GENE MODULATION IN PEROXISOME PROLIFERATOR-INDUCED HEPATOCARCINOGENESIS

By

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DEDICATION

I dedicate this thesis to my daughter Lindsey, to my parents, Paul and Theda Anderson, and to the memory of Gina, Walter, and Monica.

BIOGRAPHY

Steven Paul Anderson was born on December 18, 1960 in Amarillo, Texas to Paul and Theda Anderson. He attended primary and secondary schools in west Texas and eastern Oklahoma, and graduated from Memorial High School, Tulsa, Oklahoma in 1979.

Steve attended the University of Oklahoma at Norman, where he earned the degree of Bachelor of Science in Zoology in 1985. Following graduation, he was a research associate in the Biochemistry department of the School of Medicine at Oral Roberts University and also a veterinary laboratory technician at Woodland Animal Hospital, both in Tulsa. Steve entered veterinary medical school in 1988, and spent several months as a visiting scientist in the laboratory of Dr. Isaiah J. Fidler at M.D. Anderson Cancer Center in Houston, Texas. In 1992, Steve graduated from Oklahoma State University at Stillwater with a Doctor of Veterinary Medicine. From 1992 to 1994, he was a resident in Anatomic Pathology at North Carolina State University College of Veterinary Medicine at Raleigh. During this time, he spent several months as a visiting pathology resident at the University of North Carolina School of Medicine. After the residency he began his dissertation research at the CIIT Centers for Health Research, under the direction of Dr. Russell Cattley. Following his tenure at CIIT, Steve became a Fellow in Molecular Pathology in the Toxicogenomics Group of GlaxoSmithKline in Research Triangle Park, North Carolina.

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Chapter 1

Review: The Medical Significance of Peroxisome Proliferators

Peroxisomes

Peroxisomes are single membrane-limited cytoplasmic organelles that are present in every eukaryotic cell except mature erythrocytes. They exhibit a high degree of evolutionary conservation, as the basic machinery for peroxisomal function is similar among yeast, plants, insects, and vertebrates (1). In mammals, peroxisomes are most abundant in kidney and liver, where they were first detected by electron microscopy of the proximal convoluted tubule (2), and later in hepatocytes (3) of mice and rats. In rat liver, peroxisomes account for approximately 2.5% of liver protein (4) and occupy about 1.5% of the parenchymal cell volume (5,6). Studies by de Duve *et al.* established that peroxisomes contain several enzymes, including catalase, D-amino acid oxidase, and urate oxidase (7). To stress the association of one particle of hydrogen peroxide (H_2O_2)-producing oxidases and catalase, which decompose H_2O_2, they proposed the name 'peroxisome'. Peroxisomes contain no DNA (4), so nuclear genes encode all peroxisomal proteins—including upwards of 40 enzymes. Most of these enzymes are involved in lipid metabolism, where the primary objective is the β-oxidation of fatty acids and synthesis of ether glycolipids, cholesterol and dolichols. In eukaryotic microorganisms and plants peroxisomes are the sole site of β-oxidation (8,9). Animal cells, in contrast, also enlist the help of mitochondria for this task. Rat hepatic peroxisomes, for example, contribute as much as 50% of the oxidative activity for fatty acids longer than 16 carbons (8,9).

Peroxisome Proliferation

The first description of peroxisome proliferation was in rats treated with a member of the fibrate (phenoisobutyrate) class of chemicals by Hess and colleagues (10). In 1975, Reddy and Krishnakantha reported that exposing rats to the hypolipidemic drug clofibrate also led to hepatic peroxisome proliferation (11). Later studies confirmed this response in rats exposed to another hypolipidemic drug, nafenopin (12), the industrial plasticizer di-(2-ethylhexyl) phthalate (DEHP) (13), and the solvent trichloroethylene (TCE) (14). Presently, we are aware of over 100 chemicals that elicit similar responses. In addition, physiological stimuli, such as starvation, diabetes mellitus, or high fat diets, induce increases in the size and number of peroxisomes (15,16). This change can be dramatic: whereas peroxisomes in hepatocytes normally occupy less than 2% of the cytoplasmic volume this may increase to 25% after exposure to certain stimuli. Interest in these 'peroxisome proliferators' (PP) was heightened after Reddy and associates proposed a link between peroxisome proliferation and carcinogenesis in rodent bioassays (17). PP are structurally and pharmacologically diverse, and comprise endogenous physiological mediators as well as commercially important chemicals (Table 1).

Coincident with hepatic peroxisome proliferation after PP exposure, there is also increased liver growth, as manifest by cellular hypertrophy (peroxisome and smooth endoplasmic reticulum proliferation) and hyperplasia (hepatocyte proliferation), and transcriptional induction of a number of genes. The hyperplastic response is seen within the first few days of PP administration, and it reaches steady-state levels by two weeks. At this point, liver weights can be increased by as much as 120% compared to those of untreated rodents. After PP withdrawal, the hepatomegaly completely regresses within 10 to 14 days (18,19). Transcriptional induction occurs as early as two hours after PP

exposure, and such increases can be dramatic. mRNAs for Acyl-CoA oxidase (*Aco*), the enzymatic rate-limiting step for β-oxidation, and the ω-oxidation enzyme *Cyp4a1*, for example, are typically increased 10- to 30-fold (7,20,21).

