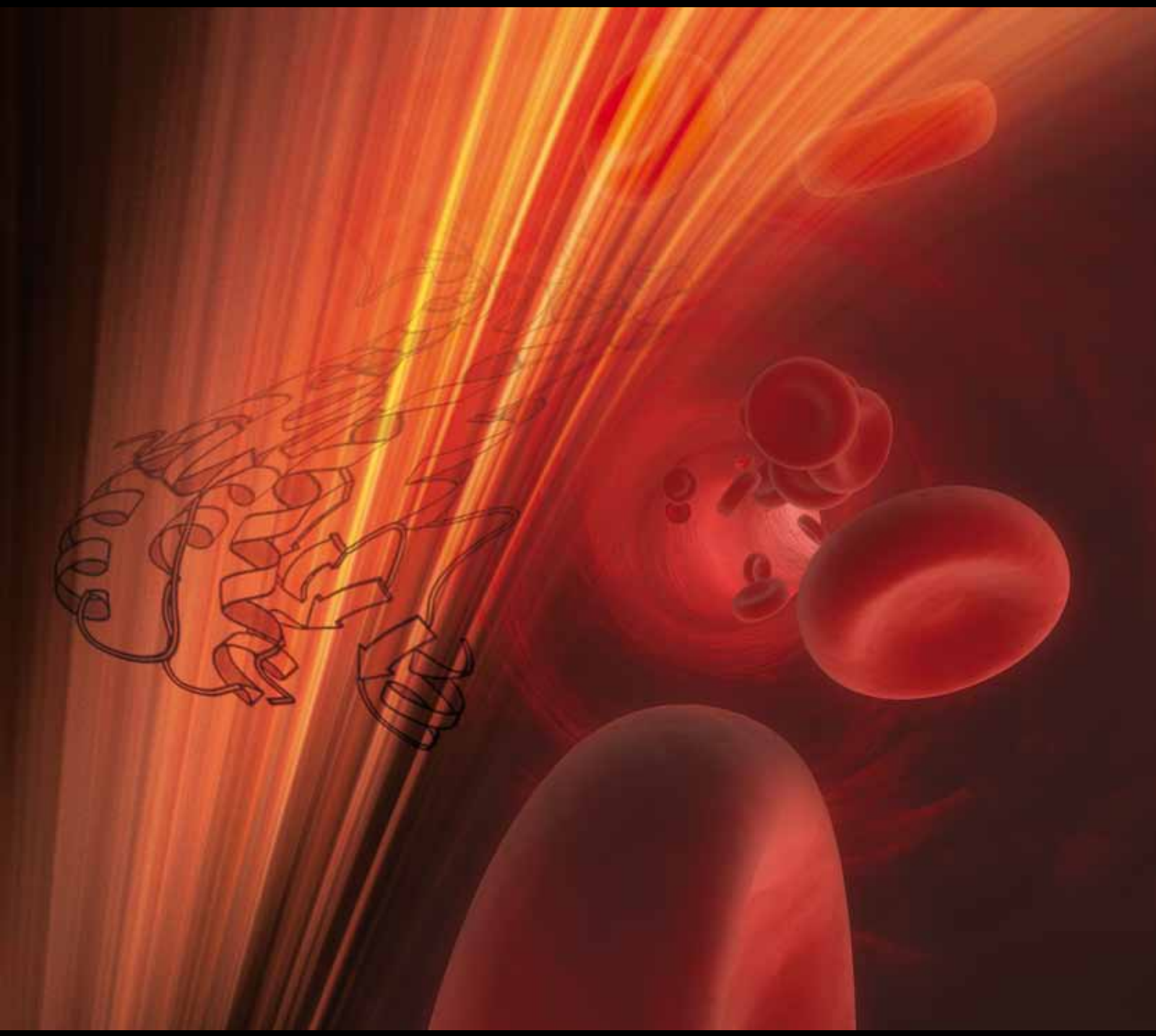
PPAR Research

PPARs, RXRs, and Drug-Metabolizing Enzymes

Guest Editors: James P. Hardwick and John Y. L. Chiang



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Editorial

PPARs, RXRs, and Drug-Metabolizing Enzymes

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This special issue of PPAR Research is dedicated to "PPARs, RXRs and Drug Metabolizing Enzymes". Knowledge of PPAR biology, over the past five years, has dramatically increased our understanding of the potential therapeutic usefulness of these receptors in metabolic alterations associated with disease process of alcoholic and non-alcoholic fatty liver disease and metabolic syndrome. In addition, the utility of PPAR agonist in the treatment of liver disease by normalizing hypertriglyceridemia, dyslipidemia, and the toxic effects of bile acids has a sound scientific basis in the ability of PPAR receptors to control lipid oxidation and disposal as well as regulators of hepatic inflammation. Further insight into both the indirect and direct effects of dual and pan PPAR agonist may potentiate the development of new therapeutic modalities to treat fatty acid oxidation disorders, dyslipidemia, inflammation, and bile acid accumulation associated with several liver diseases and metabolic syndrome.

Articles included in this special issue highlight the importance of PPARs and RXR in drug metabolism and hepatic diseases associated with metabolic disorders. Alterations in drug metabolizing gene expression in different disease states due to the differential expression of PPAR isoforms highlights the importance of PPAR in disease progression and as therapeutic target in the amelioration of disease progression. The first review summarizes past and new developments in alcoholic fatty liver disease (ALD) and how PPAR/RXR regulates phase I enzymes in alcohol metabolism and the redox balance of cells. The direct impairment of PPAR by acetaldehyde results in reduction of NAD+ pool leading to alterations in lipid metabolism, increased oxidative stress, and increased pro-inflammatory cytokines, chemokines and acute phase proteins, which may be central in the onset and perpetuation of mechanism in the clinical progression of alcoholic liver disease (ALD). A second review focuses on the role of PPAR isoforms in the regulation of bile acid and cholesterol metabolism, with specific insight into how PPARα regulates bile acid synthesis, conjugation, and transport by phase II and III enzymes. This review also provides an updated report on how PPARs regulate cholesterol synthesis, absorption, and reverse cholesterol transport, and how PPAR agonist may be used to treat cholestatic liver disease. This review also raises important questions concerning the use of PPAR agonist in treating bile acid accumulation in several liver diseases, which leads to hepatocyte injury, impaired liver metabolic function, progressing to liver fibrosis and cirrhosis. Further research needs to focus on how selective PPAR agonist and isoforms regulated bile acid conjugation and thereby prevent bile acid toxicity observed in cholestastic disease. The conjugation of bile acids by UDP-glucuronosyltransferase, a PPAR target gene further emphasizes the importance of PPAR in bile acid toxicity. In addition, induction of the sulfotransferase genes by PPAR and sulfonation of cholesterol in keratinocyte differentiation suggests that these phase II genes have an important role in epidermal wound healing. Finally, although numerous studies in animal models of disease have dramatically increased our understanding of how PPARs not only regulate drug metabolism and the elimination of drugs and toxic endogenous metabolities in disease progression, the application of these results to human in the design of effective PPAR agonist has all too often led to adverse drug interactions and unacceptable drug toxicity. A fourth review in this series details the exciting possibility of using a human hepatocyte chimeric mouse model to predict metabolism and possible effectiveness of new PPAR agonist in humans. The investigators clearly show the utility of

this model system by the increased phosphatidylcholine transport into bile canaliculi through induction of human ABCB4 transporter by benzafibrate activation of PPAR. There are marked species differences in genes and proteins associated with the absorption, distribution, metabolism, and excretion (ADME) of xenobiotics and drugs, thus the human hepatocyte chimeric mice may not only be used for increasing the safety and effectiveness of lead drugs, but may also serve as a model system to study human liver disease and assess the potency and efficacy of dual and pan PPAR agonist in preventing or delaying disease progression. The final review in these series details how fatty acids are partitioned by fatty acid transport proteins to either anabolic or catabolic pathways regulated by PPARs, and explores how medium chain fatty acid (MCFA) *CYP4A* and long chain fatty acid (LCFA) *CYP4F* ω-hydroxylase genes are regulated in fatty liver. The authors propose a hypothesis that increased *CYP4A* expression with a decrease in *CYP4F* genes may promote the progression of steatosis to steatohepatitis.

We thank the editors for the opportunity to share with other investigators these interesting reviews on drug metabolizing genes regulated by PPAR/RXR. It is apparent that drug metabolizing genes not only play a pivotal role in drug efficacy, drug toxicity, and adverse drug reactions, but also a critical and crucial role in the development and progression liver disease. Therefore, understanding how the PPAR genes and other hormone nuclear receptors are regulated during disease processes will provide us the opportunity to design effective therapeutic modalities to treat disease by the inactivation, conjugation, and transport of toxic endogenous metabolities.

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Review Article

Peroxisome Proliferator-Activated Receptor and Retinoic X Receptor in Alcoholic Liver Disease

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A growing number of new studies demonstrate that nuclear receptors are involved in the development of alcoholic liver disease (ALD). Ethanol metabolism and RXR/PPAR functions are tightly interconnected in the liver. Several ethanol metabolizing enzymes are potently regulated by RXR and PPARα after alcohol consumption. The increased ethanol metabolism, in turn, leads to alteration of the redox balance of the cells and impairment of RXR/PPAR functions by direct and indirect effects of acetaldehyde, resulting in deranged lipid metabolism, oxidative stress, and release of proinflammatory cytokines. The use of animal models played a crucial role in understanding the molecular mechanisms of ALD. In this paper we summarize the reciprocal interactions between ethanol metabolism and RXR/PPAR functions. In conclusion, RXR and PPAR play a central role in the onset and perpetuation of the mechanisms underling all steps of the clinical progression in ALD.

1. Introduction

Alcoholic liver disease, which includes a spectrum of liver injury that covers from the relatively benign alcoholic fatty liver to the potentially fatal alcoholic hepatitis and cirrhosis, has a known etiology but a complex pathogenesis resulting from a combination of genetic, environmental, nutritional, metabolic, and more recently, immunologic factors as well as cytokines [1–4].

Fatty liver is the most frequent hepatic abnormality found in alcoholics as a toxic manifestation of ethanol ingestion. Fatty liver may occur alone or be part of the picture of alcoholic hepatitis or cirrhosis and the development of these late alterations is not clearly understood.

In the liver, ethanol is metabolized to acetaldehyde by two systems: the cytosolic, largely uninducible, aldehyde dehydrogenase (ADH) and the ethanol inducible microsomal cytochrome P4502E1 [5, 6]. Mitochondrial acetaldehyde dehydrogenase (ALDH) is then responsible for the further oxidation of acetaldehyde to acetate (a nontoxic metabolite) using NAD+ as a substrate.

Ethanol complete degradation produces a large amount of reducing agents in the form of NADH (from ADH and ADLH catalyzed reactions) and NADPH (from cytochrome P4502E1) that overwhelm the hepatocyte's ability to maintain redox homeostasis. Moreover the altered redox state impairs gluconeogenesis, diverts acetyl-CoA toward ketogenesis and fatty acid synthesis, and diminishes lipid oxidation disrupting fatty acids β-oxidation [7]. Recent works indicate that reactive oxygen species play a major role in alcohol induced liver injury: CYP2E1 ethanol degradation in the presence of iron generates reactive oxygen species (ROS) thus increasing oxidative stress and leading to membrane-lipids oxidation [8], furthermore oxidative stress depletes the cell reservoir of reduced glutathione (GSH), vitamin E, and S-adenosyl methionine (SAM) [9–12]. These altered mechanisms, along with oxidative stress, are however insufficient to account for all the effects of ethanol consumption and recent works highlight the importance of nuclear receptors and transcription factors in the pathogenesis of liver disease.

Inflammation is important in the progress of alcoholic liver disease with Kupffer cell being the master regulator

(see for recent reviews [1, 2]). In alcoholics and in ethanol-treated animals plasma LPS levels increase; moreover steatosis and ethanol consumption bring to intrahepatic inflammation due to Kupffer cells deregulated release of inflammatory cytokine (especially TNF-α). Several possibilities have been proposed to explain the increase of plasma LPS induced by alcohol. Clinical and experimental studies demonstrated a bacterial outgrowth after ethanol administration [13]; however LPS diffuses from intestine at very low levels. The main mechanism involved appears to be an increased intestinal permeability. Numerous studies demonstrated that ethanol disrupts the functional and structural integrity of intestinal epithelial cells resulting in cellular hyperpermeability and gut leakiness [14–16]. Oxidative stress and toxic metabolites accumulation in intestine may explain the increased permeability. Acetaldehyde, produced by ethanol oxidation in intestine, disrupts intestinal epithelial tight junctions and increases paracellular permeability to endotoxins in Caco-2 cell monolayer [17]. Furthermore, ethanol induces, in vitro and in vivo, the overproduction of nitric oxide, mediated by the inducible nitric oxide synthase [18, 19] that causes intestinal barrier dysfunction trough oxidation and nitration of cytoskeletal proteins [20].

Liver depleted of Kupffer cells shows a decrease in tissue damage induced by ethanol feeding [21]. Kupffer cells produce a large amount of inflammatory citokines, especially TNF-α. Kupffer cells from fed-ethanol animals are more susceptible to LPS due to increased expression of Toll-like receptor 4 (TLR-4) [22] that binds LPS and regulates TNF-α secretion [23]. Accumulating evidences suggest that ethanol sensitizes Kupffer cells to LPS through the production of reactive oxygen species (ROS) by NAPDH oxydase and CYP2E1 [24].

Other important factors for the development of alcoholic liver disease whose role became more clear in the last years are some adipokines, such as adiponectin [2] and leptin [25, 26], that are involved in the control of the alcohol induced inflammatory and fibrogenic response.

Chronic exposure to ethanol inhibits the activity and/or downregulates the expression of several lipid metabolism regulating enzymes, foremost AMP-activated kinase (AMPK) [27], peroxisome proliferator activated receptors (PPARs) [28], retinoid X receptors (RXRs) [29], and sirtuin1 (SIRT1) and 5 (SIRT5) [30, 31], whereas up-regulates the sterol regulatory element-binding protein 1 (SREBP-1) [32]. The mechanisms by which ethanol consumption causes accumulation of hepatic triacylglycerols are complex. AMP-activated protein kinase (AMPK) has a pivotal role in the regulation of lipid metabolism; its activation increases fatty acid oxidation and reduces their synthesis. AMPK activity in liver of ethanol-fed rats is decreased and less sensitive to changes in the AMP/ATP ratio facilitating triacylglycerol accumulation [27]. Activation of SREBP-1 by ethanol feeding is associated with increased expression of hepatic lipogenic genes as well as the accumulation of triglycerides in the livers [32].

Alcohol significantly reduces SIRT1 and SIRT5 expression [30, 31, 33–35]: cytoplasmic SIRT1 and mitochondrial SIRT5 are (NAD^+)-dependent deacetylase that regulate the activity of histonic and nonhistonic proteins [36, 37]. They are important regulators of energy metabolism controlling the gluconeogenic genes and hepatic glucose output through PGC-1α deacetylation (and hence the gluconeogenesis/glycolitic pathway) [30, 31, 38–40]; in addition, SIRT1 modulates the effects of PGC-1α repression of glycolytic genes in response to fasting and pyruvate [39]. Knockdown of SIRT1 in liver causes mild hypoglycemia, increases systemic glucose and insulin sensitivity, and decreases glucose production. SIRT1 knockdown also decreases serum cholesterol and increases hepatic free fatty acids (FFAs) and cholesterol content [40]. Ethanol administration induces PGC-1α and p53 hyperacetylation that could be partially ascribed to SIRT1 and SIRT5 reduced expression; posttranslational modifications of these proteins inactivate PGC-1α and p53 physiological functions and are associated with mitochondrial dysfunction [30, 31]. Sirtuins may also regulate lipid metabolism: SIRT-1 mediates SBREP-1 activation by ethanol regulating its acetylation. In fact, inhibition of hepatic SIRT1 activity is associated with an increase in the acetylated active nuclear form of SREBP-1c in the livers of ethanol-fed mice [33]. Moreover in ethanol-fed mice, resveratrol, a potent SIRT agonist, prevents alcoholic liver steatosis suppressing SREBP-1 and activating PGC-1a [35].

PPARα forms heterodimers with RXR to regulate and bind to PPAR response elements (PPREs) of genes involved in the regulation of fatty acid oxidation and transport. Ethanol reduces PPARα activity and RXR protein levels; these reductions are associated to the inhibition of fatty acid oxidation [28, 29].

A growing number of new studies demonstrate that PPAR and RXR nuclear receptors are involved in many aspects of the development of alcoholic liver disease, ranging from ethanol oxidation to regulation of ethanol-induced inflammatory responses.

In this paper we will summarize the progress in the understanding of ethanol metabolism regulation by PPAR and RXR nuclear receptors in alcoholic liver disease.

2. Role of PPAR in Alcoholic Liver Disease

In the past decade the role of PPAR receptors in the development of ALD has been intensively investigated, both in cell culture systems and in ethanol-fed rodents. The emerging picture is a complex network of alcohol-induced deregulation of all PPAR isoforms, involving different cell types and mechanisms.

2.1. PPARα in Alcoholic Liver Disease. PPARα is a master regulator of lipid metabolism in the liver, controlling the expression of genes involved in the transport, oxidation, and export of free fatty acids. Increased levels of FFA in the hepatocyte activate PPARα by direct binding to the receptor and thus inducing the expression of genes involved in the mitochondrial and peroxisomal FFA β-oxidation pathways. In addition to increasing fatty acids disposal through oxidative degradation, PPARα also inhibits the lipogenic pathway by induction of the malonyl Co-A

decarboxylase, thus promoting the degradation of malonyl Co-A, a precursor of fatty acid biosynthesis [9].

Since fatty liver represents a very common finding in ALD, the effect of ethanol metabolism on PPARα regulated processes has been intensively investigated in the past ten years.

In cultured hepatoma cells ethanol affects PPARα transcriptional activity by inhibiting the ability of the receptor to bind its PPRE consensus sequences. This effect is dependent on the ability of the cell to metabolize ethanol to acetaldehyde as it was abolished by the ADH inhibitor 4-methylpirazole and enhanced by the ALDH inhibitor cyanamide. Moreover, administration of acetaldehyde alone to cultured cells inhibited PPARα binding to DNA, strongly suggesting that acetaldehyde is responsible for the effects of ethanol [28].

In vivo experiments on ethanol-fed rodents reported animal-specific differences on the effects of ethanol on hepatic PPARα protein levels. In Sv/129 mice and in rats, ethanol administration decreased PPARα protein levels [41–43]. Activation of PPARα by clofibrate in ethanol-fed rats ameliorates fatty liver and decreased necroinflammatory injury [43]. On the other hand, in ethanol-fed C57BL/6J mice PPARα protein levels did not change substantially; however PPARα/RXR binding to DNA was significantly impaired and some PPARα target genes (as medium chain acyl CoA dehydrogenase) were downregulated [29]. Although the observed reduction in RXR protein level in ethanol-fed mice could certainly account for the reduced PPARα/RXR DNA binding, induction of PPARα alone by its agonist WY14,643 restored PPARα/RXR binding activity by inducing PPARα but not RXR protein levels, thus revealing that also a reduced activation of PPARα mediates the ethanol effects in vivo. The restored PPARα/RXR binding by WY14,643 administration to ethanol-fed mice was accompanied by an increase of the mRNA of PPARαtarget genes, some of which were actually either not downregulated by ethanol feeding, such as acyl-CoA oxidase, carnitine palmitoyl transferase-1 (CPT-1), very-long chain acyl CoA dehydrogenase and synthetase, or even induced by ethanol, such as L-fatty acid binding protein (L-FABP) [29]. The induction of PPARα target genes by WY14,643 was accomplished even with concomitant administration of ethanol, indicating that this ligand prevents the effect of ethanol on PPARα, possibly by increasing the fraction of DNA-bound PPAR/RXR and thus minimizing the post-translational modifications of the PPARα DNA binding domain by acetaldehyde [2, 29]. PPARα activation by WY14,643 restored the fatty acid β-oxidation, normalized serum fatty acid and trygliceride levels, and prevented fatty liver in ethanol-fed mice.

Taken together these data suggests that if on one hand ethanol may not completely impair the basal expression of many PPARα target genes, on the other hand it severely reduces the ability of the receptor to induce the lipid oxidative metabolism and detoxification systems, as it is required in response to increased fatty acids intake and alcohol consumption. This line of thought seems to be supported by the experiments conducted in PPARα null mice. In fact in these animals several PPARα regulated genes like the peroxisomal acyl-CoA oxidase, the bifunctional enzyme enoyl-CoA:hydratase-3-3-hydroacyl-CoA dehydrogenase, CYP4A and L-FABP were constitutively expressed at levels comparable to wild-type animals [44, 45].

However, the induction of both peroxisomal and mitochondrial β-oxidation pathways by clofibrate or WY14,643 was completely abolished in PPARα−/− mice [41, 45, 46]. Moreover, PPARα null mice were found to have a marked reduction in the expression of ALDH, which led to increased acetaldehyde levels, increased lipid peroxidation and oxidative stress. PPARα null mice were thus significantly more sensitive to ethanol induced liver damage than wild-type animals [41].

A role for PPARα in hepatic inflammation has also been well established. PPARα null mice have a prolonged inflammatory response through the leukotriene B4 (LTB4) and its receptor compared to wild-type mice [47]. In fact LTB4 is a ligand of PPARα, that acts as an anti-inflammatory receptor stimulating LTB4 degradation in the β-oxidative pathways [48]. Moreover, activation of PPARα antagonizes NF-kB signalling, thus preventing the expression of several proinflammatory genes, such as C-reactive protein, fibrinogen –α and –β, acute-phase response proteins, serum amyloid A [49]. Therefore inhibition of PPARα transcriptional activity by acetaldehyde not only deranges the physiologic lipid metabolism leading to increased ROS and lipid peroxide production, but also stimulates the release of pro-inflammatory cytokines from hepatocytes.

2.2. PPARγ in Alcoholic Liver Disease. While ethanol metabolism in the liver occurs largely in hepatocytes, its oxidative product, acetaldehyde, easily diffuses to neighbours cells altering their physiologic processes. In the progression of ALD the activation of Hepatic Stellate Cells (HSCs) is a key step that leads to fibrosis and cirrhosis. Works by our group and others [50–52] demonstrated the importance of PPARγ in the activation of HSC. Active PPARγ is required for the maintenance of the resting "fat storing" phenotype by HSC, and its expression and transcriptional activity decrease during cell activation in culture. Moreover PDGF, a potent inducer of HSC proliferation and migration, induces inactivation of PPARγ by phosphorylation [50, 51]. The decrease in PPARγ transcriptional activity results in an increased synthesis of fibrillary collagens, while activation of the receptor by thiazolidinediones (TZDs) ligands was able to reduce the collagen synthesis in HSC both in vitro and in vivo [53]. The mechanism of PPARγ inhibition in human HSC by ethanol metabolism is rather complex. We found that acetaldehyde inhibits PPARγ by a MAPK mediated phosphorylation on Ser84. The phosphorylation of PPARγ was demonstrated to be dependent on a pathway involving c-Abl, PKCδ, and ERK1/2, and to be initiated by acetaldehyde through a H_2O_2 dependent mechanism [54]. The signalling axis acetaldehyde-H_2O_2-PKC-ERK1/2 in mediating the profibrogenic response of HSC is well established [55–57], and H_2O_2 produced in Kupffer cells induces collagen deposition by HSC [58]. Therefore, inhibition of PPARγ by phosphorylation can potentially occur as a consequence of several

diverse processes taking place during the ethanol-induced liver injury.

Inflammation plays a central role in the onset and progression of ALD. PPARγ ligands inhibited the pro-inflammatory behaviour of HSC downregulating the monocyte chemotactic protein-1 (MCP-1) [51]. Pioglitazone prevents liver injury by ethanol and LPS in rats [59, 60]. In this model, activation of PPARγ by pioglitazone was shown to reduce the production of TNFα by activated Kupffer cells. Kupffer cells are able to metabolize ethanol [61, 62] and chronic ethanol consumption induces CYP2E1 expression in Kupffer cells [63]. Although in acute alcohol induced-liver injury acetaldehyde has been shown to inhibit TNF-α release by Kupffer cells through inhibition of the NF-kB pathway [64, 65], in chronic alcohol abuse the role of Kupffer cell activation and TNF-α release in promoting liver injury is well established. It is therefore tempting to speculate that in ALD acetaldehyde, produced in Kupffer cells or hepatocytes, could lead to suppression of PPARγ transcriptional activity and promote the release of TNF-α. Indeed, enhanced release of TNF-α in response to acetaldehyde or LPS by Kupffer cells isolated from ethanol-fed rats has been reported [66, 67]. Moreover, in ALD increased TNF-α release by Kupffer cells does occur by LPS stimulation due to increased intestinal permeability (described elsewhere in this review). Circulating LPS binds to LPS binding protein (LBP) [68] which promotes its interaction with the Kupffer cells' CD14 cell-surface receptor. This complex interacts mainly with the toll-like receptor 4 (TLR4), which in turn transduces the down-stream signal through activation of the PCK, MAPK, and NF-kB signalling pathways [69]. Furthermore, ethanol induces the expression of CD14/TLR-4 receptors in kupffer cells, thus "priming" the liver macrophage population to respond to LPS stimulation, and this sensitization was found to depend upon NADPH oxidase mediated-oxidative stress and activation of the NF-kB pathway [22, 70]. TNF-α is also known to downregulate PPARγ function by several mechanisms (see Ye 2008 for a recent review [71]), and LPS treatment was shown to downregulate PPARγ expression in Kupffer cells both in vitro and in an animal model of sepsis through a TNFα -dependent mechanism [72].

Finally, a link between CYP2E1 and TNF-α production has been described *in vitro* in a macrophage cell line with stable expression of CYP2E1 [24]. In this model, increased expression of CYP2E1 was accompanied by increased levels of CD14/TLR-4, NADPH oxidase, and H_2O_2. The higher production of hydrogen peroxide resulted in activation of the MAPKs ERK1/2 and p38, which stimulated TNF-α production via activation of NF-kB and stabilization of TNF-α mRNA, respectively [24].

Although these interlinked events still need to be substantiate in an animal model of ALD, the emerging picture describes a likely redundant mechanism by which ethanol induction of CYP2E1 and NADPH oxidase systems enhances oxidative stress and sensitizes Kupffer cells to endotoxins, thus promoting inflammation by inhibition of PPAR$\boldsymbol{\gamma}$ function and activation of NF-kB pathways. This line of thought seems to be supported by recent evidences demonstrating that TLR-4 mediates the LPS-induced downregulation of PPAR$\boldsymbol{\gamma}$ by a NF-kB dependent mechanism in macrophages [73]. Loss of PPAR$\boldsymbol{\gamma}$ activity was sufficient to induce a pro-inflammatory state, indicating that PPAR$\boldsymbol{\gamma}$ suppress inflammation under basal conditions by repressing NF-kB activity, while upon activation of TLR4, NF-kB drives down PPAR$\boldsymbol{\gamma}$ expression and thereby obviates any potential anti-inflammatory effects of PPAR$\boldsymbol{\gamma}$ in LPS-stimulated macrophages [73].

2.3. PPARβ/δ and Ethanol. PPARβ/δ is probably the less characterized isoform of the PPAR family. It is expressed in a large array of tissues, including central nervous system, liver, adipose tissue, muscles, and the gastrointestinal tract. In skeletal and cardiac muscle PPARβ/δ is expressed at much higher levels than PPARα and PPARγ [74, 75]. Recent studies suggest that, in the adult, PPARβ/δ plays important roles in maintaining the glucose-lipid homeostasis by stimulating fatty acid oxidation and uncoupling of the respiratory chain in the muscle, by inhibiting glucose and VLDL secretion from the liver, by stimulating the cholesterol efflux increasing circulating HDL levels; moreover PPARβ/δ is also implicated in the regulation of the inflammatory activity of macrophages (for recent reviews on the metabolic action of PPARβ/δ see [76, 77]).

Very little is known about the effect of ethanol on PPARβ/δ. In rat hepatoma cells, acetaldehyde inhibited PPARβ/δ DNA binding activity but at a much higher concentration than that required for PPARα inhibition [28]. PPARβ/δ is also expressed in hepatic stellate cells, and its expression increases with cell activation in vitro and in vivo [78, 79]. However, the role of this PPAR isoform in stellate cells is of difficult interpretation, since if on one hand it promotes hepatic stellate cells proliferation [79], on the other it induces genes involved in the esterification of Vitamin A such as CRBP-1 and LRAT, possibly reflecting a compensatory mechanism aimed to counterbalance the loss of retinol storage during activation [78]. Very recently, the mRNA levels of PPARβ/δ have been shown to increase in livers of ethanol fed-rats [80]; however in this model activation of PPARβ/δ by the specific agonist L165,041 resulted in an attenuation of the ethanol-induced hepatic injury and in an improvement of liver regeneration [80].

Ethanol is known to disrupt insulin signalling [81–83] and to impair liver regeneration [84]. According to recent data demonstrating a role for PPARβ/δ in the regulation of glucose metabolism and insulin sensitivity [85], the protective effects of PPARβ/δ agonist on ethanol-induced liver injury were shown to be dependent on the amelioration of the insulin binding to insulin and IGF-1 receptors and activation of the down-stream signalling pathways [80].

An important role for PPARβ/δ in the regulation of the inflammatory profile of tissue macrophages has been recently discovered [86, 87]. Odegaard and coworkers showed that PPARβ/δ is required for alternative activation of Kupffer cells, and alternatively activated macrophages suppress inflammation and promote tissue repair [87, 88]. Moreover, alternatively activated Kupffer cells promote the β-oxidative

pathways and suppress lipogenesis in hepatocytes via a paracrine cross-talk [87]. The mouse model used by Odeggard and coworkers was developed to study obesity-induced insulin resistance and type-2 diabetes; however since several parallelisms exist between alcoholic and nonalcoholic liver injury [23], it would be of great interest to test the hypothesis that macrophage specific inhibition of PPARβ/δ would play an important role in alcohol-induced steatohepatitis.

PPARβ/δ null mice show defective myelination of the corpus callosum, reduced adipose tissue and altered inflammatory response in the epidermis [89]. In B12 oligodendrocyte-like cells ethanol was shown to selectively reduce PPARβ/δ expression by increased mRNA degradation without affecting PPARα and PPARγ [90]. These observations could possibly undercover a mechanism underling the ethanol-induced myelination defects and neurological impairment in the foetus [91]. In a recent study by Venkata et al. PPARα and β/δ were shown to be differentially affected by acetaldehyde and ethanol, respectively. The authors show that in MCF-7 breast cancer cell line acetaldehyde inhibits PPARα but not PPARβ/δ, while the latter is inhibited directly by ethanol [92]. A growing body of evidence suggests an association between ethanol consumption and increased cancer risk in different tissues, including breast [93, 94]. More studies are needed to elucidate the effects of ethanol metabolism on PPARβ/δ and the underling mechanisms, but it is intriguing to speculate that some effects of ethanol on peripheral tissues could be mediated by this poorly understood PPAR isoform.

3. RXRs, Ethanol Metabolism, and Alcoholic Liver Disease

RXR is a nuclear receptor expressed in almost every cellular type and tissue. Three isoforms of RXR have been found in human, named RXRα, β, and γ, being the α-isoform the most abundant in the liver [95]. Unique among the other nuclear receptors, RXRα play a major modulatory role across multiple cellular pathways forming mandatory heterodimers with other nuclear receptors.

RXR β and γ apparently play minor roles in the liver: it has been demonstrated in mice that RXRαnull/RXRγ null and RXRβ null/RXRγ null mutant phenotypes were indistinguishable from those of RXRα null and RXRβ null mutants, and that the presence of a single allele of RXRα is sufficient to perform most of RXR functions [96].

In the last years there has been an increasing interest in the role of RXR, especially RXRα in alcoholic liver disease. Accumulating evidence suggests that RXR may play a major role in many aspects of ethanol metabolism being its expression downregulated by ethanol [42, 97]. In fact, mice fed with ethanol show reduced ability of PPAR/RXR heterodimers to bind DNA and reduced RXR expression [29]. These effects are acetaldehyde dependent because blocking ADH reduces, while blocking ADLH increases, the observed phenotype [28]. Moreover, the human aldehyde dehydrogenase-2 promoter contains a retinoid response element (designated FP330-3′), which may contribute to the regulation of the gene. Heterodimers of retinoic acid receptor (RAR)α, β, and γ with RXRα bound the FP330-3′ site, stimulating the expression of reporter constructs containing the FP330-3′ sites in a 9-*cis* retinoic acid-dependent fashion in cultured cells.

More insights on RXR specific role in alcoholic liver disease come from experiments with animal models mainly from the group of Professor Wan JY that demonstrated an altered ethanol methabolism in RXRα null mice.

