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

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The mechanisms by which peroxisome proliferators are able to regulate metabolic processes such as fat metabolism, while at the same time creating an environment for the development of hepatocellular carcinomas, is a central issue in the non-genotoxic carcinogenesis field. The convergence of two members of the steroid receptor family (peroxisome proliferator-activated receptor, PPAR; and retinoid X receptor, RXR) has provided strong support for an oxidative stress component in this carcinogenesis process, but has yet to define clearly a pathway for the classical tumor promotion events associated with peroxisome proliferation. The findings presented here integrate a third member of the steroid receptor family into this process and suggest a novel autocrine loop and mechanism for creating both oxidative stress and tumor promotion. A central regulatory component in this pathway is farnesol which has recently been shown to induce transcription mediated by the steroid receptor family member, farnesoid X receptor (FXR). In this report, it is clearly demonstrated that farnesol can also upregulate the transcriptional events of PPAR, but most likely through a different farnesoid metabolite, resulting in the regulation of an entirely different set of genetic components. Deregulation of the activities of these receptors offers a provocative mechanism for explaining the hepatocarcinogenic effects of peroxisome proliferators in chronically treated rodents.

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-

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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 lead 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 selectivity 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-transfections assays were performed as previously described (22). Cell lines, as grown in a monolayer in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum, were plated at 70% confluency 24 h prior to transfection. The recombinant DNA constructs were transiently transfected into CV-1 cells by calcium-phosphate co-precipitation. Medium was removed from transfected cells after 6 h, cells were washed with PBS, lysed with lysis buffer and luciferase activities in cell extracts were assayed using a Dynatech luminometer following the addition of a MgCl2/ATP/NAD luciferin substrate solution. To determine the efficiency of transfection and to standardize the expression of activity, a plasmid containing a β-galactosidase expression plasmid was included in all co-transfections and 30 μl of each extract was mixed with 200 μl of a β-galactosidase ONPG substrate solution and analyzed for β-galactosidase activity at 415 nm on an ELISA plate reader (BioRad). Each transfaction was performed in triplicate and the average luciferase response for the three independent transfactions was normalized to the average β-galactosidase rate (average luciferase response/average β-galactosidase response/min).

**Acyl-CoA oxidase assay**

Acyl-CoA oxidase assays were performed essentially as previously described. Briefly, H4IEEC cells were grown in Swinn's S-77 medium containing 5% 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 for protein content (31) and acyl-CoA oxidase activity (32). 850 μl of sonicate was incubated at 37°C for 20 min in a 1 ml volume containing; 35 μM palmitoyl CoA, 50 μl FAD, 1 μM coenzyme Q1, 0.6 mg BSA, 60 mM Tris–HCl pH 8.3. The reactions were terminated by adding 4 ml 0.1 M 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 H2O2/min/g protein.

**Yeast transcription assay**

Construction of the FXR yeast expression plasmid involved subcloning the cDNA for the FXR gene (33) into the Nco I–Sac I sites downstream of the CUP1 promoter of the yeast expression plasmid YEpE2 (34). This vector expresses a ubiquitin fusion protein which is subsequently cleaved by endogenous yeast ubiquitinase. 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 rPPArα and RXRα yeast expression constructs the yeast YRpC2-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 (MATα, leu2D, his3D200, ura3-52, trp1, gal). The integrity of constructions was verified by DNA sequencing.

Yeast transcription assays were performed essentially as previously described (22,36). Briefly, protoprotic 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 mM CaSO4), 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/(A415/A600) BJ5409 cells.

### 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 isoform 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 peroxisomal 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 peroxisomal 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 squaleane synthetase inhibitor, squalestatin 1, had no detectable effect on peroxisomal 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 stereospecific 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.
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 immaterialized. 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). Furthermore, FXR is specifically activated by a classical regulator of insect differentiation and development. The mammalian developmental equivalence to metamorphosis is observed in the events of tissue remodeling and functional reorganization which occur during the morphogenetic periods of fetal and perinatal life. Therefore, it can be speculated that the function of juvenile hormone has some ancestral relationship to a mammalian isoprenoid-related transcriptional signaling pathway. Finally, the available limited data suggests that humans are refractory to peroxisome proliferation and the hepatocarcinogenic effects of peroxisome proliferators (47). A key to the mechanism described above is the linkage of fatty acid breakdown and cholesterol synthesis. This linkage is mediated by the duality of thiolase activity. A comparison of human and rat thiolase genes reveals the presence of two genes in rats displaying differential sensitivities to peroxisome proliferators (48–50), while in humans only one gene has been identified (51). Although it remains to be seen whether or not the human thiolase promoter binds and/or is sensitive to peroxisome proliferators, this deficiency would logically explain the insensitivity of humans to peroxisome proliferator-induced hepatocarcinogenesis.

In summary, the data presented here provide a novel hypothesis to explain peroxisome proliferator-induced rodent non-genotoxic hepatocarcinogenesis. The role of RXR in the above hypothesis has not been addressed other than its function as a common heterodimer partner. RXR 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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