Although the response is most dramatic in rodent livers, PP also affect other tissues. Peroxisome proliferation occurs in the intestine (22,23), adrenal gland (24), and cardiac and skeletal muscle (25,26). PP exposure also leads to peroxisome proliferation in the kidney, where the spectrum and time course of induction of enzymes are similar, but not identical, to those found in the liver (27). The lung is also affected: clofibrate reportedly increases the number of peroxisomes in type II pneumocytes by 60% (28). The PP response seems to be somewhat species-specific. Hamsters and rabbits also exhibit peroxisome proliferation, albeit to a lesser degree than that observed in rats and mice. In contrast, guinea pigs, dogs, and marmosets are either weakly- or non-responsive (29).

Peroxisome Proliferators Influence Gene Expression: the PPARs

The rapid and coordinate induction of ACO and CYP4A1 following PP exposure suggested that these enzymes might share a common mechanism of induction. In 1983, the Reddy laboratory reported identification of a peroxisome proliferator binding protein in rat liver, and suggested this molecule might mediate PP action (30). Several years later, Alvares and co-workers identified this protein as heat-shock protein 72 (31). After Reddy and Rao postulated that PP might act in a manner similar to steroid hormones (32), Issemann and Green screened a mouse liver cDNA library with a probe derived from the combined nucleotide sequences of several hormone receptors and identified four new orphan members of the nuclear hormone-like receptor family (33). Because one of these receptors could be activated by a variety of PP they called it the

peroxisome proliferator-activated receptor (PPAR); it is now known as PPARα. PPARα was identified as a putative member of the nuclear receptor family because it had the characteristic modular structure including the conserved DNA-binding domain (DBD) and ligand-binding domain (LBD). Other members of this superfamily include the retinoid, steroid, and thyroid hormone receptors. Two years later, the Wahli laboratory reported cloning the *Xenopus laevis* (a frog) ortholog of PPARα, and also two closely related orphan receptors, encoded by distinct genes, which they named PPARβ/δ and PPARγ (34). The three isoforms share approximately 90% amino acid identity in their DBDs, and about 80% in the LBDs. The nomenclature for these isoforms is a misnomer, since neither the PPARβ/δ nor the PPARγ have typically been associated with peroxisome proliferation. However, a recent report indicates that highly selective PPARγ or dual PPARγ/PPARβ/δ agonists, lacking murine PPARα agonist activity, cause peroxisome proliferation in CD-1 mice (35).

In the following years, PPARs were cloned from mammals (rat, dog, rabbit, mink, cow, pig, hamster, human), reptiles (crocodile, turtle), fish (*Pleuronectes*, *Amphioxus*, Atlantic Hagfish, Zebrafish), an amphibian (*Xenopus*), and a bird (domestic chicken) (36). Each of the three PPAR subtypes, or isoforms, is expressed in a distinct, tissue-specific manner. PPARα is expressed at high levels in liver, kidney, brown adipose tissue, heart and skeletal muscle (29). PPARα operates primarily in the catabolism of fatty acids in the liver, and its targets include a homogenous group of genes participating in aspects of lipid metabolism such as fatty acid uptake though membranes, fatty acid binding in cells, fatty acid oxidation (peroxisomal and mitochondrial), and lipoprotein assembly and transport. Conversely, PPARγ influences the storage of fatty acid in adipose tissue (37). Most of the PPARγ target genes in adipocytes are directly implicated

in lipogenic pathways, including lipoprotein lipase, liver fatty acid-binding protein (L-FABP), ACO, and fatty acid transport protein (FATP). PPARγ is found mainly in white and brown adipose tissue, the large intestine, immune system cells, and the retina. PPARβ/δ is much more ubiquitously expressed, but highest levels are found in the gut, kidney, heart, and brain (38) (Table 2).

Like several other members of the nuclear receptor superfamily, PPARs only bind to DNA after heterodimerization with the 9-*cis* retinoic acid receptor (RXR). This complex recognizes two half sites of the consensus sequence TGACCT organized as a direct repeat with a single nucleotide spacer, a so-called DR-1 motif (Figure 1). PPREs have been identified in the transcriptional regulatory sequences of numerous genes involved in carbohydrate and lipid metabolism, where they typically facilitate increases in transcription. Others, however, are negatively regulated by PPAR/RXR binding of PPREs (reviewed in 39).

Genetic Models of PPAR function

Further clarification of PPAR function has been facilitated by the generation of mice bearing inactivating mutations in the genes encoding these receptors. The first PPAR knockout mouse developed was the PPARα-null mouse. In 1995, the Gonzalez laboratory reported that although these mice were viable and lacked grossly discernable morphologic alterations, they failed to display the pleiotropic response to PP exposure (40). Additional studies revealed that they had altered hepatic expression of Apo-AI and Apo-CII (41). Older PPARα-null mice have increased circulating triglycerides, cholesterol, and phospholipids, a male-specific hepatomegaly, and a late-onset obesity

that was more pronounced in females (42). Perhaps most importantly, PPARα-null mice failed to develop liver tumors after chronic PP exposure (43).

