ACCELERATED PAPER

Convergence of three steroid receptor pathways in the mediation of nongenotoxic hepatocarcinogenesis

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Introduction

Peroxisomes are subcellular organelles that functionally compartmentalize cellular β-oxidation reactions. The oxidative enzymes found in peroxisomes are involved in a large variety of metabolic pathways, including: respiration, lipid metabolism, cholesterol metabolism, and gluconeogenesis. Of increasing interest is the large group of compounds that are capable of inducing the proliferation of peroxisomal structures in rodent livers. This list includes hypolipidemic drugs, environmental pollutants, analgesics, urocosuric drugs, and phthalates (1). Rodent chemical hepatocarcinogenesis can also be closely correlated with the proliferation of liver peroxisomes, and although a few peroxisome proliferator-inducing agents have been shown to be genotoxic (2,3), the majority of compounds show no detectable mutagenic activity (4), thus falling into the classification of non-genotoxic carcinogens. Over the years, a number of mechanisms have been proposed to explain peroxisome proliferator-induced cancer. Their non-genotoxic nature and the observed ability to stimulate fatty acid β-oxidation enzymes led Reddy and co-workers to propose oxidative stress, in the form of H2O2 buildup and the generation of free radicals, as a possible mechanism for DNA damage and tumor initiation (4–7). Although sound in many respects, theories using oxidative stress as a mechanism for tumor initiation leave unexplained the process by which these chemical compounds might also be influencing the checks and balances on cell cycle events and providing an environment for perpetuating the mutations (tumor promotion). Identifying a link between peroxisome proliferator-induced tumor initiation and tumor promotion would be a major advance in defining a mechanism for non-genotoxic carcinogenesis.

The concept of peroxisome proliferation resulting from the stimulation of a variety of oxidative pathways suggests a metabolic disturbance of transcriptional regulation events. From this premise, Reddy and co-workers developed a receptor-based working hypothesis to explain non-genotoxic tumor induction by peroxisome proliferators (4–7). In their original hypothesis, they proposed a ligand-receptor mediated mechanism in which a chemical agent binds with a specific receptor which is then altered to activate a set of genes. This mechanism is strongly supported by the recent discovery of a peroxisome proliferator activated receptor (PPAR*) and its association with members of the retinoid X family (RXR) of receptors. Issemann and Green (8) cloned a mouse peroxisome proliferator-responsive transcription factor (mPPAR) that belongs to the steroid hormone receptor superfamily of genes. This family of receptors consists of a group of ligand-activated DNA transcription factors that bind regulatory sequences upstream of their target gene(s) resulting in the activation or repression of specific gene transcription (9,10). Subsequently, it has been shown that PPAR is a small family of genes with reports of at least α, β and γ isoforms in mouse (11,12), Xenopus (13), rat (14) and human (15,16). An examination of PPAR regulatable promoters suggests that this receptor family is intimately involved in fat metabolism, including its breakdown (17,18), storage (19) and synthesis (20). The complexity of the PPAR activation pathway has been substantially enhanced by the demonstration that PPAR DNA binding is linked to heterodimerization with a member of the RXR family of receptors (21), and the recent observations that this regulation is contingent upon associations with RXR and perhaps other mammalian cell-specific factor(s) (22,23). Whether PPAR-

*Abbreviations: PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; FXR, farnesoid X receptor; AOX, acyl-CoA oxidase; AOX-RE, acyl-CoA oxidase response element; EcRS, ecdysone response element; CFA, clofibric acid; JH, juvenile hormone; FPP, farnesylpyrophosphate; F-OH, farnesol; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.
mediated transcription is a cause or a result of peroxisome proliferation has not been fully established, but these findings circumstantially implicate PPAR-regulated transcriptional events in the propagation of peroxisome proliferation.

Although PPAR transcriptional regulation induced by peroxisome proliferators is well established, the pathway and mechanism for this event is still unresolved. Of critical concern has been the inability to identify an endogenous PPAR ligand from which an established pathway to carcinogenesis might be developed. A variety of in vitro activators of PPAR (8,24) have been identified but the direct binding of these activators to PPAR has not been established. This complication has led to the proposal that PPAR activators mediate their activity through some common endogenous factor or ligand. The most recent hypothesized mechanism (25) suggests that peroxisome proliferators interact with fatty acid binding protein complexes creating an increase in intracellular free fatty acids which in turn bind and activate PPAR/RXR complexes. Evidence supporting this hypothesis include: (i) the recent observation that several of these peroxisome proliferators can bind selectively to rat liver fatty acid binding protein (26), (ii) the observation that a variety of long chain fatty acids can stimulate PPAR-mediated in vitro transcription events (27), and (iii) the identification of PPAR transcriptional regulatory sequences in the promoter of the rat fatty acid binding protein gene (28).

