Rous-Whipple Award Lecture

Peroxisome Proliferators and Peroxisome Proliferator-Activated Receptor α

Biotic and Xenobiotic Sensing

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The morphological phenomenon of peroxisome (microbody) proliferation, induced in hepatic parenchymal cells of rats and mice by the hypolipidemic drug clofibrate,¹ fascinated me ever since I ventured into the research laboratory of the late Donald J. Svoboda at the University of Kansas Medical Center as a pathology resident more than 35 years ago. The elegant ultrastructural illustrations of liver cell cytoplasm suffocated with “microbodies” Don Svoboda displayed² left an indelible impression that propelled me to pursue the cellular, biochemical, and molecular underpinnings of that phenomenon of peroxisome proliferation.^{3–8} The central effort of my research since then revolves around the analysis of causes and consequence of the induction of peroxisome proliferation by structurally diverse classes of chemicals, which we designated as peroxisome proliferators.⁹ This longstanding interest and avocation enabled my colleagues and me to make certain factual and conceptual advances, among which the following deserve special mention. First, the demonstration that the phenomenon of peroxisome proliferation can be induced by many structurally diverse agents led to our proposal that peroxisome proliferation is linked to lipid metabolism^{8,9} and this formed the impetus for the identification of the peroxisomal β -oxidation system.¹⁰ Second, the consistent observation that peroxisome proliferation induced by peroxisome proliferators is carcinogenic in rats and mice,^{11–14} resulted in the proposal that peroxisome proliferators form a novel class of nonmutagenic chemical carcinogens.^{15,16} This resulted in the dictum that all peroxisome proliferators are potentially hepatocarcinogenic and that peroxisome proliferation can serve as a biological marker or predictor of carcinogenicity of a nongenotoxic chemical.^{15,17–19} Third, sustained disproportionate induction of hydrogen peroxide generating- and hydrogen peroxide-degrading enzymes in livers by peroxisome proliferators resulted in the postulation that hepatocarcinogenicity of peroxisome

proliferators is due to oxidative stress leading to oxidative DNA damage from metabolic perturbations.^{15,19–23} Fourth, the tissue/cell specificity of pleiotropic responses and the coordinated rapid transcriptional activation of peroxisomal β -oxidation system genes led to the hypothesis that peroxisome proliferators exert their action by a receptor-mediated mechanism.^{15,18,19,24,25} This formed the stimulus for the identification of a subfamily of nuclear receptors called peroxisome proliferator-activated receptors (PPARs).^{26,27} PPARs play a central role in regulating the combustion and storage of dietary lipids, essentially by serving as sensors for fatty acids and their metabolic intermediates. In addition, they sense certain classes of exogenous chemicals and, in the process, transcriptionally regulate sets of target genes that control energy (lipid) metabolism. Thus, PPARs function as sensors for both endogenous and exogenous stimuli and this sensing mechanism regulates lipid homeostasis. I summarize here some of our studies dealing with the biological effects of peroxisome proliferators, an area of research we “nurtured” with some proprietary pride for many years. I am greatly honored that this work has been recognized by the Rous-Whipple Award this year, and dedicate this to all those who were part of our efforts and others who contributed to the developments in this field. My purpose in this paper is to review our own studies with inclusion of

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Figure 1. Mouse liver processed for the cytochemical localization of peroxisomal catalase. Brown dots in hepatocyte cytoplasm represent catalase containing peroxisomes. Normal mouse (left); mouse treated with Wy-14,643 a peroxisome proliferator for 2 weeks (right).

relevant literature citations although they are in no way comprehensive.

Microbody to Peroxisome

The morphological descriptions of an organelle called “microbody”²⁸ and biochemical and subcellular fractionation studies that culminated in the “peroxisome” concept^{29,30} originated in the 1950's. Rhodin²⁸ first described the presence of a special type of cytoplasmic organelle, characterized by a single membrane and a finely granular matrix, in the proximal convoluted tubule cells of the mouse kidney, which he called “microbody.” Subsequently, Rouiller and Bernhard³¹ described the existence in rat liver cells of “microbodies” that also contained a dense core with a regular crystalloid structure. This crystalloid core in liver peroxisomes is formed by urate oxidase.^{30,32} The elegant biochemical studies of De Duve and his co-workers³² led to the discovery that H_2O_2 -generating flavin oxidases coexist with the H_2O_2 -degrading enzyme, catalase, in the same cellular compartment. Subsequent morphological studies of these cellular fractions provided the unequivocal evidence that this organelle fraction indeed consists of so-called “microbodies.”³⁰ In 1965, De Duve proposed the name “peroxisome” for this organelle to bring attention to its H_2O_2 generation and degradation properties.²⁹ Gradually the noncommittal morphological descriptive name “microbody” became a historical relic. Although an earlier assumption was that peroxisomes existed almost exclusively in liver and kidney cells,³² and in the protist *Tetrahymena pyriformis*,³³ the development, by Novikoff and Goldfischer,³⁴ of a cytochemical staining procedure for selectively demonstrating the localization of catalase at the subcellular level, led to the discovery that these organelles are present in virtually all eukaryotic cells.^{35–37} Using this procedure, we established the presence of peroxisomes in Leydig cells and Leydig cell tumors of testis.^{36,37} This technique is routinely used for visual assessment of alterations in peroxisome population density in liver parenchymal cells under a variety of experimental conditions and in disease models (Figures 1 and 2). The subsequent advent of protein A-gold immunocytochem-

Figure 2. Electron micrographs representing normal (left) and Wy-14,643 treated (right) mouse livers cells. Peroxisomes are stained as dark osmophilic organelles.

ical technique further enabled the visualization of the presence of various proteins in different peroxisomal compartments such as the membrane, matrix, and crystalloid core (Figure 3). Immunocytochemical approaches also permitted evaluation of qualitative and quantitative changes in specific enzymes under a variety of experimental conditions.³⁸

Peroxisomes contain more than 60 proteins and perform a variety of biological functions. By virtue of the presence of an array of oxidases, such as urate oxidase, D-amino acid oxidase, L- α -hydroxyacid oxidase A and B, polyamine oxidase, glutaryl-CoA oxidase, pipecolic acid oxidase, oxalate oxidase, acyl-CoA oxidase, trihydroxycholestanoil-CoA oxidase, and pristanoyl-CoA oxidase,³⁹ these organelles may be responsible for as much as 20% of oxygen consumption in liver.³² Thus, formation of H_2O_2 is one of the critical byproducts of these peroxisomal oxidases as a result of oxidation of a variety of substrates. Peroxisomal oxidases participate in purine metabolism, fatty acid oxidation, amino acid metabolism, and polyamine metabolism, among others. Absence of urate oxidase enzymatic activity in humans and anthropoid apes and the clear lack of urate oxidase containing crystalloid cores in human liver peroxisomes provided the stimulus for us to clone rat urate oxidase gene.^{40,41} Recombinant urate oxidase expressed in insect cells or in African green monkey kidney (CV-1) cells, using the full-length rat cDNA, exhibited typical crystalloid structure.^{42,43} We and others also cloned human urate oxidase gene and found that it contains multiple mutations, especially two nonsense mutations, one at amino acid position 33 and the other at amino acid position 187.^{44,45} These mutations are the result of a single nucleotide change from a C to T in the second and fifth exons of the gene, converting a CGA arginine codon to TGA stop codon. The stop codon at amino acid position 33 is also found in the three great apes, chimpanzee, gorilla, and orangutan, suggesting that this may be the original mutation responsible for silencing or inactivation of the urate oxidase gene during hominoid evolution.⁴⁴ Of the variety of other oxidases present in peroxisomes, fatty acyl-CoA oxidase and the other enzymes of the fatty acid β -oxida-