Hepatocyte RXRα-deficient mice possess a phenotype partially overlapping both PPARα and PPARγ knockout-mice phenotypes. Like the PPARα-null mice, hepatocyte RXRα-deficient mice are obese, have a larger fat mass, and higher serum cholesterol and leptin levels compared with wild-type mice; similar to PPARγ +/− mice, hepatocyte-RXRα-deficient mice also have reduced food intake and increased serum leptin levels [98]. On the contrary, hepatocyte RXRα deficiency results in an improved glucose tolerance without altering insulin level or insulin sensitivity [98].

RXRα deletion increases liver enzymatic activity of ADH1 isoform without affecting ADH2 and ADH3 [12]. It has been suggested that RXRα may regulate ADH1 translation because mRNA levels were the same between null and wild-type mice, while there is increased abundance of ADH1 mRNA in the polysome fraction, indicating higher translation in null mice. This latter effect may be mediated by leptin whose levels are increased in RXR null mice [98] and has been reported capable of regulating ADH activity [99].

RXR may regulate acetaldehyde oxidation to acetate and its clearance. ALDH mRNAs (especially ALDH1 mRNA) are reduced in null mice indicating a possible direct regulation that would parallel with the above mentioned finding that human ALDH promoter responds to RXR. Increased ADH1 activity accounts for the rapid oxidation of ethanol to acetaldehyde; reduced ALDH activity observed in RXR deficient mice impairs the further oxidation to acetate sensitizing the mice to the ethanol-induced damage.

RXR is able to regulate the expression of many xenochemical metabolizing enzymes of the cytochrome P450 family acting as a dimerization agent for PPAR, LXR, PXR [100–103], but its ability to regulate the cytochrome P450 CYP2E1, involved in ethanol oxidation to acetaldehyde, needs further investigation. The promoter region of cytochrome P4504A contains an imperfect direct repeat sequence recognized by PPARα/RXR heterodimers and P4504A is induced by peroxisome proliferators [104] whereas CYP2B is induced by androstane receptor/RXR heterodimers [105]. It has been demonstrated that ethanol-fed animals show a reduced RXR expression whereas CYP2E1 mRNA increases [42, 97]. In an RXR null background, male mice but not female have a reduced expression of CYP4A, 3A, CYP2A and CYP2B mRNA [10, 106] but not of CYP2E1 [106] compared to male wild-type mice. Furthermore, when RXR null mice were challenged with ethanol, induction of CYPs expression was lower in mutant mice compared to wild-type mice [10]. In RXR KO mice CYP2E1 activity is reduced [12] and the enzyme is not induced by ethanol [107]. The apparent discrepancy between wild-type and RXR

KO mice in the activation of cytochrome CYP2E1 may be due to residual RXR expression in ethanol-fed animals compared to RXR KO mice where RXRα has been knocked out. It also may suggest that RXR may play an important role in P450 regulation at the transcription and translational levels.

The altered expression of cytochrome enzymes in RXR KO mice suggests that these mice could be less sensitive to ethanol damage with respect to wild-type mice; however, ethanol treated KO mice still show induced liver damage. Toxic acetaldehyde accumulation due to altered ADL/ADLH activities (as described above) may account for that damage but other mechanisms are also involved.

It is well known that ethanol alters phase II metabolism of xenobiotics agents [108]. Depletion of GSH reservoirs reduces the liver ability to withstand oxidative stress from altered redox equilibrium and to prevent lipids peroxidation as a result of ethanol ingestion. Dai et al. [10] and Gyamfi and Wan [109] demonstrated that GSH levels are reduced in wild-type mice after ethanol administration; this effect is exacerbated in RXR null mice [10, 109]. Compared to wild-type mice, hepatocyte RXRα-deficient mice have significant lower levels of SAM (a precursor for GSH synthesis) and GSH, which is further reduced after alcohol treatment [10, 11]. The Glutathione S-transferase (GSTs) is a multigene family of enzymes that bind GSH to xenobiotics agents facilitating theirs dissolution in the aqueous cellular and extracellular media, and, from there, out of the body.

GSTs are encoded by at least 9 gene family: eight of them encode for cytosolic enzymes (namely, alpha, mu, theta, pi, zeta, sigma, kappa and chi—also called omega) whereas a ninth family, composed at least by six genes encode for microsomal enzymes [110]. GST enzymes of alpha, mu, and pi families account for the majority of cytosolic GST activity.

In RXR null mice GST activity and transcription is reduced compared to wild-type mice [11, 12]. In hepatocytes, RXRα deficiency changes the gene expression profiles of the GSTs: it has been reported that basal expression of 13 out of 15 GST genes was altered in hepatocyte-specific RXRα-deficient mice [11], being either down or upregulated. The enzymatic activity of both mitochondrial and cytosolic GSTs is reduced in null mice [12]. Acute ethanol exposure of primary mouse hepatocytes reduces GSH levels and cytosolic GST enzymes activity along with the release of GST into the culture medium. Specific substrates for the mu and pi class demonstrate that ethanol significantly decreases the mu and pi class GST activity by 53% and 13%, respectively. These biochemical changes elicited by ethanol were also accompanied by increased lipid peroxidation and a decreased SAMe to SAH ratio, early biochemical features of ethanol toxicity. Moreover, hepatocytes isolated from RXR KO mice show a decrease of mu and pi class GST activity compared to wild-type mice.

In H4IIE hepatocytes GSTA2 gene is induced by PPARγ activator and 9-*cis*-retinoic acid. The TZD PPARγ agonists, troglitazone, rosiglitazone, and pioglitazone, in combination with retinoic acid, increase GSTA2 induction, confirming that the activation of PPARγ/RXR heterodimer contributes to GSTA2 expression [111]. Unique among the GSTs, the alpha class isoenzymes have selenium independent antiperoxidase activity [112]. It has been demonstrated that alpha class GST [113–115] plays an important role in the protection mechanisms against oxidative stress induced by lipid peroxidation.

This latter finding suggests that in alcoholic liver disease and ethanol metabolism, RXR regulation of GSTs expression may be important not only for conjugation of toxic metabolites to GSH but also for the protection against the deleterious effects of lipid peroxidation.

Deranged lipid metabolism and steatosis is one of the hallmark of heavy ethanol consumption. The role of nuclear receptors (especially PPARα) in the control of lipid metabolism is well known and has been already extensively reviewed [1, 2, 116, 117]; however, some specific effects, observed in ethanol fed-animals and previously ascribed to the PPARα pathway, could indeed be RXR specific [98, 118]. When fastened or treated with WY14,643, RXR null mice show a phenotype in several aspects different from PPAR null mice. Apolipoprotein A-I and C-III mRNA levels, serum cholesterol and triglyceride levels are markedly induced in untreated RXR null but not in PPARα-null mice. Moreover, fasting-induced PPARα activation and WY14,643 effects are reduced in RXR KO mice. Acyl-CoA oxidase, medium chain acyl-CoA dehydrogenase, and malic enzyme induction by WY14,643 are reduced in null mice without altering their mRNA levels [118].

In RXR null mice L-FABP levels are dramatically reduced compared to wild-type animals [107]. L-FABP is a PPARα target gene and is necessary for the transport of fatty acids. Ethanol feeding increases hepatic FFA levels both in wild-type and null mice; however in RXR null mice L-FABP is not induced by ethanol and this effect is associated with accumulation of FFA and ethanol induced liver damage [107]. A recent paper from Razny et al. further demonstrated the RXR specific role on lipid metabolism and angiogenesis. Microarray studies on RXRα deficient mice fed with a high-fat diet for 7 weeks demonstrated a down-regulation of genes related to angiogenesis, whereas genes involved in adipogenesis, apoptosis, and inflammation were upregulated. Based on these results Razny et al. suggested that impaired fatty acid metabolism in liver leads to impaired angiogenesis due to lipotoxicity and promotion of adipogenesis [119].

Liver damage resulting from chronic ethanol consumption is also caused by inflammatory processes. As previously reported, in alcoholics increased LPS levels and altered metabolism induce inflammation, worsening alcoholic liver disease, and Kupffer cells are more sensitive to LPS due to increased expression of Toll-like receptor 4 [23]. In the negative acute hepatic phase response, LPS induces a down-regulation of lipid metabolism associated with increased serum triglyceride levels and reduced lipid β-oxidation. These effects appear to be mediated by a reduction of nuclear receptors expression mediated by TNF-α and IL-1 but not IL-6 [120]. RXRα expression is lower in LPS negative acute hepatic phase response [101] and HepG2 IL-1 β-cells showed a marked decline in RAR/RXR binding to the Ntcp gene that regulates bile flow [121]. The alteration of RXR pathways is associated with changes in its subcellular localization characterized by increased cytoplasmic

and reduced nuclear levels [122]. The nuclear residence of RXRα is maintained inhibiting c-jun N-terminal kinase or CRM-1-mediated nuclear export, while IL-1 increases the proteasome mediated RXR degradation [123].

Interestingly, hepatocytes from ethanol-fed RXR null mice show an increased NF-kB activation and a strong and higher induction of inflammatory cytokines TNF-α, IL-1 and IL-6, compared to wild-type mice [107]. Apoptosis in RXR null mice increases after ethanol ingestion due to a lower expression of antiapoptotic proteins Bcl2 and Bcl-XL, even in the presence of higher levels of IL-6. RXR seems to influence IL-6/STAT3 mediated signalling circumventing STAT3 phosphorylation [107]. This suggests that RXR is an important regulator of inflammatory processes in response to ethanol; it may also be speculated that some of the effects in response to LPS (as describer above) may be ascribed to RXR reduced expression.

Finally, ethanol and retinoid metabolisms are widely interconnected [124]. Liver from alcoholics shows a marked depletion of Vitamin A that correlates with the activation of stellate cells, their loss of lipid droplets and differentiation in myofibroblast-like cells. The role of nuclear receptors in stellate cells activation is widely studied and it is reported elsewhere in this paper. Key enzymes in ethanol metabolism may act also on retinol pathway [125]. Enhanced activities of ADH, ALDH, and CYP2E1 in alcoholics may account for the observed reduction of Vitamin A in liver cells. Even if retinol it is not their supposed primary substrate it is well known that ADHs and ALDHs may catalyze the conversion of Vitamin A to retinoic acid accelerating retinol metabolism; furthermore alcohol induction of cytochrome CYP2E1 determines a higher rate of Vitamin A catabolism in polar metabolites in the liver. Ethanol may also increase retinol mobilization from liver increasing hydrolization of retinyl esters [126–128]. Alcohol-induced effects on retinol-regulated genes are further increased by RXR downregulation observed in alcohol-fed animals. This establishes a complex regulatory mechanism between RXR, retinol metabolism, and ethanol that is deregulated during heavy ethanol consumption.

4. Summary and Conclusions

Ethanol metabolism and RXR/PPAR functions are tightly interconnected in the liver. Several ethanol metabolizing enzymes are potently regulated by RXR and PPARα after alcohol consumption. The increased ethanol metabolism, in turn, lead to alteration of the redox balance of the cell and impairment of RXR/PPAR functions by direct and indirect effects of acetaldehyde, resulting in deranged lipid metabolism, oxidative stress, and release of pro-inflammatory cytokines. In this paper we summarized the reciprocal interaction between ethanol metabolism and RXR/PPAR functions.

The use of alcohol-fed rodents played a crucial role in understanding the molecular mechanisms of ALD. However, important differences exist in the regulation of the oxidative metabolism between rodents and humans. In fact, while in rodents the need for increased oxidative metabolism induces peroxisome proliferation, humans exclusively respond by increased mitochondrial β-oxidation. Moreover, the levels of PPARα receptor are markedly lower in humans than in rodents, and this may be even more striking during alcohol abuse. PPAR and RXR KO mouse models highlighted the reciprocal regulation of PPAR, RXR and ethanol in alcoholic liver disease. In conclusion, RXR, and PPAR play a central role in the onset and perpetuation of the mechanisms underlying all steps of the clinical progression in alcoholic liver disease.

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Tommaso Mello and Simone Polvani contributed equally to this paper.

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Review Article

Regulation of Sulfotransferase and UDP-Glucuronosyltransferase Gene Expression by the PPARs

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During phase II metabolism, a substrate is rendered more hydrophilic through the covalent attachment of an endogenous molecule. The cytosolic sulfotransferase (SULT) and UDP-glucuronosyltransferase (UGT) families of enzymes account for the majority of phase II metabolism in humans and animals. In general, phase II metabolism is considered to be a detoxication process, as sulfate and glucuronide conjugates are more amenable to excretion and elimination than are the parent substrates. However, certain products of phase II metabolism (e.g., unstable sulfate conjugates) are genotoxic. Members of the nuclear receptor superfamily are particularly important regulators of SULT and UGT gene transcription. In metabolically active tissues, increasing evidence supports a major role for lipid-sensing transcription factors, such as peroxisome proliferator-activated receptors (PPARs), in the regulation of rodent and human SULT and UGT gene expression. This review summarizes current information regarding the regulation of these two major classes of phase II metabolizing enzyme by PPARs.

1. Introduction

Phase II, or conjugative, metabolism is defined as the covalent attachment of an endogenous molecule to a functional group on a substrate molecule. Although a substrate containing a suitable functional group can directly undergo phase II metabolism, conjugation often occurs subsequent to a phase I reaction (e.g., catalyzed by a cytochrome P450), during which the functional group is added to the substrate. The conjugating moiety is most often a sulfonate or glucuronate group, although other conjugating moieties include glutathione, glycine, acetate, and the methyl group. Phase II metabolism usually increases the hydrophilicity of the substrate molecule, which facilitates transport and elimination of the product. Phase II sulfonation and glucuronidation reactions are catalyzed by the cytosolic sulfotransferase (SULT) and the UDP-glucuronosyltransferase (UGT) families of enzymes, respectively, (Figure 1). The SULT and UGT enzymes represent a highly responsive defense system against the mutagenicity of carcinogenic environmental chemicals and the toxicity of xenobiotics and endogenous metabolic intermediates. Members of the nuclear receptor superfamily are particularly important regulators of UGT and SULT gene transcription. In metabolically active tissues, increasing evidence supports a major role for lipid-sensing transcription factors, such as peroxisome proliferator-activated receptors (PPARs), in the regulation of rodent and human SULT and UGT gene expression. This review summarizes current information regarding the regulation of these two major classes of phase II metabolizing enzyme by PPARs.

2. PPARs

The PPAR nuclear receptor network represents a central determinant of cellular energy balance. In heterodimeric partnership with the retinoid X receptor (RXR), PPAR forms a ligand-activated nuclear receptor transcription factor that is capable of integrating the expression of a wide spectrum of target genes involved in cellular lipid metabolism, energy homeostasis, and inflammation (Figure 2). The three known PPAR isoforms, PPARα, PPARδ (also called PPARβ) and PPARγ are products of separate genes and are well conserved across species. PPARα expression is the most prominent

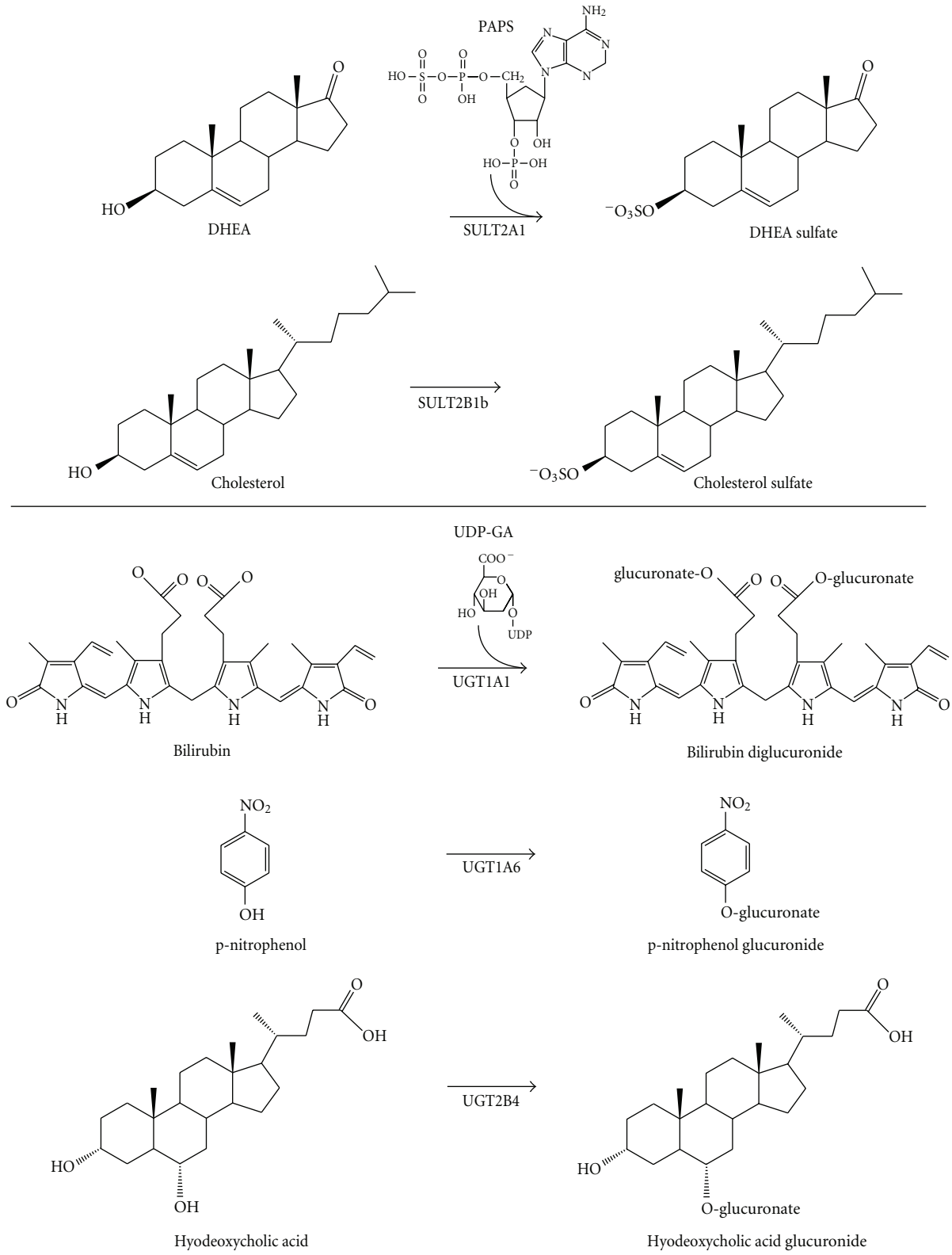
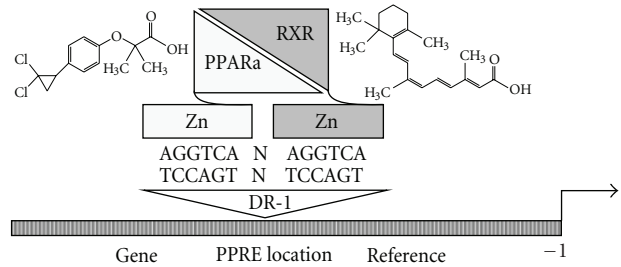


Figure 1: *Example reactions catalyzed by PPAR-regulated SULT and UGT enzymes.* The upper panel shows the SULT2A1- and SULT2B1b-catalyzed 3-sulfonation of the prototype substrates, dehydroepiandrosterone and cholesterol, respectively. Human SULT2A1 is transcriptionally regulated by PPARα in human hepatocytes, while SULT2B1b is regulated by PPARα, PPARδ and PPARγ in keratinocytes. The lower panel shows the glucuronidation of bilirubin, *p*-nitrophenol and hyodeoxycholic acid, which are prototype substrates for UGT1A1, UGT1A6, and UGT2B4, respectively. UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, and UGT2B4 have all been identified as PPAR target genes. PAPS, 3′-phosphoadenosine-5′-phosphosulfate; UDP-GA, uridine-5′-diphospho-α-D-glucuronic acid.

in the liver, kidney, and heart where it is engaged in the regulation of fatty acid oxidation [1]. By contrast, PPARγ is most widely expressed in adipose tissues where it plays a role in adipocyte differentiation and the control of insulin sensitivity [1]. PPARδ is ubiquitously expressed and has been implicated as a regulator of a range of physiological functions from the modulation of insulin resistance to embryo implantation during pregnancy [1]. Fibrates and thiazolidinediones are well-characterized ligand-activators of PPARα and PPARγ, respectively, [1]. Fatty acids represent a major source of cellular energy and are important physiological activators of PPARα [1]. Relative to fatty acids, oxidized fatty acid intermediates are more short-lived species and thus are well poised to serve as both endogenous signaling intermediates and physiological PPARγ agonists [2]. As a key integrator of cellular energy metabolism in a wide spectrum of tissues, the PPAR·RXR transcription factor network is being increasingly recognized for its potential as a therapeutic target and for its expanded role in gene regulation.



Gene	PPRE location	Reference
SULT2A1	−5949	53
UGT1A1	−3272	125
UGT1A3	−5790	125
UGT1A6	−2692	125
UGT1A9	−719	78
UGT2B4	−1199	97

Figure 2: *Regulation of SULT and UGT gene transcription byPPARα.* The PPARα and RXR nuclear receptors are each depicted as a zinc module-containing DNA-binding domain (rectangle) that is joined to a ligand-binding domain (LBD, triangle) through a hinge region. PPARα and RXR bind as a heterodimer to a peroxisome proliferator response element (PPRE) in the regulatory region of a target gene. The consensus PPRE is a nuclear receptor hexamer motif (i.e., (A/G)G(G/T)TCA) in a DR-1 configuration (direct repeat with one intervening nucleotide, N). Binding of an agonist to the LBD of PPARα (e.g., chemical structure for the potent PPARα agonist, ciprofibrate, is shown) evokes a conformational change in the receptor that results in coactivator recruitment and increased target gene transcription. In contrast to its silent role in partnership with some nuclear receptors, RXR functions as an active partner with the PPARs, whereby binding of an agonist to the LBD of RXR (e.g., chemical structure for the prototype RXR ligand, 9-*cis*-retinoic acid, is shown) activates target gene transcription and enhances PPAR ligand-activated transcription. The locations of functional PPREs that have been identified in the 5′-flanking regions of the human *SULT2A1*, *UGT1A1*, *UGT1A3*, *UGT1A6*, *UGT1A9,* and *UGT2B4* genes are shown (positions are relative to the transcription start site).

3. SULTs

3.1. The Role of SULTs in Metabolism. Sulfonation reactions are catalyzed by two distinct families of enzymes, the cytosolic SULTs and the membrane-bound sulfotransferases. Of these, only the SULTs participate in phase II drug metabolism; the membrane-bound sulfotransferases catalyze the sulfonation of proteins and complex carbohydrates [3]. The SULTs catalyze the transfer of a sulfonate moiety (SO_3^-) from the physiological donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to a small molecule substrate containing a nucleophilic moiety such as a susceptible hydroxyl group [4] (Figure 1). The SULTs are widely expressed in hepatic and extrahepatic tissues [5] where they represent key components of the xenobiotic defense system. They also function prominently in physiological processes by metabolizing endogenous substrates, including estrogens [6], thyroid hormones [7], bile acids [8], and neurosteroids [9–12]. In xenobiotic metabolism, sulfate conjugation is recognized as a double-edged sword. As a rule, sulfate conjugates are more polar than the parent substrate and, hence, more amenable to excretion and elimination. However, the production of unstable sulfate conjugates can lead to the focused generation of genotoxic species and carcinogen activation [13, 14].

Like other classes of xenobiotic-metabolizing enzymes, the SULTs exist as a superfamily of related proteins with each enzyme exhibiting a characteristic expression pattern and substrate specificity profile. The cytosolic SULTs are categorized into two major groups, the arylsulfotransferases (SULT1 family) and the hydroxysteroid sulfotransferases (SULT2 family) [5, 15]. The SULT1 family is divided into five subfamilies, designated SULT1A, SULT1B, SULT1C, SULT1D, and SULT1E. As a brief generalization, SULT1A subfamily enzymes metabolize phenolic substrates and function in drug metabolism. For example, SULT1A1 readily catalyzes the sulfonation of simple phenols, such as 1-naphthol and *p*-nitrophenol [4], and detoxifies common phenolic pharmaceuticals, such as acetaminophen [16, 17] and troglitazone [4]. Consistent with its role in drug metabolism, SULT1A1 is abundantly expressed in liver, although it is also expressed in numerous extrahepatic tissues [5]. Rodent enzymes of the SULT1B subfamily catalyze the sulfonation of 3,5,3′-triiodothyronine [18], an important step in thyroid hormone metabolism, although SULT1A1 appears to perform this function in humans [19, 20]. SULT1C enzymes are best known for their abilities to bioactivate the heterocyclic amine procarcinogen, N-hydroxy-2-acetylaminofluorene, to its ultimate carcinogenic form [21]. SULT1D enzymes, described thus far chiefly in the canine, sulfonate phenols, amines, and eicosanoids [22]. SULT1E1 is the physiologic estrogen sulfotransferase, catalyzing the 3-sulfonation of estradiol at nanomolar concentrations [5]. SULT1E1 is expressed most highly in tissues that are actively engaged in estrogen metabolism, such as breast, uterus, and placenta [23–25].

The SULT2 family is divided into two subfamilies, SULT2A and SULT2B. In general, these SULTs most effectively metabolize molecules containing a steroid or

sterol nucleus (e.g., dehydroepiandrosterone (DHEA), pregnenolone, cholesterol, and bile acids) [26]. The SULT2A subfamily members, including human SULT2A1, are most highly expressed in liver, intestine, and adrenal cortex [27–30], and the prototypical SULT2A substrate is DHEA [27] (Figure 1). SULT2A-catalyzed DHEA sulfoconjugation within the adrenal cortex provides high circulating levels of DHEA sulfate, which serves as a reservoir of a precursor molecule that can be converted into potent androgens and estrogens within various tissues (e.g., prostate) [31].

The SULT2B subfamily consists of two gene products, SULT2B1a and SULT2B1b, that are transcribed from the same gene locus through the utilization of different promoters and incorporation of different first exons [11, 32, 33]. SULT2B1b preferentially catalyzes the sulfonation of cholesterol (Figure 1), and SULT2B1b expression has been demonstrated in skin [11, 33–35], prostate [11, 34, 36], placenta [34, 36], lung [34, 37], intestine [11, 33, 36], endometrium [36, 38], breast [39], ovary [36], platelets [40], kidney [33], and muscle [11]. SULT2B1b protein has not been detected in liver [34]. SULT2B1b has been detected in both the cytosol and nuclei of human cells [34, 39, 41].

By comparison to SULT2B1b, SULT2B1a has minimal activity toward cholesterol but readily catalyzes the sulfonation of pregnenolone [42]. Pregnenolone sulfate is a neurosteroid that is synthesized in glial cells [43]. It is, therefore, noteworthy that SULT2B1a mRNA expression has been detected in brain [11] as well as in rat C6 glioma cells [44]. However, to date SULT2B1a protein has not been detected in any human tissues or cell lines [41].

In addition to the major SULT1 and SULT2 enzymes, SULT3A1, SULT3A2, SULT4A1, SULT5A1, and SULT6B1 have been described [45–48] but are not highly characterized. Of these, only SULT4A1 and SULT6B1 have been identified in humans [48–50].

3.2. Regulation of SULTs by PPAR

3.2.1. Regulation of Mouse Liver SULT Expression by PPARα Agonists. A comprehensive survey of SULT regulation by PPARα-activating treatments has been performed by the Klaassen research group, which evaluated the sex-dependent regulation of hepatic SULT expression following in vivo treatment of mice with a panel of prototypical nuclear receptor activators, including three PPARα agonists [51]. Hepatic transcript levels were evaluated for murine SULTs 1a1, 1b1, 1c1, 1c2, 1d1, 1e1, 2a1/2a2, 2b1, and the lesser characterized SULT family members 3a1, 4a1, and 5a1 [51]. In addition, treatment effects on the mRNA expression of both forms of PAPS synthase (PAPSs1 and PAPSs2), the enzymes responsible for synthesizing the PAPS cofactor for sulfate conjugation, were examined [51]. Male and female 8 week old C57BL/6 mice were treated for 4 days with either corn oil vehicle or one of the prototypical PPARα ligands, clofibrate (500 mg/kg IP), ciprofibrate (40 mg/kg IP), or diethylhexylphthalate (1000 mg/kg IP), and euthanized for hepatic SULT mRNA content analysis [51]. Overall, the effects of PPARα activation on murine hepatic SULT expression were not striking [51]. SULT expression in male mouse liver was not appreciably perturbed in response to in vivo treatment with PPARα agonists [51]. In the rat, SULT1E1, which is more abundantly expressed in male relative to female liver, was previously reported to decrease substantially following in vivo treatment with any of the PPARα agonists, WY-14,643, gemfibrozil or di-*n*-butylphthalate [52]. In Klaassen's study, the mRNA levels of several SULTs were suppressed in female mouse liver following PPARα agonist treatment, including SULTs 1c1, 1c2, 1e1, 2a1/2a2, 3a1, and 5a1 [51]. Treatment with clofibrate increased PAPSs2 mRNA content in male mouse liver [51]. However, such induction was not produced by ciprofibrate or diethylhexylphthalate treatment, suggesting that regulation of murine PAPSs2 expression is not an effect common to PPARα agonists [51]. These studies demonstrate that PPARα activation produces gene- and sex-dependent effects on the hepatic expression of SULT enzymes in the mouse.

3.2.2. Transactivation of Human Hepatic SULT2A1 Transcription by PPARα. The above-described data suggest that PPARα does not function as a positive regulator of murine hepatic SULT expression. However, our laboratory has demonstrated that human hepatic SULT2A1 expression is increased by PPARα activation, and that this effect is conferred through a functional PPARα-response element (PPRE) located in the distal 5′-flanking region of the SULT2A1 gene [53]. The treatment of primary cultured human hepatocytes with ciprofibrate induced SULT2A1 mRNA, protein and enzyme activity levels by ~ 2-fold [53]. This finding was in marked contrast to the rat counterpart of SULT2A1, which was not inducible in primary cultured rat hepatocytes following treatment with a PPARα agonist [53]. Analysis of a series of SULT2A1 5′-flanking region-luciferase reporter constructs in HepG2 cells revealed the presence of a functional direct repeat with one intervening nucleotide (DR-1) located at nucleotides −5949 to −5929 relative to the transcription start site [53] (Figure 2). Further site-directed mutagenesis, EMSA and chromatin immunoprecipitation analyses confirmed the functionality of this PPRE in the human SULT2A1 gene [53]. These investigations reveal that SULT2A1 represents a target for lipid signaling in human hepatocytes and suggest that rodent models do not capture the significance of PPARα as a modulator of SULT2A expression.

3.2.3. Regulation of SULT2B1b in Human Keratinocytes by PPAR Agonists. Maintenance of the skin requires a well orchestrated program of keratinocyte proliferation and differentiation during which the cells pass through several phenotypic phases identifiable as distinct layers (Figure 3). As keratinocytes progress through this differentiation program, a major change that occurs is the production of progressively larger amounts of lipid. Cholesterol 3-sulfate has been detected at a level of 2.4% (weight percent of total lipids) in a combined preparation of basal/spinous cells, and 5.5% in the granular layer [54]. The amount of cholesterol 3-sulfate is then reduced in the stratum corneum, due to the conversion of cholesterol 3-sulfate to free cholesterol

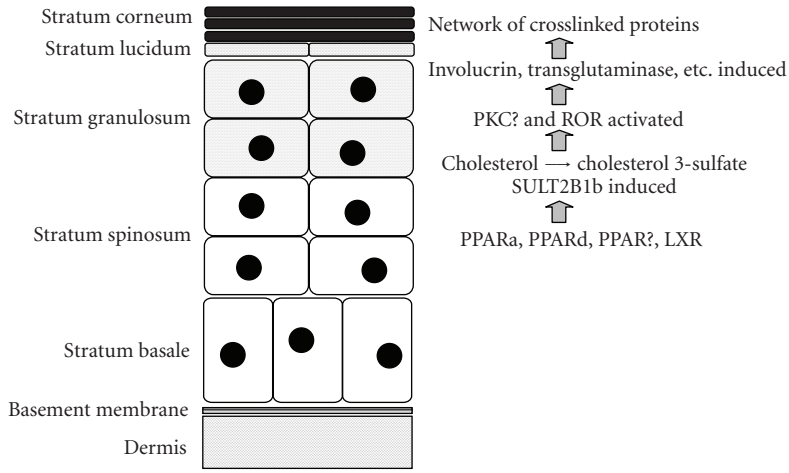


Figure 3: *Representation of keratinocyte differentiation and role of SULT2B1b-mediated cholesterol sulfonation*. During formation of the epidermis, replicating basal keratinocytes give rise to progeny that move progressively upwards and pass through several histologically distinct strata. During this process, SULT2B1b expression becomes activated at approximately the level of the stratum granulosum. SULT2B1b-catalyzed cholesterol sulfonation produces cholesterol 3-sulfate that, in addition to its role as a lipid component of the outer barrier, functions as a signaling molecule that activates PKCη and possibly RORα. These signaling events result in the induction of proteins that are involved in formation of the barrier. PPARα, PPARδ, PPARγ, and LXR have all been reported to positively regulate SULT2B1b expression in cultured keratinocytes.

through the action of steroid sulfatase [54]. Cholesterol 3-sulfate production in the lower levels of the epidermis and its hydrolysis in the stratum corneum has been termed the epidermal cholesterol cycle [55].