Deletion of PPARγ is lethal to the embryo, and this lethality occurs in two distinct phases (44). Initially, there is interference with the terminal differentiation of the trophoblast and subsequent placental vascularization leading to severe myocardial thinning and death by embryonic day 10. The second phase was discovered after rescue of one of the mutant pups. This pup exhibited another lethal combination of lesions, including lipodystrophy and multiple hemorrhages.

PPARβ/δ-null mice also show impaired fetal development, but it is mild compared to PPARγ-null mice. In addition, these mice had altered myelination of the corpus collosum, and showed some evidence of altered cell cycle control and modulation of inflammation in the skin (45). All of these models confirm the central role of the PPARs as endogenous regulators of lipid metabolism in mice.

Peroxisome Proliferators and Disease

It was first realized several decades ago that several genetic diseases, such as Refsum's disease and Zellweger's Syndrome, are the result of impaired peroxisomal function (reviewed in 46). The first indication that the PPARs may be relevant to human health was the recognition that PPARγ is involved in adipogenesis (47). Research over the last seven years indicates that all three PPAR isoforms likely have enormous impacts on human health.

Diseases of Affluence

In Westernized nations, metabolic disorders such as hyperlipidemia, atherosclerosis, hypertension, diabetes, and obesity often exist as comorbidities. Individuals with this phenotype, also known as 'Metabolic Syndrome X' (48), have increases in serum triglycerides and low-density lipoproteins (LDLs) and decreased high-density lipoprotein (HDL). The fibrate ligands for PPARα and the thiazolidinedione (TZD) ligands for PPARγ are useful adjuncts for treatment of this syndrome. Although less well characterized, a recent report indicates that PPARδ agonists also show therapeutic promise for this condition. (49).

Fibrates are widely prescribed drugs used to lower serum triglyceride levels in patients at risk for cardiovascular disease. This appears to be a result of PPARα-stimulation of hepatic fatty acid oxidation and by stimulating Apo-CIII. Concurrently, fibrates elevate circulating levels of HDLs, primarily by increasing active levels of Apo-AI and Apo–AII. The observation that PPARα-null mice have increased plasma levels of cholesterol, and become progressively obese with age, supports the contention that these effects are mediated via the PPARα (41,42). PPARα agonists also show varying degrees of hypoglycemic activity, and may be a useful adjunct therapy in the treatment of diabetes.

About 3% of the world population, or about 100 million people, suffer from diabetes. Type I, or juvenile-onset, insulin-dependent diabetes, accounts for about 10% of the cases, while the remaining 80 to 90% suffer from Type II diabetes mellitus. Eighty percent of the latter patients are obese and owe their pathology to resistance of peripheral tissues, such as skeletal muscle, liver and fat, to the actions of insulin (50). In

1982 Sohda and colleagues reported a new class of insulin-sensitizing, antidiabetic compounds known as the glitazones (51). Two of these drugs, rosiglitazone (Avandia™) and pioglitazone (Actos™) are currently approved in the U.S. for treatment of type II diabetes. Lehmann and colleagues reported in 1995 that the glitazones are ligands for PPARγ. The current model for the mechanism of these compounds involves PPARγ regulation of genes in adipocytes that leads to a flux of fatty acids away from skeletal muscle and liver and into adipocytes where they are stored or metabolized (reviewed in 37). The net effect of this fatty acid repartitioning is an increase in glucose oxidation in muscle and a decrease in glucose production by the liver. PPARγ may also effect gene expression in the liver and muscle.

Inflammation

Therapeutic modulation of the PPARs also shows great promise in treating inflammatory diseases such as atherosclerosis. In addition to their roles in regulating plasma lipoprotein concentration, the PPARs may affect foam cell formation, modulate the inflammatory response, and influence plaque stability. PPARα dampens the inflammatory response (52) and decreases the concentration of pro-atherosclerotic proteins, such as fibrinogen and C-reactive protein (53), as well as hepatic expression of several other acute-phase, inflammatory response proteins (54). PPARγ decreases macrophage secretion of interleukin –1-beta (IL1β), IL6, and tumor necrosis factor-alpha TNF (55), by inhibiting AP-I, Stat, and Nf-κB transcription factors (56).

Cancer

Modulating expression of at least two of the three PPARs affects neoplastic processes. Activated PPARγ, for example, has anti-proliferative effects in preadipocytes

and possibly in several malignant cell types. PPARγ induces terminal differentiation of human liposarcoma cells *in vitro* and in patients suffering from advanced liposarcoma (57). It also promotes terminal differentiation of malignant breast epithelial cells *in vitro* and induces apoptosis and fibrosis of breast tumor cells injected into mice (58). Furthermore, decreased tumor incidence was observed after PPARγ activation in rats treated with nitrosomethlurea (59) and in mice treated with prostate tumor cells (60). PPARγ effects on colon cancer are less clear, however, since activation has been reported to both promote (61,62) and protect (63) against colon cancer in mice.