Figure 3. Protein A-gold immunocytochemistry. **A**, urate oxidase; **B**, acyl-CoA oxidase; **C**, α -hydroxyacid oxidase; **D**, d-amino acid oxidase; **E**, catalase, normal liver; **F**, catalase, Wy-14643 treated liver; **G**, L-PBE, normal liver; **H**, L-PBE, DEHP-treated liver.

tion system have been well characterized and are of major importance in any consideration of xenobiotic-induced peroxisome proliferation and PPAR α activation. Likewise, the fatty acid oxidation system enzymes appear crucial for energy combustion, necessitating a cross-talk between fatty acid oxidation substrates and PPAR α .⁴⁶

Peroxisome Proliferation and Peroxisome Proliferators

Peroxisomes are single membrane-limited cytoplasmic organelles which are present in a wide variety of cells in animals and plants. In liver parenchymal cells, peroxisomes measure ~ 0.2 to $1 \mu\text{m}$ in diameter and are very few in number, accounting for less than 2% of cytoplasmic volume under physiological conditions. Peroxisomes in liver parenchymal cells of several species of animals are easily identifiable due to the presence of crystalloid cores or nucleoids containing the enzyme urate oxidase. As mentioned above, in some species, including the human, peroxisomes lack nucleoids, signifying the absence of the enzyme, urate oxidase.³⁸ In liver and other tissues that are processed for the localization of catalase, peroxisomes appear as dense brown granules at the light microscopic level (Figure 1), and at the ultrastructural level as dark osmiophilic structures (Figure 2). The catalase cytochemistry provides a dramatic distinction to appreciate the distribution of peroxisomes in normal liver and compare with livers with peroxisome proliferation (Figures 1 and 2). In livers with peroxisome proliferation, these organelles can occupy up to 25% of hepatocyte cytoplasmic volume. Since the first description of the induction of microbody (peroxisome) proliferation in liver parenchymal cells of rats fed a diet containing clofibrate, a lipid lowering drug in 1965,¹ we systematically screened several chemicals that induce hepatomegaly

and/or lower serum lipids for peroxisome proliferative property.^{6–8,47} Structure biological activity studies with clofibrate analogues established a link between hypolipidemic property and peroxisome proliferative effect.⁴⁷ Several of the structural analogues of clofibrate are extremely potent inducers of hepatic peroxisome proliferation in rats and mice. These included methyl clofenapate, and nafenopin, which are several orders of magnitude more potent than the prototype compound, clofibrate, in inducing hepatic peroxisome proliferation. It could not be decided if this indicates a structure-function relationship or coincidentally related properties of structurally closely related clofibrate-like compounds.⁴⁷ We then studied the nature of the hepatomegaly effects of two novel compounds, [4-chloro-6-(2, 3-xylylidino)-2-pyrimidinylthio] acetic acid (Wy-14, 643) and 2-chloro-5-(3, 5-dimethylpiperidinosulfonyl) benzoic acid (tibric acid) (Figure 4). Both of these compounds exhibited a potent lipid lowering effect and also caused massive hepatomegaly with profound proliferation of peroxisomes in rat and mouse liver cells (Figures 1 and 2). The stimulation of hepatic peroxisome proliferation by these structurally unrelated hypolipidemic compounds suggested that the peroxisome proliferative property and hypolipidemic responses are interrelated.⁹ Because of the structural diversity of these agents, we decided to call them peroxisome proliferators, to highlight their common biological property.⁹ We subsequently identified that certain phthalate-ester plasticizers, such as di-(2-ethylhexyl)-phthalate (DEHP), and di-(2-ethylhexyl) adipate (DEHA), used in the manufacture of polyvinyl chloride plastics, also induce peroxisome proliferation and exhibit a lipid lowering property.⁴⁸ Several other clofibrate analogues, such as fenofibrate, gemfibrozil, and ciprofibrate were also identified as potent peroxisome proliferators.^{17,49,50} These are currently used as lipid lowering drugs. The frequent association of hepatic

Figure 4. Structural diversity of peroxisome proliferators. **a**, clofibrate; **b**, nafenopin; **c**, ciprofibrate; **d**, Wy-14,643; **e**, tibric acid; **f**, di-(2-ethylhexyl)phthalate (DEHP).

peroxisome proliferation with drug-induced hypolipidemia suggested that yet unidentified peroxisomal enzymes might be responsible for the hypocholesterolemic and hypotriglyceridemic effects.⁹ These observations served as a solid foundation for the concept that peroxisomes participate in lipid metabolism and for the subsequent discovery of peroxisomal β -oxidation by Lazarow and De Duve.¹⁰

Peroxisome Proliferator-Induced Pleiotropic Responses: Immediate and Delayed

Peroxisome proliferation is a unique phenomenon generated by a wide spectrum of a diverse group of chemicals that include certain hypolipidemic drugs, industrial solvents, phthalate ester plasticizers, herbicides, and others.^{9,17} Despite the structural diversity, peroxisome proliferators, as a group, induce qualitatively predictable pleiotropic responses. These include hepatomegaly, proliferation of peroxisomes in hepatic parenchymal cells, and the induction of several hepatic enzymes, especially those associated with the peroxisome as well as others that participate in lipid metabolism.^{17–19,50} Since all peroxisome proliferators tested thus far in long-term studies have been found to induce liver tumors, the hepatocarcinogenicity of peroxisome proliferators is considered a delayed component of the receptor-mediated pleiotropic responses resulting from prolonged exposure to these agents.^{15,18,19,50} We characterized the peroxisome proliferator-induced hepatic alterations in rats and mice demarcated into two phases, immediate and delayed (or carcinogenic). Short-term treatment results in the immediate or early, adaptive changes, typified by hepatomegaly including liver cell proliferation, hepatic peroxisome proliferation, with attendant increases in selected enzyme activities.^{9,13,17,51} Hepatomegaly is due to massive hypertrophy of hepatocytes in most part resulting from increases in the number of peroxisomes (Figures 1 and 2). We described the initial burst of hepatocellular prolifera-

Figure 5. A: Northern blot showing increases in fatty acyl-CoA oxidase, L-PBE, and thiolase mRNA levels in peroxisome proliferator treated mouse liver as compared to untreated controls. **B:** SDS-PAGE of liver homogenates reveals increases in 78-kd L-PBE protein in peroxisome proliferator treated livers.

tion induced by peroxisome proliferators as primary mitogenic response, and not a reparative hyperplasia, as these agents are not necrogenic at the doses stimulating peroxisome proliferation.¹³ These early hepatic effects remain invariant as long as the peroxisome proliferators are administered and regress gradually within a few days of withdrawal of treatment.⁵²

Early hepatic effects induced by peroxisome proliferators are associated with increased transcription of genes responsible for the peroxisomal β -oxidation.²⁴ Proliferation of peroxisomes in liver is associated with up to ~30-fold or greater increase in the activities of the enzymes required for peroxisomal β -oxidation of fatty acids.²⁴ We showed that the increased activities of the three enzymes of the peroxisomal β -oxidation system, fatty acyl-CoA oxidase (AOX), enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (L-PBE), and 3-ketoacyl-CoA thiolase (Figure 5A) are due to the rapid and coordinated transcriptional activation of the nuclear genes encoding these enzymes.²⁴ Protein profiling studies, using traditional SDS-PAGE analysis or high

Figure 6. Hepatocellular carcinomas in rat and mouse liver (**top left** and **bottom left**) and metastases to lungs (**right**).