In 1984, an enzyme capable of sulfonating cholesterol was reported to be present in mouse epidermis [56], and several subsequent pieces of evidence have supported the concept that "cholesterol sulfotransferase" expression and cholesterol 3-sulfate production are events that are closely linked to keratinocyte differentiation. For example, the specific activity of cholesterol sulfotransferase, but not of steroid sulfatase, paralleled both the accumulation of cholesterol 3-sulfate and the formation of the multilayered structure of the epidermis during mouse development [57, 58]. Cholesterol sulfotransferase activity was also induced during the culture of epidermal tissues isolated from 13.5-days, postcoitus mouse embryos with a time course that paralleled in vitro stratification [58]. In monolayer cultures of normal human keratinocytes, confluence-mediated differentiation was accompanied by increased cholesterol sulfotransferase activity and accumulation of cholesterol 3-sulfate, with parallel increases in transglutaminase-1 [59]. Calcium-mediated differentiation of normal human keratinocytes was accompanied by the induction of two distinct sulfotransferase activities, cholesterol sulfotransferase, and minoxidil sulfotransferase [60]. Exposure of fetal rat skin explants to air caused the accelerated expression of cholesterol sulfotransferase along with several other markers of keratinocyte differentiation, including filaggrin, loricrin and involucrin [61]. A single topical administration of a tumor promoter (e.g., phorbol ester) to mouse skin caused the induction of cholesterol sulfotransferase, the accumulation of cholesterol 3-sulfate, and the induction of transglutaminase-1 [62, 63]. Cholesterol sulfotransferase activity was also elevated during epidermal wound healing [62].

SULT2B1b preferentially sulfonates cholesterol and is the chief SULT2B1 enzyme that is expressed in human keratinocytes [64]. Higashi et al. [35] reported that SULT2B1b, but not SULT2B1a or SULT2A1, was present in normal human epidermal tissue, as well as in cultures of normal human keratinocytes following calcium-induced differentiation. By immunohistochemistry, SULT2B1b was largely localized to the granular layer of normal skin where cholesterol sulfate content is highest [35]. Altogether, the combination of substrate specificity and expression properties establish SULT2B1b as the "cholesterol sulfotransferase" of differentiating keratinocytes [35].

In addition to its role as structural component, cholesterol 3-sulfate is an important signaling molecule that plays an essential role in keratinocyte differentiation (Figure 3). For example, cholesterol 3-sulfate has been proposed to be a physiological activator of the novel protein kinase C (PKC) isoform, PKCη, which is localized to the granular layer where cholesterol sulfate concentrations are the highest [65–67]. Activation of PKCη signals the continuance of the keratinocyte differentiation program, resulting in the expression of late differentiation markers, such as transglutaminase1 [65, 66, 68, 69]. In addition, it has been demonstrated that cholesterol sulfate is a preferred ligand for the retinoid-related orphan nuclear receptor-α (ROR-α). This nuclear receptor, particularly the RORα4 splice variant, is robustly expressed in the epidermis, where it is present in suprabasal differentiating keratinocytes but not in proliferating basal keratinocytes [70]. Until recently, RORα was considered to

be a true orphan receptor, since no firm ligand activator for RORα had been identified. However, recent X-ray crystallography data revealed the presence of either cholesterol or cholesterol sulfate within the RORα ligand binding pocket domain [70]. Furthermore, pharmacological manipulation of cellular cholesterol levels altered RORα transcriptional activity in human osteosarcoma cells [70]. These studies led to the recognition that RORα may represent a novel regulatory pathway in the control of cholesterol homeostasis [71]. Compared to cholesterol, cholesterol sulfate demonstrated a greater affinity for the RORα ligand binding domain and more profound effects on RORα-mediated transcriptional activation, suggesting a major role for cholesterol sulfate as a physiologic activator of RORα in tissues such as the epidermis where both cholesterol sulfate and RORα are abundantly coexpressed [71, 72].

In light of the functional role of SULT2B1b in keratinocyte differentiation, corneoctye desquamation and dermal lipid homeostasis, it is reasonable to expect that SULT2B1b should be a target for regulation by lipid-sensing nuclear receptors in the skin. The effects, on SULT2B1b expression, of treating cultured human keratinocytes with chemical activators of PPARα, PPARδ, PPARγ, and of the liver X receptor (LXRα and LXRβ) have been examined [73]. Measurable levels of PPAR and LXR transcripts were detected in cultured human keratinocytes, and the levels of PPARγ, LXRα, and LXRβ increased following calcium-induced differentiation [73]. By contrast, PPARα and PPARδ mRNA levels did not change demonstrably with keratinocyte differentiation [73]. Treatment with PPARα, PPARδ, PPARγ, and LXR agonists significantly induced SULT2B1b mRNA and enzyme activity in cultured human keratinocytes [73]. The PPARα agonist clofibric acid increased SULT2B1b mRNA content by ~39.9% [73]. Activators of PPARδ (GW501516) and PPARγ (ciglitazone) produced even greater increases (~ 9.8-fold and ~25.1-fold, resp.) in SULT2B1b mRNA content, and these increases were further augmented by calcium-induced keratinocyte differentiation [73]. As modulators of SULT2B1b expression in differentiating keratinocytes, these results underscore the central role of PPAR transcription factors as integrators of skin physiology and barrier function (Figure 3).

4. UDP-Glucuronosyltransferases

4.1. The Role of UGTs in Metabolism. The UGTs catalyze the transfer of glucuronic acid from a high-energy cofactor, UDP-glucuronic acid, to a xenobiotic or endogenous substrate containing an available nucleophilic center such as a hydroxyl, carboxyl, amino, or thiol group [74–77] (Figure 1). The UGTs are membrane-bound enzymes localized on the luminal surface of the endoplasmic reticulum [74]. Relative to the parent substrate, the end-products of glucuronidation are typically more polar and better suited for excretion and elimination through the urine or bile [74]. Endogenous UGT substrates include bilirubin, neutral steroids, bile acids, fatty acids, and retinoids [74, 78, 79]. Xenobiotic UGT substrates range from environmental toxicants such as benzo[a]pyrene to common pharmaceuticals such as acetaminophen, nonsteroidal anti-inflammatory agents, fibrates, thiazolidinedione-class insulin sensitizers, and opioids [75, 78–84]. Individual UGT enzymes display distinctive patterns of substrate specificity and inducible regulation, but as with the SULTs, some UGTs display overlapping substrate specificities [74, 85]. Different UGTs are expressed in a species- and tissue-specific manner [86, 87]. Overall, the broad metabolic range of the UGTs distinguishes this class of conjugating enzyme as a major detoxicating system in rodents and humans.

Though over 50 individual UGT enzymes have been described [86, 88], comparisons of cDNA and amino acid sequences have revealed two major UGT gene families, UGT1 and UGT2 [75, 86, 88, 89]. The UGT1A multigenic locus is unusual in that it is comprised of a tandem series of thirteen promoter regions on human chromosome 2 [75, 86, 90]. Each of nine functional UGT1A proteins is produced as a result of transcription initiation at a particular promoter, which results in the transcription and splicing of a unique exon 1 sequence to a cassette of common exons (exons 2–5) that share the same 3′ end [75, 86, 88, 90, 91]. As an additional layer of complexity, recent evidence indicates that alternative splicing events generate UGT1A isoforms with different exon 5 sequences. For each of these UGT1A proteins, isoform 1 (containing exon 5a) is the classical catalytically active enzyme, while isoform 2 (containing exon 5b) is a smaller protein that lacks UGT activity but can inhibit the activity of the corresponding isoform 1 [92, 93]. By contrast to the UGT1A locus, UGT2 enzymes are products of individual genes [75].

Like sulfonation, glucuronidation plays a physiological role in the modulation of biologically active endogenous hormones and metabolic intermediates. For example, the activity of UGT1A1 conjugation in the tight control of bilirubin metabolism has established the importance of UGT1A1 genetic polymorphisms in the pathogenesis of toxic hyperbilirubinemias such as the Crigler-Najjar and Gilbert's syndromes in humans [75, 89, 94]. Thyroxine is metabolized by O-glucuronidation in addition to deiodination and sulfonation [95]. Studies using recombinant UGTs and human liver microsomes revealed that human hepatic UGT1A1 and UGT1A3 are the UGT principals most catalytically active toward thyroxine [95]. Bile acids represent the end-products of hepatic cholesterol metabolism and in the absence of sufficient detoxication metabolism, particularly in the face of cholestasis, the detergent properties of bile acids produce significant hepatotoxicity [96]. Human UGT2B4 [97], UGT2B7 [96, 98], UGT1A4 [96, 98], and UGT1A3 [98, 99] are all bile acid-metabolizing enzymes. The formation of chenodeoxycholic acid (CDCA) glucuronide by UGT1A3 has been shown to decrease farnesoid X receptor (FXR) activation by CDCA, the prototypical FXR ligand [99], suggesting that UGT1A3 induction in human liver would be expected to have down-stream consequences for an array of gene expression networks that are transcriptionally regulated by FXR.

4.2. Regulation of UGTs by PPAR.

4.2.1. Studies of the Effects of Clofibrate and Other Perixosome Proliferation-Inducing Agents on UGT Activities. It has been more than 40 years since the discovery that clofibrate treatment causes peroxisome proliferation in rat liver [100, 101]. After clofibrate was subsequently shown to cause hepatic induction of a unique cytochrome P450 with a reduced carbon monoxide-bound absorbance peak at 452 nm and catalytic activity toward lauric acid 12-hydroxylation, clofibrate became accepted as the prototype of a novel class of microsomal enzyme inducers [102]. Clofibrate, therefore, was used in a number of studies designed to evaluate the effects of microsomal enzyme inducer treatments on glucuronidation activities. The most commonly reported finding in these studies was that treatment with clofibrate or a structurally related compound increased hepatic glucuronidation activity toward bilirubin in rats [103–110], mice [111], and primary cultured rat hepatocytes [112], with the clofibrate-mediated increases generally in the range of two-fold over control. In some of these studies, use of multiple compounds provided evidence for correspondence between induction of rat hepatic bilirubin conjugation activity and induction of lauric acid 12-hydroxylation [104, 105], implying probable identity of the induction mechanism. In addition, human subjects with Gilbert's syndrome treated with clofibrate had decreased serum total bilirubin concentrations [113], and hepatic microsomes prepared from humans who had received clofibrate contained elevated bilirubin UGT activity [114].

Clofibrate treatment of rats has also been reported to increase hepatic UGT activity toward 4′-hydroxy-N,N-dimethyl-4-aminoazobenzene [106], thyroxine and reverse triiodothyronine [109, 115], retinoic acid [116], and the antithrombotic drug LF 4.0212 [117]. In agreement with these findings, clofibric acid treatment also increased thyroxine UGT activity in primary cultured rat hepatocytes [112]. However, a species difference in UGT induction was suggested by the finding that clofibrate treatment of male OF-1 mice displayed no increase in hepatic microsomal thyroxine UGT activity [111]. Likewise, clofibric acid treatment of primary cultured mouse hepatocytes (OF-1 strain) did not increase bilirubin or thyroxine UGT activity [112]. In a porcine model, in vivo treatment with clofibrate induced hepatic glucuronidation of thyroid hormones sufficiently to reduce circulating plasma 3,3′,5-triiodothyronine and thyroxine concentrations [118].

Clofibrate treatment has been consistently reported not to increase UGT activity toward *p*-nitrophenol in rat liver [106, 109, 110], mouse liver [111], primary cultured rat hepatocytes [112, 119], or primary cultured mouse hepatocytes [112]. Clofibrate treatment of rats or mice (OF-1 strain) has also been shown to have little effect on hepatic microsomal UGT activities toward triiodothyronine and androsterone [109, 111].

In another study, treatment of rats with a single dose of the nonfibrate peroxisome proliferator, the fully-fluorinated ten-carbon fatty acid perfluorodecanoic acid, induced hepatic bilirubin UGT activity two-fold, and this induced a state persisted for 3 weeks [120]. This single-dose treatment also decreased hepatic UGT activities toward 1-naphthol, morphine, and testosterone, with maximal reductions occurring 12 days after treatment and recovery to control activities occurring at 3 weeks [120].

These early findings, in which peroxisome proliferator treatments produced differential effects on various UGT activities, provided a clear indication of the multiplicity of the UGT superfamily. As seen for the cytochromes P450, only certain UGT activities displayed peroxisome proliferator inducibility, predicting the later demonstration that particular UGT genes would be targets of PPARα-mediated transactivation.

4.2.2. UGT1A Regulation by PPARs. UGT1A1 is the major catalyst of bilirubin glucuronidation [121] (Figure 1). Therefore, based on the above-described observation that clofibrate treatment consistently caused induction of hepatic bilrubin UGT activity, UGT1A1 is expected to be a PPARα target gene, possibly along with other UGTs of the "bilirubin-like" portion of the UGT1A subfamily (i.e., UGTs 1A2-1A5).

In a study using antipeptide antibodies to examine the effects of drug treatments on rat hepatic microsomal UGT levels, clofibrate treatment was found to increase the immunoreactive protein levels of UGT1A1 and UGT1A5 (termed UGT1B1 and UGT1B5 in that study) along with bilirubin UGT activity [110]. In a more recent study, four-day treatments of male rats with PPARα activators produced modest increases in hepatic UGT1A1 and UGT1A3 mRNA levels [122]. In addition, clofibric acid treatment of primary cultured rat hepatocytes has been reported to increase the amount of UGT1A1 protein by western blot analysis [119] and the amount of UGT1A1 mRNA by microarray analysis [123]. Therefore, as predicted by the bilirubin glucuronidation activity data, UGT1A1 is a target of PPARα activation. Clofibrate treatment has also been reported to decrease the amount of rat hepatic microsomal UGT1A6 protein (termed UGT1A1 in that study) [110]. This finding is also in agreement with the above-mentioned earlier finding that clofibrate treatment did not induce *p*-nitrophenol glucuronidation activity in rodent liver or cultured hepatocytes, since UGT1A6 is a major catalyst of that activity [124] (Figure 1).

In a recent study by Senekeo-Effenberger et al. [125], Wy-14,643 treatment of primary cultured human hepatocytes increased the levels of UGT1A1, UGT1A3, UGT1A4, and UGT1A6, but not UGT1A9, mRNAs. In the same study, experiments conducted with transgenic mice engineered to express the complete human UGT1 gene locus demonstrated that oral Wy-14,643 treatment resulted in prominent induction of human UGT1A1 and UGT1A6, and observable induction of UGT1A4, immunoreactive protein content in liver microsomes. At the mRNA level in liver, very strong (>100-fold) induction of UGT1A1 and UGT1A3, significant ($\sim$ 3- to 4-fold) induction of UGT1A4 and UGT1A6, and no induction of UGT1A9 were seen, in general agreement with the above-described effects in the human hepatocyte cultures [125]. In small intestine, Wy-14,643 treatment produced

induction of UGT1A1 and UGT1A4, but not UGT1A6, while in kidney only UGT1A6 was induced [125]. Additional experiments confirmed the presence of functional PPREs in the 5′-flanking regions of UGT1A1, UGT1A3 and UGT1A6 [125] (Figure 2).

In another study, treatment of primary cultured human hepatocytes with activators of PPARα increased the expression of UGT1A3 and UGT1A3-catalyzed glucuronidation of chenodeoxycholic acid (CDCA) and demonstrably tempered the transactivation of FXR by CDCA [99]. Promoter analysis of the human UGT1A3 gene revealed coregulation by two lipid-sensing transcription factors, LXR [126] and PPARα [125].

UGT1A4 was also identified as a significantly upregulated gene in clofibric acid-treated primary cultured human hepatocytes by Affymetrix microarray analysis [123]. In this same study, clofibric acid treatment of primary cultured mouse hepatocytes failed to cause significant alteration of any UGT transcripts [123], consistent with the above-described apparent lack of sensitivity of mouse to PPARα-mediated regulation of UGT activities.

By comparison to the results described by Senekeo-Effenberger et al. [125], Barbier et al. [78] previously reported that mouse and human UGT1A9 are transcriptionally activated by PPARα and PPARγ, and that human UGT1A9 contains a functional PPRE located at nucleotides −719 to −706 (Figure 2). UGT1A9 mRNA induction was observed in primary cultured human hepatocytes treated with fenofibric acid, HepG2 cells treated with Wy-14,643, 3T3-L1 adipocytes treated with rosiglitazone, primary human macrophages treated with Wy-14,643, or differentiated THP-1 macrophages treated with Wy-14,643 or rosiglitazone [78]. As expected, the inducing effect of fenofibric acid on mouse hepatic UGT1A9 was ablated in PPARα-null mice [78].

4.2.3. UGT2B Regulation by PPARα. In human hepatocytes, treatment with a PPARα agonist, fenofibric acid or Wy-14643, increased the expression of UGT2B4 mRNA and stimulated the glucuronidation of hyodeoxycholic acid, a model substrate for UGT2B4 [97] (Figure 1). Transient transfection and EMSA studies revealed a functional DR-1 PPRE at nucleotides −1199 to −1175 nt relative to the UGT2B4 transcription start site and solidified UGT2B4 as a transcriptional target of PPARα [97] (Figure 2).

5. Regulation of SULTs and UGTs by Other Nuclear Receptors

In addition to regulation by PPARs, the SULTs and UGTs receive transcriptional input from multiple other nuclear receptors. Here, we do not attempt to be comprehensive but present several findings related to the regulation of hepatic SULT2A expression as an example. Our previous investigations suggested roles for both the glucocorticoid receptor (GR) and the pregnane X receptor (PXR) in the mediation of glucocorticoid-inducible rat hepatic SULT2A expression [127]. GR-activating concentrations of glucocorticoid transactivated SULT2A transcription indirectly, through intermediary steps involving GR-inducible liver-enriched CCAAT enhancer binding protein [128], while pharmacological concentrations of dexamethasone induced rat hepatic SULT2A expression via a PXR-mediated mechanism [127]. Rodent and human SULT2A are differentially regulated by the xenobiotic-sensing receptor, PXR. In mice and rats, hepatic SULT2A transcription is activated by PXR through the direct binding of PXR to the 5′-flanking regions of SULT2A genes [127, 129]. However, unlike rodent hepatic SULT2As, treatment of human liver cells with rifampicin, the prototypical ligand activator of human PXR [130], produces a biphasic effect on SULT2A1 expression [131]. Treatment with PXR-activating concentrations of rifampicin causes PXR-dependent suppression of SULT2A1 expression, whereas treatment with higher rifampicin concentrations induces SULT2A1 expression through a PXR-independent mechanism [131]. In addition, the nuclear receptor hepatocyte nuclear factor 4α (HNF4α) is a positive regulator of SULT2A1 expression, and both the suppressive and activating effects of rifampicin appear to be transduced through interactions with HNF4α [131].

The constitutive androstane receptor (CAR) also partners with RXR and transactivates murine hepatic SULT2A, and possibly human SULT2A1 [132]. The vitamin D receptor (VDR, NR1I1) is activated not only by 1α,25-dihydroxyvitamin D_3 but also by bile acids [133], and emerging evidence suggests that the VDR regulates murine hepatic SULT2A transcription, and can also drive the transcription of SULT2A1 in vitro [134]. The role of SULT2A as an integrator of endogenous lipid metabolism is just emerging. Oxysterol intermediates of cholesterol metabolism are physiological ligands for LXR, an RXR heterodimer transcription factor that regulates a number of genes involved in the maintenance of lipid homeostasis [135]. LXR-inducible murine hepatic SULT2A gene transcription has been described and has been shown to confer a protective effect against bile acid toxicity during cholestasis [136]. Certain sulfonated auto-oxidized sterols, such as 5α,6α epoxycholesterol-3-sulfate and 7-ketocholesterol-3-sulfate, have been shown to interact with the LXRα ligand-binding domain and inhibit LXRα-mediated transactivation in vitro [137]. A suppressive role for FXR in the regulation of hepatic SULT2A expression has also been identified, as SULT2A expression was increased 8 fold in FXR-null mice as compared to wild-type mice, and CDCA feeding decreased SULT2A expression in wild-type mice [138]. Also, SULT2A1 expression was reduced following treatment of HepG2 cells with FXR agonists [138].

6. Conclusions

Emerging evidence supports an under-appreciated physiological role for members of the SULT and UGT gene families to serve as modulators of biologically active lipids and to undergo transactivation by lipid-sensing transcription factors such as the PPARs. Particularly in keratinocytes which rely on lipid signaling for the progression of pro-

grammed cellular differentiation, the inducible expression of cholesterol sulfotransferase (SULT2B1b) by PPAR activators has been demonstrated. Studies in primary cultured human but not rat hepatocytes clearly demonstrate that PPARα-inducible human hepatic SULT2A1 expression occurs through a distal PPRE in the 5′-flanking region of the SULT2A1 gene. Several human hepatic UGTs also demonstrate inducible transcription in response to PPARα activation, and transgenic mice expressing the human UGT1 gene locus display transcriptional regulation of human UGT1A transgenes in liver and intestine by PPARα. In view of the coordinated integration between phase II metabolism and PPAR lipid signaling networks, future investigations will likely focus on the disturbances in hepatic and gastrointestinal lipid homeostasis that significantly alter SULT and UGT expression sufficiently to disrupt the down-stream metabolism of environmental xenobiotics, pharmaceuticals, or biologically active intermediates of metabolism.

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Review Article

Regulation of Bile Acid and Cholesterol Metabolism by PPARs

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Bile acids are amphipathic molecules synthesized from cholesterol in the liver. Bile acid synthesis is a major pathway for hepatic cholesterol catabolism. Bile acid synthesis generates bile flow which is important for biliary secretion of free cholesterol, endogenous metabolites, and xenobiotics. Bile acids are biological detergents that facilitate intestinal absorption of lipids and fat-soluble vitamins. Recent studies suggest that bile acids are important metabolic regulators of lipid, glucose, and energy homeostasis. Agonists of peroxisome proliferator-activated receptors (PPARα, PPARγ, PPARδ) regulate lipoprotein metabolism, fatty acid oxidation, glucose homeostasis and inflammation, and therefore are used as anti-diabetic drugs for treatment of dyslipidemia and insulin insistence. Recent studies have shown that activation of PPARα alters bile acid synthesis, conjugation, and transport, and also cholesterol synthesis, absorption and reverse cholesterol transport. This review will focus on the roles of PPARs in the regulation of pathways in bile acid and cholesterol homeostasis, and the therapeutic implications of using PPAR agonists for the treatment of metabolic syndrome.

1. Introduction

Fibrates have been used for decades to treat hypertriglyceridemia or mixed hyperlipidemia for their ability to significantly reduce plasma triglyceride levels [1]. Fibrate treatments also modestly elevate plasma HDL-C and slightly decrease plasma LDL-C. Studies in the past have revealed that the hypolipidemic effects of fibrates are mainly a result of activation of the nuclear receptor peroxisome proliferator-activated receptor α (PPARα), which belongs to the nuclear hormone receptor superfamily [2]. PPARα can be activated by natural fatty acids and fibrates. PPARα forms a heterodimer with nuclear receptor RXR and recognizes a consensus PPAR responsive element (PPRE) on its target gene promoters. PPARα regulates a network of genes that promote lipolysis and fatty acid β-oxidation, the major mechanisms mediating the lipid lowering effects of fibrates. Based on the sequence homology, two additional PPAR isoforms were identified and named PPARγ and PPARβ/δ [3, 4]. PPARγ plays critical roles in adipocyte differentiation, lipid storage, inflammation, and energy metabolism. PPARγ is activated by the thiazolidinediones (TZDs) drugs, which improve insulin sensitivity and lower plasma glucose levels in diabetes [1]. PPARδ plays a role in fatty acid and energy metabolism in the muscle. Activation of PPARδ has been shown to prevent dyslipidemia and obesity in animal models of metabolic syndromes [5, 6]. PPAR agonists have been extensively investigated for their therapeutic benefits in improving diabetes, dyslipidemia, and features of metabolic syndromes.

Bile acids are amphipathic molecules derived from cholesterol in the liver [7, 8]. Bile acid synthesis generates bile flow from the liver to the intestine. The transport of bile acids between liver and intestine is referred to as the enterohepatic circulation of bile, which plays important roles in liver function, liver physiology, and metabolic regulation. Bile acids are detergent molecules that facilitate biliary excretion of cholesterol, endogenous metabolites and xenobiotics, and intestinal absorption of lipids and nutrients. In cholestatic liver diseases, bile acids accumulate at high concentrations in the liver, resulting in hepatocyte injury, impaired liver function, fibrosis and cirrhosis. The liver plays a central role in maintaining cholesterol homeostasis by balancing multiple pathways including de novo cholesterol and bile acid synthesis, dietary

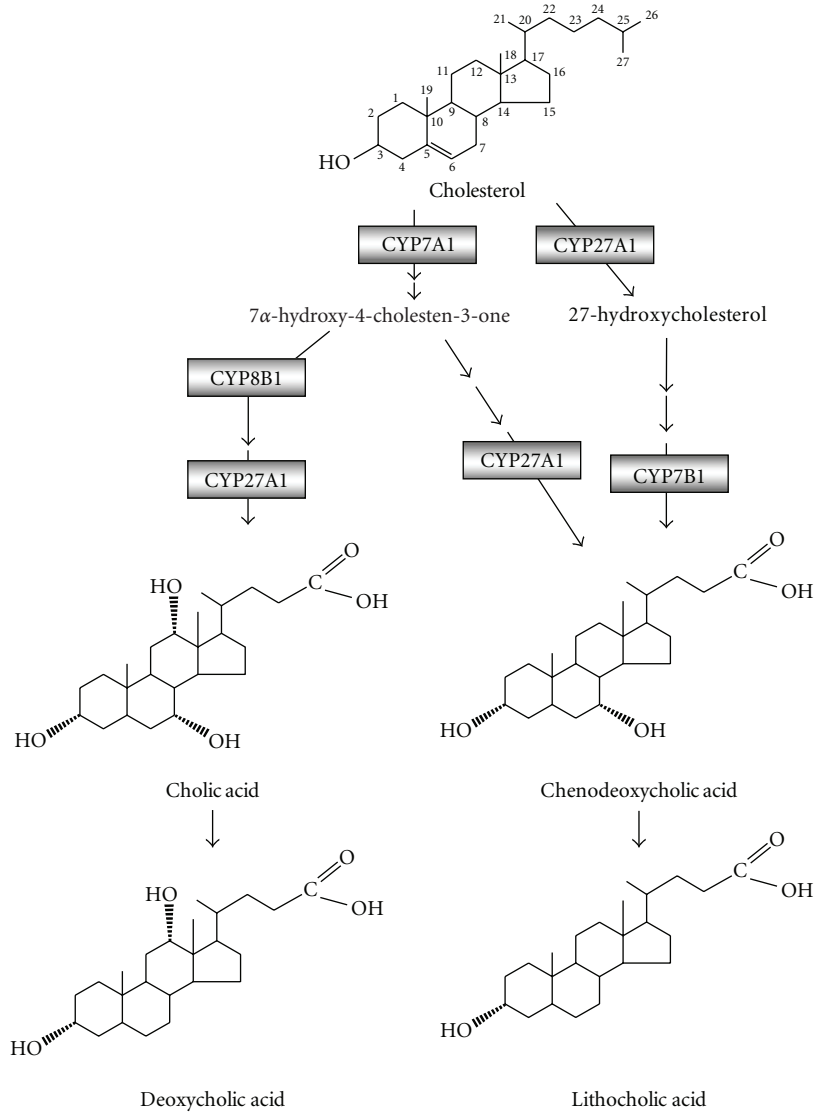

Figure 1: *Bile acid synthesis.* Bile acids are synthesized from cholesterol in the liver through two pathways: the classic pathway and the alternative pathway. In human liver, bile acid synthesis mainly produces two primary bile acids, cholic acid (CA), and chenodeoxycholic acid (CDCA). Key regulatory enzymes in both pathways are indicated. CYP7A1 catalyzes the first the rate-limiting step in the classic pathway to convert cholesterol into 7α-hydroxycholesterol, while CYP27A1 initiates the alternative pathway by converting cholesterol into 27-hydroxycholesterol, which is then 7α-hydroxylated by oxysterol 7α-hydroxylase (CYP7B1). CYP8B1 regulates the cholic acid synthesis in the classic pathway. In the intestine, primary bile acid CA and CDCA are dehydroxylated at the 7α-position by the bacterial enzymes to produce the secondary bile acids, deoxycholic acid (DCA), and lithocholic acid (LCA), respectively.

cholesterol uptake, biliary cholesterol excretion, lipoprotein synthesis, and reverse cholesterol transport. Defects in bile acid synthesis due to mutations in bile acid biosynthetic genes caused both abnormal cholesterol metabolism and bile acid metabolism, which led to cholesterol gallstone disease, dyslipidemia, and cardiovascular diseases in humans [9]. This review will summarize the recent development in understanding the role of PPARs in regulation of bile acid and cholesterol homeostasis, and the therapeutic implications in using PPAR agonists for treating metabolic dyslipidemia and reducing the risk of cardiovascular and heart diseases.

2. Bile Acid Synthesis and Transport

2.1. Bile Acid Synthesis. In humans, bile acid pool consists of primary bile acids (cholic acid, CA, and chenodeoxycholic acid, CDCA) and secondary bile acids (deoxycholic acid, DCA, and lithocholic acid, LCA) [7]. Primary bile acids are synthesized from cholesterol exclusively in the liver through two general pathways, the classic pathway and the alternative pathway (Figure 1) [9]. Secondary bile acids are derived from primary bile acids in the intestine by bacterial enzymes. Enzymes that catalyze these multistep reactions are located in the endoplasmic reticulum, mitochondria, cytosol, and

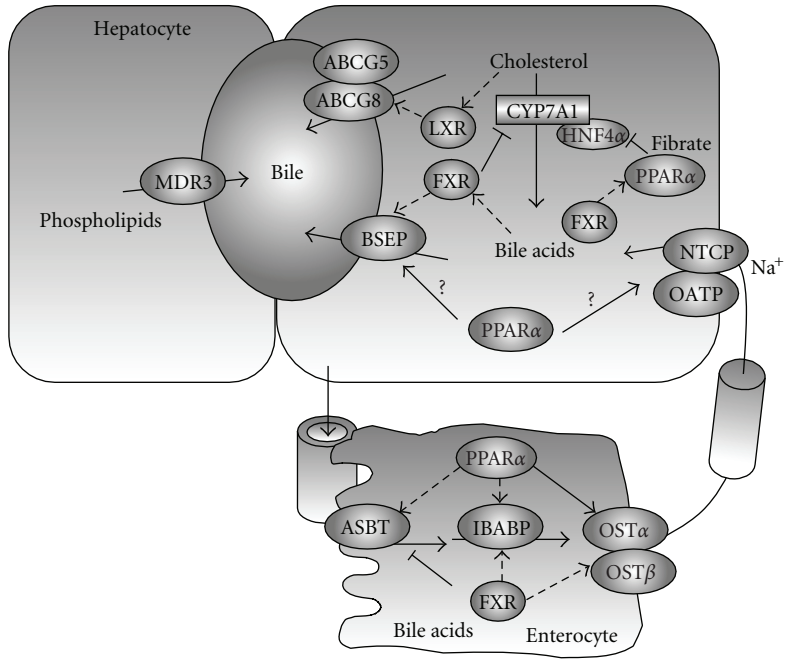

Figure 2: *Enterohepatic circulation of bile.* Bile acids are transported across the canalicular membrane of hepatocytes by BSEP. Cholesterol is either converted into bile acids for biliary secretion or transported by ABCG5/G8 into the bile. MDR3 mediates biliary phospolipids secretion. Cholesterol, bile acids and phospholipids form mixed micelles to solublize cholesterol and reduce bile acid cytotoxicity. After food intake, gallbladder contracts and releases bile acids into intestine. Approximately 95% of bile acids are reabsorbed into the enterocytes. OSTα/OSTβ heterodimeric transporter mediates basolateral bile acid efflux into the portal circulation. NTCP and OATPs mediate hepatic basolateral uptake of bile acids, which are then resecreted into the bile. In the hepatocytes, bile acid-activated FXR feedback inhibits CYP7A1 and NTCP, and thus bile acid synthesis and uptake. Bile acid-activated feed-forward stimulates BSEP and biliary bile acid secretion. Cholesterol derivatives oxysterols activate LXR, which induces ABCG5/G8 and biliary cholesterol secretion. In the intestine, FXR inhibits ASBT and stimulates IBABP and OSTα and OSTβ.

peroxisomes. The classic pathway is also known as the neutral pathway for most of its intermediates are neutral sterols. In human, this pathway produces CA and CDCA in approximately equal amounts. Cholesterol 7α-hydroxylase (CYP7A1), a microsomal cytochrome p450 enzyme, catalyzes the first and rate-limiting step in the classic pathway to convert cholesterol into 7α hydroxycholesterol [10]. Microsomal 3β-hydroxy-27-steroid dehydrogenase/isomerase (3β-HSD) then converts 7α-hydroxycholesterol into 7α-hydroxy-4-cholestene-3-one, which is the common precursor for both CA and CDCA. 7α-hydroxy-4-cholestene-3-one can be hydroxylated at C-12 position by microsomal sterol 12α-hydroxylase (CYP8B1) and modified by other enzymes and eventually converted to CA. Alternatively, without 12α-hydroxylation, 7α-hydroxy-4-cholestene-3-one is converted to CDCA. Mitochondrial sterol 27-hydroxylase (CYP27A1) mediates the steroid side chain oxidation and cleavage to give carboxyl groups in both CA and CDCA synthesis [11]. The alternative pathway, also called the acidic pathway, was originally revealed by the identification of several acidic intermediates which are not present in the classic pathway [12, 13]. The alternative pathway mainly produces CDCA. CYP27A1 catalyzes the first two steps and converts cholesterol into 27-hydroxycholesterol and then 3β,7α-dihydroxy-5-cholestenoic acid [14]. Oxysterol 7α-hydroxylase (CYP7B1) then catalyzes the hydroxylation reaction at C-7 position of these two intermediates, which are subsequently converted to CDCA by the same enzymes in the classic pathway. In humans, the classic pathway is thought to be the major bile acid biosynthesis pathway in normal physiology in humans.