As provocative as this mechanism sounds, the hydrophobic nature of fatty acids has made it experimentally difficult to establish them as high affinity ligands for PPAR.

Materials and methods

Mammalian cell co-transfection assay

The PPAR transcription assay plasmid constructs were generated as previously described (22). Briefly, the rat PPARα receptor expression plasmid and β-galactosidase normalization plasmid used in the mammalian co-transfection assay were constructed by directionally cloning the cDNA structure for these genes downstream from the constitutive RSV-LTR promoter in the pRSV eukaryotic expression plasmid (29). The acyl-CoA oxidase response element (AOX-RE) driven reporter plasmid construction was generated by inserting synthetic oligonucleotides into the polylinker located 5' of the minimal tk promoter in the previously described pBL luciferase vector (30). The integrity of all constructs was verified by DNA sequencing. Transient co-transfection assays were performed as previously described (22) Cell lines, grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 20% horse serum. Agents were added to cells for 72 h and cells were scraped from plates, sonicated and the sonicates analyzed sodium borate and fluorescence was read at an excitation wavelength of 395 nm and an emission wavelength of 470 nm. Enzyme activity was expressed as nmol H_{2}O_{2}min\(^{-1}\)mg protein.

Yeast transcription assay

Construction of the FXY yeast expression plasmid involved subcloning the cDNA for the FXY gene (33) into the Neo I–Sac I sites downstream of the CUP1 promoter of the yeast expression plasmid YEP2 (34). This vector expresses a ubiquitin fusion protein which is subsequently cleaved by endogenous yeast ubiquinase. The construction of the yeast Ecre β-galactosidase reporter vector involved fill in blunt ending of a Hind III/Bam HI fragment (containing the Ecre response element domain), derived from a previously described pBL-Ecre-Luc vector, and subsequent subcloning of this fragment into the blunt ended Xho I site on the YRpC2 yeast reporter vector (35). The construction of pRRARα and RXXRα yeast expression constructs the yeast YRCP2-AOX-RE reporter vector were as previously described (22,36). Receptor and reporter constructs were transformed by the lithium acetate method (37), into BJ5409 yeast (MATa, leu2Δ, his3Δ200, ura3-52, trpl, gal). The integrity of constructions was verified by DNA sequencing. Yeast transcription assays were performed essentially as previously described (22,36). Briefly, prototrophic yeast transformants were grown under selection to an A600 of 0.5–1.0, cells were plated (100 ul) into 96 well plates, ligand was added (< 10 nM CaSO\(_4\)), and plates were incubated at 30°C. After 22 h, A600 nm readings were taken, the cells were lysed, ONPG substrate was added, the plates were incubated at 37°C for 30 min, stop buffer was added, and the plates were read at A415 nm. Normalized β-galactosidase values were determined from triplicate samples as a measure of (A415/A600) transformant cells/µg.

Results

A cholesterol intermediate as an activator of PPAR

The regulation of peroxisomal fatty acid catabolism pathways by PPARα-mediated transcriptional activities has been well established (17,18). More recently it has been demonstrated that the PPARγ isomerase may be intimately involved with adipocyte-specific lipid storage (19). The first suggestions of PPAR involvement in fatty acid synthesis are implied in the identification of PPAR responsive promoters in the rat peroxisomal-specific thiolase gene and the rat mitochondrial HMG-CoA synthetase gene (20,21). These two enzymes catalyze the condensation of three molecules of acetyl CoA into the six carbon 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) molecule, a precursor in the synthesis of cholesterol. Interestingly, the initial step in cholesterol synthesis is mediated by the same enzyme (thiolase) responsible for the final step in peroxisome-specific fatty acid breakdown. As it turns out, a further examination of the literature reveals that many of the intermediates involved in cholesterol synthesis have been identified in peroxisomes (38). Cholesterol is an essential component of membrane structure and function and is the precursor to a variety of steroid based regulators of metabolic function. To explore the possibility that PPAR may be mediating cholesterol synthesis events, specific inhibitors of the cholesterol synthesis pathway were analyzed for their ability to stimulate or inhibit peroxisome proliferator-induced PPAR transcription (Figure 1A). Using an in vitro co-transfection assay containing a rPPARα expression construct and an acyl-CoA oxidase response element-driven reporter vector (AOX-RE), it was observed that peroxisome proliferator-induced PPAR activity could be repressed by the HMG-CoA reductase inhibitor lovastatin, and that this repression could be overcome by the addition of mevalonate. Furthermore, the squalene synthetase inhibitor, squalatin 1, had no detectable effect on peroxisome proliferator-induced PPAR activity. These data implicate the presence of a PPAR activating intermediate in the farnesylpyrophosphate (FPP) synthesis pathway.