resolu13tion two-dimensional gel electrophoresis, revealed predictable alterations in the amounts of several proteins, including marked induction of peroxisome proliferation-associated protein L-PBE in the livers of rats treated with ciprofibrate, Wy-14, 643, or DEHP (Figure 5B).^{53,54} It is also of interest that the concentration of catalase mRNA in liver did not change appreciably, suggesting differential regulation of peroxisomal enzymes in livers with peroxisome proliferation.⁵⁵ Catalase activity increases ~2- to 3-fold in livers with peroxisome proliferation and recent protein profiling data show up to ~6-fold increase in the amount of this protein.⁵⁴

Peroxisome Proliferators Form a Novel Class of Chemical Hepatocarcinogens

Sustained hepatomegaly and increase in hepatic peroxisome proliferation in rats and mice exposed to peroxisome proliferators suggested early on that we undertake detailed studies to ascertain the long-range consequences. We reported the development of hepatocellular carcinomas in mice fed a diet containing nafenopin, a peroxisome proliferator in 1976.¹¹ Subsequently, we demonstrated the hepatocarcinogenicity of other, structurally dissimilar, peroxisome proliferators and proposed that these agents constitute a novel class of nongenotoxic carcinogens.¹²⁻¹⁵ These tumors are generally multiple in the liver and show characteristic morphology of hepatocellular carcinomas (Figure 6). Some of these also metastasize to the lungs. Since our initial postulation that peroxisome proliferators are carcinogenic, several other peroxisome proliferators, including hypolipidemic compounds and the plasticizers, DEHP and DEHA, have been shown to induce liver tumors in rats and mice.^{56,57} The latency period and the incidence of tumors correlated well with the effectiveness of the compound to induce hepatomegaly and peroxisome proliferation.^{56,58} Potent peroxisome proliferators, such as Wy-14, 643, ciprofibrate, nafenopin, BR-931, and tibric acid, induced liver tumors in nearly 100% of rats and or mice within 50 to 60 weeks when administered at dietary concentrations

ranging from 0.2 to 0.025% (w/w).¹⁵ With less potent peroxisome proliferators (clofibrate and DEHP), liver tumors developed between 70 and 104 weeks at dietary levels ranging from 0.5 to 1.2% (w/w).⁵⁶⁻⁵⁸ A close concordance with the magnitude of hepatic peroxisome proliferation and liver development has been demonstrated and it is now fairly well established that all peroxisome proliferators are potentially carcinogenic.^{50,56}

Peroxisome Proliferators are Nonmutagenic Chemical Carcinogens

Based on the mechanism of action, chemical carcinogens are classified as genotoxic (mutagenic) and non-genotoxic (nonmutagenic) agents.⁵⁹ The genotoxic chemicals covalently react with the DNA and are identifiable using several short-term *in vitro* and *in vivo* assays that measure DNA damage, mutagenic effects, and chromosomal aberrations.⁵⁹ We wanted to ascertain whether the structurally diverse peroxisome proliferators induce mutations/DNA damage in short-term mutagenesis and DNA damage-repair assays.¹⁶ None of these compounds caused detectable mutagenic activity in the *Salmonella*/microsome assay, or produced DNA damage in the lymphocyte ³H-thymidine incorporation assay.¹⁶ Since our initial proposal that peroxisome proliferators are nongenotoxic/nonmutagenic agents and do not cause DNA damage, either directly or after metabolic activation, none of the peroxisome proliferators have been shown to exert mutagenicity in any prokaryotic or eukaryotic short-term bioassays.^{16,60,61} We also examined the potential interaction of two structurally diverse peroxisome proliferators on rat liver DNA using the ³²P-post-labeling assay and found no peroxisome proliferator-DNA adducts.^{62,63} Thus, the dictum that none of the carcinogenic peroxisome proliferators interact with DNA or damage DNA still holds, raising intriguing questions about the mechanisms of their carcinogenic action. The functional class of peroxisome proliferators, which includes structurally diverse chemicals, emerged as the first major prototype of nongenotoxic carcinogens.^{15,17,60}

Peroxisome Proliferation as a Biological Marker for Hepatocarcinogenicity

There are no short-term *in vitro* screening tests to identify whether a chemical that is not genotoxic may prove to be a nongenotoxic carcinogen in animals after long-term exposure or will prove to be a noncarcinogenic chemical. Since all carcinogenic peroxisome proliferators are non-mutagenic chemicals, we proposed that evaluation for the induction of peroxisome proliferation would serve as a useful and practical short-term *in vivo* test to screen nongenotoxic chemicals and also to predict their carcinogenic effect. Evidence suggests that when peroxisome volume density reaches or exceeds 20% of cytoplasmic volume of hepatocytes in rats and mice, by the administered dose of a peroxisome proliferator, the liver tumor incidence can be expected to reach nearly 100%

level.^{15,56} The liver tumor incidence will be expected to be proportionately lower if the dose levels are far below the maximum dose for the induction of peroxisome proliferation. Accordingly, to test whether a nongenotoxic chemical is a peroxisome proliferator, it would be important to assay using several dose levels.

Peroxisome Proliferator-Induced Liver Tumors Exhibit Unique Phenotype

The liver tumors induced by peroxisome proliferators in rats and mice are usually multiple. These tumor-bearing livers are generally enlarged and grayish and nontumorous areas appear as intensely dark-brown. Histologically, these hepatocellular carcinomas are trabecular to poorly differentiated in appearance. Metastases in lungs are encountered in some rats and mice with peroxisome proliferator-induced liver tumors (Figure 6). These tumors are not distinguishable from those induced by genotoxic hepatocarcinogens, but we discovered that the hepatocellular carcinomas induced by peroxisome proliferators in rat liver do not express the classical liver tumor markers γ -glutamyltranspeptidase and the placental form of glutathione S-transferase (GST- π).^{64,65} Protein profiling studies also confirmed the reduction in GST- π content in livers with peroxisome proliferation, suggesting that peroxisome proliferators are negative regulators of GST- π expression.⁵⁴ We conclude that the expression of stress proteins, such as γ -glutamyltranspeptidase and GST- π genes, is not a prerequisite for the initiation and progression of hepatocarcinogenesis.⁶⁵ Using cDNA microarrays, we investigated the expression profiles of hepatocellular carcinomas and found distinguishing features between tumors induced by genotoxic and nongenotoxic hepatocarcinogens.⁶⁶ These differences may prove to be molecular fingerprints depicting different carcinogenic mechanism(s).