2.2. Bile Acid Transport

2.2.1. Enterohepatic Circulation. Bile acids, once produced in the liver, are transported across the canalicular membrane of the hepatocytes into the bile and stored in the gallbladder. After each meal, bile acids are released into the intestinal tract, efficiently reabsorbed in the ileum, and transported back to the liver via portal blood for reexcretion into the bile. This process is referred to as enterohepatic circulation of the bile (Figure 2) [8]. Bile acid transporters play important roles in this transport process [15]. The biliary excretion of bile acids is the major driving force of bile flow. The bile acid pool size is defined as the total amount of bile acids circulating in the enterohepatic circulation. In humans, bile acid pool consists of CA, CDCA, and DCA in an approximate 40 : 40 : 20 ratio, with a mass of around 2.5–3 gm. After reaching the small intestine, approximately 95% of the bile acids are reabsorbed and only 5% is lost into the feces. The daily loss of bile acids is compensated by de novo synthesis in the liver and thus, a constant bile acid pool is maintained.

2.2.2. Hepatic Bile Acid Transport. Hepatocytes are polarized epithelial cells with basolateral (sinusoidal) and apical (canalicular) membrane domains. Hepatocytes take up bile acids through the basolateral membrane, which is in direct contact with the portal blood plasma, and excrete bile acid at the canalicular membrane into the bile [16]. Bile acids are conjugated with taurine or glycine in the peroxisomes and present as bile salts. They cannot cross the cell membrane and need active transport mechanisms for cellular uptake [17]. Two bile acid transporters, Na^+-dependent taurocholate transporter (NTCP) and organic anion transporters (OATPs) are responsible for basolateral bile acid transport into the hepatocytes (Figure 2). The NTCP cotransports two Na^+ down its gradient into the hepatocytes along with one molecule of conjugated bile acid [18]. Na^+-dependent bile salt uptake pathway accounts for 80% of the total taurocholate uptake and is considered as the major bile acid transport system located at the basolateral membrane [19]. The Na^+-independent bile salt uptake is mediated by several members of OATP family. These transporters have wide substrate preference. Besides conjugated and unconjugated bile acids, many amphipathic organic compounds such as bilirubin, selected organic cations and numerous drugs are also taken up by these transporters [20]. In rat liver, Oatp-1, -2, and -4 account for the bulk Na^+-independent bile acid uptake while OATP-C is the most relevant isoform in humans [21–24].

Since the biliary bile acids concentration is about 100 to 1000 fold higher in the bile than in the hepatocytes, canalicular bile acid transport represent the rate-limiting step in bile formation. Several members of the ATP-binding cassette (ABC) transporter family are responsible for transporting bile acids and other organic compounds across the canalicular membrane against their concentration gradients. The bile salt export pump (BSEP, ABCB11), originally identified as the sister of P-glycoprotein (SPGP), is mainly responsible for bile acid transport at the canalicular membrane (Figure 2) [25]. Mutations in *BSEP* were first identified in patients with progressive familial intrahepatic cholestasis subtype 2 (PFIC-2). The absence of functional BSEP in the hepatic canalicular membrane and less than 1% of normal biliary bile acid concentration found in these patients suggested that BSEP is the major canalicular bile acid transport system [26].

After bile acids are pumped into the bile, they stimulate phospholipids and cholesterol secretions into the bile, followed by passive inflow of water [27]. Phospholipids are excreted via the phospholipid flippase MDR3 (ABCB4), and the major phospholipid in the bile is phosphatidylcholine [28, 29]. Biliary free cholesterol secretion mediated by ABCG5/G8 transporters is an important route for hepatic cholesterol elimination. Mice lacking ABCG5 and ABCG8 showed decreased biliary cholesterol concentration, while transgenic expression of ABCG5 and ABCG8 in mice resulted in increased biliary cholesterol secretion [30]. Bile acids, phospholipids, and cholesterol are three major organic solutes of the bile and once secreted, they form mixed micelles to increase cholesterol solubility and reduce their toxicity to the bile duct. Normal bile formation depends largely on balanced secretion of these constituents. Impaired secretions will disrupt the bile flow and result in cholestasis or cholesterol gallstone disease.

2.2.3. Intestine Bile Acid Transport. In the intestine lumen, bile acids are reabsorbed mostly at the terminal ileum. Like the hepatic basolateral uptake system, intestinal bile acid uptake is also mainly mediated by the apical sodium-dependent bile salt transporter (ASBT) [31]. This transporter has substrate specificity for both primary and secondary conjugated and unconjugated bile acids. Unlike some hepatic bile acid transporters that also mediate the secretion of other organic compounds, the substrates for ASBT is strictly limited to bile acids [32].

Once absorbed into the enterocytes, bile acids bind the intestinal bile acid binding protein (I-BABP) and are transported to the basolateral membrane for secretion (Figure 2) [33]. Recently identified heterodimeric organic solute transporters OSTα/OSTβ appeared to be the major basolateral bile acid transport system in the intestine and many other epithelial cells [34]. This is supported by studies showing in mouse that overexpression of OSTα and OSTβ enhanced basolateral efflux of taurocholate, while mice lacking *Ostα* showed marked decreases in intestinal bile acid absorption, serum bile acid concentration, and bile acid pool size [35].

3. PPAR Regulation of Bile Acid Synthesis and Transport

3.1. PPAR Regulation of Bile Acid Synthesis. Early clinical studies have found consistent increases in biliary cholesterol saturation and the risk of cholesterol gallstone formation in hyperlipidemic human patients following long-term fibrate therapies [36–39]. Despite the observed decrease in plasma LDL-C and increase in plasma HDL-C by fibrate treatments, biliary cholesterol secretion was found to be increased in both normal and hyperlipidemic individuals after fibrate treatments. Biliary bile acid secretion was also reported to be decreased by fibrates [36, 38]. In contrast, biliary phospholipid secretion, which may also affect normal bile flow and cholesterol gallstone formation, seemed to be unaffected [38, 39]. Fibrate treatment has been found to associate with decreased CYP7A1 mRNA expression and enzyme activity. In one study, bezafibrate reduced hepatic CYP7A1 enzyme activity by 60% in normolipidemic gallstone patients [40]. In another study, both gemfibrozil and bezafibrate reduced the rate of cholesterol 7α-hydroxylation by 55% in patients with hypercholesterolemia [41]. Human with genetic defects in *CYP7A1* developed premature hypercholesterolemia and gallstone disease [42]. It is likely that inhibition of hepatic CYP7A1 activity following long-term fibrate treatments may account for the reduced cholesterol catabolism and bile acid output, leading to imbalanced bile acid and cholesterol secretion, increased biliary cholesterol saturation, and the incidence of cholesterol gallstone formation.

Consistent with these observations, studies performed in cell-based models or animal models revealed that fibrate

inhibition of hepatic CYP7A1 activity might be a result of PPAR-dependent repression of *Cyp7a1* transcription. Using cell-based gene reporter assays, two groups showed that PPARα/RXR and Wy14643 repressed both human and rat *CYP7A1* promoter reporter activities [43, 44]. Although a putative PPRE was mapped in the human *CYP7A1* promoter, this site did not bind PPARα/RXR, but was a previously identified binding site for nuclear receptor HNF4α, a positive regulator of *Cyp7a1* transcription. The mechanistic studies further revealed that PPARα inhibited *Cyp7a1* by decreasing the cellular levels of HNF4α. Consistent with these in vitro studies, ciprofibrate treatment was shown to inhibit CYP7A1 mRNA expression and enzyme activity in both rat and mouse livers in vivo [45]. Although various fatty acids may act as the endogenous PPARα agonists, it seems that PPARα may not play a critical role in controlling CYP7A1 gene expression under normal physiology, as genetic knockout of *Pparα* in mice did not affect the basal *Cyp7a1* transcription [44, 45]. However, the repressive effect of ciprofibrate on CYP7A1 mRNA expression and enzyme activity was completely abolished in mice lacking *Pparα*, providing an in vivo evidence that fibrates inhibition of *Cyp7a1* was PPARα-dependent [44, 45]. As mentioned earlier, CYP27A1 is the rate-limiting enzyme in the alternative bile acid synthetic pathway, and is also responsible for the side chain oxidation in the classic bile acid synthetic pathway (Figure 1). The *Cyp27a1* transcription was also repressed by fibrate treatment in mice, despite a much weaker reduction in the mRNA level and enzyme activity when compared to those of *CYP7A1* [45]. The fibrate inhibition of *CYP27A1* was also likely to be PPARα-dependent, but the molecular mechanism of this regulation is still not clear. Simultaneous inhibition of both bile acid synthetic pathways may result in decreased hepatic cholesterol catabolism and overall bile acid production in the liver. Unlike CYP7A1, which is specifically expressed in the liver, CYP27A1 is also expressed in peripheral tissues such as macrophages and intestines and is thought to play a role in cellular cholesterol efflux by converting cholesterol into oxysterols [46, 47]. It was found that *CYP27A1* was upregulated by PPARγ activation in human macrophages [48, 49]. A PPRE was identified in the human *CYP27A1* promoter that specifically bound PPARγ/RXR heterodimer. Treatment of a PPARγ agonist caused an increased cholesterol efflux from human macrophages (Figure 3). Although how activation of PPAR isoforms led to tissue specific regulation of *CYP27A1* in the liver and macrophages is not clear, these findings are in general consistent with the roles of PPARs in inhibition of overall hepatic bile acid synthesis and stimulation of reverse cholesterol transport (see Section 4.1).

The ratio of CA to CDCA in the bile determines the hydrophobicity of the overall bile acid pool in humans, and may affect biliary cholesterol solubility in the bile. Hydrophilic bile acid ursodeoxycholic acid has been used clinically to dissolve cholesterol gallstones [50]. CYP8B1 regulates CA formation in the classic bile acid synthesis pathway and plays an important role in controlling the CA : CDCA ratio. Clofibrate treatment has been shown to increase CYP8B1 activity and mRNA level in rat liver microsomes [51]. Treating mice a PPARα agonist Wy14643 resulted in an up regulation of CYP8B1 mRNA levels and increased CA to CDCA/β-muricholic acid ratio, and knockout of *Pparα* reversed that [52]. A functional PPRE was identified in both mouse and rat *CYP8B1* promoter, suggesting a direct transcriptional activation of *CYP8B1* by PPARα. Bezafibrate treatment has been shown to increase the CA to CDCA ratio in human patients, which further suggests that PPARα regulation of CYP8B1 may likely be conserved in humans [40]. The observation that reduction in bile acid output by gemfibrozil in human was mainly a result of decreased CDCA in the biliary bile acid pool, while CA level was not significantly changed further suggested that PPARα activation of CYP8B1 could compensate the reduction in overall bile acid synthesis and maintain CA levels after fibrate treatment [38]. Since increased CA to CDCA ratio may favor cholesterol solubilization, the direct induction of CYP8B1 by PPARα may not contribute to the increased lithogenic index of the bile by synthetic PPARα agonists.

3.2. PPAR Regulation of Bile Acid Transport. Limited studies have implicated that PPARs may play a role in regulation of bile acid conjugation and transport in the liver and intestine. An early study showed that ciprofibrate feeding for two weeks resulted in a significant decrease of hepatic NTCP, OATP1 and BSEP in mice, and these effects were largely abolished in *Pparα* null mice [53]. This was supported by another study showing that down regulation of OATP and NTCP by perfluorinated fatty acids were PPARα-dependent [54]. Consistent with decreased expression of hepatic bile acid transporters, biliary bile acid concentration was also decreased by ciprofibrate [53]. Although this study did not evaluate the biliary cholesterol saturation, the reported increase in bile flow and decreased biliary cholesterol concentration following ciprofibrate treatment seemed to contradict previous observations in humans. Further studies are required to evaluate the role of PPARα in regulation of the hepatic and biliary bile acid transport systems. In the intestine, ASBT was found to be upregulated upon PPARα activation by ciprofibrate in Caco2 cells [55]. The intestinal bile acid binding protein (I-BABP) was also found to be induced upon PPAR activation in Caco2 cells [56]. Upregulation of ASBT and I-BABP presumably increases intestinal bile acid uptake and intracellular transport. However, it is unclear how PPARα may regulate intestinal OSTα/OSTβ and basolateral efflux of bile acids. Mice lacking functional OSTα/OSTβ heterodimer due to *Ostα* knockout showed significantly decreased bile acid pool and decreased serum bile acid concentrations [35]. Changes in bile acid concentration in hepatocytes and enterocytes may affect the activity of nuclear receptor FXR. FXR deficiency in the liver has been implicated in the gallstone formation in mice due to imbalanced expressions of cholesterol, bile acid, and phospholipid transporters [57]. Decreased basolateral bile acid efflux in *Ostα* null mice was associated with significantly decreased hepatic *Cyp7a1* expression, likely due to induction of intestinal fibroblast growth factor 15 (FGF15), which inhibits *Cyp7a1* expression via bile acid activation of FXR [35].

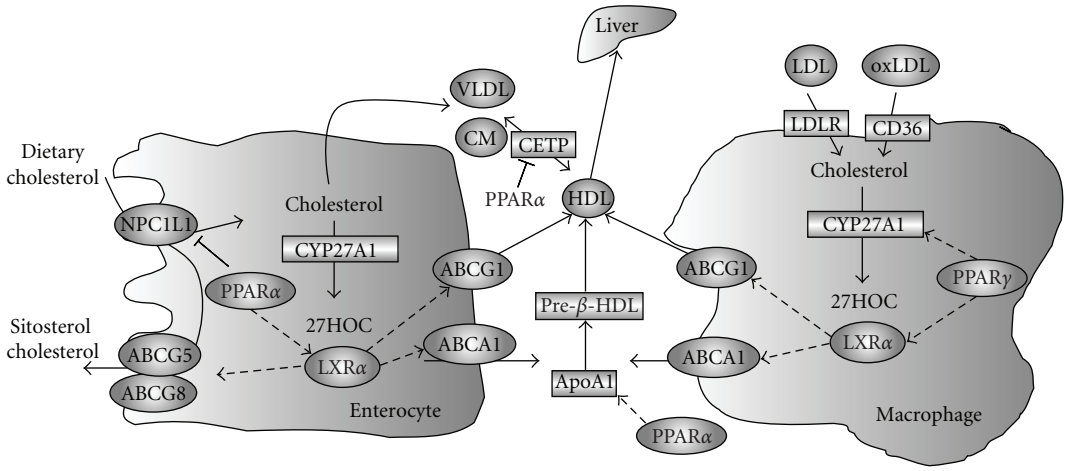

Figure 3: *Reverse cholesterol transport.* In the intestine, dietary uptake of cholesterol is mediated by NPC1L1. ABCG5/G8 effluxes sitosterols and cholesterol back to the intestine lumen and limits intestinal sterol absorption. Oxysterols activate LXR, which induces ABCA1 and ABCG1 to transport cholesterol to ApoA1 and HDL, respectively. PPARα activation reduces NPC1L1 and fractional cholesterol absorption, and may promote cholesterol secretion by stimulating CYP27A1 and LXR activation of ABCA1 and ABCG1. In macrophages, LDLR and CD36 mediate LDL and oxidized-LDL uptake, respectively. CYP27A1 converts cholesterol into 27-hydroxycholesterol, which may activate LXR and cholesterol efflux via ABCA1 and ABCG1. Cholesterol can also be secreted in the form of 27-hydroxycholesterol. PPARγ induces CYP27A1 and LXR, and positively regulates the cholesterol efflux from macrophages. PPARα induces ApoA1 and inhibits CETP, and thus increases circulating HDL-C levels.

4. PPAR Regulation of Cholesterol Metabolism

Cholesterol is not only an essential cell membrane component for maintaining normal cell functions but also the precursor to all steroid hormones, bile acids, and oxysterols, which are important regulators in diverse metabolic pathways. High intracellular cholesterol is toxic to the cells, and high serum cholesterol built up in the arterial walls will lead to the plaque formation, one of the initial steps in atherosclerosis development. Hypercholesterolemia is considered as one of the leading causes of many cardiovascular and heart diseases and has become a major health concern worldwide. Fibrates are used to treat dyslipidemia mainly for its ability to stimulate fatty acid oxidation, while TZDs are used to improve insulin sensitivity and glucose homeostasis. It is suggested that PPARs may play a role in the development of atherosclerosis by modulating cholesterol metabolism as well as alleviating inflammation in the liver and vasculature [58]. The PPAR regulation of the pathways related to whole body cholesterol homeostasis will be summarized below.

4.1. PPAR and Reverse Cholesterol Transport. Plasma lipoproteins are macromolecules that carry triglycerides, cholesterol, and other lipids for tissue distribution and metabolism. In the blood circulation, cholesterol is carried on LDL and HDL particles. Studies in the past decades have linked elevated plasma LDL-C to higher risks of cardiovascular incidence. Thus, developing therapeutic agents that efficiently decrease plasma LDL-C has been a major pharmacological effort for the prevention and treatment for coronary heart diseases. So far, the use of HMG-CoA reductase inhibitor statins has consistently shown adequate reduction of plasma LDL-C levels by inhibiting the de novo cholesterol synthesis in the liver and increasing LDL receptor-mediated clearance of serum cholesterol [59–62]. However, even with an adequate control of plasma LDL-C, only an approximate 20–35% reduction in major cardiovascular events was seen in a randomized clinical trial [63]. In fact, a significant percentage of patients had normal plasma LDL-C levels at the onset of major cardiovascular events [64]. Early clinical trials have found that the risk of cardiovascular disease shows an inverse correlation with plasma HDL-C levels, and low HDL-C has been considered as a risk factor for cardiovascular diseases [65, 66]. Compared to the efficacy of statin therapies in lowering plasma LDL-C, no therapies have been established so far to raise plasma HDL-C, and current studies are in search for therapeutic agents that raise plasma HDL-C levels as a means to achieve further risk reduction of cardiovascular events in human patients.

The ability of plasma HDL in reducing the risk of coronary heart disease resides in its physiological function to transport excess cholesterol from peripheral tissues to the liver for excretion or reutilization, a process that is referred to as reverse cholesterol transport (Figure 3). The role of PPARα in regulating HDL metabolism and promoting reverse cholesterol transport is supported by the clinical studies showing that fibrate treatments not only led to a marked reduction in plasma triglycerides but also caused about 5–15% increase in plasma HDL-C levels, with a modest reduction in LDL-C [67]. Accordingly, such induction of HDL-C can be translated into an approximately 25% reduction of the risk of coronary heart disease [68].

At the molecular level, fibrate effects on plasma HDL-C level are thought to be at least in part mediated by PPARα induction of the Apolipoprotein AI (ApoA-I). ApoA-I and ApoA-II consist of the major protein moiety on HDL

particles and serve as the receptor ligands for hepatic HDL uptake and metabolism [69]. HDL is initially synthesized by the liver and intestine. ApoA-rich and lipid-poor pre-β-HDL particles acquire cholesterol and phospholipids from peripheral tissues and circulating VLDL and chylomicrons, and then mature into HDL particles. Increased plasma ApoA-I by ApoA-I infusion or transgenic expression of *ApoA-I* were associated with increased plasma HDL-C and decreased atherosclerosis in experimental animal models [70–72]. PPARα activation by fibrates induced ApoA-I mRNA expression in human hepatocytes [73]. A PPRE has been identified in the human *ApoA-I* promoter [74]. Interestingly, PPARα effect on *ApoA-I* seems to be species-specific as PPRE is not conserved in rodent *ApoA1* genes [74]. PPARα activation in rodents actually decreased plasma HDL-C [73, 75], whereas genetic knockout of *Pparα* in mice showed increased ApoA-I mRNA expression and plasma ApoA-I and HDL levels [76]. Using human *ApoA-I* transgenic mice, Bertou et al. demonstrated that gemfibrozil increased hepatic human ApoA-I mRNA expression and plasma human ApoA-I and HDL levels [73], which provided an in vivo evidence that PPARα activation positively regulates plasma HDL and reverse cholesterol transport in humans.

The ATP-binding cassette transporter A1 (ABCA1) is expressed in liver, intestine, and macrophages. ABCA1 plays a central role in HDL formation by transporting intracellular cholesterol to pre-β HDL particles (Figure 3). Both human patients with nonfunctional ABCA1 due to autosomal recessive disorder (Tangier disease) and ABCA1 knockout mice showed extremely low plasma HDL levels, underscoring the importance of ABCA1 in HDL metabolism [77, 78]. Several independent studies evaluating the PPAR effect on macrophage cholesterol efflux have found that both human and mouse ABCA1 are induced upon PPARα and PPARγ activation, suggesting PPAR may have an anti-atherogenic function by regulating cholesterol efflux from macrophages and thus reducing foam cell formation [79, 80]. However, no PPRE has been identified in *ABCA1*, and PPAR induction of *ABCA1* expression may be an indirect effect. *ABCA1* is a direct target of the oxysterol receptor, liver orphan receptor α (LXRα), which induces ABCA1 in response to high cellular cholesterol activation [81]. *LXRα* expression is induced by both PPARα and PPARγ agonists in human and murine macrophages [82]. In *Pparγ* knockout mice, both ABCA1 expression and cholesterol efflux were reduced in macrophages [80]. PPRE has been identified in both human and mouse *LXRα* promoter [82, 83]. Results from these studies supported a PPARγ-LXRα-ABCA1 signaling cascade that mediates cholesterol efflux in macrophages. However, despite the critical role of cholesterol-laden macrophage in foam cell formation and development of atherosclerosis, it is believed that cholesterol efflux from macrophages may not contribute significantly to the total plasma HDL-C levels. Instead, liver and intestine represent the major sources of plasma HDL-C [84, 85]. PPARα activation by Wy14643 has been shown to induce *Abca1* expression in the mouse intestine [86]. However, as discussed in the next section, it seems that PPARs exert a negative effect on LXRα-dependent gene transcription in the hepatocytes via physical interaction with LXRα (next section). Thus, the relative contribution of this PPAR cascade in overall plasma HDL metabolism need to be further defined.

PPARs may also regulate several genes that are involved in HDL modification and metabolism. An important step in HDL metabolism is the cholesteryl ester transfer protein-(CETP-)mediated transport of triglycerides from VLDL and LDL to HDL in exchange for cholesterol esters. Mutations in *CETP* has been shown to increase plasma HDL levels with a modest reduction in LDL [87]. CETP is expressed in human but not mice. A recent study of human *CETP* transgenic mouse model showed that fenofibrate significantly reduced plasma CETP activity, which was correlated with elevated plasma HDL-C levels [88]. This study suggests that PPARα activation may inhibit plasma CETP activity in humans and may contribute to elevated HDL-C by fibrate treatment. However, the association between CETP inhibition and cardiovascular risk reduction remains controversial, as clinical trials showed that although inhibition of CETP significantly increased plasma HDL levels, further reduction of atherosclerotic progression was not seen in patients receiving torcetrapib/atrovastatin combined therapy compared to patients receiving atorvastatin alone [89, 90].

Given the potential role of fibrates in raising plasma HDL-C, the statin and fibrate combined therapy has been tested in several clinical trials. In these studies, addition of fibrate significantly increased plasma HDL when compared to statin alone [91–93]. Certain fibrate/statin combination therapies were well tolerated by the patients, while others showed side effects. Larger trials are needed to further evaluate the benefit and safety for using fibrate and statin combined therapies in the treatment of hyperlipidemia.

4.2. PPAR and Cholesterol Synthesis. To elucidate the mechanisms of cholesterol lowering effect by fibrates, a limited number of studies have been carried out to investigate the effect of PPARs on hepatic de novo cholesterol synthesis. It was shown that feeding wild-type mice a diet containing the Wy14643 significantly decreased hepatic cholesterol synthesis rate, as measured by in vivo ^3H_2O incorporation. Such reduction in cholesterol synthesis was not seen in *Pparα* knockout mice [94]. Similar reduction of HMG-CoA reductase activity and hepatic cholesterol synthesis was also seen in rats receiving clofibrate treatment [95]. Consistent to these studies, PPARγ agonist troglitazone has been shown to reduce cholesterol synthesis in hepatoma HepG2 cells and intestine Caco2 cells [96].

Recently, a few studies have indicated that the PPAR effect on de novo cholesterol synthesis may be mediated by PPAR-dependent inhibition of sterol response element binding protein-2 (SREBP-2) protein cleavage and maturation. SREBPs are transcriptional factors that regulate the expression of genes in cholesterol, fatty acid and triglyceride synthesis [97]. Three isoforms have been identified in mammals, SREBP-1a, SREBP-1c and SREBP-2. While SREBP-1 is believed to be mainly responsible for activation of genes in fatty acid and triglyceride synthesis, SREBP-2 preferentially stimulates genes in cholesterol synthesis and

uptake, including HMG-CoA reductase and LDL receptor (LDLR). SREBPs are synthesized as a 120 KDa precursor protein that forms a complex with SREBP cleavage activating protein (SCAP) and is localized in the ER membrane. Upon sterol depletion, SREBP is translocated to the Glogi apparatus where a two-step proteolytic cleavage process releases a mature form of SREBP that enters the nucleus and activates gene expression by binding to a consensus SRE sequence in the gene promoters. The retention of SREBP/SCAP complex in the ER depends on its binding to the endoplasmic reticulum resident proteins, insulin-induced gene-1 (Insig-1), and Insig-2. Insig-1 and insig-2 are highly expressed in the liver [98, 99]. Increased Insig-1, but not Insig-2, was associated with increased endoplasmic reticulum retention of SREBPs under high sterol conditions [99]. Kast-Woelbern et al. first reported that a PPARγ agonist rosiglitazone induced Insig-1 expression in the adipose tissue of diabetic db/db mice [100]. A functional PPRE was identified in *Insig-1* promoter and binds PPARγ. A similar induction in Insig-1, but not Insig-2, mRNA expression and a reduction of nuclear SREBP-2 by clofibrate was also reported in rats [101]. In a more recent study, Qin et al showed that PPARδ activation also induced Insig-1 in HepG2 cells [102], which correlated with a reduced amount of SREBP-1 mature form. The study by Qin et al. also showed that expression of PPARδ in *db/db* mice by adenovirus-mediated gene transfer induced Insig-1 expression, inhibited SREBP-1c maturation, and alleviated hepatic lipogenesis. Although increased Insig-1 expression represses the cleavage of both SREBP-1 and SREBP-2, these authors did not observe reduced expression of SREBP-2 target genes including LDLR and HMG-CoA reductase, indicating PPARδ may preferentially regulate SREBP-1c and hepatic fatty acid metabolism, but not cholesterol metabolism. It seems that three PPAR isoforms may regulate *insig-1* expression. However, since three PPAR isoforms exhibit different tissue expression profiles, activation of different PPAR isoforms by pharmacological agents may lead to somewhat distinct and tissue-specific effect on the activity of SREBPs, and thus fatty acid and cholesterol metabolism.

4.3. PPAR and Intestinal Cholesterol Absorption. Intestinal cholesterol absorption is thought to be coordinately regulated by Niemann Pick C1-Like1 protein (NPC1L1) and the ATP binding cassette half transporters ABCG5/G8 (Figure 3). NPC1L1 was first identified as a candidate gene for cholesterol transport based on its sequence homology to NPC1 [103]. NPC1L1 is highly expressed in the mouse small intestine. Genetic knockout of NPC1L1 in mice resulted in markedly decreased fractional cholesterol absorption [104]. In addition, fractional cholesterol absorption in *Npc1l1* knockout mice was insensitive to the inhibition by ezetimibe, a potent cholesterol absorption inhibitor, suggesting that NPC1L1 plays a central role in intestinal cholesterol absorption. ABCG5 and ABCG8 are expressed on the canalicular membrane of hepatocytes and the apical membrane of the proximal small intestine. They form functional heterodimers and transport dietary plant sterols and cholesterol into the bile or intestine lumen. ABCG5/G8 were identified as the defective genes in a rare genetic disorder called sitosterolemia, where patients showed markedly increased plasma and organ plant sterol levels due to increased intestinal absorption and decreased biliary secretion [105]. Consistent with the proposed function of ABCG5/G8 in cholesterol transport, transgenic overexpression of ABCG5/G8 in mice caused a significant increase in biliary cholesterol secretion and decreased intestinal fractional cholesterol absorption [106].

Gemfibrozil or Wy14643 have been shown to inhibit intestinal cholesterol absorption in rats and mice [86, 107]. Similarly, a potent PPARδ agonist GW610742 also reduces intestinal cholesterol absorption, which is correlated with decreased mRNA expression of NPC1L1 in the mouse intestine [108]. Recently, Valasek et al. showed that long-term fenofibrate administration inhibits NPC1L1 mRNA expression and fractional cholesterol absorption. These effects were abolished in *Pparα* knockout mice and further confirmed the role of PPAR in intestinal cholesterol absorption [109]. The molecular mechanism of PPAR inhibition of NPC1L1 is not clear, and is likely to be an indirect effect, secondary to changes caused by PPAR activation [109].

The mechanism of PPARs regulation of ABCG5/G8 is not known. Valasek reported that intestinal ABCG5 and ABCG8 are not involved in reduced cholesterol absorption in fenofibrate-fed mice [109]. In contrast, PPARα was implicated in the fasting-induced hepatic ABCG5/G8 expression in mice [110].

5. Crosstalk of PPAR with Other Nuclear Receptors in Cholesterol and Bile Acid Metabolism

5.1. PPAR Crosstalk with LXR. The LXR subfamily of nuclear receptor consists of two isoforms: LXRα and LXRβ. LXRα is expressed at high levels in liver, intestine and macrophages, while LXRβ is universally expressed in most tissues. LXRα is activated by oxysterols such as 22(S)-hydroxycholesterol, 24(S), 25-epoxycholesterol, and 27-hydroxycholesterol, whose levels are thought to be proportional to cellular cholesterol levels [111, 112]. Extensive studies in the past have established LXRα as a central regulator of tissue cholesterol homeostasis by regulating a network of genes in cholesterol metabolism and excretion. In rodent, but not human livers, LXRα stimulates conversion of excess cholesterol to bile acids by activation of hepatic *Cyp7a1* expression [113]. LXRα also stimulates the cholesterol efflux transporters ABCG5/G8 for biliary free cholesterol secretion [114]. In the intestine and liver, LXRα induces ABCA1 and ABCG1, which transport cholesterol to ApoA-I and HDL and thus promote reverse cholesterol transport [81, 115]. In macrophages, LXRα-dependent activation of ABCA1 and ABCG1 prevents cholesterol accumulation and atherosclerosis progression. Mice lacking LXRα are susceptible to high cholesterol diet induced hypercholesterolemia, while activation of LXRα by synthetic agonists show protective effects in hypercholesterolemic mice, demonstrating the

critical role of LXRα in maintaining whole body cholesterol homeostasis [116–118]. However, the development of potent LXRα agonist for treating hypercholesterolemia was hindered due to the lipogenic effect of LXRα activation [119, 120]. Mice receiving LXRα agonists showed significantly increased hepatic fatty acid synthesis and elevated plasma triglyceride levels. It is now clear that the lipogenic effect of LXRα is mainly due to its transcriptional activation of SREBP-1c [121, 122].