The 15 carbon FPP structure, a major branch point in the cholesterol synthesis pathway, is produced by the condensa-
Convergence of three steroid receptor pathways

Fig. 1. Modulation of PPAR activity by cholesterol precursors. (A) Lovastatin and Squalestatin were analyzed in an in vitro co-transfection assay for their ability to stimulate or inhibit peroxisome proliferator-induced PPAR transcription. rPPARα and β-galactosidase expression plasmids, and an AOX-RE driven luciferase reporter plasmid were transfected into CV-1 cells and assayed for the effects Lovastatin, Squalestatin and mevalonate + Lovastatin had on clofibric acid (CFA) stimulation of PPAR-mediated transcription. (B) In a similar assay, farnesol was analyzed for its stereo-specific activation of PPAR-mediated transcription. rPPARα and β-galactosidase expression plasmids, and an AOX-RE driven luciferase reporter plasmid were transfected into CV-1 cells and the naturally occurring t,t-farnesol (t,t-F-OH) and a plant derived c,c-farnesol (c,c-F-OH) stereoisomer were assay for stimulation of PPAR-mediated transcription. Activity is expressed as the average of triplicate luciferase responses normalized to their respective β-galactosidase rate.

PPAR activation appears to be mediated by a metabolite of farnesol

In mammalian cells, free farnesol may be derived from exogenous sources, or alternatively, formed by the dephosphorylation of FPP (44) and possibly during the turnover of isoprenylated proteins. Recent observations with rodents fed large amounts of farnesol or a squalene synthase inhibitor (39,45) support the hypothesis that excess farnesol can be converted into farnesoic acid and farnesoic dicarboxylic acids. Under the supposition that fatty acids are endogenous regulators of PPAR-induced fatty acid catabolism, t,t-F-OH was evaluated for its effects on acyl-CoA oxidase (AOX) enzyme activity (Figure 2A). The incubation of a rat hepatoma cell line (H4IIEC3) with 0.1 mM t,t-F-OH was observed to significantly upregulate the specific activity of endogenous AOX. To further evaluate these observations, the ability of farnesoic acid to stimulate PPAR-mediated in vitro transcription was analyzed (Figure 2B). Farnesoic acid was observed to be more than twice as active as farnesol and 10-fold more potent than...
Clofibric acid. The results of these studies strongly suggest farnesol stimulated PPAR activity is mediated through its lipophilic properties.

**Convergence of PPAR and FXR pathways**

These data suggest that PPAR is regulated by farnesol or a metabolite of farnesyl pyrophosphate, a precursor of cholesterol and a common intermediate in other branches of isoprenoid metabolism. This activation pathway interestingly converges with the pathway of another steroid receptor family member FXR, a recently described orphan member of the steroid receptor superfamily (33). Like PPAR, FXR-mediated transcription can be upregulated by farnesol. A close comparison of in vitro transcriptional parameters for these two receptors in mammalian and yeast-based assays reveals common and unique characteristics that define their functional activities.

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**Fig. 3.** Comparison of FXR and PPAR activation in mammalian and yeast assays. (A) rPPARα or FXR mammalian expression plasmids, a β-galactosidase expression plasmid and/or a designated luciferase reporter construct (AOX-RE or EcRE) were transfected into CV-1 cells and analyzed for farnesol (F-OH), CFA, and juvenile hormone (JH) stimulation of receptor activities. Activity is expressed as the average of triplicate luciferase responses normalized to their respective β-galactosidase rates. (B) Yeast cells transformed with rPPARα or FXR yeast expression plasmids, and/or a designated yeast β-galactosidase reporter construct (AOX-RE or EcRE) were analyzed for farnesol (F-OH), CFA, and juvenile hormone (JH) stimulation of receptor activities. Activity is expressed as the average of triplicate β-galactosidase responses normalized to cell number.