Peroxisome Proliferator-Induced Carcinogenesis: Oxidative Stress and Oxidative DNA Damage

The nongenotoxic nature of peroxisome proliferators poses an important challenge to delineate their carcinogenic mechanisms. We proposed that disproportionate increases in H_2O_2 -generating and -degrading enzymes contribute to sustained intrahepatic oxidative stress resulting in indirect oxidative DNA damage contributing to carcinogenesis.^{15,17-19,46,67} At least three potential sources of reactive oxygen radical production contributing to oxidative overload are possible in livers with peroxisome proliferation. First, the activity of fatty acyl-CoA oxidase, the rate-limiting enzyme of the classical inducible β -oxidation system, increases significantly. We initially considered this as the major source of H_2O_2 in the livers of rats and mice exposed chronically to peroxisome proliferators, but development of liver tumors in mice with disrupted acyl-CoA oxidase gene suggested the exis-

tence of other potential contributing sources. Second, and equally important, the CYP4A subfamily of enzymes, which are also markedly upregulated, can also contribute to the superoxide and H_2O_2 generation. In general, P450 enzymes catalyze the insertion of oxygen into a wide variety of substrates and generate reactive oxygen species. The magnitude of induction of fatty acid metabolizing CYP4A family of enzymes in livers with peroxisome proliferation, parallels the increases noted for peroxisomal fatty acid oxidation enzymes.⁶⁷ The third source of reactive oxygen species production is the overall increases, albeit subtle, of other peroxisomal oxidases, such as urate oxidase in livers with peroxisome proliferation. As pointed out above, in these livers with higher levels of oxidants, increases in catalase activity appear modest, leading to the suggestion of disproportionate increases in enzymes capable of producing and degrading H_2O_2 .^{24,55} Equally important is that protein profiling data points to marked down-regulation of several proteins critical for countering the deleterious effects of reactive species, such as selenium binding protein 2 (SBP2).^{54,68} Nearly 18-fold decrease in SBP2 content has been found in livers. SBPs are believed to play a crucial role in the growth inhibitory and anticarcinogenic effects of selenite by acting as growth regulatory proteins.⁵⁴ In normal liver, peroxisomes appear to account for 20% of oxygen consumption and this level of oxygen utilization is expected to be higher in livers with increased levels of peroxisomal and microsomal fatty acid oxidation.^{20,32} Both short- and long-term treatment with various peroxisome proliferators resulted in increased levels of 8-hydroxydeoxyguanosine in livers.⁶⁹ Furthermore, ³²P-post-labeling studies have confirmed the presence of unidentified DNA adducts in the livers of rats treated with a potent peroxisome proliferator, ciprofibrate, but the DNA adducts were not due to chemical-DNA adduction.^{62,63} Consistent with this oxidative DNA damage mechanism is that livers with chronic peroxisome proliferation manifest high levels of lipid peroxidation and lipofuscin pigment, a hallmark of chronic oxidative damage to macromolecules.^{20,22,23} Our work also established significant inhibition of peroxisome proliferator-induced liver carcinogenesis with simultaneous administration of ethoxyquin, a potent antioxidant.²¹ Recent work demonstrates marked induction of genes specific for the long-patch base excision DNA repair, a predominant pathway that removes oxidized DNA lesions, in livers with peroxisome proliferation.⁷⁰ These observations clearly establish that DNA-damaging oxidants are generated by enzymes induced in association with peroxisome proliferation in liver and provide further support for our oxidative stress hypothesis. Oxidative DNA damage most likely contributes to genetic alterations and the smoldering low level cell proliferation that generally occurs in livers with peroxisome proliferation may serve as a contributory and synergistic factor but is unlikely to be the primary cause of cancer.^{25,50,71} Peroxisome proliferators induce liver cell proliferation mostly during the first week or so,¹³ and chronic treatment does not lead to this level of sustained mitogenic action.⁷¹ Overall, liver cell proliferation *per se* is unlikely to be a key ingredient in the

causation of hepatocarcinogenesis, but it might certainly fix a mutation induced by reactive oxygen species.^{18,19} It should be noted that neoplastic transformation of hepatocytes does not occur despite the near unlimited potential of hepatocytes to divide and yet not become neoplastic.⁷² Thus, in liver one can certainly question the proposition that mitogenesis *per se* is mutagenesis.

Overexpression of Peroxisomal Oxidases and Neoplastic Transformation

We examined the role of overexpression of peroxisomal fatty acyl-CoA oxidase and urate oxidase in carcinogenesis by generating cell lines stably overexpressing these enzymes. Rat peroxisomal acyl-CoA oxidase, under the transcriptional control of the cytomegalovirus promoter, was transfected into African green monkey kidney (CV-1) cells and a cell line stably overexpressing the enzyme was derived.⁷³ When exposed to a fatty acid substrate, this cell line formed transformed foci, grew efficiently in soft agar, and developed into adenocarcinomas when transplanted into athymic nude mice.⁷³ Similar results were obtained when this enzyme was overexpressed in a nontumorigenic rat urothelial cell line, MYP-3.⁷⁴ These fatty acyl-CoA overexpressing urothelial cells, when exposed to fatty acid substrate, demonstrated strikingly higher H₂O₂ levels and formed colonies in soft agar in proportion to the duration of exposure to linoleic acid.⁷⁴ In another study, we stably overexpressed fatty acyl-CoA in a nontumorigenic mouse fibroblast cell line (LM-tk-) under the control of mouse urinary protein promoter.⁷⁵ These cells, when treated with a fatty acid for 6 to 96 hours, exhibited ~10-fold increase in intracellular H₂O₂ and apoptosis.⁷⁵ Prolonged exposure to a fatty acid resulted in the transformation of these cells, including tumor formation, when introduced into nude mice.⁷⁵ Overall, these studies clearly establish that prolonged overexpression of H₂O₂-generating fatty acyl-CoA oxidase is oncogenic.⁷⁵ Finally, we examined the role of stable overexpression of urate oxidase, another peroxisomal enzyme, in the transformation of CV-1 cells. Urate oxidase converts uric acid to allantoin and, in the process, generates H₂O₂.^{43,76} We expressed urate oxidase under the control of constitutively active human peroxisomal fatty acyl-CoA oxidase gene promoter we cloned⁷⁷ and found this promoter functionally intact, unlike claims to the contrary.^{78,79} CV-1 cells stably expressing urate oxidase were exposed to the substrate uric acid and continued exposure to this substrate resulted in transformation and tumorigenicity.⁷⁶ These studies clearly establish the carcinogenic potential of overproduction of H₂O₂ by peroxisomal oxidases. Others also obtained similar results by overexpressing superoxide-generating oxidase Mox1 in NIH3T3 cells.⁸⁰

Evolution of the Peroxisome Proliferator Receptor Concept

In 1983, we proposed that peroxisome proliferators induce the predominantly liver-specific effects by two pos-

Figure 7. Receptor model for peroxisome proliferator induced pleiotropic responses (adapted from reference 86).

sible mechanisms. These include exerting their effects through a ligand receptor-mediated mechanism and by increasing the influx of lipids into the liver.¹⁷ We subsequently accumulated convincing evidence, in favor of the receptor-mediated signal transduction hypothesis.¹⁷⁻¹⁹ From a historical perspective, it would be appropriate to recapitulate the following: the similarity of biological/pleiotropic responses induced by dissimilar peroxisome proliferators;⁹ hepatocyte-specific induction of these pleiotropic effects and the fact that similar magnitude of peroxisome proliferation in other cell types does not occur;⁵⁵ fidelity of the response of extrahepatic hepatocytes (transplanted either subcutaneously⁸¹ or in the anterior chamber of eye,⁸²) and hepatocytes developing in pancreas in rats with copper deficiency induced acinar cell depletion⁸³ to the inductive effects of peroxisome proliferators implying that hepatocytes irrespective of their location can recognize and respond to peroxisome proliferators; induction of specific changes in protein composition in the livers of rats/mice treated with structurally different peroxisome proliferators;⁵³ rapid and coordinated transcriptional activation of the peroxisomal β -oxidation system genes by different peroxisome proliferators,²⁴ and the detection of a specific peroxisome proliferator binding moiety in liver cytosol.⁸⁴⁻⁸⁶ Based on this information, we proposed a model that peroxisome proliferators act by receptor-mediated mechanism and that synthetic (chemical peroxisome proliferators) and natural (fatty acids) ligands act by this mechanism to induce specific genes and that cancer is due to oxidative stress caused by the induction of enzyme systems (Figure 7). Since peroxisome proliferation occurs in extrahepatic locations but not in the adjacent nonhepatic cells in these locations it became clear that liver cells possess a mechanism to recognize these xenobiotics. Thus, the hepatocytes show a biological response to peroxisome