PPARα was identified as an interacting partner of LXRα in a yeast-two hybrid assay [123]. It has been shown that LXRα interaction with PPARα blocked PPARα/RXR heterodimer binding to the PPRE and resulted in inhibiting PPARα target genes. Another study showed that PPARα/LXRα interaction was enhanced by addition of an LXRα agonist TO901317, and PPARα/LXRα interaction reduced PPARα/RXRα heterodimer formation [124]. In mice fed TO901317, PPARα-regulated genes in hepatic fatty acid oxidation were repressed suggesting that activation of LXRα may repress hepatic fatty acid oxidation via inhibition of PPARα transcriptional activity. In contrast, LXRα activation induces PPARα in mouse intestine [125, 126]. Further more, activation of LXRα by a specific agonist induced not only LXRα target genes but also PPARα target genes in mouse intestine. As discussed in the previous section, PPARα is also implicated in the intestinal cholesterol absorption and transport process. Thus, the identification of intestine-specific LXRα-PPARα signaling cascade may provide an additional pathway for LXRα/PPARα in coordinated regulation of cholesterol metabolism in the intestine.

The effect of PPARs on LXRα-dependent transcriptional network has also been studied, and both positive and negative effects have been reported. Polyunsaturated fatty acids (PUFA) inhibit hepatic lipogenesis by decreasing SREBP-1c mRNA and protein in cultured hepatocytes and animal livers [127, 128]. A study by Yoshikawa et al. suggested that activation of PPARα caused decreased LXRα/RXR binding to the SREBP-1c gene promoter and resulted in down regulation of SREBP-1c and lipogenic gene expression [121, 129]. The finding that PPARα activation inhibits SREBP-1c is in agreement with the known function of PPARα in stimulating hepatic fatty acid oxidation and its lipid lowering effect in humans. Consistent with this notion, Matsusue et al. reported that activation of PPARδ downregulated angiopoietin-like protein 3 gene in lipid metabolism via an LXRE on the angiopoietin-like protein 3 gene promoter [130]. However, these findings seem to contradict the existing reports that activation of both PPARα and PPARγ in macrophages induces LXRα gene expression and LXRα-dependent cholesterol efflux [79, 80, 130]. Because functional PPRE has been identified in both human and mouse LXRα gene promoter, the lack of activation of LXRα by PPARs in the liver is still not fully understood [82, 83]. In general, fibrate therapy showed protective effect against atherosclerosis in men. However, studies with fibrate administration in mice yielded mixed results. In hyperlipidemic LDLR knockout mice, activation of PPARα or PPARγ have been shown to prevent atherosclerosis and foam cell formation, and such protective effect seems to involve ABC-dependent cholesterol efflux pathways [131]. Similar antiatherogenic effects of PPARs were also found by studies using ApoE knockout mice [132, 133]. In contrast, genetic deletion of PPARα in ApoE knockout mice resulted in more severe atherosclerosis [134]. Another study reported that ciprofibrate treatment in ApoE knockout mice promoted the progression of atherosclerosis [135].

5.2. PPAR Crosstalk with FXR. It is reported that bile acids, acting through nuclear receptor FXR, induced human PPARα gene in HepG2 cells [136]. It is known that activation of FXR by bile acids or a synthesis FXR agonist negatively regulates hepatic fatty acid synthesis and plasma triglyceride levels [137]. The lipid lowering effects of bile acids are thought to be attributable to the inhibition of SREBP-1c activity in the liver. In concert, FXR induction of PPARα may be an additional mechanism to antagonize hepatic SREBP-1c activity and promote hepatic fatty acid oxidation. However, the FXRE on the human *PPARα* is not conserved in murine *Pparα* gene promoter [136]. Consistent with this finding, mice fed a diet supplemented with bile acids antagonized PPARα agonist effect [138]. It seemed that bile acid-activated FXR/SHP pathway was not involved in such regulation as bile acids still inhibited PPARα activity in *Fxr* knockout mice [138]. Bile acid-activated cellular signaling pathways may be implicated in the negative regulation of PPARα in mice. SHP is generally considered as a negative regulator by interacting with other nuclear receptors and transcriptional factors. Both PPARα and PPARγ physically interact with SHP [139, 140]. Surprisingly, both studies found that SHP was able to enhance PPARα- and PPARγ-mediated transcriptional activity. The study by Nishizawa et al. showed that SHP competed with corepressrors for the binding to PPARγ, which provided a possible explanation of SHP effect on the transcriptional activity of PPARs [140]. As SHP does not possess intrinsic transcriptional activity, the positive effect of SHP on PPAR transcriptional activity was somehow unexpected, and the physiological relevance of the role of SHP in regulating PPAR signaling needs to be further defined in future studies.

6. Conclusion and Future Perspectives

In the past decades, the roles of PPARs have been extended from stimulating fatty acid oxidation and glucose metabolism to regulating cholesterol and lipoprotein metabolism, bile acid metabolism, energy homeostasis and inflammation, and so forth. Currently, most of the regulatory roles of PPARs turned out to be beneficial in improving dyslipidemia and glucose homeostasis and reducing the risks of major cardiovascular and heart events, while others may represent adverse effects associated with the use of certain PPAR agonists. With the ability of PPARs to crosstalk with other protein factors and cellular signaling pathways, it is not surprising that more regulatory roles of PPARs have been revealed. Long-term fibrate therapy represses hepatic bile acid synthesis and increases the incidence of cholesterol gallstone. However, the role of PPAR in bile acid

transport are still not clear. The use of PPAR agonists for treating steatohepatitis has also been considered due to their known effects in fatty acid metabolism, inflammation, and hepatic fibrosis [141–145]. In addition to the repressive effect on hepatic bile acid synthesis, PPARα have recently been implicated in the positive regulation of bile acid conjugation and toxicity [146–149]. Whether these regulatory roles of PPARα represent any beneficial effects in cholestatic liver injury needs to be further explored. PPAR agonists have been proven to be a group of drugs with great therapeutic potentials in treating metabolic syndromes. Clinical trials are being conducted to evaluate the efficiency and safety of fibrate/statin combined therapy. Given the increasingly recognized epidemic of obesity, diabetes, and chronic liver diseases associated with metabolic disorders, more potent and selective PPAR agonists need to be developed to achieve desirable biological effects and to avoid adverse effects. The development of such agents will depend on a better understanding of the regulatory roles of PPARs in diverse biological processes beyond triglyceride and glucose metabolism.

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Review Article

A Human Hepatocyte-Bearing Mouse: An Animal Model to Predict Drug Metabolism and Effectiveness in Humans

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Preclinical studies to predict the efficacy and safety of drugs have conventionally been conducted almost exclusively in mice and rats as rodents, despite the differences in drug metabolism between humans and rodents. Furthermore, human (*h*) viruses such as hepatitis viruses do not infect the rodent liver. A mouse bearing a liver in which the hepatocytes have been largely repopulated with *h*-hepatocytes would overcome some of these disadvantages. We have established a practical, efficient, and large-scale production system for such mice. Accumulated evidence has demonstrated that these hepatocyte-humanized mice are a useful and reliable animal model, exhibiting *h*-type responses in a series of in vivo drug processing (adsorption, distribution, metabolism, excretion) experiments and in the infection and propagation of hepatic viruses. In this review, we present the current status of studies on chimeric mice and describe their usefulness in the study of peroxisome proliferator-activated receptors.

1. Introduction

The human (*h*)-body consists of approximately 30 organs, each of which fulfills a specific function, autonomously yet cooperatively with other organs, to maintain life. The liver is essential to (*h*)-life, as it participates in the control of energy balance and plays central roles in the metabolism and excretion of ingested food and chemicals. Knowledge of the mechanisms underlying the functions of the *h*-liver is important for understanding the biology of the liver as well as for clinically treating liver-damaged patients and for studying drug pharmacology in humans. The ideal approach to elucidating the mechanisms responsible for liver functions would be to perform experiments using the *h*-liver *in situ*, but of course this approach is not realistic. Therefore, scientists have taken two other approaches: in vitro examination of samples isolated from the *h*-body (in vitro/human), and in vivo examinations using animals (in vivo/animal). Although these two approaches, separately and together, have revealed much about the mechanisms governing the functions and morphology of the *h*-liver, they are inherently limited by the complexity of the biological phenomena and the species differences in homologous mechanisms between animals and humans.

The complexity of a biological phenomenon results from the required mutual interactions of multiple different components. The specific cells that represent an organ's functions are collectively termed parenchymal cells. For example, the parenchymal cells of the liver are hepatocyte, because they perform liver-specific functions such as the synthesis and secretion of serum proteins and the synthesis of metabolism-related enzymes, including liver-specific cytochrome P450 (CYP450) proteins. However, hepatocytes by themselves are unable to fulfill liver functions and require the cooperation of nonparenchymal liver cells such as hepatic blood vessels, bile duct biliary cells, Kupffer cells, and stellate cells in the space of Disse, located between the hepatic plate and the sinusoids [1]. The portal vein is the major import route for nutrients to the liver, via the hepatic sinusoids, from the small and most of the large intestine, the spleen, and the pancreas. Nutrients and oxygen in the sinusoids and secretory proteins in the hepatocytes are exchanged through the space of Disse. Stellate cells, the major cell type producing extracellular matrix components in the liver, are located

adjacent to the hepatocytes and the sinusoidal endothelial cells [2]. Hepatocytes, endothelial cells, and stellate cells represent 65, 21, and 6%, respectively, of the *h*-liver and are the main cells responsible for liver functions [1].

Interactive cooperation among different cells types is a principal way in which a multicellular entity is able to function as a living system. It is also a major source of the limitations in in vitro/human studies. To date, no studies have successfully reconstituted an in vitro/*h*-liver system that perfectly mimics the events that occur in the *h*-liver in vivo. This limitation has prompted a search for an in vivo/animal experimental system appropriate for providing animal data that can be extrapolated to humans. However, animal models must address the challenge of species differences in the genes and proteins associated with a biological phenomenon.

The liver processes nutrients from the gut and intestines into proteins, lipids, and carbohydrates. It also serves an endocrine function by secreting albumin (Alb), most coagulation factors, several plasma carrier proteins, and lipids into the blood. In addition, the liver synthesizes bile and secretes it into the digestive tract. The elaborate histological structure of the liver optimizes these functions [3]. Hepatocytes are well organized in an aggregated association (the hepatic epithelium) of polarized hepatocytes, creating small apical domains that line the channels between cells (canaliculi). These channels connect to the bile ducts, which drain into the intestine. The basal sides of the hepatocytes are juxtaposed to the fenestrated endothelium of the sinusoids, into which blood flows from the arterial and intestinal portal circulations before emptying into the venous circulation [4].

h-Hepatocytes are indispensable for an in vitro/human liver study. Nevertheless, the preparation of *h*-hepatocytes in sufficient numbers for experimental purposes is difficult because the source is very limited and because *h*-hepatocytes do not abundantly proliferate and grow in vitro. This led us to create a mouse (*m*) bearing a liver composed almost entirely of *h*-hepatocytes. This approach may simultaneously abolish the limitations of both in vitro/human and in vivo/animal studies. With this *m*-model, a small number of available *h*-hepatocytes could abundantly proliferate in the *m*-liver for use in in vitro/human studies. Furthermore, these mice would provide a superior new type of model animal for in vivo/animal studies, because fewer species differences would exist with respect to liver functions.

We have called this type of mouse a "liver-humanized mouse," or simply a chimeric mouse, although the correct name should be "hepatocyte-humanized mouse." The idea of a *h*-liver chimeric mouse was originally described by Brinster's group in 1995 [5] and was actualized by the two groups in 2001 to study *h*-hepatitis B virus (*h*-HBVs) [6] and *h*-HCV infections [7], and later, in 2004, by us to study the in vivo growth capacity of *h*-hepatocytes and the gene and protein expression of CYPs [8]. One year later, a detailed morphological study of a chimeric *m*-liver was reported by Meuleman et al. [9]. Kneteman and Mercer briefly reviewed the current chimeric mouse studies [10]. In this article, we review the studies on chimeric mice, including their short historical background, usefulness in testing *h*-type metabolism of clinically usable drugs, and potential use in examining *h*-type peroxisome proliferator-activated receptors (PPARs), especially PPARα, which plays key roles in the metabolism of xenobiotics in an animal species-dependent manner. We demonstrate that *h*-hepatocytes propagated in a chimeric *m*-liver and then isolated can serve as normal *h*-hepatocytes for an in vitro/human model [11].

2. A Mouse Bearing Transplanted Homogenic and Xenogenic Hepatocytes

To study neonatal bleeding disorders, transgenic mice ($Tg_{Alb\text{-}uPA}$) carrying a tandem array of about four Albumin promoter/enhancer-driven urokinase-type plasminogen activator (uPA) genes were created [12]. Their hepatocytes over-produce murine urokinase, and the liver becomes severely hypofibrinogenemic, which accelerates hepatocyte death. Sandgren et al. [13] developed a model of liver regeneration in $Tg_{Alb\text{-}uPA}$ mice, in which a chronic stimulus for liver growth was generated due to a functional liver deficit. When a hepatocyte stochastically deleted the deleterious transgene, the hepatocytes of mice hemizygous for the transgene started to replicate and selectively expanded to regain the original size of the liver. Transgene expression in the replicating hepatocytes was abolished because of a DNA rearrangement that affected the transgene tandem array. This permitted the individuals to survive beyond birth, and the plasma uPA concentrations gradually returned to normal by 2 months of age. The transgene-deficient cells formed clonal colonies called hepatic nodules. These nodules expanded and replaced the surrounding transgene-active cells, which could not replicate because of cellular damage. Eventually, the transgene-deficient cells replaced the entire liver. This study demonstrates the usefulness of the $Tg_{Alb\text{-}uPA}$ mouse for examining the replicative capacity of not only *m*-hepatocytes, which was successfully done by transplanting hepatocytes isolated from adult mice into the transgenic mice [14], but also hepatocytes of mammals that acquire immunotolerance as follows.

Rhim et al. [5] introduced the Alb-uPA transgene into immunotolerant nu/nu mice by mating $Tg_{Alb\text{-}uPA}$ mice with Swiss athymic nude mice, generating immunotolerant $Tg_{Alb\text{-}uPA}$ mice ($Tg_{Alb\text{-}uPA}$/NUDE mice). Rat (*r*) liver cells were transplanted into the livers of $Tg_{Alb\text{-}uPA}{}^{+/+}$/NUDE mice homozygous for the transgene. The host livers that had not been transplanted with *r*-liver cells were completely pale (white). In contrast, those with *r*-liver cells had white regions, representing the area composed only of transgene-expressing host *m*-cells, and red regions, representing the area composed of transgene-deleted host *m*-cells, repopulated *r*-cells, or both. Immunohistochemical analysis with antibodies against *r*-hepatocytes confirmed that the red region was composed primarily of *r*-hepatocytes. The completely regenerated transgenic *m*-livers resemble normal *m*-livers in color, shape, and size. Southern blot analysis demonstrated that up to 56% of the DNA was of rat origin, which agreed well with the parenchymal cell occupancy rate in the liver. These findings strongly support the idea that the host liver was chimeric, with *r*-parenchyma and *m*-nonparenchymal cells,

which included vessels, bile ducts, and associated connective tissues. The ratio of the liver weight to the body weight was 6.8%, which was similar to that of the non-transgenic control mice (5.8%), indicating that the rat-mouse (*r*/*m*) chimeric livers were able to normally terminate growth. The successful generation of a healthy mouse with a chimeric liver indicates that *r*-parenchymal and *m*-nonparenchymal cells were able to communicate with each other to reconstitute a functional liver, despite the species difference.

Hepatocytes initiate and terminate proliferation under the influence of nonparenchymal cells [1]. Thus, the normal progression and termination of *r*/*m*-chimeric liver regeneration implies that *r*-hepatocytes produce surface proteins that interact correctly with soluble *m*-factors, *m*-extracellular matrix, and *m*-surface proteins on *m*-nonparenchymal cells. The successful replacement of $Tg_{Alb\text{-}uPA}{}^{+/+}$/NUDE*m*-livers with *r*-hepatocytes raised the exciting possibility that *m*-livers could also be reconstituted with *h*-hepatocytes [5].

3. Repopulation of *h*-Hepatocytes in *m*-Liver

In two previous studies to generate a mouse with a *h*-hepatocyte-mouse (*h*/*m*) chimeric liver, Rug-2-knockout mice [6] and severe combined immunodeficient (SCID) mice [7] were used as immunodeficient mating partners for uPA transgenic mice. We mated SCID mice ($mice_{SCID}$) with $Tg_{Alb\text{-}uPA}{}^{+/+}$ mice to yield liver-injured SCID mice ($mice_{Alb\text{-}uPA/SCID}$) [8]. Normal *h*-hepatocytes, $\sim 10^6$ viable cells per mouse, were transplanted into the livers of these mice at 20–30 days after birth. The *h*-hepatocytes engrafted the liver at rates as high as 96% and progressively repopulated it. The repopulation after *h*-hepatocyte transplantation was easily monitored by the increase in the *h*-Alb concentration in the host blood, and the expansion of *h*-hepatocyte colonies was visualized by immunohistological staining of liver sections with *h*-specific anti-cytokeratin (CK) 8/18 antibodies. The ratio of the number of engrafted *h*-hepatocytes to total hepatocytes (*m*- and *h*-hepatocytes) in the host liver, which is the replacement index (RI), was determined by calculating the ratio of the area occupied by hCK8/18-positive hepatocytes to the entire area examined in immunohistochemical sections of seven lobes. It was demonstrated that sustained engraftment of *h*-hepatocytes occurs in homozygous Alb-uPA transgenic ($Tg^{+/+}_{Alb\text{-}uPA}$) mice, but not in hemizygous transgenic ($Tg^{+/-}_{Alb\text{-}uPA}$) mice. The *h*-hepatocytes started to proliferate around 7 days after transplantation. Their colonies gradually became larger and were almost confluent at around 70 days, when the RI was as high as 96%. Immunohistological staining of liver sections for type IV collagen, laminin, stabilin (a liver endothelial cell marker), BM8 (a Kupffer cell marker), and desmin (a hepatic stellate cell marker) demonstrated the chimeric nature of the liver (Figure 1). The interactions between hepatocytes and stellate cells are critical for physiological and pathological conditions of the liver [15]. Close and seemingly normal associations of *h*-hepatocytes with *m*-stellate cells were immunohistologically visualized by staining with specific antibodies against *h*-CK8/18 (*h*-hepatocytes) and *m*-desmin (*m*-stellate cells) (Figure 2). These results clearly show that the chimeric *m*-livers with a high RI consisted of parenchymal cells (mostly *h*-cells with a low percentage of *m*-cells), *m*-nonparenchymal cells, and *m*-ECMs, in agreement with a previous study [9]. There was good correlation between the RI and the mRNA expression levels of housekeeping genes such as *h*-Alb and *h*-transferrin, supporting the notion that transplanted *h*-hepatocytes are functional [16]. In our experience, mice with >6 mg/mL *h*-Alb in the blood had an RI >70%. Our histological studies illustrated that the *h*-hepatocytes were well organized and surrounded by *m*-nonparenchymal cells, and they reconstituted the normal tissues specific to a normal functional liver (described in Section 1), despite the large species difference between humans and mice.

We chose robustly growing young mice as hosts. These mice were able to not only survive but also grow, although relatively slowly, and increase their body weight by >50% of their original weight, during the replacement of host *m*-hepatocytes with *h*-counterparts. These simple animal experiments made us realize that *m*-cells and *h*-hepatocytes were able to mutually communicate to maintain life: *m*-cells supported the proliferation of *h*-hepatocytes, and *h*-hepatocytes supported the growth of the young mouse. The host liver of a $mouse_{Alb\text{-}uPA/SCID}$ is congenitally damaged owing to uPA overproduction, low blood levels of Alb, and significantly high levels of alanine aminotransferase (ALT). Repopulation of the *h*-hepatocytes in the liver increased the blood Alb concentration and decreased the ALT level, indicating that *h*-hepatocytes contributed to the improvement of *m*-liver function [8]. Based on these considerations and findings, we expect that a *m*-liver made of *h*-hepatocytes would function as an apparently normal liver, metabolizing and detoxifying endogenous and exogenous biomolecules.

4. Expression Profiles of *h*-Cytchrome P450s in Relation to Phase 1 Metabolic Enzymes

Biochemical treatment of foreign substances (xenobiotics) that have been absorbed into the body is one of the major tasks of the liver. In hepatocytes, xenobiotics are processed to more stable and hydrophilic derivatives by groups of enzymes, collectively called xenobiotic-metabolizing enzymes (XMEs), via two phases: phase 1, which is accomplished by oxidative enzyme, and phase 2, performed by conjugating enzymes [17]. Ingested drugs, toxicants, and chemical carcinogens are metabolized in phase I by CYP and the flavin-containing monooxygenase superfamily. Notably, CYP is the key enzyme in the elimination of clinical drugs.

Humans and rodents respond differently to xenobiotics, and this is explained in part by species differences in CYP subfamilies. These species differences raise serious issues in research for clinically usable medicines, because the results of xenobiotic metabolism studies with mice and rats, which are the most commonly used experimental models for pharmacological and toxicological studies, cannot be extrapolated to humans. Thus, information about the expression of CYP families and subfamilies should be valuable from two viewpoints. First, the expression in *h*/*m*-chimeric mice of

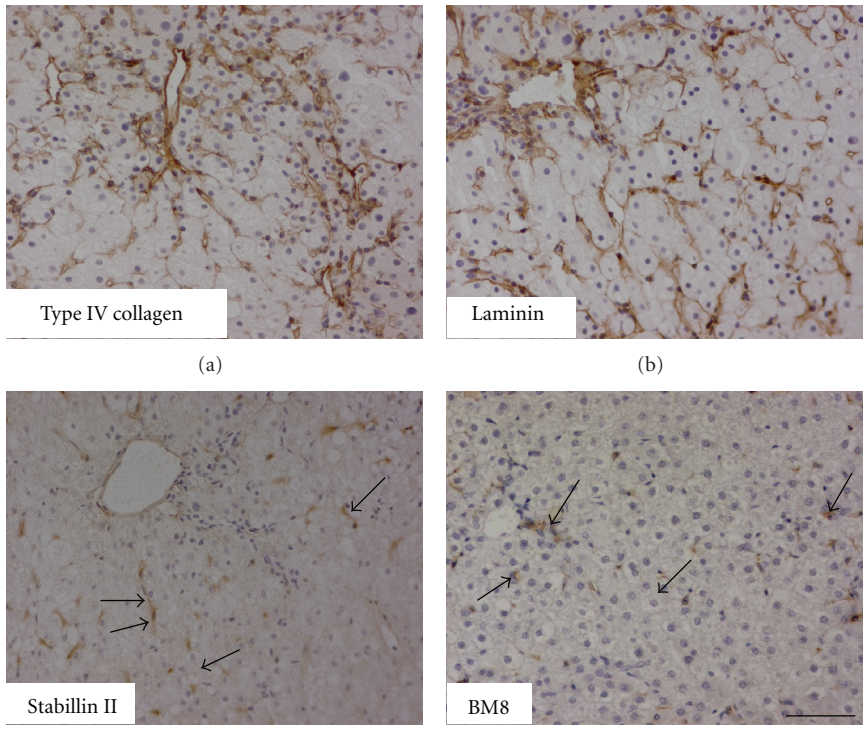

Figure 1: The histological harmonization of *h*-hepatocytes with *m*-nonparenchymal cells. uPA/SCID mice were transplanted with *h*-hepatocytes and allowed to grow until the repopulation of the liver was complete. Then, liver sections were prepared from the *h*-hepatocyte-chimeric mice. Sections were immunostained with *m*-specific antibodies for type IV collagen (a); laminin (b); stabillin (c), a marker of liver endothelial cells (a gift from Dr. A. Miyajima, Tokyo University); and BM8 (d), a marker of Kupffer cells. The immunosignals are brown. The arrows in (c) and (d) point to typical immunopositive cells. Bar, 100 μm.

a CYP subtype that is found in *h*-hepatocytes, but not in mice, would be a good indication that the *h*-hepatocytes are biochemically functional in the *m*-iver. Second, the *h*-CYP-expressing chimeric mouse is a useful experimental model for studying *h*-type metabolic responses to xenobiotics, including clinically valuable drugs. Of note, CYP3A4 is the most abundantly expressed CYP in *h*-liver and metabolizes >60% of all therapeutic drugs; collectively, CYP2D6 and CYP3A4 metabolize >70% of the drugs on the market [17].

Species differences in the CYP2C subfamily are well known and have been characterized intensively [18, 19]. The *h*-liver contains four CYP2C isoforms, CYP2C8, CYP2C9, CYP2C18, and CYP2C19, all of which are absent from mice and rats. Western blot analyses using *h*-specific antibodies against CYP2C9 revealed positive signals with hepatocytic microsomal fractions from *h*/*m*-chimeric mice with an RI >34% and from the donor, but not with hepatocytic microsomal fractions from chimeric mice with an RI <28% or from mice that had not been transplanted with *h*-hepatocytes. CYP2C9 catalyzes the 4′-hydroxylation of diclofenac, and the microsomal fractions from the chimeric mice showed diclofenac 4′-hydroxylation activity that depended on the RI of the mouse, strongly suggesting that the *h*-hepatocytes in chimeric livers retain *h*-type pharmacological activity toward drugs. One of the clearest and best-defined examples of a difference in a CYP between mice and humans is CYP2D6 [20, 21], which is involved in the metabolism of a large number of clinically used drugs [22, 23]. In humans, CYP2D6 is the only active member of the CYP2D subfamily, whereas rats and mice do not express a protein with the enzyme activity of *h*-CYP2D6, although they do have at least five other CYP2D genes [20, 24]. The enzymatic activity of *h*-CYP2D6 in the chimeric mouse was demonstrated by orally administering debrisoquin, a *h*-CYP2D6 substrate, to the mice and subsequently detecting 4′-hydroxydebrisoquin, a major debrisoquin metabolite produced by *h*-CYP2D6, in the blood of the mice. Pretreatment of the mice with quinidine, a typical *h*-CYP2D6 inhibitor, decreased the level of the metabolite. Thus, a CYP enzymatic activity in the chimeric mice was specifically induced by a CYP2D6-metabolized drug and specifically suppressed by a CYP2D6 inhibitor [25].

Among the known CYP families, four families (CYP1–4) play primary roles in XMEs. We compared the mRNA and protein expression profiles of six *h*-CYPs, CYP1A1, 1A2, 2C9, 2C19, 2D6, and 3A4, in the chimeric *m*-liver with those in the donor liver [8]. Total RNA was prepared from the livers of chimeric mice with different RIs and of donors, and the mRNA for the six *h*-CYPs was amplified in a quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR).

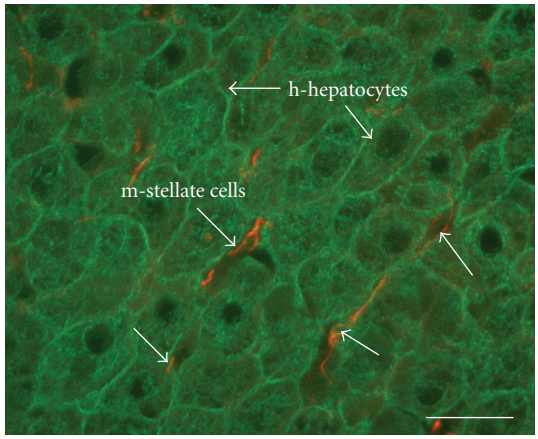

Figure 2: Close natural apposition of *m*-stellate cells and *h*-hepatocytes in a chimeric liver. A serial section shown in Figure 1 was doubly stained with *h*-CK8/18 (green) for *h*-hepatocytes and *m*-desmin (orange) for *m*-stellate cells. The *h*-hepatocytes are well organized and closely apposed to *m*-stellate cells in Disse's space. Arrows indicate representative *h*-hepatocytes (green) and *m*-stellate cells (orange). Bar, 10 μm.

All six mRNAs were amplified to detectable levels, which were higher in mice with higher RI values. Thus, the *h*-hepatocytes in the chimeric mice appeared to express the six *h*-CYP genes in a manner similar to their expression in the *h*-body.

We then asked whether these normally expressed *h*-CYPs in the *h*/*m*-chimeric liver were inducible in a drug-specific manner. The *h*-CYP3A4 and *h*-CYP1A subfamilies specifically respond to rifampicin and 3-methylcholanthrene (3-MC), respectively [26]. Chimeric mice with *h*-hepatocytes were injected intraperitoneally with rifampicin or 3-MC, once per day for 4 days. The mRNA levels of the six *h*-CYPs in the liver tissues were measured 24 h after the last injection. Rifampicin treatment enhanced the expression of *h*-CYP3A4 by 5.8-fold, but did not affect the levels of the other five *h*-CYPs. The administration of 3-MC enhanced CYP1A1 and CYP1A2 mRNA levels by 10.0-fold and 6.4-fold, respectively, but had no effect on the other four CYPs. Neither rifampicin nor 3-MC induced the expression of any of the six *h*-CYPs in mice$_{\text{Alb-uPA/SCID}}$ that had not been transplanted with *h*-hepatocytes. Rifabutin, an analogue of rifampicin, also specifically induced *h*-CYP3A, but not the host murine Cyp3a, in the chimeric *m*-liver [27]. The degree of CYP3A4 induction in the chimeric mouse has practical applications in drug testing, because many drugs are CYP3A4 substrates and the induction of CYP3A4 decreases the pharmacological potency of these drugs [17].

Rifampicin is a ligand for the pregnane X receptor (PXR), which forms a heterodimer with retinoid X receptor a (RXRa). Rifampicin/PXR/RXRa subsequently binds to a xenobiotic response element (XRE) composed of the direct repeat of alpha and beta half-sites separated by four nucleotides on the CYP3A4 gene, thereby upregulating its expression in phase 1 [28]. Rifampicin is a potent activator of human and rabbit PXR, but has little activity in the rat and mouse [29]. Thus, that the liver data of *h*/*m*-chimeric mice faithfully reflect those in humans. The binding of 3-MC to the aryl hydrocarbon receptor (AHR) forms a AHR/3-MC complex, which upregulates CYP1A1, CYP1A2, and CYP1B1 expression by binding, together with the AHR nuclear translocator (ARNT), to the XREs of these genes [30]. Our studies suggest that these known ligand-activated receptor signaling pathways activated by rifampicin and 3-MC are functional in the *h*/*m*-chimeric *m*-liver. Thus, we propose that the hepatocyte-humanized mouse will be a useful animal model in studies of *h*-type signaling pathways that regulate gene expression induced by xenobiotics.

5. Humanization of Phase II Conjugation Pathway of a Drug in *h*/*m*-Chimeric Mice

It is estimated that phase II conjugation accounts for approximately >30% of drug clearance [31], especially of compounds with polar groups. The major hepatic phase II enzymes in humans are UDP-glucuronosyltransferase (UGT), which is responsible for glucuronidation; sulfotransferase (SULT), for sulfation; *N*-acetyltransferase (NAT), for acetylation; and glutathione *S*-transferase (GST), for glutathione conjugation. We examined the mRNA and protein expression and the enzyme activity of the *h*-forms of these enzymes in chimeric mice with livers having RI values ranging from 0 to 90% [32]. The chimeric livers expressed *h*-UGT, *h*-SULT, *h*-NAT, and *h*-GST mRNA and the UGT2B7, SULT1E1, SULT2A1, and GSTA1 proteins at levels that correlated with their RI values. Activities of related enzymes such as morphine 6-glucuronosyltransferase and estrone 3-sulfotransferase were also detected in an RI-dependent manner. The protein content and enzyme activities of phase II-associated enzymes in chimeric *m*-livers with an RI of approximately 90% were similar to those in the donor liver. In a separate study, we systematically compared the mRNA expression profiles for 26 phase II *h*-enzymes, including GST, SUL, NAT, and UGT members, between livers of chimeric mice with RIs of 71–89% and donor livers [16]. All of the tested enzyme genes were detected. For 65% of the tested genes, the expression levels in the chimeric livers were 30 to 55% of the levels in the donor livers; although lower, these values are comparable to the RI values. These results indicate that the hepatic phase II biotransformation of a drug is appreciably humanized in the *h*/*m*-chimeric mouse.

There are groups of drugs in clinical use that bind to PXRs or constitutive androstane receptors (CARs). The ligand-activated PXRs and CARs are involved in the regulation of some phase II XME genes such as SULT1A, UGT1A, and GST [33–35]. Thus, it is likely that these *h*-type ligand-activated transcriptional regulators are functional in *h*/*m*-chimeric *m*-livers, suggesting that these chimeric mice will contribute to studies on the regulation of gene and protein expression of these transcription factors in relation to xenobiotic metabolism.