The role of PPARα in carcinogenesis, at least in rodents, is more clearly defined: all PPARα ligands that have been adequately tested for carcinogenicity in chronic rodent feeding studies caused liver tumors (64). This is somewhat surprising, since even the most sensitive assays fail to detect evidence of DNA binding or mutation by PP (reviewed in 29). Morphologically, these tumors arise in a sequential fashion (65). The earliest lesions are discrete areas or foci of either eosinophilic or basophilic hepatocytes with cytomegaly and karyomegaly. These cells have prominent nucleoli, are arranged in cords that are 1 to 2-cells thick, and have variable mitotic activity. After 6 to 12 months grossly visible nodules arise that range in size from 1 mm to 10 mm. These lesions are composed of hepatocytes with increased mitotic activity that are arranged in plates greater than 2 cells thick. Nodules that compress adjacent parenchyma are labeled as hepatocellular adenomas. After 12 months of PP exposure, discrete areas exhibiting features of malignancy arise. These hepatocellular carcinomas are often large, sometimes more than 4 cm in diameter, and exhibit marked cytological and nuclear pleomorphism. In lifetime feeding studies, 20 to 40% of these animals will develop pulmonary metastases.

In rats and mice treated with PP, the orderly progression from preneoplastic liver lesions to benign adenomas and, ultimately, invasive cancers suggests the sequential appearance of subpopulations of cells differing in several phenotypic attributes (e.g., invasiveness, rate of growth, hormonal responsiveness). Indeed, epidemiologic, experimental, and molecular studies indicate that malignant tumors arise from a protracted sequence of genetic and epigenetic events occurring in a particular temporal order. Studies of oncogenes and tumor suppressor genes in many types of cancers support this concept of tumor progression. For example, we have known for decades that no single oncogene can fully transform cells *in vitro*. Furthermore, every human cancer that has been analyzed harbors multiple alterations involving activation of several oncogenes and loss of 2 or more tumor suppressor genes. The orderly appearance of hepatic lesions in mice and rats treated chronically with PP suggests that these tumors exhibit a similar evolution in which the altered hepatocytes are progressively selected for malignant properties.

Although liver lesions induced by PP appear similar to those induced by other hepatocarcinogens, PP-induced tumors have several distinguishing features. In contrast to rodent liver tumors induced by other carcinogens, PP-induced tumors do not overexpress fetal marker enzymes, such as γ-GGT, placental GST or α-fetoprotein, and they have markedly less UDP-glucuronyltransferases, sulfotransferases and serum epoxide hydrolase. In other respects they are similar, since tumors induced by PP have decreased expression of glucose-6-phosphatase, adenosine triphosphatase, and a number of the cytochrome P450 enzymes (65,66,67,68,69,70,71,72,73,74).

The specific sequence of molecular alterations associated with the various premalignant or malignant lesions induced by PP is unknown. However, *Ppar*α plays a

central role in this process. Ligand binding studies of PP of different affinities show a good correlation between receptor activation and potency as a hepatocarcinogen. Furthermore, short-term treatment of *Ppar*α-null mice with one of several PP failed to induce classical short-term responses associated with PP exposure, including peroxisome proliferation, hepatocyte hyperplasia and hypertrophy, increased hepatocellular DNA synthesis, and transcriptional activation of peroxisomal β-oxidation and microsomal ω-oxidation genes (40). Chronic feeding studies demonstrated a 100% tumor incidence in wild type mice fed WY (0.1% in the diet) and no tumors in *Ppar*α-null mice fed the same diet (43). Thus, *Ppar*α plays a necessary role in hepatic mitogenesis and carcinogenesis in mice exposed to PP.

Observations of consistent cellular adaptations following PP exposure have led to at least 2 or 3 hypotheses about PP-induced hepatocarcinogenesis. The first maintains that the carcinogenesis arises as an indirect effect of increased lipid metabolism (75). During the cyclic oxidation of fatty acids, hydrogen peroxide, which is generated in peroxisomes by *Aco*, is quickly converted to water and molecular oxygen by a catalase enzyme. PP treatment disrupts this equilibrium by preferentially inducing *Aco* over catalase. Under these conditions, hydrogen peroxide accumulates in the peroxisome until it eventually diffuses through the membrane and mutates DNA, either directly or after conversion to hydroxyl radicals. Experiments showing increases in lipofuscin, conjugated dienes, and 8-OH deoxyguanine (all measures of oxidative damage) in total liver DNA following PP exposure provided early support for this hypothesis (76,77,78,79). Recent work, however, including: a lack of correlation between peroxisomal endpoints and carcinogenicity potency; lack of initiating ability; lack of increases in liver nuclear DNA oxidation levels (80,81,82,83,84); combined with the

reversibility of PP-induced lesions following cessation of PP treatment (85,86), has largely weighed against oxidative stress as being obligatory for the carcinogenesis induced by PP.