proliferators irrespective of their location in the body. This glaring biological fact, together with rapid coordinated transcriptional activation of lipid metabolizing genes and the specific, albeit weak, binding moiety in liver cytosol laid a solid foundation for the peroxisome proliferator receptor mechanism.^{24,81–86} Our biochemical purification approaches yielded several peroxisome proliferator binding proteins, including a 55-kd protein, heat-shock 70 protein, and a few others but the nature of receptor was far from clear.^{85,86} Nonetheless, these observations and the concept formed the impetus for molecular cloning of the receptor. A mouse peroxisome proliferator-activated receptor (PPAR) was cloned from the liver, in 1990, by Issemann and Green²⁶ using transactivation assays. A high-level expression of PPAR mRNA was noted in liver and kidney, and its expression in other tissues was less pronounced, confirming the documented pattern of tissue-specific effects of peroxisome proliferators.^{26,55} In essence, the molecular cloning of this nuclear receptor for peroxisome proliferators confirmed our hypothesis and a series of other concepts generated earlier. In retrospect, multiple peroxisome proliferator binding proteins identified by us initially by affinity purification approach,⁸⁵ in some part, represented a complex which we recently identified as peroxisome proliferator interacting protein complex.⁸⁷

PPAR Family of Nuclear Receptors and PPAR α as a Sensor for Xenobiotic Peroxisome Proliferators

The cloning of the mouse PPAR in 1990 heralded a new era of biotic and xenobiotic sensing by liver.²⁶ This also paved the way for finding ligands for other orphan nuclear receptors in rapid succession during the past decade.⁸⁸ Soon after the cloning of the mouse PPAR (now PPAR α), three isoforms of PPAR, designated α -, γ -, β (also called δ), have been cloned from *Xenopus*.²⁷ We then reported the cloning of mouse PPAR γ from the liver in 1993.⁸⁹ This was followed by cloning of PPAR γ 2 from adipocytes⁹⁰ and other PPAR isoforms from other mammalian sources, including human.^{91,92} The PPAR isoforms are encoded by separate genes, with PPAR γ gene yielding two major transcripts, PPAR γ 1 and PPAR γ 2, resulting from differential mRNA splicing and alternate promoter usage (Figure 8).⁹³ Like other nuclear receptors, PPARs possess a highly conserved DNA binding domain, with two zinc fingers, that recognizes peroxisome proliferator response elements (PPREs) in the promoter regions of target genes.^{50,92} PPARs also contain two transcriptional activation function (AF) domains, termed AF-1 in the N-terminal domain and AF-2 in the ligand binding domain.⁹² After ligand binding, PPARs heterodimerize with another nuclear receptor, the retinoid-X receptor (RXR), and the PPAR/RXR heterodimers bind to DNA sequences containing direct repeats of the hexanucleotide sequence AGGTCA separated by one nucleotide, known as direct repeat 1 (DR-1) response element.⁹⁴ The promoter regions of PPAR target genes

Peroxisome Proliferator-Activated Receptor (PPAR) family

PPAR α : Energy Combustion; Peroxisome Proliferator-induced pleiotropic responses

PPAR γ : Energy Conservation; Adipogenesis

PPAR β/δ : Widespread fat burning; Macrophage VLDL sensor; Other functions?

Figure 8. Peroxisome proliferator-activated receptor family consists of three members: PPAR α , PPAR γ , and PPAR β/δ (adapted from reference 104).

contain this PPRE sequence or minor variants of the consensus sequence: 5'-AGGNCA A AGGTCA-3'.⁹²

PPARs are expressed in all tissues, and are activated by a variety of synthetic ligands, such as peroxisome proliferators, and biological molecules, such as polyunsaturated fatty acids and eicosanoids.^{50,92,95} Differing levels of expression of these molecules, raises the question as to which factors determine the cellular- and gene-specific responses to a wide variety of PPAR ligands and how PPREs of a given target gene recognize a particular PPAR isotype. Differences in the tissue distribution and in the ligand binding domain attest to the fact that they perform different functions.⁹² PPAR α is expressed in the liver, kidney, intestine, and heart and the gene-knockout mouse model (PPAR $\alpha^{-/-}$) unequivocally establishes that it is the critical player in peroxisome proliferator-induced pleiotropic responses and in the hepatic combustion of energy, mainly fatty acids.⁹⁶ Absence of PPAR α abrogates the pleiotropic responses in liver induced by peroxisome proliferators, including the development of liver cancer.^{96,97} The cloning of PPAR α and the generation of PPAR $\alpha^{-/-}$ mouse, which establishes the role of this receptor in peroxisome proliferator-induced pleiotropic responses, clearly validates the receptor concept we proposed in 1983.¹⁷ All synthetic peroxisome proliferators, including clofibrate family of fibrates, Wy-14, 643, phthalate ester plasticizers, and herbicides, function as PPAR α ligands. Thus, PPAR α functions as the hepatic sensing mechanism to recognize xenobiotic peroxisome proliferators and endogenous ligands, in particular fatty acid intermediates generated during fatty acid oxidation among others.

Of the other two members of PPAR family, PPAR β/δ is expressed broadly in many tissues and appears to participate in growth, development, and in up-regulating energy combustion, in particular, in extrahepatic tissues similar to that of PPAR α .^{98,99} However, PPAR β/δ does not function as a receptor for peroxisome proliferator-mediated induction of pleiotropic responses in liver in the absence of PPAR α .^{96,100} PPAR γ , unlike PPAR α and PPAR β/δ , plays a major role in adipogenesis and energy storage, with PPAR γ 2 expressed predominantly in adipose tissue and PPAR γ 1 in many tissues at relatively low

levels.⁹⁰ The synthetic chemicals that function as PPAR γ ligands include the thiazolidinedione class of drugs such as troglitazone, rosiglitazone, and pioglitazone.^{91,92} PPAR γ increases adipocyte conversion to accommodate the storage of excess energy in the body as triacylglycerols by increasing adipocyte-specific gene expression and differentiation.^{91,92} In this context, overexpression of either PPAR γ 1 or PPAR γ 2 leads to adipogenic conversion of fibroblasts and overexpression of PPAR γ 1 in liver has resulted in adipogenic hepatic steatosis.^{101,102} Gene expression profiling of livers overexpressing PPAR γ revealed induction of adipocyte-specific and lipogenesis-related genes in association with the onset of adipogenic hepatic steatosis.¹⁰² Induction of adipsin, adiponectin, aP2, caveolin-1, fasting-induced adipose factor (angiopoietin-like 4), and others in liver point to adipogenic transformation of liver cells.¹⁰² These observations are of potential significance in that PPAR γ ligands may induce adipocyte-specific differentiation in certain neoplastic and nonneoplastic epithelial tissues, including liver.