6. Drug Transport through the Chimeric *m*-Hepatocyte Membrane

Drug transport in the liver is largely performed by two systems: extrahepatic-to-hepatic transport using transporters such as organic cation transporter 1 (OCT1), organic anion transporting polypeptide (OATP) 1B1, and OATP1B3; and hepatic-to-bile duct transport using adenosine 5′-triphosphate-binding cassette (ABC) proteins, including P-glycoprotein, bile salt export pump (BSEP/ABCB11), breast cancer resistance protein (BCRP/ABCG2), and multidrug resistance-associated protein 2 (MRP2) [36]. The former transporters are located on the sinusoidal membrane and are responsible for the uptake of drugs into hepatocytes; the latter are on the canalicular membrane and are responsible for biliary excretion of the metabolites. The *h*-genes of these transporting systems were preferentially expressed compared with the *m*-counterpart genes in chimeric mice with RIs >60% [36]. Cefmetazole (CMZ), a cephalosporin antibiotic, is excreted without any chemical modification, through urinary and biliary pathways. The urinary pathway is dominant in humans [37], whereas rats [38] and mice [36] use the biliary pathway. Before receiving *h*-hepatocytes, the host mice excreted CMZ primarily through the biliary pathway, but the urinary pathway was dominant in chimeric mice with RIs >60% [36].

The *h*-ABCB4 transporters have been characterized in relation to fibrate-metabolism [39]. In addition, we examined the expression levels of 21 *h*-transporter genes, including members of the ABC, solute carrier (SLC), and OATP families, in the livers of chimeric mice with RIs ranging from 71 to 89%, with respect to the levels in donor livers [16]. For 62% of the tested genes, the expression ratios in the chimeric livers were 0.35 to 0.75. From these limited data, it appears that most of the *h*-type transporter genes were expressed in the chimeric *m*-liver.

7. Infectivity of Chimeric Mice with *h*-Hepatitis Viruses

h-Liver diseases caused by HBV and HCV, especially HCV, are targets for the discovery of efficient antivirus drugs, worldwide [40]. However, the development of effective therapeutics has been hampered by the lack of useful in vitro and in vivo models of viral replication. For example, cultured *h*-hepatocytes are not appropriate as recipient cells for viral propagation, and rodents are not useful animal models because of the strict species specificity of viral infection [41]. Viral infectivity and propagative potential in the *h*/*m*-chimeric mouse would be persuasive evidence for concluding that it was actually "humanized." A research group led by Kneteman first challenged chimeric mice with an inoculation of HCV-infected *h*-serum, which produced a virus-infected model mouse [7]. Owing to their substantial advantage in both magnitude and duration of *h*-hepatocyte engraftment, homozygous animals were superior to their hemizygous counterparts in this regard. Initial increases in total viral load were up to 1950-fold, with replication confirmed by the detection of negative-strand viral RNA in transplanted livers. HCV viral proteins were localized to *h*-hepatocyte nodules, and infection was serially passed through three generations of mice, confirming both synthesis and release of infectious viral particles. Using *h*-hepatocyte-chimeric Rug-2-knockout mice as test animals, Dandri et al. was the first to succeed in producing in vivo HBV infection [6].

We studied HBV infectivity in the chimeric mice [42]. After mice were inoculated with *h*-serum containing HBV, a high level of viremia occurred in mice for up to 22 weeks. Passage experiments showed that the serum of these mice contained infectious HBV. As shown previously for HCV, the level of HBV viremia tended to be high in mice with a continuously high RI. Furthermore, lamivudine, an anti-HBV drug, effectively reduced the level of viremia in the infected mice. Thus, the chimeric mouse may be an ideal model in which we can develop and evaluate anti-*h*-hepatitis virus drugs.

8. The *h*/*m*-Chimeric Mouse as an Animal Model for the Study of *h*-Type Peroxisome Proliferator-Activated Receptors

8.1. Drug Metabolism under The Control of Ligand-Activated Receptors. Biochemical systems in the liver manage not only endogenous (homobiotic), but also xenobiotic molecules. These molecules are first recognized by specific protein receptors on the hepatocyte surface. In general, the binding of a ligand to its receptor generates a signal that ultimately changes gene expression, producing a cellular response. Hepatocytes possess four types of receptors [16], all of which are ligand-activated transcriptional regulators: CAR; PXR [also called steroid X receptor (SXR)]; peroxisome proliferator-activated receptor (PPAR); and aryl hydrocarbon receptor (AHR). The first three belong to the nuclear receptor (NR) superfamily, which consists of seven subfamilies, 1 to 6 and 0 [43]. AHR is a member of the Per-AhR/Arnt-Sim homology sequence (PAS)/basic helix-loop-helix (HLH) superfamily, which also represents the period regulator of circadian rhythm (PER), Ah receptor nuclear translocator (ARNT), and single-minded regulator of midline cell differentiation.

Historically, the roles of PPARs have been studied using liver. They belong to NR subfamily 1, along with thyroid hormone receptor, retinoic acid receptor (RAR), and vitamin D receptor (VDR). As transcription factors, these receptors share a similar process. They are activated by ligand binding; form heterodimers, usually with the retinoid X receptor (RXR); translocate to the nucleus; bind to a *cis*-acting XRE consisting of a direct repeat of two hexanucleotides, separated by one or two nucleotides, in the promoter region of the target gene; and enhance target gene expression [17]. Generally, in the absence of ligand, subfamily 1 NR heterodimers are bound to co-repressor proteins and repress transcription when bound to the *cis*-acting element [44]. Upon ligand binding, the receptor dissociates from the co-repressors and associates with coactivator proteins, which enables the NRs to promote gene expression.

Three PPAR subtypes are currently known [45]: PPARα (or NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3). When continuously exposed to certain xenobiotics such as hypolipidemic drugs, plasticizers, and herbicides, which have little apparent structural relationship, rats and mice may show hepatic peroxisome proliferation (increase in volume and number) leading to hepatic tumors; this suggests a correlation between the stimulation of genes for fatty acid β- and ω-oxidation enzymes and the hepatic neoplastic process [46, 47]. Reddy and Rao [48] proposed that specific soluble binding sites for these drugs, collectively termed peroxisome proliferators (PPs), were present in liver and kidney cell extracts [49, 50]. The PPAR gene was first cloned as a member of the steroid hormone receptor superfamily from a *m*-hepatic cDNA library [51]. This gene corresponds to PPARα, according to the current nomenclature. Two years later, three closely related members of the PPAR family (xPPARα, β, and γ) were isolated from a *Xenopus* ovary cDNA library and were shown to activate the promoter of the acyl coenzyme A oxidase (ACO) gene, which encodes the key peroxisomal fatty acid β-oxidation enzyme [52]. xPPARα is homologous to Issemann's PPARα [51], and xPPARγ is currently placed in the PPARγsubfamily, together with other homologous members found in mammals. Mammalian PPARδ was in a new PPAR group because of a difference in amino acid sequence compared with xPPARβ; however, it is presently considered to be a PPARβ and is designated as PPARβ/δ [45]. Of the three PPARs, PPARα is the most critical in the present review, because it is expressed at high levels in the liver, activates fatty acid catabolism, stimulates gluconeogenesis and ketone body synthesis, and participates in the control of lipoprotein assembly [45].

8.2. Species Differences in PPARα-Associated Signaling. The PPARα isotype has prime importance for studies with animal models to predict the effects of hepatic PPs in humans, because PPARα agonists induce seemingly quite different actions in rodents and humans [53]. Originally, as the name indicates, PPARs were studied because of their ability to bind PPs and consequently induce PP-metabolizing enzymes. In rats and mice, but not in humans, PPs such as hypolipidemic drugs, industrial plasticizers, and herbicides are non-genotoxic carcinogens that cause liver tumors [54]. In humans, these drugs function to maintain lipid homeostasis and do not induce peroxisome proliferation. Thus, the toxicity and carcinogenicity of PPs are highly species specific [55]. The species differences may be attributable to lower PPAR mRNA expression levels in *h*-hepatocytes compared with rodent cells [56, 57]. Alternatively, or additionally, species differences may be the result of different sensitivities of the genes associated with the peroxisome proliferation response to low levels of PPs, owing to structural differences in PPARα [54]. There are both similarities and differences in responses to xenobiotics among not only different species (interspecies) but also individuals of the same species (intraspecies). Interspecific PPARα diversity between rodents and humans is well known and has been studied with respect to drug metabolism. NR subfamily 1 members have at least two functions in mammals. One is to regulate peroxisome proliferation through binding to PPAR response elements (PPREs) in the promoters of genes such as ACO [58, 59], bifunctional dehydrogenase/hydratase (BFE) [60], and microsomal CYP4A1 [61]. The other is to modulate the serum cholesterol level by targeting genes such as the lipoprotein lipase gene [62] and the apolipoprotein regulating genes AI, AII, and CII [63]. The former mechanism appears to function in rodents, but not in humans, and is responsible for the induction of peroxisome proliferation and hepatocarcinogenesis, whereas the latter mechanism controls basic lipid metabolism in both rodents and humans [56]. This species difference in xenobiotic receptor/ligand signaling may be attributable to differences in the expression level of a receptor, or to differences in receptor/ligand binding affinity, and causes difficulty in determining responses in humans based on rodent data [56].

8.3. PPARα Gene-Humanized Mice. One approach to overcoming species differences is to generate "humanized" transgenic mice (gene-humanized mice), in which a *h*-gene of interest is introduced into the *m*-genome [17]. A PPARα gene-humanized *m*-line that expresses the *h*-PPARα gene [64] under the control of the tetracycline responsive regulatory system in the liver of murine PPARα gene-null mice [65] has been created. These mice functionally responded to the expected ligands as wild-type mice, but did not exhibit the hepatocellular proliferation, including increases in peroxisomes, seen in wild-type mice. Thus, this approach may help overcome species differences and provide animal models suitable for studying *h*-responses regulated by genes of interest.

8.4. PPAR Signaling in Chimeric Mice. Considering the prominent roles and the species divergence of PPARs in the response to xenobiotics, it is important to study *h*-PPAR-related responses of the *h/m*-chimeric mouse. We examined the effects of fibrates (antihyperlipidemic drugs and PPAR agonists) [63, 66] in the chimeric mice. Given the central role of the liver in PPAR-regulated lipid metabolism and the use of fibrate compounds in a variety of clinical drugs, the responses of *h/m*-chimeric mice to fibrates and PPARs may have important practical implications.

Hepatocytes secrete biliary phospholipids, composed largely of phosphatidylcholine (PC), through multidrug-resistance 2 P-glycoprotein (MDR3, or ABCB4) embedded in the canalicular membrane. MDR3 was shown to translocate PC in a study using mdr2 gene (a murine homolog of *h*-MDR3) knockout mice. These mice completely lack phospholipids in their bile [67], but the bile PC is fully recovered with the overexpression of *h*-MDR3 [68]. The expression level of the *h*-MDR3 gene affects the development of hepatobiliary diseases [69].

Fibrates upregulate mdr2 gene expression [70], which is associated with an increase in biliary phospholipid secretion [71]. Benzafibrate (BF), a second-generation fibrate analog, was clinically shown to reduce elevated serum biliary enzyme levels in patients with chronic cholestatic liver disease

[72]. It was shown to bind to PPARβ/δ and α, with a higher affinity for the former, and was thus said to be a bona fide PPAR ligand [73]. Other researchers created a coactivator-dependent receptor-ligand in vitro interaction assay and demonstrated that BF was a ligand for PPARα, β/δ, and γ [74]. The same researchers also showed drug-induced activation of PPARα/RXRα, PPARβ/δ/RXRα, and PPARγ/RXRα [74].

BF induces an increase in ABCB4 (MDR3), and its redistribution in the cell membrane. This induction was associated with an enhanced capacity of *h*-hepatocytes to direct PC into bile canaliculi [75]. Furthermore, ABCB4 redistribution was attenuated when PPARα expression was suppressed by small interfering RNA or morpholino antisense oligonucleotides in cultured HepG2 cells (hepatoblastoma cells) [75], strongly suggesting the necessity for PPARα in the BF-induced activation of PC secretion in *h*-hepatocytes.

We tested the ability of the *h*/*m*-chimeric *m*-liver to exhibit *h*-type PPAR-dependent responses by administering BF to the chimeric mice. Mice with RIs of 60–80% were fed a standard laboratory chow containing 0.3% (wt/wt) BF for 7 days, and their livers were analyzed for MDR3 mRNA and protein expression [39]. The mRNA level in the BF-treated mice was approximately 2-fold the level in non-treated control mice. The protein level was approximately 3.5-fold that in the controls. The fibrate induced a robust redistribution (exocytosis and insertion) of MDR3 proteins into the bile canaliculi.

Although studies on the expression and function of PPARs in the *h*/*m*-chimeric *m*-liver are limited, we conclude, based on the studies described above, that the chimeric *m*-liver exhibits the phenotypes of PPAR-regulated physiological and pathological processes, including responses to xenobiotics, that are normally present in the *h*-liver in vivo.

9. Summary and Prospective

After administration, a xenobiotic is generally and largely absorbed by the liver, intracellularly distributed, metabolized, and secreted through the bile or urinary ducts. These steps, collectively termed absorption, distribution, metabolism, and excretion (ADME), are interdependent, and drug pharmacokinetics are determined by the parameters resulting from these interactive processes. There are marked species differences in the many genes and proteins associated with ADME of a xenobiotic. The differences between humans and rodents dictate that pharmacokinetic data determined in rodents must be very cautiously, deliberately, and correctly extrapolated to humans in order to ensure that the drug will be safe and effective in patients. Until recently, *h*-hepatocyte-chimeric mice have been studied primarily in relation to CYP-associated metabolism, representing the M of ADME, and HCV/HBV infection. These studies have shown that the chimeric mice are significantly and appreciably humanized, providing a reliable and promising animal model for predicting drug metabolism and efficacy in humans. Although data have also been accumulated for the A, D, and E steps of ADME, more work is required before reaching an appropriate conclusion concerning the humanization of a chimeric mouse with respect to these processes. Nevertheless, currently available data appear to demonstrate that these processes are also well humanized.

Based on our studies and experiences to date, the *h*-hepatocyte-chimeric mice exhibit *h*-type liver responses at the gene and protein levels. These mice can mimic the steady-state expression in the *h*-liver in the absence of exogenous stimuli and exhibit the expected *h*-type responses upon stimulation. However, we must consider the limitations of chimeric mice. Current chimeric mice carry hepatocytes only of human origin, but all other cells are of *m*-origin. To perform liver functions, parenchymal cells require non-parenchymal cells, which are of mouse, and not of human, origin in the chimeric mice. Some interactions between *h*-hepatocytes and *m*-nonparenchymal cells may proceed as normal homogeneous interactions, and some may not.

In addition, endocrinological regulation is crucial for hepatocytes to achieve normal metabolic homeostasis and to return to normal conditions after endogenous or exogenous factors have caused metabolic parameters to extend beyond the normal range. Chimeric livers are under the influence of the *m*-endocrinological system, and some *m*-hormones such as growth hormone (GH) are not able to act on *h*-cells, because a hormone-receptor complex does not form between *m*-GH and *h*-hepatocyte receptors [76]. In support of this notion, *h*-hepatocytes administered with *h*-GH showed enhanced expression of liver growth-associated *h*-genes, including IGF-1, STAT-3, Cdc 25A, and cyclinD1, and repopulated the host liver at a rate approximately 6-fold that in the control. Despite these possible limitations, we consider the chimeric mouse to be the best animal model to date for *h*-liver function studies, because the chimeric mice with high RI values not only expressed *h*-liver proteins but also mimicked *h*-liver functions. Five years ago, we started mass production of homogenous populations of the hepatocyte-humanized mice with high RIs to facilitate research activities in the academic and industrial communities, including examinations of *h*-type metabolism of new drugs for *h*-use, the study of *h*-HCV infection mechanism and propagation, and the development of new anti-HCV-drugs. However, we are still in the initial stages of characterizing various aspects of the chimeric mice. Further study will systematically reveal the advantages and limits of this newly developed hepatocyte-humanized mouse.

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We are much obliged to a recent review [1] on historical and histological aspects of chimeric mice, some parts of which are cited herein, and to an excellent review [17] that presented information on recent progress in xenobiotic metabolism studies, especially those using gene-engineered humanized mice to evaluate *h*-type xenobiotic metabolism. Our study of chimeric mice with livers composed of *h*-hepatocytes was supported by three programs, the Yoshizato project, Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER), Japanese Ministry of Education, Culture, Sports, Science, and Technology, the Hiroshima Prefecture to K. Yoshizato, Hiroshima University 21st Century COE Program for Advanced Radiation Casualty Medicine to K. Kamiya, and Advanced Medical Technology, Health, and Labor Sciences Research Grant from the Ministry of Health, Labor, and Welfare of Japan to T. Yokoi.

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Review Article

PPAR/RXR Regulation of Fatty Acid Metabolism and Fatty Acid ω-Hydroxylase (CYP4) Isozymes: Implications for Prevention of Lipotoxicity in Fatty Liver Disease

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Fatty liver disease is a common lipid metabolism disorder influenced by the combination of individual genetic makeup, drug exposure, and life-style choices that are frequently associated with metabolic syndrome, which encompasses obesity, dyslipidemia, hypertension, hypertriglyceridemia, and insulin resistant diabetes. Common to obesity related dyslipidemia is the excessive storage of hepatic fatty acids (steatosis), due to a decrease in mitochondria β-oxidation with an increase in both peroxisomal β-oxidation, and microsomal ω-oxidation of fatty acids through peroxisome proliferator activated receptors (PPARs). How steatosis increases PPARα activated gene expression of fatty acid transport proteins, peroxisomal and mitochondrial fatty acid β-oxidation and ω-oxidation of fatty acids genes regardless of whether dietary fatty acids are polyunsaturated (PUFA), monounsaturated (MUFA), or saturated (SFA) may be determined by the interplay of PPARs and HNF4α with the fatty acid transport proteins L-FABP and ACBP. In hepatic steatosis and steatohepatitis, the ω-oxidation cytochrome P450 *CYP4A* gene expression is increased even with reduced hepatic levels of PPARα. Although numerous studies have suggested the role ethanol-inducible *CYP2E1* in contributing to increased oxidative stress, *Cyp2e1*-null mice still develop steatohepatitis with a dramatic increase in *CYP4A* gene expression. This strongly implies that *CYP4A* fatty acid ω-hydroxylase P450s may play an important role in the development of steatohepatitis. In this review and tutorial, we briefly describe how fatty acids are partitioned by fatty acid transport proteins to either anabolic or catabolic pathways regulated by PPARs, and we explore how medium-chain fatty acid (MCFA) *CYP4A* and long-chain fatty acid (LCFA) *CYP4F* ω-hydroxylase genes are regulated in fatty liver. We finally propose a hypothesis that increased *CYP4A* expression with a decrease in *CYP4F* genes may promote the progression of steatosis to steatohepatitis.

1. Introduction

Disorders of lipid metabolism are closely dependent on genetic factors, exposure to drugs, and many common life-style choices (e.g., diets and alcohol) that often lead to metabolic syndrome in which patients exhibit obesity, dyslipidemia, hypertension, hypertriglyceridemia, and insulin resistance diabetes [1, 2]. Common to obesity-related dyslipidemia and hypertriglyceridemia is the excessive storage of fatty acids in the liver (steatosis) frequently referred as nonalcoholic fatty liver disease (NAFLD). Increased hepatic fatty acids can cause lipotoxicity and initiate fatty liver inflammation (steatohepatitis) commonly referred as nonalcoholic steatohepatitis (NASH) [3]. Excessive fatty acids in

the liver dramatically alter lipid metabolism by decreasing mitochondrial β-oxidation, while increasing peroxisomal β-oxidation and microsomal ω-oxidation of fatty acids resulting in lipotoxicity [4, 5]. During hepatic steatosis, members of *CYP4* family of fatty acid ω-hydroxylase are induced even with the downregulation of PPARα, which regulates *CYP4A* gene expression. Numerous reports have indicated that the ethanol-inducible CYP2E1 is induced in both hepatic steatosis and steatohepatitis [6, 7] even though *Cyp2e1*-null mice develop steatohepatitis with a markedly increased *CYP4A* gene expression, suggesting that *CYP4A* P450 may also play an important role in the progression of NAFLD to NASH. Since elevated levels of long chain fatty acids (LCFAs) and LCFA coenzyme A esters (LCFA-CoAs) are observed in hepatic steatosis and several metabolic disorders, including obesity, diabetes and hyperlipidemia, it is of necessity that we understand the mechanism that regulates fatty acid (FA) transport and partitioning of free fatty acids (FFA) and fatty acid-CoA in the initiation of fatty liver lipotoxicity in the progression of NAFLD to NASH.

Intracellular fatty acids (FAs) and their metabolites coordinate physiological processes by several transcriptional factors controlling energy metabolism. For instance, several transcriptional factors include: peroxisome proliferator activated receptors (PPARα, PPARδ, PPARγ), sterol regulatory binding proteins (SREBP-1 and SREBP-2), liver X receptor (LXRα), and carbohydrate response element binding protein (ChREBP), all of which are activated or repressed by different fatty acids [8]. Both polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) and LCFA-CoA bind and activate PPARα to increase fatty acid oxidation, gluconeogenesis, and ketogenesis [9], while PUFAs suppress activation of SREBP-1c, ChREBP, and LXRα through diverse mechanisms [10]. In contrast, saturated fatty acids activate HNF4α through binding to acyl-CoA binding protein (ACBP) containing LCFA while SREBP-1c activation by LCFA occurs by recruiting SREBP-1c and PPARγ coactivator-1β (PGC-1β). In addition, intracellular fatty acids produced from triglyceride hydrolysis or de novo lipogenesis (DNL) can regulate gene expression, suggesting that not only the type of fatty acids (saturated versus unsaturated), sources (intracellular versus exogenous), fatty acid association with different fatty acid transport proteins (L-FABP and ACBP), and type of fatty acid metabolites (FATP/ACSVL) have selective effects in regulating genes involved in oxidation, synthesis, and storage of fatty acids [10, 11]. Perturbations in these pathways by nutritions, drugs, alcohol, or genetic factors lead to fatty acid disorders often associated with dyslipidemia, obesity, and diabetes [12–14].

2. Causes of Hepatic Steatosis in NAFLD

Convincing evidence has shown that fatty liver is closely associated with insulin resistance and metabolic syndrome. Although, life style choices of a high carbohydrate or a high fat diet and excessive alcohol consumption are specific causes of hepatic steatosis and NAFLD, nutritional factors (e.g., malnutrition and rapid weight loss) [15], drug exposure (e.g., glucocorticoids, methotrexate, Amiodarone, Tamoxifen, HIV protease inhibitors, etc.) [12, 16], specific diseases (e.g., inflammatory bowel disease, primary biliary cirrhosis, Cushing's syndrome, Hematochromatosis, etc.), and genetic factors [17] are also relevant causes of fatty liver diseases [16, 18–22].

The major sources of fatty acids that contribute to hepatic steatosis include fat stored in adipose tissues and released during fasting by lipolysis to increase plasma levels of nonesterified fatty acid (NEFA), which provides the majority of fatty acids secreted by liver as VLDL. During adipose insulin resistance, the increased release of fatty acids leads to elevated accumulation of TAG in fatty liver. A second source of increased plasma NEFA is through liver de novo lipogenesis (DNL) while dietary fatty acid, from intestinal-derived chylomicron remnants, also increases the plasma pool of NEFAs. In patients with NAFLD, 59% of TAG fatty acid is derived from adipose lipolysis while 26% is from DNL and 15% from the dietary NEFA pool [21]. The elevated DNL in patients with NAFLD compared to patients without NAFLD does not change after a meal. In contrast, in normal control individuals it increases from 5% to 28% 4 hours after a meal [22]. In NAFLD, the NEFA pool contributes equally to the liver TAG and VLDL TAG even though NAFLD patients have a reduced ability to increase VLDL production during fasting, suggesting that NAFLD patients have a limited capacity to adapt to metabolic changes that occur during cycles of fasting and feeding [23].

Thus, plasma NEFAs from adipose stores or dietary sources provide most of the hepatic lipid in NAFLD. Therefore, the regulatory mechanism of fatty acid uptake by hepatocytes and cellular distribution of esterified and nonesterified fatty acids have an important role in the initiation of hepatic steatosis in NAFLD. There are five mechanisms responsible for fatty acid uptake that initiates of fatty liver. Facilitated transport is the primary mechanism for the uptake of free fatty acids (FFAs) into hepatocytes under normal plasma NEFA levels (225–700 uM). However, higher plasma NEFA concentrations may overload this saturable system, resulting in the activation of a passive nonsaturable pathway to reduce plasma NEFA levels, unfortunately with the consequence of hepatic steatosis [24]. The saturable systems for plasma membrane fatty acid transport are caveolins, fatty acid transport proteins (FATPs), fatty acid translocase (FAT/CD36), and fatty acid binding proteins (FABPs). Caveolin-1 of lipid rafts is localized within plasma membrane invaginations that have a critical role in cell signaling, protein trafficking, and uptake of fatty acids; caveolin-1 deficient mice are resistant to diet induced obesity [25]. FATPs are a family of six integral membrane proteins with an extracellular/luminal N-terminal and C-terminal domain with *fatty acyl-CoA synthetase* activity and therefore FATP proteins have the ability to trap FA intracellularly [26] (Table 1). FATP2 (ACSVL1), FATP5 (ASCLV6), and FATP4 (ACSVL5) are expressed in the liver and are regulated by PPARα and PPARγ [27]. Both FATP2 and FATP4 have a strong substrate preference in transport and activation of C16:0 to C24:0 straight chain and branched-chain fatty

acids while FATP5 (ACSVL6) preferentially transports and activates bile acids [26]. FATP5 knockout mice show a 50% decrease in hepatocyte fatty acid uptake with reduced caloric uptake, and improved whole body glucose homeostasis. Consequently, these mice were protected from high fat induced hepatic steatosis [28]. FATP5 also exhibits *bile acid CoA synthetase* activity and therefore *Fatp5*-null mice display a dramatic increase in unconjugated bile acids. As expected *Fatp5*-null mice are resistant to obesity and hepatic accumulation of TAG with improved insulin sensitivity [28, 29]. In adenovirus FATP4 infected rat hepatocytes, there was a 30% increase in fatty acid uptake and 2-fold increase in acyl-CoA activity with a 42% increase in TAG synthesis, indicating that FATP4 partitions fatty acids towards TAG synthesis and storage [30–32]. In human hepatocytes, FATP4 knockdown decreased $C_{18:1}$ incorporation into phospholipids and VLDL synthesis, suggesting an anabolic role of FATP4 in energy metabolism [33]. In contrast, overexpression of FATP2 in primary hepatocytes results in $C_{18:1}$ channeling toward diacylglycerol and phospholipid synthesis with shift away from cholesterol esterification [33]. FATP2 is regulated by PPARα agonists as well as high carbohydrate and high fat diets which increase FATP2 expression 8-fold in rat liver. However, surprisingly the PPARγ ligand BRL-49953 induces FATP2 expression in adipose tissue, which is similar with insulin induced FATP2 expression [34]. It is therefore not surprising that FATP2 is induced in obese Zucker (fa/fa) rats and that both FATP2 and FATP4 mRNA are increased in hepatocytes by carbohydrates feeding and by insulin through SREBP-1c [35]. FAT/CD36 is expressed in a broad range of tissues and cell types. It promotes fatty acid release from albumin and insertion into the plasma membrane by facilitated diffusion with the assistance of L-FABP. Although expression of CD36/FAT is low in hepatocytes, this transport protein is unique since it mediates the uptake of VLCL and oxidized LDL. *Cd36*-null mice show a higher level of plasma NEFA, higher liver TAG, and severe hepatic insulin resistance [36]. The induction of CD36/FAT in mice fed a high fat diet results in fatty liver [37], and patients with increased expression of this protein have a higher level of hepatic fatty acids and display NAFLD [38]. CD36/FAT expression is controlled by the pregnane X receptor (PXR), PPARγ, and the LXRα [37].

FABP and ACBP bind a diverse array of fatty acids including eicosanoids [39] and facilitate intracellular transport of FA and FA-CoAs from the cytosol to different organelles including the nucleus. Unlike FATP, this protein does not exhibit *acyl-CoA synthetase* activity (Table 1). There are nine different FABPs each of which shows distinct tissue specific distribution with L-FABP being prominently expressed in the liver. *L-Fabp*-null mice show a 2-fold increase in hepatic TAG compared to a 10-fold increase in wild type mice after a 48-hour fasting, which was due to decreased TAG secretion and reduced fatty acid oxidation [40]. *L-Fabp*-null mice fed a high fat western diet are resistant to diet induced obesity and show a similar increase in TAG secretion as the wild type mice [41], suggesting either that other fatty acid transport proteins compensate for L-FABP or increased plasma NEFAs initiate passive diffusion. A high fat diet potently increases the expression of L-FABP [42] and microsomal triglyceride transfer protein (MTP) via PPARα, thereby leading to efficient delivery of FA for VLDL assembly and secretion [43]. In contrast, the repression of these enzymes would reduce VLDL secretion without causing TAG accumulation in the liver [44]. ACBP, responsible for transporting acyl-CoA esters intracellularly, is downregulated during fasting but induced by insulin through SREBP-1c, and fibrates through PPARα [45]. Thus ACBP is a dual PPARα and SREBP-1c target gene where fasting reduces SREBP-1c expression and increases PPARα in hepatocytes, thus reflecting a dual role of ABCP in lipogenesis and lipid oxidation.

The apparently similar mechanism that regulates FATP2, FATP4, ACBP, and L-FABP by PPARα during fasting induced fatty acid influx with the paradoxical upregulation by insulin through SREBP-1c identifies an ideal mechanism to prevent lipotoxicity [46]. Cellular acyl-CoA levels correlate with hepatic insulin resistance and have been suggested to mediate lipotoxicity. Because the normal acyl-CoA levels in cytosol are between 1 and 20 uM, and ACBP and FABP comprise up to 5% of the total hepatic cytosol proteins [34], it is likely that most acyl-CoAs are bound to ACBP and/or FABP and thus FFAs may be the major culprit in causing lipotoxicity. Since LCFA-CoA production is controlled by FATPs, which channel acyl-CoAs to the synthesis of phospholipids, cholesterol esters, and triglycerides or oxidation by the mitochondrial and peroxisomal β-oxidation pathways or microsomal ω-oxidation, it is highly likely that elevated NEFAs in serum during fasting and hepatic steatosis significantly increase the intracellular pool of unesterified fatty acids in hepatocytes. The increased synthesis of TAG observed in hepatic steatosis may be a compensatory mechanism to reduce intracellular FFAs and lipotoxicity. Therefore, the upregulated FATP2, FATP4 and FATP5 expression in obese rats increases TAG synthesis and storage, while increased FATP expression promotes the cellular importation of FFAs [33, 47], leading to FFA overload and hepatic steatosis. However, the knockdown of FATP3 (ACSLV3) in primary rat hepatocytes revealed a significant reduction in the expression of several lipogenic transcription factors, PPARγ, ChREBP, SREBP-1c, and LXRα as well as their target genes [46, 48].

Since the knockdown (or knockout) of different FATP isoforms reduces hepatic steatosis and in some cases increases insulin sensitivity and glucose handling, it is imperative that we understand the mechanism through which various FATP isoforms interact with different metabolic enzymes and/or intracellular transport proteins (L-FABP and ACBP) to regulate lipogenesis, TG synthesis and storage, and fatty acid oxidation pathways. These mechanistic studies will provide valuable insights into how hepatic steatosis causes lipotoxicity and the progression of steatosis to steatohepatitis.

3. Role of PPAR in the Regulation of Fatty Acid Metabolic Fate

The overexpression of various fatty acid transport proteins in different cell lines, and their ability to channel FA to different

Table 1: Nomenclature and properties of fatty acid transport proteins.