An alternative hypothesis suggests that PP have a more direct effect on the cell cycle and act by inappropriately stimulating cell division (83) and/or inhibiting apoptosis (87). In a seminal study, Marsman and co-workers correlated hepatocellular DNA replication in rats with PP potency (83). Rats were fed either the very potent PP and experimental hypolipidemic drug, Wyeth-14,643 (WY) at a level reported to give 100% incidence of hepatocellular carcinoma (HCC) at 60 weeks, or the less potent PP di-(2-ethylhexyl) phthalate (DEHP) at a level that results in a 10% incidence of HCC after 2 years. Elevations in peroxisomal fatty acid oxidation were similar for the two groups up to 40 days, and both carcinogens caused a marked burst of DNA replication during the first week of feeding. After one week, WY but not DEHP caused a 10-fold elevation in DNA replication for up to one year. These results argued that chronic persistent cell replication is more closely correlated with the carcinogenic activity than is peroxisomal proliferation. Several groups have reported that WY induces discordant expression of proliferating cell nuclear antigen (*Pcna*) and certain cyclin-dependent kinases (*Cdk*) (88,89,90). More recent evidence suggests that *Ppar*α does indeed modulate the cell cycle regulatory apparatus. WY administration to wild type, but not *Ppar*α-null mice, led to increased *Cdk1*, *Cdk2*, *Cdk4*, and *Pcna* proteins, as well as *Cdk1*, *Cdk4*, cyclin D1 (*Ccnd1*), and *cMyc* mRNAs (91). How cell proliferation alone would lead to cancer is yet unclear, but there is evidence that mitogenesis itself is mutagenic, since cell division allows DNA adducts, formed by either exogenous or endogenous processes, to be fixed as mutations (92).

PP also decrease the rate of apoptosis in hepatocytes, possibly setting the stage for clonal expansion of genetically damaged cells. Treating cultured primary rat hepatocytes with nafenopin resulted in a ~4-fold increase in viability when compared to untreated cells (87). Furthermore, the treated cells had significantly less evidence of nuclear condensation and fragmentation. The same study showed that increases in apoptosis induced by transforming growth factor-beta (*Tgfβ*) could be prevented by nafenopin. The anti-apoptotic effect of PP may require *Pparα* since transfecting cells with the dominant-negative regulator of Pparα-mediated gene expression, hPPARα-6/29, prevented the suppression of apoptosis by nafenopin, but not that seen in response to phenobarbitone, a nongenotoxic carcinogen whose action does not involve PPARα (87). Altered regulation of the Bcl2 protein family has recently been demonstrated in livers, and liver tumors, from mice acutely and chronically treated with PP (93).

Several investigators have reported that PP are only weakly mitogenic *in vitro*, inducing a two- to three-fold increase in cell replication (94,95,96,97), suggesting that the mitogenesis requires the cooperation of other soluble factors or cell types. One cell type that is postulated to contribute to hepatic mitogenesis is the resident macrophage in the liver, the Kupffer cell. Kupffer cells produce a number of mitogenic factors with known roles in liver regeneration following partial hepatectomy, including epidermal growth factor (*Egf*), tumor necrosis factor-alpha (*Tnfα*), hepatocyte growth factor (*Hgf*), and prostaglandin E (*Pge*) (98). Kupffer cells are reported to be activated *in vivo* by treatment with nafenopin or WY (99,100), and inactivation of Kupffer cells with methyl palmitate prevents the 8-fold increase in hepatocellular proliferation normally seen *in*

vivo following WY treatment (100). PP treatment does not appear to induce expression of *Hgf*, *Egf*, or their receptors (101,102,103), and may actually repress *Hgf* expression after chronic treatment and in tumors (104). *Tnfα*, however, is an attractive candidate mitogenic cytokine released from Kupffer cells following PP exposure. *Tnfα* reportedly stimulates hepatocellular growth (105,106,107), and antibodies to *Tnfα* block liver regeneration following partial hepatectomy (106). Other investigators report that *Tnfα* either kills cells or impairs their growth (108). The discrepancy may be attributed to concentration, since treating cultures of human airway smooth muscle cells with high concentrations of *Tnfα* inhibits the proliferative effects of growth factors, whereas low concentrations stimulate DNA synthesis (109). WY treatment of rats reportedly results in a 2-fold induction of hepatic *Tnfα* mRNA, and this induction does not occur if Kupffer cells are ablated by methyl palmitate pretreatment (100). Interestingly, *Tnfα* administration to rats led to roughly 50% reductions in hepatic mRNAs, relative to controls, for the PP marker enzymes catalase, *Aco*, and multifunctional enzyme, and about 25% less *Pparα* mRNA. *Pparα* protein levels were decreased by about 50% (110). This evidence is inconclusive, however, because other investigators report no induction of hepatic *Tnfα* mRNA following PP treatment (101,111,112).