PPAR α Target Genes

Peroxisome proliferators induce immediate (short-term) and delayed (chronic) pleiotropic responses, which were attributed to be mediated by a postulated receptor before the cloning of PPAR α .¹⁷ As a result, all peroxisome proliferators essentially function as PPAR α ligands. Genes that were transcriptionally activated by peroxisome proliferators are now identified as PPAR α target genes and, in most of these, functional PPRES have been found in their promoter regions.^{50,92} Peroxisomal β -oxidation genes were first identified as PPAR α target genes because of their rapid and coordinated transcriptional induction in liver.^{24,50} Peroxisomal β -oxidation consists of four steps.⁴⁶ Each metabolic conversion can be performed by at least two distinct enzymes. The classical peroxisome proliferator-inducible pathway (Figure 9) utilizes straight-chain acyl-CoAs as substrates, whereas the second, mostly noninducible, pathway catalyzes the oxidation of 2-methylbranched fatty acyl-CoAs. In the classical inducible β -oxidation pathway, dehydrogenation of acyl-CoA esters to their corresponding *trans*-2-enoyl-CoAs is catalyzed by fatty acyl-CoA oxidase (AOX) and the second and third reactions, hydration and dehydrogenation of enoyl-CoA esters to 3-ketoacyl-CoA, are catalyzed by a single enzyme, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (L-bi-/multifunctional enzyme (L-PBE/MFP1)).⁴⁶ The third enzyme of this inducible system, 3-ketoacyl-CoA thiolase, converts 3-ketoacyl-CoA to acetyl-CoA and an acyl-CoA that is two carbon atoms shorter than the original molecule and the two carbon-shortened acyl-CoA then re-enters the β -oxidation cycle.⁴⁶ The three genes encoding for these three proteins of the inducible β -oxidation system are regulatable by PPAR α .^{24,96} We reported the cloning of human AOX gene using the P1 clone no. 177 that we obtained from the human foreskin fibroblast P1 bacteriophage library (Genome Systems, St. Louis, MO).^{77,103} The original PPRES published from this human P1 bacteriophage

Peroxisomal Fatty Acid β -oxidation

Figure 9. Inducible classical peroxisomal β -oxidation system. Straight chain acyl-CoA oxidase uses oxygen to generate hydrogen peroxide. Disruption of this gene results in massive spontaneous peroxisome proliferation in liver cells.

clone no. 177 had a typographical error, CTG instead of GCT, in the reported sequence AGGTCA C TGGTCA. The corrected sequence published after resequencing of the original P1, one freshly obtained from Genome Systems and of the pHACOXUOX,⁷⁷ and DNA obtained from three human genomic DNA samples showed identical PPRES consisting of 5'-AGGTCA G CTGTCA-3'.⁷⁷ Unfortunately, this corrected evidence was ignored by Roberts and her co-workers^{78,79} and a mistaken notion that human AOX promoter was nonfunctional has been promulgated, in part, to assert that a noninducible human AOX will possibly reduce the potential risk to humans of chronic exposure to PPAR α ligands. If human AOX gene is uninducible, as claimed by Roberts and her colleagues,^{78,79} one would expect severe metabolic syndromes and hepatic dysfunction in humans, since they would not be able to sense the influx of fatty acids into the liver and respond accordingly.^{46,104}

As pointed out above, L-PBE, the second enzyme of the peroxisomal β -oxidation system, becomes most inducible in the liver of rats and mice in response to PPAR α ligands.⁵⁴ The genetic basis of this hyperinduction may be due to four imperfect TGACCT half-sites in its promoter that constitute an unique binding with two DR1 elements overlapping a DR2 element.¹⁰⁵ The PPRES of L-PBE gene appear to bind with one or two PPAR/RXR heterodimers providing the peroxisome proliferator-signaling pathway with two levels of response. The peroxisomes also possess a second, generally noninducible, β -oxidation system, which acts on branched fatty acyl-CoA esters.⁴⁶ Although the oxidases and D-PBE of this system are not inducible by PPAR α ligands, the gene encoding for the third enzyme, sterol carrier protein x (SCPx), which possesses thiolase activity, is inducible.⁶⁶ The gene encoding for SCPx (58-kd protein) also codes for a smaller 5.3-kd protein (SCP2 or nonspecific lipid transfer protein).^{66,106}

Genes encoding for microsomal CYP4A family (CYP4A1 and CYP4A3) ω -oxidation enzymes, mitochondrial β -oxidation enzymes (especially medium chain acyl-CoA dehydrogenase), liver fatty acid binding protein, lipoprotein lipase, apolipoprotein A-I, A-II, and C-III, and some other cytosolic proteins were subsequently identified as PPAR α target genes.^{50,92} The livers with peroxisome proliferation and PPAR α activation are transcriptionally geared toward fatty acid combustion. Recent work, using cDNA microarray and protein profiling approaches, has identified several novel PPAR α target genes in liver.¹⁰⁷ These include pyruvate dehydrogenase kinase-4 (PDK4), peroxisomal biogenesis factor 11 β , as well as several cell recognition surface proteins including annexin A2, CD24, CD39, CD36, lymphocyte antigen 6D, and many others. Enhanced expression of several of these genes identified in microarray screening was confirmed in Wy-14, 643-treated mouse livers by Northern analysis and an obligatory role for PPAR α in the induction of these genes has been demonstrated.¹⁰⁷ Additional studies are necessary to ascertain the presence of PPRE in the promoter regions of these newly regulated genes in livers with peroxisome proliferation.

Peroxisomal Fatty Acid Oxidation: Gene Knockout Mouse Models

Of the three PPAR isotypes, PPAR α plays prominent role in the hepatic catabolism (combustion) of energy. PPAR α tightly regulates constitutive and peroxisome proliferator-inducible levels of expression of genes involved in peroxisomal, mitochondrial, and microsomal fatty acid oxidation.^{24,96,108} Mice deficient in PPAR α exhibited reduced levels of mitochondrial β -oxidation in liver as compared with wild-type mice.¹⁰⁸ On the other hand, the constitutive expression of enzymes involved in peroxisomal β -oxidation of very long-chain fatty acids was unaffected in PPAR α ^{-/-} mouse livers. These observations clearly point to the criticality of maintaining basal unaltered expression of peroxi-

Figure 10. Fatty acyl-CoA oxidase null mouse liver. Note the progressive replacement of steatotic hepatocytes with regenerating hepatocytes that extend toward the centrilobular region (**left**) and nuclear labeling of bromodeoxyuridine incorporation to show cell proliferation (**left**) resulting in the emergence of hepatocytes that are devoid of fat but rich in peroxisomes (see Figure 11, **right**).

Figure 11. Spontaneous peroxisome proliferation in fatty acyl-CoA oxidase null mouse (**right**) as compared with normal mouse liver (**left**). Sections processed for catalase localization.

somal enzymes in that any drastic reductions in peroxisomal fatty acid β -oxidation may lead to adverse effects. In fact, disturbances in peroxisomal fatty acid oxidation are known to lead to serious abnormalities including death in children.¹⁰⁹ To investigate the impact of disturbances in peroxisomal fatty acid oxidation we generated mice lacking: AOX (AOX^{-/-});¹¹⁰ both AOX and PPAR α (AOX^{-/-}/PPAR α ^{-/-});¹⁰⁰ L-PBE,¹¹¹ and L-PBE and D-PBE (L-PBE^{-/-}/D-PBE^{-/-}).¹⁰⁶ The genetically altered mouse models we generated underscore the importance of PPAR α and of the criticality of peroxisomal AOX and other enzymes of the enzyme β -oxidation system.¹⁰⁴ The following represents a brief summary of some important observations.

FASTING → ADIPOSE TISSUE / LIPOLYSIS
FATTY ACID INFLUX
PPAR α SENSING / OXIDATION

Figure 12. Mice deficient in both PPAR α and fatty acyl-CoA oxidase (PPAR α ^{-/-}AOX^{-/-}) develop severe steatosis when subjected to fasting, as they cannot upregulate PPAR α -dependent fatty acid oxidation systems. **A** and **C**, wild-type fasted; **B** and **D**, double null mouse liver fasted (adapted from reference 113).