Gene id	Nomenclature	Tissue	Regulation	Substrate, ligand, or binding protein	Subcellular location	Function
SLC27A1	FATP1-ACSVL4	Heart, adipose, muscle, brain	PPARγ	C16:0, C18:1, C24:0	Mitochondria Plasma membrane	β-oxidation TAG synthesis
SLC27A2	FATP2-ACSVL1	Liver, kidney	PPARα, PPARγ	C16:0, C24:0 Phytanic acid, pristanic acid,	Endoplasmic reticulum Peroxisome	TAG synthesis β-oxidation
SLC27A3	FATP3-ACSVL3	Kidney, ovary, lung, brain, adrenal, testis		C16 :0, C18:1, C24:0	Cytosolic vesicles	unknown
SLC27A4	FATP4-ACSVL5	Liver, kidney, heart, adipose, skin, muscle, small intestine	PPARγ, SREBP1c	C16:0, C24:0	Endoplasmic reticulum Peroxisome	TAG synthesis β-oxidation
SLC27A5	FATP5-ACSVL6	Liver		Cholate, THCA Chenodeoxycholate Lithocholate, C24:0 Deoxycholate	Endoplasmic reticulum Peroxisome	Bile acid conjugation Bile acid synthesis
SLC27A6	FATP6-ACSVL2	Heart, placenta		C18:1, C20:4, C24:0	Plasma membrane	unknown
FABP1	L-FABP	Liver, Intestine	PPARα, HNF4α	Acyl-CoA, PPARα, γ	Cytosol, nucleus	
FABP2	I-FABP	Intestine		Acyl-CoA	Cytosol	TAG synthesis
FABP3	H-FABP	Heart, kidney muscle, thymus	c/EBPα, SREBP1 AP-1	Acyl-CoA, PPARα	Cytosol	β-oxidation
FABP4	A-FABP	Heart, adipose, Epidermis, nerve	cJun, PPARγ	Acyl-CoA, PPARγ	Cytosol	Chylomicron assembly
FABP5	E-FABP	Eye, adipose, testis	PPARδ	Acyl-CoA, PPARδ	Cytosol	lipogenesis
FABP6	Il-FABP	Ileum		Acyl-CoA, FXRα	Cytosol	
FABP7	B-FABP	Liver, brain	POU	Acyl-CoA		
FABP8	N-FABP	Myelin		Acyl-CoA	Cytosol	Vesicle assembly
FABP9	T-FABP	Testis		Acyl-CoA	Cytosol	
FABP12	R-FABP	Retina,testis		Acyl-CoA	Cytosol	
ACBP	L-ACBP	Liver, multiple tissues	PPARα, c/EBPα SREBP1c, Sp1 PPARγ	C14:0–C22:0 acyl-CoA esters, HNF4α	Cytosol	Glycerolipid, cholesterol synthesis
ACBP	T-ACBP	Testis, adrenal		C14:0–C22:0 acyl-CoA esters	Cytosol, endoplasmic reticulum	
ACBP	B-ACBP	Brain		C14:0–C22:0 acyl-CoA esters	Cytosol	

Characteristics of fatty acid transport protein (FATP-ACSVL), fatty acid binding protein (FABP), and acyl-CoA binding protein (ACBP). This table summarizes tissue specific expression, regulation by transcription factors, substrate, ligand binding, and interaction with nuclear receptors and putative function in the metabolism of fatty binding proteins.

metabolic fates within the cell [49] may be determined by the energy demands of specific metabolic pathways. Indeed, FATP proteins are not only associated with the plasma membrane but are also present in specific organelles (Table 1). FATP5 is intimately associated with the mitochondria while FATP2 (ACSVL1) is localized in peroxisomes. CD36/FAT is closely correlated with the degree of mitochondrial fatty acid oxidation, while L-FABP, also known as the mitochondrial aspartate aminotransferase (mAST), may have distinct functions at different subcellular sites. Unlike FATP which activates FA to FA-CoA, FABP proteins transport fatty acids to different intracellular compartments. The association of FATP with different subcellular organelles may also function in preventing lipotoxicity through increased metabolism. This mechanism may be important in hepatic fatty acid partitioning to different metabolic pathways during hepatic steatosis. Recent studies have shown that L-FABP also functions to shuttle lipids to the nucleus to allow them to directly interact with PPARα [49], suggesting an important role of L-FABP in controlling the metabolism of LCFAs. The ability of fatty acids to regulate metabolic pathways through activation of nuclear receptors has long been known; however the observation that L-FABP assists in supplying lipid ligands for nuclear receptors suggests an important feedback regulatory mechanism of L-FABP and FAs in controlling lipid metabolism.

The ability of FATPs to target fatty acids to specific cellular organelles for either fatty acid oxidation or synthesis and their ability to direct fatty acid ligands to activate selective nuclear receptors represent an efficient mechanism to control both metabolic pathways at the transcriptional and substrate levels. The activation of hepatocyte nuclear factor 4α (HNF4α) and PPARα that bind similar direct repeat DNA elements (DR1) by different fatty acids suggests an efficient mechanism to control globally fatty acid metabolism [50, 51] (Figure 1). It has been shown that saturated LCFA and VLCFA are extremely poor or nonactivators of PPARα. Therefore it was uncertain how increased hepatic fatty acids during fasting would increase PPARα target genes involved in fatty acid β-oxidation, VLDL production, and ketogenesis. This was resolved by demonstration that LCFA-CoA and VLCF-CoA are high affinity PPARα ligands. PPARα has a high affinity for polyunsaturated fatty acids (PUFAs) and LCFA-CoA, while these saturated LCFAs or VLCFAs are nonselective in the activation of PPARα, PPARδ, and PPARγ. In vivo, the importance of acyl-CoA in the activation of PPARα target genes was apparent when it was found that CoA esters of peroxisome proliferator chemicals or drugs were activating ligands for PPARα mediated expression of peroxisomal fatty acid β-oxidation genes, Acyl-CoA oxidase (AOX1), bifunctional protein and thioesterase [52–54]. It was also found that peroxisome proliferator-CoA esters (PP-CoAs) are potent inhibitors of HNF4α activation. The importance of VLCFA-CoAs in activation of PPARα was shown in the in vivo animal model of *Aox1*-null mice, which show elevated plasma levels of VLCFAs and increased hepatic levels of VLCFA-CoAs. The inability of peroxisomal β-oxidation system to metabolize VLCFA-CoAs results in hyperactivation of PPARα and increased expression of target genes [55]. In contrast, HNF4α has a high affinity for saturated LCFAs, and VLCFAs, but not PUFA-CoAs or LCFA-CoAs, suggesting that fatty acid CoA, chain length, and degree of unsaturation determine whether HNF4α or PPARα will be activated and therefore which metabolic pathway controlled by these nuclear receptors [10, 56]. Thus, the elevated uptake and transport of LCFA-CoAs or PUFAs to the nucleus by L-FABP activate PPARα and increase fatty acid oxidation. In contrast, ACBP binds saturated LCFAs, which preferentially bind and activate HNF4α. Therefore, PPARα/L-FABP and HNF4α/ACBP would mediate a differential association with coactivators and corepressors to their respective target genes to control fatty metabolism. This suggests that binding of saturated LCFAs to ACBP and its association with HNF4α would increase HNF4α transcription activity and inhibit PPARα, while transactivation of PPARα with PUFAs or LCFA-CoAs would decrease HNF4α activation [10]. Thus, in hepatic steatosis, the ratio of saturated to unsaturated fatty acids with increased expression of selective fatty acid transport proteins (FATP/L-FABP/ACBP) may mediate metabolic defects accounting for hepatic steatosis. Consequently, LCFA activation of HNF4α with ACBP would increase plasma levels of lipid rich lipoproteins (VLDL, LDL, and HDL) and their constituent apolipoproteins (AI, AII, B, and CIII). In contrast, dietary PUFAs associated with L-FABP would activate PPARα, resulting in decreased transcription of these apolipoproteins and lipoproteins with increased fatty acid oxidation [52, 53, 57]. It will be of significance to determine if selective fatty acids and fatty acid transport proteins regulate the expression of PPARγ, LXRα, and SREBP-1c in hepatic steatosis. Recently, the suppression of FATP3 (ACSVL3) was shown to significantly decrease the expression of lipogenic transcription factors, PPARγ, ChREBP, LXRα, and SREBP1c and their respective target genes, resulting in decreased de novo lipogenesis (DNL) [46]. In hepatic steatosis, many of the fatty acid transporter proteins are upregulated, yet it is uncertain how this common event of increased hepatic fatty acid levels seen in fasting leads to increased fatty liver and lipotoxicity in the progression of NAFLD to NASH.

4. Fatty Acid-Induced Lipotoxicity

Elevated intracellular fatty acids in hepatocytes lead to lipotoxicity characterized by increased oxidative-stress and lipid peroxidation, thus promoting the progression of simple hepatic steatosis to steatohepatitis. Normal cellular fatty acid homeostasis represents a balance between fatty acid uptake, utilization, and export from the liver, which is controlled by an elaborate transcriptional network that is finely tuned to meet the energy needs of cells to prevent the accumulation of fatty acids and toxic intermediates. However, when this system is overwhelmed by excessive free fatty acids (FFAs), hepatic steatosis commonly referred as NAFLD can progress to steatohepatitis often referred as NASH. NASH is characterized by cellular damage, inflammation, and varying degrees of fibrosis [58]. Untreated NASH can progress to liver fibrosis, contributing to hepatorenal portal hypertension, liver cirrhosis, hepatic encephalopathy, and liver failure or

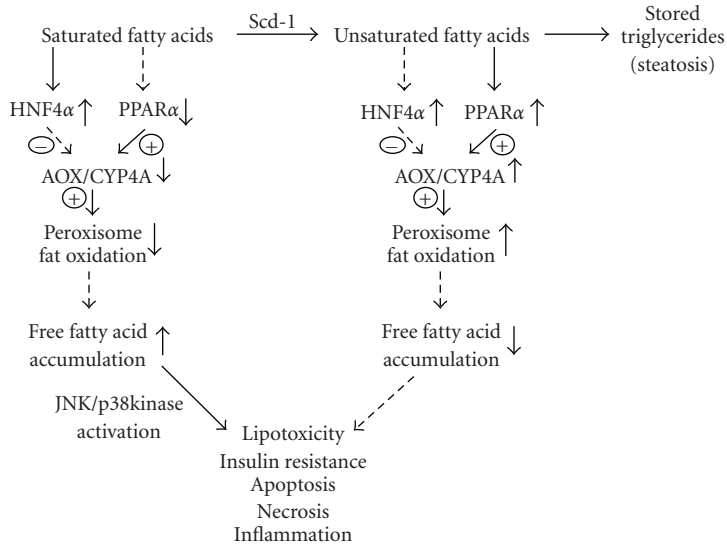

Figure 1: Schematic diagram of the role of saturated fatty acids in causing nonalcoholic fatty liver diseases and lipotoxicity. The positive signs with solid lines represent activation and/or upregulation of the downstream targets while the negative signs with broken lines indicate the opposite effects. Abbreviations used are HNF4α, hepatocyte nuclear factor 4; PPARα, peroxisome proliferator activator receptor α; AOX, acyl-CoA oxidase; CYP4A, cytochrome P450 4A; Scd-1, stearoyl-CoA desaturase.

progress to hepatocellular carcinoma [59, 60]. In the liver, FFAs are considered the causative agents for hepatic steatosis and also for obesity, and diabetes. Therefore, elucidating the mechanism by which excessive hepatic FFAs induce hepatic insulin resistance, hepatic gluconeogenesis, and fatty acid disposal or intracellular partitioning is critical for understanding the progression of steatosis to steatohepatitis. Serum FFAs levels are increased in obese individuals in both the fed and fasting states and have been shown to play a critical role in the progression of obesity to Type II diabetes [61]. FFAs in the liver desensitize insulin signaling by dampening suppression of hepatic gluconeogenesis through activation of p38 mitogen-activated kinase [62]. FFAs increase the transcription of phosphoenolpyruvate carboxykinase (PEPCK), glucose 6-phosphatase (G6Pase), peroxisome proliferator-activated receptor coactivator α (PGC-1α), and cAMP-responsive element binding protein (CREBP). Activation of p38 kinase by FFAs is mediated by upstream activation of protein kinase Cδ (PKCδ), which might be activated by ACSLV-directed FFA diversion to diacylglycerol (DAG), which is known to activate PKCδ in the presence of calcium.

Hepatic insulin resistance represents a paradox in hepatic steatosis since insulin receptor activation of insulin receptor substrate-1 (IRS-1) is active and accounts for increased DNL, yet insulin does not inhibit insulin receptor substrate-2, which controls liver gluconeogenesis [63]. Both IRS-1 and IRS-2 inhibit the transcription factor FOXO-mediated gene transcription of PEPCK, G6Pase, and PGC-1α [64]. Increased activation of IRS-1 during hyperinsulinemia increases expression of SREBP-1c, which normally blocks IRS-2 expression and CREBP critical for hepatic gluconeogenesis. Excessive FFAs induce hepatic insulin resistance by activation of c-Jun N-terminal protein kinase (JNK), which is known to be activated by oxidative stress and cytokines, and is abnormally elevated in the diabetics [7, 65]. Thus, inhibition of JNK dramatically improves insulin resistance and markedly decreases blood glucose levels. JNK adenovirus overexpression increases serine phosphorylation of IRS-1 with decreased IRS-1 tyrosine phosphorylation and increased SREBP-1c activation, resulting in increased fat synthesis and storage. JNK-mediated decreased activation of IRS-1 also results in reduced suppression of FOXO1, leading to increased hepatic gluconeogenesis. However, a recent study reported that specific ablation of JNK1 in hepatocytes exhibits glucose intolerance, insulin resistance, and hepatic steatosis [66]. This study suggests that JNK1 has opposing actions in liver and adipose tissue to promote and prevent hepatic steatosis. Liver specific *Jnk1*-null mice display increased gluconeogenesis and lipogenesis and, therefore, have the paradox of selective insulin resistance that is a central characteristic of type 2 diabetes. JNK activation has also been implicated in FFA-induced hepatocyte lipoapoptosis by activation of proapoptotic BCL-2 proteins Bim and Bax, triggering the mitochondrial apoptotic pathway [4, 67]. Furthermore, IRS-2 signaling was repressed in hepatic insulin resistance by short-term feeding of FFAs, which caused a 3-fold increase in triglycerides and acyl-CoAs. Not only was tyrosine phosphorylation of IRS-2 decreased, but also IRS-1 activation was suppressed in mice fed a high fat diet. Consequently, the reduced tyrosine phosphorylation of IRS-1 and IRS-2 in rat hepatoma cells exposed to palmitic acid can be reversed by inhibition of acyl-CoA synthesis of palmitoyl-CoA, suggesting an important role of FATP (ACSLV) proteins in insulin signaling [50].

Although liver insulin resistance and hepatocyte apoptosis are induced by excessive FFAs, the question of which types

of fatty acids promote each process has not been explored in mouse models of hepatic steatosis. Recent data suggested that partitioning of saturated and unsaturated fatty acids through *stearoyl CoA desaturase* (SCD-1) determines the degree of fatty acid induced liver injury [68]. SCD-1 knockout mice are resistant to hepatic steatosis and hepatic insulin resistance [69, 70]. SCD-1 regulates partitioning of saturated fatty acids (SFAs) between MUFAs present in simple hepatic steatosis and SFAs present during hepatic steatohepatitis and fibrotic livers. In an elegant study, Feldstein and colleagues clearly showed that MUFA leads to hepatic steatosis without hepatocyte injury while SFA significantly decreases hepatocyte cell viability through caspase activation and apoptosis [71]. Furthermore, inhibition of SCD-1 sensitized hepatocytes to SFA-induced apoptosis, and mice fed with a high-fat diet showed increased SCD-1 expression and hepatic accumulation of MUFA. In contrast, mice fed with a methionine-choline deficient (MCD) diet that induces steatohepatitis had increased hepatic levels of SFAs. *Scd1*-null mice fed with the MCD diet showed decreased hepatic steatosis, but increased apoptosis and liver fibrosis, which could be prevented by feeding MUFA. Therefore, although *Scd1*-null mice are resistant to hepatic steatosis, they are more susceptible to liver injury while increased SCD-1 expression during NAFLD reflects a compensatory beneficial effect on increasing MUFA synthesis and storage of excessive FFAs as triglycerides. Therefore, a decreased fatty acid desaturation index (MUFA/SFA) may be one important trigger in the progression of NAFLD to NASH. In addition, a recent report revealed that the SFA C12:0 is a potent Toll-like receptor four (TLR4) activator [72]. Toll-like receptors are pattern-recognition receptors that induce the innate and adaptive immune responses in mammals, and therefore their activation by SFAs may initiate steatohepatitis. It is interesting that PUFAs inhibit TLR4 dimerization, which promotes the production of NADPH oxidase-dependent ROS, which is believed to be the second hit in the progression of steatosis to steatohepatitis.

5. Mitochondrial Fatty Acid Oxidation in Hepatic Steatosis

In hepatic steatosis it is believed that mitochondrial dysfunction and decreased fatty acid β-oxidation are precipitating causes for increased intracellular FFA accumulation and hepatic insulin resistance. It is of interest that mitochondrial β-oxidation is active in patients with NAFLD, and therefore the question of whether excessive influx of FFAs and overproduction of ROS promote mitochondrial injury or simply represent the consequences of abnormal fatty acid metabolism needs further investigation. Mitochondrial β-oxidation is primarily responsible for the oxidation of short chain (SCFA < C_8), medium chain (MCFA, C_8–C_{12}), and long chain (LCFA, C_{12}–C_{18}) fatty acids to acetyl-CoA, which can be condensed into ketone bodies, oxidized to CO_2 and water, or serve as the building block for lipid synthesis. Mitochondrial β-oxiation is regulated by carnitine palmitoyltransferase (CPT1), carnitine concentration, and malonyl-CoA produced by acetyl CoA carboxylase (ACC2) from cytosolic acetyl-CoA. ACC2 gene expression is induced by SREBP-1a, and therefore *Srebp-1a*-null mice have lower hepatic levels of triglycerides and higher serum levels of ketone bodies. SREBP-1a levels are increased in patients with NAFLD and may provide an important mechanism to prevent efficient β-oxidation of fatty acids by increased ACC2-mediated production of malonyl-CoA and inhibition of CPT1. ACC2 knockout mice have a greater fatty acid oxidation rate, with reduced fat mass and enhanced insulin sensitivity [73]. The initial step in mitochondrial β-oxidation is the dehydrogenation of acyl-CoA esters by a family of 4 chain length specific straight chain acyl-CoA dehydrogenases. Mice with disrupted MCFA or LCFA acyl-CoA dehydrogenase develop micro- and macrovascular hepatic steatosis [74]. In human with defects in mitochondria trifunctional protein complex (MTPr), consisting of 2-enoyl CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl CoA thiolase activities, hepatic steatosis develops quickly after birth, leading to premature death [75].

How lipotoxicity causes oxidative stress and mitochondria dysfunction is an active area of research with regards to palmitic acid toxicity. It is known that $C_{16:0}$ is a poor substrate for diacylglycerol acyltransferase, the last step in TAG synthesis; therefore, $C_{16:0}$ is used instead in the synthesis of ceramide, which can induce NADPH oxidase and disrupt mitochondrial respiration either by inducing mitochondrial release of cytochrome c or by disruption of the respiratory chain complex III [76]. However, DAG through activation of protein kinase C-dependent pathways is also able to activate NADPH oxidase and initiate FFA-induced apoptosis. However, it is uncertain whether oxidative stress from NADPH oxidase, cytochrome P450, or the mitochondria is the main contributing factor in the progression of steatosis to steatohepatitis. Treating rat H4IIEC3 hepatoma cells with either $C_{18:1}$ MUFA or $C_{16:0}$ SFA revealed that palmitic acid, but not oleate, inhibited IRS-2 tyrosine phosphorylation and serine phosphorylation of AKT, through JNK activated by mitochondria-derived ROS [77]. Thus, mitochondria-derived ROS induced by palmitic acid may be a major contributor to JNK activation and hepatic insulin resistance. However, whether a similar mechanism occurs in animal fed with a high-fat saturated fatty acid diet will have to be determined. Indeed, mitochondria isolated from mice fed a high fat diet show depressed state-3 respiration, decreased uncoupled respiration, and decreased cytochrome c oxidase activity with no change in complex-I-mediated ROS production [78]. A reduced membrane potential of mitochondria from mice fed with a high fat diet suggests that mitochondria may not be the major source of ROS in hepatic steatosis, which is contradictory to the results observed with cultured hepatoma cells [77]. Unlike animal studies, in humans with NAFLD, whole body lipid oxidation is increased because of peripheral insulin resistance, suggesting that impairment in hepatic fatty acid oxidation does not seem to contribute to hepatic steatosis in humans [79], although these data need further confirmation. Therefore, the role of mitochondrial fatty acid oxidation in the liver seems controversial, with fatty acid oxidation viewed as a

protective mechanism of disposal of potentially toxic FFAs, although increased oxidation of fatty acids can generate ROS, which may initiate steatohepatitis. Fatty acids impair mitochondrial function in human primary hepatocytes through disruption of the respiratory chain activity. This may increase ROS production in NAFLD patients, with increased fatty acid β-oxidation, resulting in uncoupling of the electron transport to oxidative phosphorylation [3, 80]. In addition to functional abnormalities in both experimental models of hepatic steatosis and in human patients with NAFLD/NASH, mitochondrial morphological changes have been observed in steatohepatitis but not steatosis. These morphological changes are believed to be due to oxidative stress-induced phospholipid phase transition that is seen as crystalline mitochondria inclusions [81]. Thus, the upstream events that lead to mitochondrial dysfunction and whether mitochondria are the source of ROS in NAFLD/NASH are largely unknown. Indeed moderate mitochondrial dysfunction in the respiratory chains may be of benefit in protecting against obesity and diabetes, by inducing the expression of mitochondrial uncoupling proteins (UCPs) and dissipation of mitochondria membrane potential.

Mitochondrial uncoupling proteins (UCP2 and UCP3) are believed to function to increase mitochondrial conductance when activated by ROS. UCP gene expression is increased in hepatic steatosis and evidence supports a role for UCP3 in fatty acid metabolism by exporting fatty acid anions, and thus maintaining mitochondria CoA levels [82]. UCP3 was found to be necessary for fasting-induced increase in fatty acid oxidation rate and capacity through mitigation of mitochondrial oxidative stress [30]. When there is an excess of FFA, either through passive transport of shorter chain fatty acids from peroxisomes or ACSVL activation and CPT1 transport that exceed the mitochondrial fatty acid oxidation potential, there is a greater risk of mitochondrial lipid peroxidation, which can be prevented by UCP3-mediated efflux of fatty acids [83, 84]. Thus, the increase in ACSLV and UCP3 proteins in hepatic steatosis is an adaptive protective mechanism to prevent mitochondrial lipotoxicity [85].

6. Peroxisomal Fatty Acid Oxidation in Hepatic Steatosis

The peroxisomal β-oxidation system metabolizes very long-chain saturated and unsaturated fatty acids (VLCFA, $>C_{18}$), prostaglandins, and leukotrienes. Peroxisomes also metabolize branched-chain fatty acids, dicarboxylic acid produced by the microsomal ω-oxidation, and C_{27} bile acid intermediates. Unlike mitochondrial β-oxidation, peroxisomal β-oxidation does not completely oxidize FAs to CO_2 and water but rather chain shortens FAs by 2 or 3 cycles of the β-oxidation. The peroxisomal β-oxidation consists of a *PPARα* inducible system that metabolizes straight-chain saturated fatty acids and a noninducible pathway that metabolizes branched-chain fatty acids and bile acid intermediates. The initial oxidation of VLCFA is performed by the *PPARα* inducible acyl-CoA oxidase (AOX1) producing trans-enoyl-CoA, which is sequentially hydrated and dehydrogenated to 3-ketoacyl-CoA by a single bifunctional enzyme, L-enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (L-BP). The ketoacyl-CoAs are converted to acyl-CoAs and acetyl-CoAs that are acted upon by a group of thioesterases, some induced by PPARα, to acetate and short- or medium- chain fatty acids that are transported to mitochondria for their complete oxidation to CO_2 and water. Branched-chain fatty acids are metabolized by a noninducible AOX and then further metabolized by the D-bifunctional enzymes (D-enoyl-CoA hydratase/D-3-hydroxyacyl CoA dehydrogenase) to acyl-CoAs, which are cleaved by a specific thiolase known as sterol carrier protein (SCP-X).

The increased expression of the PPARα-dependent peroxisomal β-oxidation pathway induced in hepatic steatosis provides an alternative mechanism to remove excessive fatty acids. Impairment of mitochondrial β-oxidation is suggested to be an important mechanism of fatty acid induced liver injury. Inhibition of mitochondrial β-oxidation can lead to dicarboxylic aciduria [86]; therefore, induction of peroxisomal β-oxidation pathway provides an adaptive protective role in reducing intracellular levels of dicarboxylic acids that inhibit the mitochondrial function. The importance of peroxisomal β-oxidation in hepatic steatosis is evident in *Aox1*-null mice, which show high levels of VLCFA in serum and serve hepatic steatosis, steatohepatitis, and hepatocellular carcinoma [56]. In lean and fatty (fa/fa) Zucker rats, obese rats showed a 50% reduction in complete mitochondria oxidation and a 3-fold increase in incomplete peroxisomal β-oxidation. The increased peroxisomal β-oxidation in Zucker obese rats accounted for up to 25% of the total mitochondrial β-oxidation by supplying shorter-chain fatty acids to mitochondria for complete oxidation [87]. Therefore, the peroxisomal oxidation of VLCFAs to SCFAs by incomplete β-oxidation provides a mechanism by which FAs can be imported into mitochondria directly without activation to a CoA ester and import by CPT1. The peroxisomes have two systems to uptake fatty acids, either as FA-CoA esters by using ATP-binding cassette transports or as free fatty acids. These systems provide a mechanism to prevent lipotoxicity of FFAs in the cytosol.

However, even though the peroxisomes have the capacity to supply shorter-chain fatty acids to the mitochondria for complete oxidation and an efficient system to remove excessive cytosolic free fatty acids, the β-oxidation system is able to generate ROS. The peroxisome-produced H_2O_2 accounts for 35% of the total cellular hydrogen peroxide produced and 20% of the total oxygen consumption in hepatocytes [88]. Although peroxisomes have numerous oxidase enzymes that produce H_2O_2, they also have several antioxidant enzymes to prevent ROS-mediated cell damage [89]. However, the massive proliferation of peroxisomes in rodents by hypolipidemia drugs and peroxisome proliferator chemicals is a viable reason why rodents develop hepatocarcinogenesis [56]. The central event in rodent hepatocarcinogenesis induced by peroxisome proliferators is the activation of PPARα since *Pparα*-null mice are refractory to peroxisome proliferation and hepatocarcinogenesis when

fed the nongenotoxic peroxisome proliferator and PPARα ligand, Wy14,643 [90]. The low levels of PPARα in human liver may be a reason why humans are resistant to the hepatocarcinogenic effects of peroxisome proliferator chemicals despite the beneficial effects of lipid lowering hypolipidemic drugs in the treatment of dyslipidemia.

The apparent beneficial effect of peroxisomal β-oxidation in hepatic steatosis is evident in *Aox1-null* mice that develop severe microvesicular steatohepatitis [91, 92]. Similarly, *Ppara*-null mice also develop steatohepatitis [93]. In both mouse models, peroxisomal β-oxidation is severely compromised, resulting in increased dicarboxylic acids that uncouple mitochondrial electron transport and inhibits mitochondrial β-oxidation pathway. However, in the *Aox1*-null mice, there a massive induction of *CYP4A* genes while *Ppar α-null* mice do not show induction of *CYP4A* genes. It is unclear about the beneficial role of CYP4A since neither the amount of ω-hydroxylated fatty acids nor intracellular level of dicarboxylic acids was determined in these studies [91–93]. However, this paradigm of severe steatohepatitis in *Aox1*-null and *Ppar α*-null mice with reduced peroxisomal and mitochondrial β-oxidation is questioned in double knock-out (DKO) mice for both genes, which develop only mild steatohepatitis [94]. These results suggest that the role of CYP4A in promoting the severity of steatosis in livers with defective peroxisomal β-oxidation is still unclear because of the lack of *PPARα*-mediated induction of *CYP4A* in DKO mice.

7. Regulation of Drug Metabolizing Cytochrome P450 Enzymes in NAFLD

The induction of cytochrome P450 drug metabolizing enzymes has been implicated as a source of ROS in the perpetuation and progression of steatosis to steatohepatitis. Microsomal oxidation of fatty acids, catalyzed by cytochrome CYP2E1, CYP4A10, CYP4A12, and CYP4A14, which are induced in mouse models of steatosis and steatohepatitis, is a potent source of ROS through uncoupling of their catalytic cycles. The production of ROS by cytochrome P450 catalytic cycle is dependent on the redox potential and spin state of the transition element iron of heme [95]. Cytochrome P450 normally functions as a monooxygenase but can work as an oxidase releasing H_2O_2 into endoplasmic environment. The P450 catalytic cycle can produce superoxide and peroxide by uncoupling of the substrate metabolism with electron transport [96]. ROS can also be produced by the P450 catalytic cycle by futile cycling and redox cycling (Fe^{+2}/Fe^{+3}) with the former producing free radical semiquinones and the latter by a similar redox cycling observed in the mitochondria electron transport chain [97].

The ethanol-inducible CYP2E1 is elevated in both rodent experimental models of steatosis and steatohepatitis and human NAFLD patients, and in vivo levels of CYP2E1 activation correlate with the severity of liver damage, suggesting that this P450 is one of the major microsomal contributors of ROS-induced hepatic injury [6, 98]. *CYP2E1* is unique among P450s since it is loosely bound to ER membrane and naturally present in the high spin Fe^{+3} state. Therefore it can produce ROS even in the absence of its potentially toxic substrates [99] while its iron is able to interact directly with molecular oxygen [96]. The induction of *CYP2E1* by many potentially toxic/carcinogenic substrates such as ethanol, benzene, and haloalkanes and their metabolisms promote ROS production [6]. Furthermore, the substrates that are poorly metabolized by CYP2E1 such as fatty acids lead to uncoupling and futile catalytic cycle with increased ROS production. Thus, the P450 catalytic cycle generates significant amounts of reactive superoxide anion, substrate radical, and protonation of peroxy-cytochromes along with a third leaky mechanism requiring two-electron transfer followed by decay of peroxycytochrome P450 with the release of superoxide. The efficiency of electron transfer from NADPH to P450 catalytic cycle is called the degree of coupling, which is less than 50% or lower in eukaryotes [96]. Due to the high uncoupling rates of the P450 catalytic cycle, the rates of NADPH and oxygen consumption are weakly dependent on the presence of a substrate. Therefore, the microsomal P450 monooxygenase system is a significant contributor of ROS formation in hepatocytes.

CYP2E1 seems to play a key role in the pathogenesis of alcoholic liver injury because of its induction in chronic alcohol drinkers and ability to produce ROS [100]. Increased CYP2E1 protein levels are observed in animal models of fatty liver diseases and in morbid obese men with NAFLD and patients with NASH [101, 102]. CYP2E1 P450 levels are increased in patients with NASH because fatty acids and ketone bodies including acetone, which increased in diabetes/ketosis [96], are substrates and inducers of CYP2E1 protein. However, even with this apparently close association of CYP2E1 with both NAFLD and NASH and in several animal models of both alcoholic and nonalcoholic steatosis, *Cyp2e1*-null mice still develop diet-induced NASH or alcoholic liver inflammatory disease indicating that *Cyp2e1* deletion neither prevented nor decreased oxidative damage [103–105]. However, these *Cyp2e1*-null mice did show a dramatic increase in the amounts of *CYP4A10* and *CYP4A14* fatty acid omega hydroxylases, and inhibition of *CYP4A14* P450 prevented oxidative damage in *Cyp2e1*-null mice [104], thus demonstrating an important role of *CYP4A* P450 isozymes in the steatohepatitis induced by a MCD diet.

The omega fatty acid hydroxylase P450s (*CYP4A*) metabolize a variety of endogenous saturated and unsaturated fatty acids that are sequentially dehydrogenated to their corresponding dicarboxylic acids in the cytosol [106]. Dicarboxylic acids are activated by *dicarboxyl CoA synthetase* (ACSVL1) CoA esters that are chain shortened by peroxisomal β-oxidation. Although microsomal ω-oxidation of fatty acids and peroxisomal β-oxidation are minor pathways for fatty acid oxidation, under fatty acid overload, significant amounts of dicarboxylic acids are formed in patients with NAFLD, obesity, and diabetes. Dicarboxylic acids are highly toxic to mitochondria and therefore efficient disposal is necessary to prevent mitochondrial dysfunction during hepatic steatosis. PPARα agonists and fatty acids elevated during fasting and hepatic steatosis could prevent dicarboxylic acid formation through induction of the genes including ω-oxidation CYP4A and peroxisomal β-oxidation.