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**Fig. 4.** Hypothetical mechanism for rodent peroxisome proliferator-induced hepatocarcinogenesis. Direct or indirect peroxisome proliferator stimulation of PPAR results in a dramatic increase in β-oxidation of fatty acids and the production of acetyl CoA. Excess acetyl CoA or acetoacetyl CoA drives the formation of farnesylpyrophosphate (FPP) which, when in excess, is converted to farnesol. Farnesol, or perhaps even FPP, is viewed to have multiple fates; one being its conversion to famesoic acid and the other to a juvenile hormone-like mediator of FXR. Famesoic acid acts to upregulate PPAR activity and in an autocatalytic fashion may also serve as source of acetyl CoA through PPAR regulated fatty acid α-oxidation, β-oxidation and/or ω-hydroxylation pathways. These maximally induced fatty acid oxidation pathways generate an oxidative stress environment and subsequent DNA damage (tumor initiation). Excess farnesol or FPP is also hypothesized to metabolize into a yet to be determined 'X' ligand for FXR, and upregulation of FXR is hypothesized to be linked to cell cycle deregulation events (tumor promotion). Convergence of three steroid receptor pathways.
This hypothesis does not eliminate the role of oxidative stress cycle or differentiation functions of the cell, thus providing a hypothesis, PPAR-linked farnesol production is thought to be mediated by the conversion of farnesol to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) in the mevalonate pathway. Interestingly, juvenile hormone, a sesquiterpene diterpene, can be activated by some metabolic product of farnesol or, alternatively, may be activated by a product of the FPP pathway. These data suggest farnesol serves as a common intermediate for distinct endogenous activators of both PPAR and FXR. A potential scenario for the generation of an endogenous ligand for PPAR is the conversion of farnesol to the fatty acid farnesolic acid, or to one of the farnesol-derived dicarboxylic acid forms. These compounds have recently been identified in the urine of rodents fed large amounts of farnesol or a squalene synthase inhibitor (39,45), and this conversion pathway is further supported by the acyl CoA oxidase and farnesolic acid data presented here. (Figure 2). FXR on the other hand, might also be activated by some metabolic product of farnesol or, alternatively, may be activated by a product of the FPP pathway. Interestingly, juvenile hormone, a sesquiterpene derivative of FPP and regulator of insect morphogenesis, selectively activates transcription of FXR. Therefore, the data presented here argue effectively that the stimulation of PPAR mediated fatty acid catabolism is ultimately going to provide the substrate (acyl CoA) for upregulating farnesol production and consequently the activities of FXR. In addition, these findings suggest that to understand the consequences of peroxisome proliferator activation of PPAR it might ultimately be necessary to understand the consequences of FXR activation.

From the above data, a novel working hypothesis to explain peroxisome proliferator-induced tumorigenesis can be generated (Figure 4). This hypothesis invokes an indirect involvement of PPAR in tumor promotion. PPAR activation of oxidative catabolism of fatty acids is proposed to generate excess acetyl CoA, driving the overproduction of FPP, which subsequently feeds into the activation pathways of both PPAR and FXR. The autocrine loop of PPAR stimulated farnesol production feeding back to restimulate PPAR is viewed as the vehicle by which oxidative stress, DNA mutations and tumor initiation are created. In the more speculative aspect of the hypothesis, PPAR-linked farnesol production is thought to modulate FXR pathways theorized to be associated with cell cycle or differentiation functions of the cell, thus providing the deregulatory phenomena associated with tumor promotion. This hypothesis does not eliminate the role of oxidative stress in tumorigenesis but rather creates a mechanism wherein the effects of oxidative stress are uncontrolled and/or are immortalized. The link between the two receptor-mediated pathways cannot be ignored. FXR expression in rodents appears to be limited predominantly to liver and kidney (33), a distribution pattern somewhat reflective of PPARα (46).

In summary, the data presented here provide a novel hypothesis to explain peroxisome proliferator-induced rodent non-genotoxic hepatocarcinogenesis. The role of FXR in the above hypothesis has not been addressed other than its function as a common heterodimer partner. FXR has been proposed to converge at the DNA binding or heterodimerization level with a variety of steroid receptor family members. Its cellular concentration as well as its subtype expression could play a significant role in determining the ultimate activity of PPAR and FXR in the proposed mechanism. Beyond the convergence of receptors at the DNA and protein levels the studies presented here extend this complex network of regulation to include endogenous ligands or ligand precursors, with the caveat that perturbation of one receptor-mediated event can have important consequences on seemingly unrelated metabolic pathways. Validation of this hypothesis will require a thorough dissection of PPAR, FXR and RXR-mediated transcriptional regulation.

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References


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