AOX Is Required for the Metabolic Degradation of PPAR α Ligands

We generated mice lacking AOX, the first enzyme of the inducible straight-chain fatty acid oxidation system by homologous recombination.¹¹⁰ These mice serve as valuable tools to investigate the functional implications of disrupting the PPAR α -independent basal peroxisomal metabolism of very long-chain fatty acids.¹¹⁰ We noted that AOX^{-/-} mice developed severe microvesicular steatohepatitis, increased intrahepatic H₂O₂ levels, and hepatocellular regeneration that eventually leads to complete replacement of steatotic hepatocytes with regenerated hepatocytes that exhibit massive spontaneous peroxisome proliferation (Figures 10 and 11). The AOX^{-/-} mouse livers also reveal marked increases in the expression of PPAR α target genes.^{110,112} Older AOX^{-/-} mice also develop spontaneous hepatocellular carcinomas due to sustained activation of PPAR α and induction of spontaneous peroxisome proliferation.¹¹² These observations clearly point to natural ligands of PPAR α that are effectively metabolized by peroxisomal AOX. The presence of this enzyme is crucial for keeping PPAR α in check; in essence, the substrates of AOX act as ligands for PPAR α , the transcription factor that regulates the inducible levels of this enzyme.^{67,112} The results obtained from AOX null mouse model also indicate that oxidases of non-inducible branched chain peroxisomal β -oxidation system are incapable of metabolizing some or all of the unmetabolized AOX substrates by crossover function.¹¹² These studies also point to the importance of the maintenance of PPAR α -independent basal peroxisomal β -oxidation activity to metabolize very long-chain fatty acids and other substrates to prevent hepatotoxicity and steatohepatitis. The AOX null mouse model clearly establishes that lack of this enzyme leads to profound changes in liver, including steatohepatitis and transcriptional activation of PPAR α target genes.¹¹²

Mice Nullizygous for Both PPAR α and AOX

We generated mice nullizygous for both PPAR α and AOX (AOX^{-/-}/PPAR α ^{-/-}) to establish that neither PPAR β / δ nor PPAR γ in liver contribute to the phenotype of steatohepatitis, and spontaneous peroxisome proliferation.^{100,112} AOX^{-/-}/PPAR α ^{-/-} mice fail to exhibit microvesicular steatohepatitis, spontaneous peroxisome proliferation, and induction of PPAR α -regulated genes by biological ligands that remain unmetabolized in the absence of AOX.¹⁰⁰ In AOX^{-/-} mice, sustained activation of PPAR α enhances the severity of hepatic steatosis by inducing CYP4A family of genes that generate dicarboxylic fatty acids that cannot be metabolized in the absence of AOX. CYP4A enzymes also generate H₂O₂, which further contribute to steatohepatitis.⁶⁷ In double null mice, the absence of PPAR α abrogates the generation of dicarboxylic acids, thus pointing to the importance of the induction of CYP4A enzymes in the pathogenesis of steatohepatitis in AOX^{-/-} mice.¹⁰⁰

The availability of PPAR α ^{-/-},⁹⁶ AOX^{-/-},^{110,112} and AOX^{-/-}/PPAR α ^{-/-}¹⁰⁰ mice enabled us to compare their responses to fasting.¹¹³ In wild-type mice, fasting for 24 to 72 hours resulted in a modest induction of hepatic expression of PPAR α target genes encoding for fatty acid oxidation enzymes to efficiently metabolize fatty acids influxed into the liver during starvation. In PPAR α ^{-/-} livers and in AOX^{-/-}/PPAR α ^{-/-} double nulls, the absence of PPAR α abrogated the induction of these acute stress responses and resulted in severe hepatic steatosis (Figure 12). AOX^{-/-} mice, on the other hand, showed no increase in hepatic steatosis in response to fasting because these animals already exhibit PPAR α hyperactivity.¹¹³ These observations establish the critical importance of PPAR α and AOX in energy combustion.

Mice Deficient in Both L-PBE and D-PBE Overexpress PPAR α -Regulated Genes in Liver in the Absence of Peroxisome Proliferation

The AOX null mouse clearly highlighted the genetic importance of this gene in the metabolic degradation of PPAR α ligands.¹¹² To further extend the role of peroxisomal β -oxidation and intermediates generated during fatty acid oxidation, we generated mice lacking L-PBE, the second enzyme of the inducible peroxisomal β -oxidation system.¹¹¹ L-PBE^{-/-} mice were viable and fertile, and exhibited no detectable gross phenotypic defects. Hepatic steatosis, hepatic peroxisome proliferation, and induction of PPAR α target genes in liver were not noted in L-PBE, suggesting that disruption of peroxisomal β -oxidation pathway distal to AOX does not interfere with the inactivation of endogenous ligands. To eliminate the possibility that the substrates of L-PBE can be metabolized by the D-PBE of the branched chain system, we generated mice lacking L-PBE and D-PBE (L-PBE^{-/-}/D-PBE^{-/-}), ie, total inactivation of peroxisomal β -oxidation at the level of second enzyme.¹⁰⁰ Mice with complete inactivation of peroxisomal β -oxidation at the level of second enzyme, exhibit severe growth retardation and postnatal mortality with none surviving beyond weaning. L-PBE^{-/-}/D-PBE^{-/-} mice that survived exceptionally beyond the age of 3 weeks exhibited overexpression of PPAR α -regulated genes in liver, despite the absence of morphological evidence of hepatic peroxisome proliferation. These double null mice show that complete disruption of β -oxidation at the level of second enzyme results in induction of many of PPAR α target genes independently of hepatic peroxisome proliferation suggesting that intermediate metabolites of very long-chain fatty acids and other substrates of peroxisomal β -oxidation act as ligands for PPAR α . This L-PBE^{-/-}/D-PBE^{-/-} mouse model also shows that hyperinducible L-PBE, and to some extent D-PBE, contribute to the morphological entity of peroxisome proliferation.¹⁰⁰

Overall, these mouse knockout models point to the criticality of PPAR α -inducible fatty acid oxidation systems in energy metabolism and in the development of hepatic steatosis. These model systems also suggest that main-

Figure 13. Model for the involvement of nuclear receptor coactivators in PPAR α mediated transcription (reproduced with permission from reference 46).

tenance of high levels of fatty acid oxidation reduced fat accumulation in liver and in extrahepatic tissues.

PPAR-Interacting Nuclear Receptor Coactivators

During the past decade, we focused our attention on the mechanisms underlying the transcriptional activation of PPARs in an effort to understand the basis for the gene-, tissue/cell- and species-specific transcriptional activation. Transcriptional activity of PPARs and other nuclear receptors is regulated by the binding of specific ligands and by the orchestrated recruitment and assembly of several cofactors that assemble into multi-subunit protein coactivator complexes on promoters.^{114–116} These coactivator proteins activate the AF-2 domain of the nuclear receptor and enhance transcription by linking the liganded nuclear receptor to the basal transcription machinery (Figure 13). After the initial cloning of SRC-1,^{117–119} two other members of this SRC-1/p160 family of coactivators, TIF2/GRIP1/SRC-2^{120,121} and ACTR/pCIP/AIB1/SRC-3,^{122–124} were cloned and it has been demonstrated that these SRC1-/p160 family of proteins form a multi-subunit complex with CBP/p300.^{114–116,118}

Subsequently, using yeast two-hybrid screening and other methodologies, several nuclear receptor coactivators and coactivator binding proteins were identified.^{114–116,125–127} We used yeast two-hybrid screen to identify and characterize coactivators that interact with and influence PPAR-mediated transcriptional activation. We cloned SRC-1,¹¹⁹ PPAR binding protein (PBP),¹²⁶ and PPAR-interacting protein (PRIP).¹²⁷ SRC-1 contained two PPAR binding regions; one located between residues 620 and 789 displays stronger interaction with PPAR than the second binding domain located at the carboxyl-terminus between residues 1231–1447.¹¹⁹ The first region contains three LXXLL motifs and the second region contains one LXXLL motif; these LXXLL motifs in coactivators appear sufficient and necessary for the binding of the coactivators to nuclear receptors.^{122,128} SRC-1 en-