These hypotheses for the carcinogenic mechanism of PP are not necessarily exclusive and may converge on a common end point (85) (Figure 2). Lacking DNA reactivity (113,114,115), PP might induce altered expression of hepatic growth and differentiation genes that contribute to the carcinogenesis. Although PPREs have yet to be identified in the regulatory regions of growth regulatory genes, *Pparα* is an attractive candidate for a PP-activated transcription factor with hepatic growth modulating effects. *In vitro* PP can directly activate, independent of the phenomenon of peroxisome

proliferation, growth regulatory signal transduction pathways leading to the expression of the immediate-early nuclear protooncogenes *fos* and *jun* (116). Moreover, emerging evidence suggests that the hepatocellular proliferation induced by PP is mediated by patterns of growth factor modulation and signal transduction that are different from those induced by compensatory hyperplasia or other hepatic mitogens. For example, growth factors [*Hgf* and transforming growth factor-alpha (*Tgfα*)], mediators of regenerative hepatocellular proliferation, the immediate-early genes [early growth response gene (*Egr*)-1, liver regeneration factor (*Lrf*)-1, and *cMyc*], are not altered by PP treatment (101). Likewise, nuclear factor (*Nf*)-*kb* activation, a regulator of the immediate-early gene response proceeding to cell division following partial hepatectomy, does not follow PP treatment (101). These data suggest a distinct, as yet uncharacterized, signaling pathway for induction of hepatocyte mitogenesis by PP, which may converge upon cell cycle control genes (91,101). Other work analyzing PP-induced tumors for differential expression of various hepatic growth factors and their receptors (*Tgfα*, *Egfr*, *cMet*, c*Myc*, *Hgf*) suggests that cell proliferation in the developing tumors similarly depends on unique signaling pathways (103,117). These data, coupled with observations that PP-induced hepatic proliferative lesions completely regress upon cessation of PP exposure by a mechanism involving increased apoptosis and decreased mitosis (85,86,118), have led us to hypothesize that prolonged and unique PP-induced alterations in transcriptional control of key hepatic genes are essential for the neoplastic development associated with exposure to these compounds.

Unlike DNA-reactive (genotoxic) carcinogens, which are generally regarded as having the potential to induce tumors in any species, and for which there appears to be no theoretical threshold dose, the mechanisms by which non-genotoxic carcinogens

cause cancer has largely remained obscure. This is problematic for regulatory agencies (119), and resolution of the controversy surrounding approval and licensing of non-genotoxic carcinogens will require a mechanistic understanding of the rodent carcinogenesis. Several non-genotoxic carcinogens, including the PP, have been identified as ligands for specific receptors. Thus, it seems plausible that altered transcription of unaltered gene products may drive the carcinogenesis (120,121,122). If so, identification and characterization of the signaling pathways induced (or repressed) by these receptors will be an essential step towards understanding the nature, and human health relevance, of these non-genotoxic rodent carcinogens.

Is there a human cancer risk associated with PP exposure? The weight of the current evidence indicates that humans exposed to PP do not have an increase in cancer risk in the liver or, for that matter, in any other organ. Because humans (123,124,125) and other primates (125) are much less susceptible to the actual proliferation of peroxisomes, some workers have questioned the relevance of extrapolating the rodent data to humans (125). However, the relationship between peroxisomal proliferation and cancer is tentative, since it is based on carcinogenicity studies that have been conducted only on species that are highly susceptible to peroxisomal proliferation (126). Gemfibrozil and clofibrate have been used on humans in the US for 15 to 30 years, and epidemiological studies on patients receiving these drugs for 5 to 8 years did not reveal a significant increase in cancer (reviewed in 127). However, the short-term follow-up periods limit the value of these studies, since the onset of human cancer after exposure to a carcinogen is estimated to be on the order of 20 years. Furthermore, PP do exert some of their effects independent of peroxisome proliferation (128). Non- or weakly responsive species treated with clofibrate do show distinct hypolipidemic effects without dramatic peroxisomal proliferation in their

hepatocytes (129,130), and these drugs can modulate gene expression in humans without the concurrent induction of peroxisomal enzymes (131).

Lastly, some nonspecific peroxisomal changes have been reported in humans on therapeutic doses of fibrates (113,124), and other histologic and biochemical changes in the liver are common, especially with long-term use (124,125). On the other hand, liver biopsies from people treated with hypolipidemic drugs or cultured hepatocytes treated with similar or dissimilar PP exhibit a uniform lack of peroxisome proliferation and induction of *ACO* (113). Furthermore, *Ppar*α is expressed at high levels in the liver of rodents, but not in humans (reviewed in 29). What little *Ppar*α exists in human liver is largely unable to bind to target gene response elements, because of the presence of competing proteins (29). Lacking any direct evidence of carcinogenicity in humans, one approach for extrapolating human cancer risk from rodent carcinogenicity data is to understand the mechanism of tumorigenesis in rodents and then determine if this mechanism is applicable to humans.

Although human tissues appear refractory to the short-term effects of PP, it would be imprudent to casually dismiss the rodent carcinogenicity data, particularly in light of the typical long-term human exposure (30 plus years) to many of these compounds (132). To resolve the controversy regarding the regulatory status of these compounds, a mechanistic understanding of the rodent carcinogenicity is needed. Since the PP are nongenotoxic carcinogens, they likely elicit their oncogenic effects via modulation of transcription, probably through the *Ppar*α (43). If the distribution, activity, or transcriptional targets of human *Ppar*α differ from those of the rodent, peroxisome proliferator-induced rodent liver tumors may have little predictive relevance to human

cancer risk. Regardless, identification of genes transcriptionally targeted by PP will be critical for resolving the issue of potential human cancer risk associated with PP exposure.

