peroxisomes to import proteins, such as thiolase, that contain PTS2 (4, 5). Since PTS1 import system is intact in this group B, morphologically discernible peroxisomes are generally visualized (10). In group C disorders, the enzymatic activity or the subcellular localization of a single peroxisomal protein, such as ACOX, is affected, and in these patients peroxisomes appear intact or their number slightly reduced (4). According to this scheme, the ACOX
−/− mouse should not display defects in peroxisome assembly. While this may be true in the kidney and in some hepatocytes of the ACOX null mutant mouse we have described here, peroxisome-deficient hepatocytes predominate during the first 2–3 months of age (lipid storage phase), and as the liver cells regenerate the number of cells without lipid and with more peroxisomes begins to rise (regenerative/repair phase). Since data on the distribution of peroxisomes in ACOX-deficient patients are usually based on a single diagnostic liver biopsy during early childhood, it would be difficult to speculate whether liver cells during the early neonatal period in this peroxisomal disorder have fewer or no peroxisomes, i.e. show mosaicism, whereas in older children with ACOX deficiency peroxisomes may be seen because of the emergence of regenerating hepatocytes. Whether hepatocellular steatosis in ACOX
−/− mice exerts a secondary effect on peroxisome assembly factor(s) needs to be examined (5, 50).

This is particularly relevant since mutations of the peroxisomal assembly factor-1 are responsible for the widespread defect in peroxisome assembly observed in Zellweger syndrome (50). Whether mice with deficiency of peroxisomal HD and thiolase show a phenotype similar to that of ACOX null mutant remains to be ascertained.

In summary, despite significant research efforts, many aspects of peroxisome biogenesis, the role of peroxisomal enzymes and membrane proteins in various peroxisomal genetic disorders, and the mechanisms of peroxisome proliferator-induced pleiotropic responses remain unresolved. The current progress, utilizing molecular genetic approaches, including the generation of transgenic and gene knock-out techniques, promises a better understanding of the functional role of peroxisomes in health and disease. In this context, the ACOX null mutant mice should provide an indispensable animal model of pseudoneonatal adrenoleukodystrophy and to investigate the relationships between peroxisomally fatty acid β-oxidation, steatosis, and peroxisome assembly. In addition, these mice can also be useful in investigating the natural progression of nonalcoholic fatty liver and the role of confounding hepatotoxins such as alcohol, in the progression of a fatty liver to cirrhosis and liver cancer. Finally, this animal model should provide information on the role of ACOX in peroxisome proliferator-induced liver tumor development in rodents.

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