Figure 14. A proposed model for the bridging role of PIMT and PRIP in the formation of multi-subunit cofactor complex (reproduced from reference 139).

hances the transactivation function of both ligand-bound PPAR α and PPAR γ .¹¹⁹ A strong interaction between PPAR γ and SRC-1 has been observed in the absence of ligand, suggesting the presence of natural PPAR ligands in the transactivation system.¹¹⁹ SRC-1 has a basic helix-loop-helix (bHLH) motif indicating that it can function as a transcription factor by itself.¹¹⁹ The PBP we cloned, using PPAR γ as bait, is not a member of SRC1/p160 family.¹²⁶ PBP contains two LXXLL motifs and it binds to PPAR γ , PPAR α , RXR, RAR, TR β 1, and ER.¹²⁶ The binding site for PBP on PPAR is located to the extreme carboxyl-terminal region of the ligand binding domain and deletion of last 12 amino acids from the PPAR γ results in the abolition of interaction between PBP and PPAR γ .¹²⁶ Subsequently, PBP was identified as TRAP220, the anchor protein for TRAP complex.¹²⁹ PBP/TRAP220 gene is amplified and overexpressed in several breast cancers suggesting that it might enhance the transcriptional activity of ER and other transcription factors important in mammary growth control and differentiation.¹³⁰ We recently showed that PBP has 6 phosphorylation sites including two extracellular signal-regulated kinase 2 sites of the mitogen-activated protein kinase (MAPK) family at threonine 1017 and threonine 1444.¹³¹ PBP phosphorylation by Raf/MEK/MAPK cascade exerts positive effect on PBP coactivator function.¹³¹ We also cloned another PPAR-interacting coactivator designated PRIP/ASC2/RAP250/NRC/TRBP^{127,132–135} and PRIP binding protein PIMT.¹³⁶ PRIP interacts with PPARs and several nuclear receptors.^{127,132–135} It is of interest that it binds CBP/p300 and the TRAP130 of the TRAP/DRIP/ARC complex.^{129,137,138} Thus, PRIP appears to function as a bridge between the first coactivator complex anchored by CBP/p300 and the downstream TRAP/DRIP/ARC mediator complex anchored by PBP. Furthermore, PRIP interacting protein PIMT forms a complex with CBP/p300 and PBP.^{133,135,139} These findings further attest to the possibility that the two multi-protein coactivator complexes merge into one megacomplex on DNA template (Figure 14). Consistent with this is the isolation of a transcriptionally active PPAR α -interacting cofactor (PRIC) complex from rat liver nuclear extracts.⁸⁷ PRIC complex from rat liver nuclear extracts, which has been isolated using full-length PPAR α in the presence of ciprofibrate, a synthetic ligand, and leukotriene B₄, a natural ligand, comprises some ~25 polypeptides. PRIC complex provides a snapshot of aggregation of cofactors on liganded

GST-PPAR α matrix but does not provide the dynamics of assembly of association and dissociation of cofactors. PRIC complex includes known coactivators and coactivator binding proteins and other known and unknown proteins that have not previously been described in association with transcription complexes.⁸⁷ One of these proteins, PRIC285, contains five LXXLL motifs and interacts with PPAR α , PPAR γ , ER α , RXR and TR β 1.⁸⁷ There are few other polypeptides in PRIC complex with LXXLL motifs that require further characterization.

PRIP Binding Protein PIMT

Coactivator-associated enhancement of transcription involves recruitment of coactivator-associated proteins such as coactivator-associated arginine methyltransferase 1 (CARM1), a member of the S-adenosyl-L-methionine-dependent protein arginine methyltransferase family.¹⁴⁰ CARM1 catalyzes the methylation of arginine residues in histone and thus is known to enhance the function of the SRC-1/p160 family of coactivators.¹⁴⁰ Another coactivator-associated protein PIMT, which exhibits RNA binding and methyltransferase properties,¹³⁶ has been shown to interact with CBP, p300, and PBP.¹³⁹ PIMT is evolutionarily highly conserved.^{136,141,142} Yeast homologue of PIMT, designated Tgs1p, appears essential for hypermethylation of the m7G caps of both snRNAs and snoRNAs.¹⁴¹ Additional studies are required to determine the role of this RNA methyltransferase in nuclear receptor-mediated transcription.

Coactivator Gene Knockout Mouse Models

We and others have initiated gene disruption studies to evaluate the biological functions of nuclear receptor coactivators.^{143–154} SRC-1, TIF/GRIP1/SRC-2, and p/CIP/SRC-3 null mice are viable but show varying degrees of developmental reproductive functional perturbations and partial hormone resistance.^{143–147} SRC-1 mice fed PPAR α ligands responded in a fashion similar to that exhibited by wild-type mice fed the drug, suggesting that SRC-1 is redundant for PPAR α transcriptional activity.¹⁴⁴ The hepatic responses of SRC-1 null mice to other nuclear receptor ligands remain to be determined. In contrast to SRC-1/p160 family of coactivators, PBP and PRIP null mutations revealed embryonic lethality.^{148–155} PBP/TRAP220 is critical for the development of placenta and for the normal embryonic development of the heart, eye, vascular, and hematopoietic systems.^{148,150,151} PBP null embryos died around embryonic day 11.5 with cardiac failure due to noncompaction of myocardium.¹⁵⁰ Phenotypic changes in four organ systems noted in PBP null mice overlapped with those in mice deficient in members of GATA, a family of transcription factors known to regulate differentiation of megakaryocytes, erythrocytes, and adipocytes.¹⁵⁰ PBP also interacts with GATA factors and this interaction is not dependent on LXXLL motifs.¹⁵⁰ PRIP null mutation also resulted in embryonic lethality between E11.5 and E12.5 with abnormalities in the development of placenta, heart, liver, and generalized

growth retardation.^{152–154} Livers in PRIP null embryos revealed marked apoptosis.¹⁵² Mouse embryonic fibroblasts derived from PRIP null embryos failed to exhibit PPAR γ -mediated adipogenesis.¹⁵⁵ Based on available data from gene knockout studies, coactivators fall into the essential (nonredundant) and nonessential (redundant) categories from the developmental considerations. Additional studies using tissue-specific conditional knockouts should provide valuable information regarding the role of these essential coactivators in specific nuclear receptor function. In this regard, it is of interest to note that PBP gene-disrupted hepatocytes in PBP liver conditional null mice fail to respond to peroxisome proliferators implying that neither PPAR α nor PBP alone is sufficient for the transcriptional activation of PPAR α target genes in liver.¹⁵⁶

In summary, our work has provided a platform to investigate the cellular and molecular mechanisms responsible for the induction of peroxisomes and peroxisomal enzymes in response to a novel class of structurally diverse xenobiotic chemicals. The studies also defined and delineated the consequences of sustained induction of enzymes associated with peroxisome proliferation. These observations laid the foundations for a xenobiotic receptor-mediated mechanism and also proposed the concept early on that reactive oxygen species and oxidative stress can contribute to carcinogenesis. The gene knockout model systems should enable further studies on the role of fatty acid oxidation and fatty acid metabolic intermediates in PPAR α gene transcription and in nuclear receptor coactivator function.

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