Research Goals

The long-term objective of this research was to improve human risk estimation for PP exposure by defining a subset of the molecular events associated with hepatic mitogenesis and carcinogenesis observed in rodents exposed to PP. Our working hypothesis was that PP-induced rodent liver tumors develop as a consequence of specific, *Ppar*α-modulated perturbations in homeostatic gene expression. To accomplish our objective, we had three specific aims. Using the tools of genomics and genetics, we sought to identify: 1) *Ppar*α target genes with altered mRNA expression in hepatic tumors induced by PP; 2) upstream signaling events modulating expression of the identified genes; and 3) the role of *Ppar*α in mediating normal hepatic growth in the absence of xenobiotic exposure. The results of our investigations are reported in this dissertation.

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TABLE 1. Representative peroxisome proliferators

Category	Compound
Physiologic	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), 8(S)-hydroxyeicosatetraenoic acid (8(S)-HETE), oxidized components of LDL,; arachadonic, eicosapentaenoic, palmitic, oleic, and linoleic acids
Hypolipidemic drugs (approved)	gemfibrozil, clofibrate, ciprofibrate. fenofibrate
Hypolipidemic drug (experimental)	Wy-14,643, nafenopin, methyl clofenapate
Antidiabetic drugs	Thiazolidinediones (e.g., rosiglitazone, pioglitazone)
Antimania drugs	valproic acid
Adrenal steroid and supplement	dehydroepiandrosterone (DHEA)
Leukotriene D4 receptor antagonist	LY-171,883
Non-steroidal anti-inflammatory drugs	fenoprofen, ibuprofen, aspirin
Plasticizers	di-(2-ethylhexyl) phthalate (DEHP), di-(2-ethylhexyl) adipate (DEHA), di-n-butyl phthalate (DBP)
Solvent	trichloroethylene (TCE)
Herbicides	lactofen, fomasafen, 2,4-Dichlorophenoxyacetic acid (2,4-D)

TABLE 2. Tissue distribution of peroxisome proliferator-activated receptors

Isoform	*Liver*	*Kidney*	*Intestine*	*Spleen*	*Fat*
α	++++	++	++++	+	–
β/δ	++	++	+++	++	–
γ	–	+/–	++	+++	++++

Figure Legends

FIGURE 1.1.— Gene modulation by Ppars. Peroxisome proliferator (PP) ligand binding induces a conformational change in the Ppar receptor that allows it to heterodimerize with the retinoic acid receptor-X (RXR) receptor-ligand (9-cis retinoic acid) complex. The heterodimer recognizes a peroxisome proliferator response element (PPRE) sequence (TGACCTnTGACCT) in the promoter region of Ppar-responsive genes. After binding the PPRE, the complex recruits co-receptors and other modulator proteins, and the basal transcriptional machinery, to alter gene expression.

FIGURE 1.2— Cancer Model for peroxisome proliferators. After peroxisome proliferator exposure, a small percentage of resting hepatocytes are morphologically altered (E_1, or event 1) by an unknown process, and undergo a clonal expansion to form preneoplastic foci. These foci increase in size and evolve into hepatocellular adenomas. Removing exposure to the peroxisome proliferator at this point results in complete reversion of the liver to the normal phenotype. If peroxisome proliferator exposure is continued past the adenoma stage a small percentage of the adenomatous hepatocytes undergo a second event (E_2) that converts them into malignant hepatocytes (hepatocellular carcinoma). E_2 is likely a mutational event(s), since these lesions persist even after peroxisome proliferator exposure is terminated.

Figure 1.1

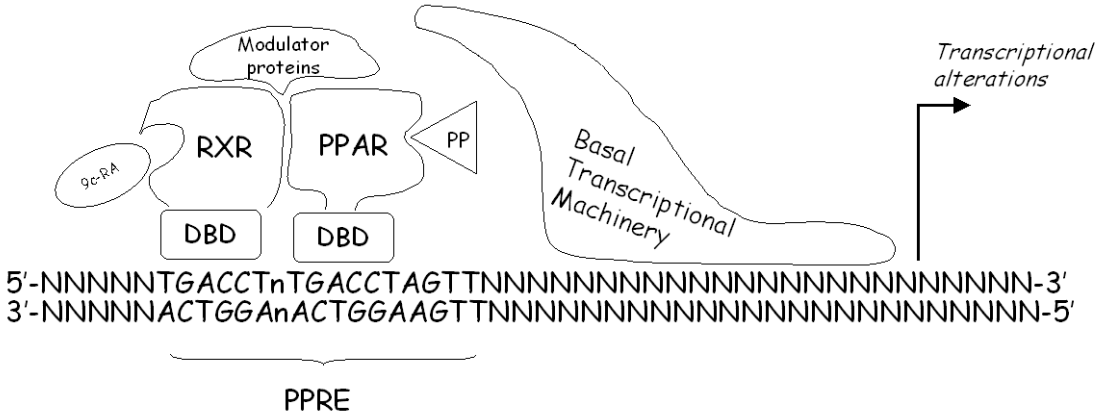
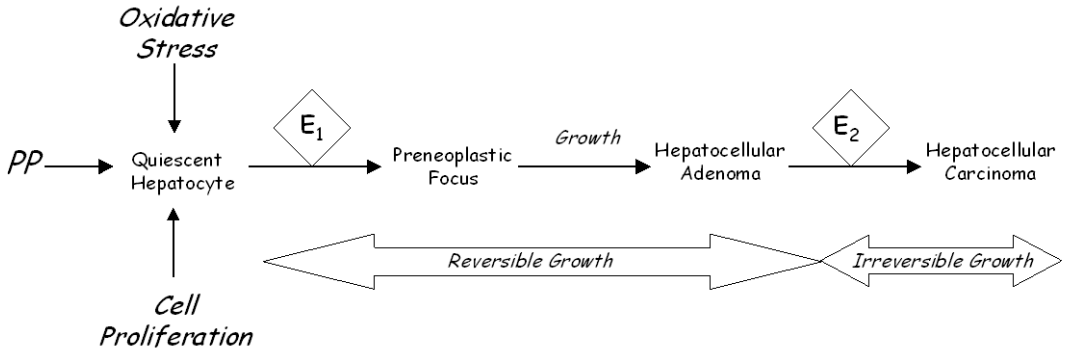