Although the ethanol-inducible *CYP2E1* and fatty acid metabolizing *CYP4A* P450s may be directly involved in the progression of steatosis to steatohepatitis by production of ROS and initiation of lipotoxicity, other drug metabolizing cytochrome P450 genes are dramatically affected in fatty liver disease and therefore have an important role in identification of appropriate drug treatment modalities to avoid the consequences of adverse drug reactions or drug toxicity. In primary human hepatocytes isolated from liver with macrosteatosis, there is a 60% to 40% reduction in 7-ethoxycoumarin *O*-deethylation (ECOD) and testosterone oxidation with a reduction in *CYP1A2*, *CYP2C9, CYP2E1,* and *CYP3A4* mRNA and their respective proteins to metabolize their specific substrate drugs [107]. A recent study analyzed changes in hepatic cytochrome P450 mRNA, protein and enzymatic activity in human patients with steatosis, steatohepatitis and hepatitis without steatosis, which reflects the beginning of liver fibrosis [108]. These livers therefore represent the progressive stages of NAFLD to NASH and ultimately fibrosis. During NAFLD progression, *CYP2E1*, *CYP2C19,* and *CYP1A2* mRNA and the corresponding P450 protein contents were decreased while those of *CYP2A6*, *CYP2B6* and *CYP2C9* mRNA and the proteins were increased [109]. During disease progression, *CYP2D6*, *CYP3A4* or *CYP2C*8 mRNA levels did not change; however CYP3A4 and CYP2D6 protein levels decreased. The changes in CYP P450 levels correlated with the changes in the enzymatic activities of the different P450s in the progression of NAFLD. The differential expression of P450 in the progression of NAFLD to NASH and/or fibrosis has an important clinical implication in patient treatment to avoid adverse drug reactions and possible drug toxicity. CYP2C9 is the second most abundant P450 expressed in human liver and is responsible for the metabolism of *S*-warfarin, Tamoxifen, fluoxetine, losartan, and the antidiabetic PPARγ agonist rosiglitazone. The increase in *CYP2C9* mRNA, protein, and enzymatic activity during NAFLD progression would suggest that the standard dose of rosiglitazone to treat patients with type II diabetes may be ineffective in managing hyperglycemia in patients with NASH or liver fibrosis. CYP3A4 is the most prominent P450 expressed in the human liver and metabolizes over 50% of all therapeutic drugs prescribed. Therefore, reduced CYP3A4 levels during the progression of NAFLD to fibrosis and liver cirrhosis put these patients at risk for adverse drug reaction and possible drug toxicity [110]. In contrast to the many studies indicating a role of *CYP2E1* in NAFLD and NASH [109], the decrease in *CYP2E1* mRNA and protein from steatosis to steatohepatitis and fibrosis needs to be confirmed independently. However, these data question the role of *CYP2E1* in oxidative damage-induced disease progression and suggest a possibility that other P450 enzymes such as CYP4A isozymes elevated in NAFLD and NASH may be involved in the progression of fatty liver diseases.

8. Microsomal Fatty Acid Oxidation in Hepatic Steatosis

The ω-hydroxylation of saturated and unsaturated fatty acids by *CYP4* family members has long been thought to be a minor pathway in the metabolism of fatty acids accounting for 5%–10%. However, its importance is dramatically increased due to their upregulation during fasting, starvation, and in several human diseases where its contribution to fatty acid metabolism increases dramatically to 15%–30%. The close association between microsomal CYP4 ω-hydroxylation of MCFAs and peroxisomal β-oxidation of LCFAs is evident by the conversion of dicaboxylic acids to succinate, an anaplerotic gluconeogenic precursor, and acetate, which can be used by peripheral tissue like ketone bodies during fasting and starvation through a dramatic induction of *CYP4A* genes. The increased expression of *CYP4A* genes during fasting, starvation, by a high fat diet and in steatohepatitis may be a mechanism to prevent lipotoxicity from FFAs, but at the expense of possibly increased uncoupling of the P450 catalytic cycle, leading to increased ROS production.

In mammals, six *CYP4* gene subfamilies have been identified: *CYP4A*, *CYP4B*, *CYP4F*, *CYP4V*, *CYP4X*, and *CYP4Z* [111–114]. Three of these subfamily members (i.e., *CYP4A*, *CYP4B*, and *CYP4F*) have been shown to ω-hydroxylate saturated, branched, unsaturated fatty acids and the eicosanoids. Members of the *CYP4B* subfamily metabolize SCFAs (C_7–C_9), while members of the *CYP4A* subfamily metabolize MCFAs (C_{10}–C_{16}), and members of the *CYP4F* subfamily metabolize LCFA and VLCFA (C_{18}–C_{26}) fatty acids.

Members of the *CYP4A* family are by far the best characterized ω-fatty acid hydroxylases in regard to their induction by peroxisome proliferators, PPARα, and regulation by fasting, high fat diet, ethanol consumption, and in diabetes in rodents. The importance of *CYP4A* P450s in the metabolism of MCFAs is evident by their upregulation during starvation, caloric restriction, and in animals fed a high fat diet, which mimics starvation-induced lipolysis and excessive fatty acid transport to the liver. In these situations, there is a dramatic induction of the *CYP4A* genes, which may function to not only prevent lipid toxicity but also provide consumable nutrients for peripheral tissue during starvation. MCFAs in hepatocytes are transported into the peroxisomes as FFAs or as dicarboxylic acids after *CYP4A* ω-hydroxylation and esterified by *peroxisomal acyl-CoA synthetase* ACSVL1 (FATP2) and ACSVL5 (FATP4) [26]. MCFA acyl-CoAs undergo 2 to 3 rounds of peroxisomal β-oxidation, producing succinyl-CoA and acetyl-CoA [115, 116]. These products are converted by several peroxisomal acyl-CoA thioesterases (ACOT), which can catalyze the hydrolysis of CoA esters of different chain-length fatty acids including succinate. Succinate can be directly used as an anaplerotic intermediate for gluconeogensis while released acetate can be taken up and oxidized by extra-hepatic tissues in the same way as ketone bodies for energy production. During starvation or administration of hypolipidemic drugs that activate PPARα, there is a rapid proliferation of peroxisomes in rodents but not humans. In humans, the *CYP4A11* and *CYPF2* genes are not induced by peroxisome proliferators (PP), and therefore the absence of peroxisome proliferation may be due to decreased levels of ωHEET, which is a high affinity ligand in the PPARα activation. In humans, the hypolipidemic effect of peroxisomal proliferators is not mediated through PPARα

activation but through the suppression of HNF4α by PPs-CoAs [55]. HNF4α controls genes involved in the production of lipoproteins [55]. Thus, the activation of PPARα in rodents by ωHEET and the suppression of HNF4α in humans by PP-CoAs may explain the absence of peroxisome proliferation in humans and why humans in contrast to rodents are resistant to the hepatocarcinogenic effects of hypolipidemic drugs.

In contrast to the *CYP4A* members, *CYP4F* P450s isozymes ω-hydroxylate a variety of LCFAs and VLCFAs, unsaturated and branched-chain fatty acids,and vitamins with long alkyl side chains, the physiologically important leukotrienes (LT), prostaglandins (PG), and hydroxyeicosatetraenoic acids (HETE) [117–121]. The human *CYP4F* P450s metabolize and inactivate the proinflammatory leukotriene B_4 (LTB_4) [118], with the myeloid-expressed CYP4F3A having a twofold greater affinity for LTB_4 than CYP4F2 expressed in liver, kidney, and skin, but not in myeloid cells. However, *CYP4F3B* splice variant of the *CYP4F3* gene expressed in liver and has a similar affinity as CYP4F2 for LTB_4 [122]. Both CYP4F3B and CYP4F2 P450 can metabolize arachidonic acid to 20-HETE while CYP4F3A has little activity towards arachidonic acid. Besides ω-hydroxylating other proinflammatory eicosanoids such as 5-HETE, 12-HETE, and 8-HETE, *CYP4F* P450s can metabolize the anti-inflammatory lipoxins, LXA_4, and LXB_4. The ability of CYP4F3 and CYP4F2 to ω-hydroxylate both pro- and anti-inflammatory leukotrienes indicates that they may function both in the activation and resolution phases of the inflammatory response [106, 113].

Similar to the MCFAs and SCFAs, omega-hydroxylated LCFAs and VLCFAs are converted to their corresponding dicarboxylic acids by the sequential action of cytosolic *alcohol* and *aldehyde dehydrogenases*. The roles of different chain length ω-hydroxylated fatty acids in lipid metabolism are indicated by increased ω-hydroxylation of MCFAs by *CYP4A* P450s in rodents during fasting, by peroxisome proliferators, and in hepatic steatosis while decreased *CYP4F* genes expression by peroxisome proliferators and during starvation results in reduced ω-hydroxylation of LCFAs and VLCFAs. Unlike the ω-hydroxylated MCFAs, which are β-oxidized to succinyl-CoA and acetate, the omega-hydroxylation of LCFAs would produce only SCFAs and acetate. Excessive acetate in the hepatocyte cytosol [123] can be used for the synthesis of cholesterol and fatty acids with malonyl-CoA inhibiting the mitochondrial CPT1 fatty acid uptake and therefore blocking mitochondrial β-oxidation. Dicarboxylic acids are almost elusively metabolized by the peroxisomal β-oxidation system since the Km value for dicarboxylic acids by the mitochondrial system is 15–40-fold higher than that of the peroxisomes [124]. Furthermore, branched and long chain saturated and unsaturated fatty acids are preferentially metabolized by the peroxisomes. The transportation of fatty acids into the peroxisomes is different for MCFAs, LCFAs, and VLCFAs. MCFAs are transported as free acids, which are esterified in peroxisomes to their corresponding CoA derivatives by FATP2 and FATP4, while LCFAs and VLCFAs are transported by ABC transporters as CoA derivatives [125]. In addition, β-oxidation in the peroxisomes does not completely oxidizes fatty acid substrates but produces shorter-chain fatty acids which are transported from the peroxisomes to mitochondria for either complete oxidation by the mitochondrial β-oxidation or usage for synthesis of other fatty acids, as seen in the conversion of C24:6n-3 to docosahexaenoic acid (C22:6n-3).

Omega-hydroxylated fatty acids and eicosanoids have several metabolic fates dependent upon the cell type and *CYP4* genes expressed. In the liver, ω-hydroxylated fatty acids can be metabolized and used for energy production, lipogenesis, the synthesis of structural lipids, and production of fatty acids that function as agonists in the regulation of hormone nuclear receptors (HNRs) (Figure 1). The diverse array of fatty acids omega-hydroxylated by members of the *CYP4* family and their functional roles in metabolism, cell signaling, inflammation, and lipid structure suggest that CYP4 members play an important role in human diseases [106]. The importance of ω-hydroxylated fatty acids and eicosanoids in human diseases is evident by the role 20-HETE has in hypertension and vascular disease and its dramatic appearance in patients with end stage liver diseases, and the possible role of ω-hydroxylated fatty acids in lipid metabolism in fatty liver diseases [113, 126–128].

9. Role of CYP4 Isozymes in the Initiation and Progression of NAFLD to NASH

NAFLD encompasses a broad disease spectrum ranging from simple triglyceride accumulation in hepatocytes (hepatic steatosis) to hepatic steatosis with inflammation (steatohepatitis) that can progress to fibrosis and cirrhosis [1]. A two-hit hypothesis has been proposed to explain the progression of NAFLD as hepatic triglycerides being the first hit, while increased oxidative stress, as the second hit, promoting liver inflammation, cell death, and fibrosis in NASH [127, 129]. Both obesity and insulin resistance are strongly associated with NAFLD, resulting in the hydrolysis of adipose triglycerides by hormone sensitive lipases, leading to elevated plasma and hepatic levels of FFAs. In the liver FFAs can be either metabolized by the mitochondrial β-oxidation system for energy production or esterified to triglycerides and incorporated into VLDL particles with cholesterol esters and phospholipids for transport and use by peripheral tissues. Through the use of mouse models of fatty liver disease and the generation of knockouts of key regulator enzymes involved in lipid and glucose metabolism, we are beginning to understand the key molecular targets responsible for increased triglyceride accumulation in hepatocytes and how fatty acids induce oxidative stress and the progression of steatosis to steatohepatitis.

Key regulatory enzymes control the ability of the liver to provide nutrients to peripheral tissues through gluconeogenesis, ketogenesis, VLDL secretion and possibly acetate. Interestingly, key regulatory enzymes are differentially affected in liver insulin resistance with the major pathway in lipogenesis being activated while the hepatic gluconeogenic pathway is active under insulin resistance. Both a*cetyl-CoA carboxylases* (ACC1 and ACC2) and *stearoyl-CoA desaturase* (SCD-1) are

activated in the insulin resistant liver. ACC1 is responsible for converting acetyl-CoA to malonyl-CoA, which functions as both a precursor of cholesterol and fatty acid synthesis and also a potent inhibitor of mitochondrial CPT1 necessary for the transport of fatty acids to the mitochondria for β-oxidation while SCD-1 functions to desaturate stearic acid ($C_{18:0}$) to oleic acid ($C_{18:1}$) necessary for synthesis of triglycerides. These genes are activated through insulin-mediated activation of two transcription factors (e.g., SREBP-1c and ChREBP). During hepatic steatosis in both humans and mouse models, there is an excessive accumulation of oleic acid either from increased de novo fatty acid synthesis or conversion of imported fatty acids from the adipose tissues. Elevated hepatic glucose production in the presence of hyperinsulinemia is a hallmark of insulin resistance in the liver even with the increased expression of the liver specific *pyruvate kinase* (L-PK), a key *regulatory* enzyme in converting phosphoenolpyruvate to pyruvate during glycolysis. Increase *L-PK* gene expression is mediated by glucose activation of the ChREBP transcription factor. ChREBP has also been shown to increase the expression of many of the fatty acid synthesis enzymes as well as SCD-1, thereby facilitating the conversion of glucose to fatty acids. It is presently unknown why hyperinsulinemia activates the lipogenic pathway, but fails to prevent the inactivation of a gluconeogenic pathway and hyperglycemia observed in insulin resistant diabetes [127, 129, 130].

The activation of both anabolic and catabolic pathways during insulin resistance in the liver suggests that different fasting and feeding signals are simultaneously controlling the metabolic responses of the liver. What these signals are and how they function either directly to activate key regulator enzymes or as agonists or antagonists to activate key transcription factors that control the expression of the genes involved in fatty acid catabolism (PPARα, HNF4α) or fatty acid synthesis (SREBP-1c, ChREBP, PPARγ) remain to be determined.

The increase in peroxisomal β-oxidation during steatosis exerts a beneficial effect in NAFLD by metabolizing excessive fatty acids to shorter-chain fatty acids that can be directly transported and completely oxidized by the mitochondrial β-oxidation system. In addition, the incomplete peroxisomal β-oxidation of fatty acids can supply the anaplerotic mitochondrial intermediate, succinate, necessary for gluconeogenesis, while acetate from acetyl-CoA can be used for the anabolic synthesis of cholesterol and fatty acid as seen in NAFLD [131–133]. The peroxisomes, unlike mitochondria, metabolize long chain fatty acids, exclusively metabolize branched-chain fatty acid, and preferentially metabolize dicarboxylic acids, which are produced by the ω-hydroxylation of fatty acids by members of the *CYP4* gene family. The increased expression of the *CYP4A* omega-hydroxylases during steatohepatitis and their induction in animals fed with a high-fat diet suggest that they may play a pivotal role in lipotoxicity [134], and may be responsible for the induction of oxidative stress as well as progression to steatohepatitis. A dramatic induction of both the mouse *CYP4A10* and *CYP4A14* genes is seen in *Cyp2e1*-null mice and likely accounts for the increased ROS that induce lipid peroxidation [103, 104]. The increased production of dicarboxylic acids during steatosis by *CYP4A* members can impair mitochondrial function by dissipation of the mitochondria proton gradient and uncoupling of oxidative phosphorylation. In addition, the uncoupling of the P450 catalytic cycle has been known to be a major source of ROS, which led to the identification of the ethanol-inducible CYP2E1 P450 as a major source of ROS-mediated microsomal lipid peroxidation [96]. CYP2E1 can metabolize fatty acids at the ω-1 position, and CYP4A, which normally ω-hydroxylates lauric acid and hydroxylates longer chain fatty acids at both the ω- and ω-1 positions. It is not known whether different chain length fatty acids assist in the uncoupling of the CYP2E1 and CYP4A catalytic cycles or whether cytochrome b_5, which increases P450 catalytic activity and prevents uncoupling, can reduce ROS formation in fatty liver disease [135]. Both cytochrome b_5 reductase and cytochrome b_5 are also used in the desaturation of stearic and palmitic acids by SCD-1. It is unknown whether increased conversion of stearic acid to oleic acid observed in NAFLD increases uncoupling of the P450 catalytic cycle, resulting in increased ROS formation by SCD-1 sequestering cytochrome b_5. Both cytochrome b_5 and cytochrome b_5 reductase have been identified as susceptibility genes in obesity [136]. While the induction of *CYP4A* genes during fasting provides both gluconeogenic precursors and acetate to supply the needs for peripheral tissues, their induction during steatosis may increase hyperglycemia, shuttle acetate for synthesis of fatty acids and cholesterol, and increase ROS formation by uncoupling of the P450 catalytic cycle.

Similar to the role of *CYP4A* genes in possibly initiating hepatocyte cell injury and steatohepatitis, *CYP4F* genes may also play a functional role in hepatic lipid accumulation and in the recruitment of inflammatory cells during progression from steatosis to steatohepatitis. Unlike *CYP4A* genes that are induced by fasting, hypolipidemic drugs, and peroxisome proliferators through PPARα activation, the *CYP4F* genes were reported to be repressed during fasting and by peroxisome proliferators possibly by PP-CoA or PUFA-CoA mediated inhibition of HNF4α [137]. Furthermore, unlike the *CYP4A* genes that are induced in fatty liver, our preliminary data suggest that the mouse *CYP4F* genes may be repressed in mice fed a high fat diet and in the leptin-deficient ob/ob mice as a model of fatty liver disease (unpublished results). The recent report of lipid accumulation in primitive liver cells (oenocytes) of Drosophila that have a mutation in the *stearic ω-hydroxylase CYP4g1* gene [138] suggests that *CYP4F* genes may play an important role in maintaining lipid homeostasis in the liver. Drosophila homozygous mutant for *CYP4g1* manifests a two-fold increase in the oleic acid : stearic acid ratio (C18 : 1/C18 : 0) with a notable imbalance in the fatty acid desaturation found in the TAG fraction but not in the phospholipid fraction. This suggests that CYP4g1 is important in the metabolic storage of fatty acids and its expression would decrease oleic acid synthesis and storage of fatty acids in TAGs. The human CYP4F2 efficiently ω-hydroxylates stearic acid and therefore may serve the same function as CYP4g1 in competing with SCD-1 in the metabolism of stearic acid [117]. It is currently unknown whether the human *CYP4F2*

gene is repressed by fatty acids in patients with NAFLD. However, one study suggested that the human *CYP4A11* mRNA was reduced in obese individuals [126]. In contrast, the *CYP4F2* gene has been shown to be downregulated by peroxisome proliferators [139], induced by retinoic acids [140], and its expression increased by lovastatin-mediated activation of SREBP-2 [141].

Therefore, the activation of the *CYP4F2* gene in fatty liver diseases may decrease the formation of oleic acid and storage of TAG in the liver, and also play a vital role in preventing recruitment of immune cells to the liver during steatohepatitis by metabolism of the proinflammatory leukotrienes. Even though there are numerous studies showing that fatty acids induce hepatocytes to produce proinflammatory cytokines and chemokines (IL-8) which attract neutrophils to the liver, the function of leukotrienes in attracting immune cells to the liver during steatohepatitis has not been explored. Whether LTB_4 or LTC_4 increases production of the potent neutrophil chemokine IL-8 during hepatic steatosis remains to be determined. The genetic association of *CYP4F2* and the neutrophil-specific *CYP4F3A* gene in Celiac disease establishes a connection between the innate immune response of neutrophil recruitment to the established Th1 adaptive immune response in disease patients [142]. Furthermore, Celiac disease has been associated with several inflammatory diseases of the liver including primary biliary cirrhosis, primary sclerotizing cholangitis, autoimmune hepatitis, hemochromatosis, and fatty liver diseases [143].

It will be of significant importance to understand how the *CYP4F* genes are regulated by fatty acids and in animal models of fatty liver disease. Whether induction of CYP4F long chain fatty acid ω-hydroxylases can prevent steatosis by inhibiting SCD-1 activity and reduce the hepatic levels of proinflammatory leukotrienes in steatohepatitis needs further study.

10. Genetic Regulation of CYP4A and CYP4F Genes and Their Roles in Lipid Metabolism during Hepatic Steatosis

The differential regulation of the *CYP4A* and *CYP4F* genes by peroxisome proliferators, during fasting and by high fat diets indicates that these P450s may have distinct roles in lipid metabolism during the fasting and feeding cycles, and that different nuclear transcription factors regulate the expression of these genes (Figure 1). It is well established that the rodent *CYP4A* genes are induced by or during hepatic steatosis and steatohepatitis [104, 134, 144, 145]. However, their functional roles in initiation and progression of NAFLD to NASH are relatively unknown. The importance of induction of *CYP4A* genes in hepatic steatosis and steatohepatitis was revealed in *Cyp2e1*-null mice that developed severe steatohepatitis with a pronounced increase in CYP4A10 and CYP4A14 associated with increased accumulation of lipid peroxides. The knockout mice deficient of the peroxisome straight chain acyl-CoA oxidase (*Aox*-null) fed a normal diet develop severe steatohepatitis with a massive increase in CYP4A protein expression. In contrast, *Ppar*α-null and *Aox*-null double-knockout mice develop only mild to moderate steatohepatitis with much less lipid peroxide accumulation because a CYP4A protein, which produces ROS and lipid peroxidation, was not induced. It is known that PPARα agonists exhibit a beneficial effect on preventing steatohepatitis by increasing fatty acids β-oxidation and indirectly by inhibiting SREBP-1c, thus protecting against obesity-induced hepatic inflammation [146, 147]. To resolve this issue, Leclercq and colleagues fed wild type and *Ppar*α-null mice with a MCD diet to induce steatohepatitis and the specific PPARα agonist, Wy14,643 [145]. MCD-diet fed *Ppar*α-null mice, which are very sensitive to oxidative stress [148], develop severe steatohepatitis in the absence of *CYP4A* induction and, Wy14,643 decreased the degree of steatohepatitis in these mice. These data suggest that *CYP4A* induction was not necessary for promoting steatohepatitis or the cause of increased microsomal lipid peroxides in *Ppar*α-null mice fed a MCD diet [145]. It is difficult to reconcile the effect of the PPARα agonist in the prevention of steatohepatitis in *Ppar*α-null mice since none of the mitochondrial, peroxisomal β-oxidation, and microsomal ω-oxidation pathways was induced by Wy14,643 in these mice. These results indicate that inflammatory cells might be responsible for increased lipid peroxidation and steatohepatitis in *Ppar*α-null mice. These data further suggests that Wy14,643 may have a *PPAR*α independent effect on the amelioration of steatohepatitis. In fact, another *PPAR*α agonist bezafibrate at clinically relevant doses decreased serum and liver triglycerides through down-regulation of SREBP-1c, revealing a clue for *PPAR*α-independent mechanism in the suppression of de novo lipogenesis [146]. In contrast, wild-type mice fed the MCD diet and Wy14,643 do not develop steatohepatitis, although both the rates of peroxisomal and mitochondrial β-oxidation are increased with a 20~50-fold-fold upregulation in *CYP4A10* and *CYP4A14* gene expression. Thus, these results further question the role of ROS generation from either mitochondrial β-oxidation or microsomal ω-oxidation in the development of steatohepatitis. To resolve this issue and to further define the role of *CYP4A* genes in production of ROS during hepatic steatosis, mouse models overexpressing *CYP4A10* and *CYP4A14* will be needed to determine if excessive fatty acid induces uncoupling of the P450 catalytic cycle and generation of ROS during the progression of NAFLD to NASH. However, most PPARα agonists activate the expression of the *CYP4A* genes, suggesting an interesting paradox whether *CYP4A* gene induction is beneficial or harmful in promoting steatohepatitis. A recent study suggests that *CYP4A14* overexpression in hyperoxia increases resistance to oxidative stress [149]. In contrast to the extensive data on the role of rodent *CYP4A* gene in animal models of steatosis and steatohepatitis, little is known about how the human *CYP4A11* gene is controlled by PPARα agonists and its functional role in NAFLD or NASH. The modest 2-fold induction of the *CYP4A11* gene by peroxisome proliferators in primary human hepatocytes compared with the 30–70 fold induction of the mouse *CYP4A* genes indicates a species difference between rodents and humans with respect to regulation of the *CYP4A* genes by peroxisome

proliferators [150]. In addition, the 60–700 fold increase in mouse *CYP4A* mRNA during fasting [144] and 2–8 fold decrease in *CYP4F* mRNA further indicate the differential regulation of these distinct genes. In humans with obesity, *CYP4A11* mRNA decreased by 50% while in NAFLD patients while *CYP4A11* mRNA increases 4-fold [126], suggesting a differential regulation in liver disease progression.

LCFAs are endogenous ligands in the activation of NHRs, PPARα and HNF4α. LCFAs and LCFA-CoAs are significant NHR ligands as shown by their presence in the nucleus, their high affinity binding (Kd values ~ nM ranges), their ability to induce conformational changes in NHRs, and their ability to induce coregulator recruitment to nuclear receptors [55]. Support for LCFA-CoAs in the hyperactivation of *PPARα* was evident in *Aox*-null mice with accumulated VLCFAs and VLCFA-CoAs, and the observation that the thio-esterification inhibitor, 2-bromopalmitate inhibits bezafibrate induced peroxisome proliferation in rodents. In humans, the importance of VLCFA-CoAs in PPARα activation was evident in adrenoleukodystrophy where there is accumulation of VLCFA in the cytosol, but no peroxisome formation of VLCFA-CoA and no hyperactivation of PPARα [120]. Serum fatty acids increase dramatically from the normal physiological range of 200 uM to 1 mM under fasting and up to 8 mM in Refsum's disease, adrenoleukodystrophy, Zellweger's syndrome, and fatty liver diseases, diabetes, and inflammation. This suggests an important link between peroxisome fatty acid metabolism and conversion of VLCFAs to VLCFA-CoAs in the activation of PPARα and control of *CYP4* gene expression. PPARα has a high affinity for polyunsaturated LCFAs, LCFA-CoAs and VLCFA-CoAs, but not saturated LCFAs or VLCFAs while HNF4α has a high affinity for saturated LCFAs and VLCFA acyl-CoAs but not polyunsaturated acyl-CoAs (Figure 1). These facts indicate that fatty acid CoA chain length and degree of unsaturation determine whether HNF4α or PPARα will be activated [48]. The mechanism of LCFA uptake and importation into the nucleus has recently been shown to be mediated by L-FABP, which binds polyunsaturated LCFAs with a greater affinity than saturated LCFAs and associates with PPARα, while ACBP preferentially binds saturated LCFAs and associates with HNF4α [55]. These studies indicate that ACBP selectively cooperates with HNF4α while L-FABP selectively cooperates with PPARα, which is believed to elicit downstream alteration in coactivator and corepressor association with NHRs. Thus, the binding of saturated LCFA-CoAs to HNF4α would increase HNF4α activity and inhibit PPARα transactivation while polyunsaturated LCFA-CoAs would decrease HNF4α activation and increase L-FABP PPARα transactivation. Since PPARα and HNF4α regulate gene transcription through similar promiscuous DR1 sequences, and compete for the same coactivators and corepressors, the specificity of receptor activation may be determined by either saturated or polyunsaturated fatty acid ligand while the cross-talk between these transcription factors would be determined by the FABP/ACBP mediated coregulator recruitment and repression of the cognate receptor. For instance, the differential regulation of the *CYP4A* and *CYP4F* genes may be determined by cross-talk between PPARα and HNFα through the type of fatty acid ligand, method of nuclear import, and receptor activation by L-FABP or ACBP. This scenario is highly likely in the regulation of *CYP4A* and *CYP4F* genes since peroxisome proliferators (PP) activate PPARα while PP-CoAs inhibits HNF4α transactivation. It is also possible that MCFAs and VLCFAs metabolized by the *CYP4A* and *CYP4F* may produce fatty acid metabolites that reciprocally regulate the expression of the *CYP4A* and *CYP4F* genes (Figure 1). The induction of *CYP4A* genes by a high fat diet leads to increased production of dicarboxylic acids that are potent inhibitors of HNF4α transactivation, which may contribute to the suppression of the *CYP4F* genes during steatosis.

11. Conclusion and Discussion

The concentrations of FFAs increase either in the blood plasma through a high-fat diet and release by adipocytes or in the liver as a consequence of lipolysis or de novo fatty acid synthesis. FFAs travel through the body mainly bound to albumin and intracelluarly bound to fatty acid transport proteins (FABP, FATP), which regulate their intracellular fate. Evidence suggests that FFAs induce insulin resistance by raising intracellular lipid metabolites, which activate protein kinase C that inhibits nuclear factor kappa β kinase and activates the inflammatory pathway. Activation JNK and/or p38 kinase especially by saturated FFAs and increased ROS [151, 152] leads to serine phosphorylation and inhibition of insulin receptor substrates IRS-1 and IRS-2, resulting in decreased insulin signaling (i.e., decreased Tyrosine phosphorylation) and increased hepatic gluconeogenesis during insulin resistance. The elevation of plasma FFAs in obesity, fatty liver diseases and insulin resistance are predictors of type 2 diabetes, and therefore understanding the mechanisms to decrease hepatocyte intracellular levels of FFAs will offer opportunities to prevent hepatic lipotoxicity and treat not only fatty liver diseases, but also metabolic syndrome associated with obesity and diabetes. The importance of understanding the mechanism by which PPARs can be used to treat hepatic steatosis is apparent considering that PPARα, although downregulated in fatty liver diseases, is a viable target to increase the mitochondrial, peroxisomal β-oxidation, and microsomal ω-oxidation of FFAs to prevent lipotoxicity despite production of some ROS by the latter two processes. In hepatic steatosis and NAFLD, hepatic PPARγ levels increase; this compensates for the decreased PPARα and presents another important target to prevent excessive intracellular FFAs by increasing de novo lipogenesis and thus preventing the lipotoxicity from FFAs. It will be of importance to determine if PPARγ is able to be activated by selective fatty acids through interaction with L-FABP or ACBP. Furthermore, recent evidence has suggested that PPARδ is a true sensor of plasma FFA levels by interacting with and stimulating the expression of lpin2 and St3gal5 genes after fasting [153]. Whether selective activation of PPARδ can activate the genes involved in either the disposal or lipogenesis to prevent FFA-mediated lipotoxicity will be important in understanding the pathogenesis of NAFLD and NASH. Although, steatosis is the first step or hit in

the progression of NAFLD to NASH, the source of ROS in the second step has not been clearly defined and will require investigations. These may include simultaneous measurement of the source of ROS generated by mitochondria, peroxisome, microsome, and inflammatory cells during the progression of steatosis to steatohepatitis. Finally, based on the similar steps of disease progress between NAFLD and alcoholic fatty liver diseases (AFLD) [7, 152], the similar mechanisms and relevant problems can be also applied to understanding of the pathogenesis mechanisms of AFLD.

Abbreviations

PUFA: Polyunsaturated fatty acid
MUFA: Monounsaturated fatty acid
SFA: Saturated fatty acid
MCFA: Medium chain fatty acid
LCFA: Long chain fatty acid
VLCFA: Very long chain fatty acid
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
FFA: Free fatty acid
PPARα: Peroxisome proliferator activated receptor α
SREBP: Sterol regulatory binding proteins (SREBP-1 and SREBP-2)
LXRα: Liver X receptor α
ChREBP: Carbohydrate response element binding protein
ACBP: Acyl CoA binding protein
MTP: Microsomal triglyceride transfer protein
FABP Fatty acid binding protein
FATP: Fatty acid transport protein
HNF4α: Hepatocyte nuclear factor 4
DNL: De novo lipogenesis
NEFA: Nonesterified fatty acids
ROS: Reactive oxygen species
LTB_4: Leukotriene B_4
20-HETE: 20-hydroxyeicosatetrenoic acid
CYP: Cytochrome P450
LDL: Low density lipoprotein
HDL: High density lipoprotein
VLCL: Very low density lipoprotein
SCD-1: Stearoyl CoA-desaturase
JNK: c-Jun N-terminal protein kinase
IRS: Insulin receptor substrate
DAG: Diacylglycerol
PEPCK: Phosphoenolcarboxylase kinase
G6Pase: Glucose 6-phosphatase
UCP: Uncoupling protein
L-BP: L-bifunctional protein
HEET: Hydroxyepoxyeicosatetraenoic acid
PP-CoA: Peroxisome proliferator-CoA
CPT-1: Carnithine palmitoyltransferase
DHA: Docosahexaenoic acid
L-PK: Liver pyruvate kinase
ACC: Acyl-CoA carboxylase
IL-8: Interleukin 8
TAG: Triacylglycerol
MCD: Methionine-choline deficient diet
Lpin2: Phosphatide phosphohydrolase
st3gal5: Monosialoganglioside synthase (GM3 synthase).

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