Modulator proteins
RXR
PPAR
PP
9c-RA
DBD
DBD
Basal Transcriptional Machinery
Transcriptional alterations
5′-NNNNNTGACCTnTGACCTAGTTNNNNNNNNNNNNNNNNNNNNNNNNN-3′
3′-NNNNNACTGGAnACTGGAAGTTNNNNNNNNNNNNNNNNNNNNNNNNN-5′
PPRE

Figure 1.2


Oxidative Stress
PP
Quiescent Hepatocyte
E1
Preneoplastic Focus
Growth
Hepatocellular Adenoma
E2
Hepatocellular Carcinoma
Reversible Growth
Irreversible Growth
Cell Proliferation

Chapter 2

Manuscript 1: Hepatic Expression of Acute-Phase Protein Genes During Carcinogenesis Induced by Peroxisome Proliferators.

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ARTICLES

Hepatic Expression of Acute-Phase Protein Genes During Carcinogenesis Induced by Peroxisome Proliferators

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Concern exists regarding peroxisome proliferator (PP) xenobiotic exposure because many PPs are potent hepatocarcinogens in rodents. The mechanism of carcinogenicity induced by PPs is atypical compared with those of other hepatocarcinogens in that the former appears to involve alterations in expression of PP-activated receptor (PPAR) target genes rather than direct mutagenicity. To begin to identify some of these genes, we used differential display to compare mRNA expression between hepatic adenomas and adjacent non-tumor liver from rats fed the potent PP Wy-14643 (WY) for 78 wk. Here, we report increased expression of the acute-phase protein (APP) gene α-1 antitrypsin (*AT*) and decreased expression of α2-urinary globulin in the tumors. Similar changes were seen in hepatic adenomas induced by a diethylnitrosamine and phenobarbital protocol, indicating a lack of specificity for PP-induced tumors. Additional APP genes, including ceruloplasmin, haptoglobin, β-fibrinogen, and α1-acid glycoprotein were also upregulated in WY-induced tumors but were downregulated in the livers of rats administered a different PP for 13 wk. Mice treated with either WY or di(2-ethylhexyl) phthalate for 3 wk had decreased hepatic AT expression but increased expression of ceruloplasmin and haptoglobin. *PPARα*-null mice showed no hepatic APP gene alteration after PP treatment but had higher basal expression than did wild-type controls. We conclude that PPARα activation by several different PPs leads to dysregulation of hepatic APP gene expression in rats and mice. This dysregulation may indicate alterations in cytokine signaling networks regulating both APP gene expression and hepatocellular proliferation. *Mol. Carcinog. 26:226–238, 1999.*

Key words: hepatocarcinogenesis; gene expression; differential display; acute-phase proteins

INTRODUCTION

Over 100 structurally diverse chemicals induce acute increases in both the number and size of peroxisomes, as well as increase synthesis of fatty acid oxidation enzymes, in rodent hepatocytes [1]. Chronic administration of these agents, known collectively as peroxisome proliferators (PPs), leads to increases in the size and number of hepatocytes and a markedly increased incidence of hepatocellular preneoplastic foci, adenomas, and carcinomas [2]. Although limited epidemiological studies, along with data generated by treating cultured human hepatocytes with PPs, indicated that humans are significantly less responsive, controversy exists over potential adverse human health effects associated with exposure to many commercially important PPs, including representative hypolipidemic drugs, plasticizers, fungicides, herbicides, and industrial solvents. In the absence of evidence for human PP responsiveness in vivo, resolution of this controversy will be greatly facilitated by a more complete understanding of the mechanism underlying the rodent carcinogenicity [3]. This mechanism remains largely obscure but is relevant because of the potential for widespread human exposure to PPs [4].

The mechanism of PP carcinogenicity in rodents is not completely clear. Numerous studies indicated that PPs demonstrate little or no direct genotoxicity [reviewed in 4], and many or all of the typical short-term adaptations to PP exposure [5], as well as the carcinogenicity [6], may be mediated by a specific xenobiotic-activated orphan nuclear receptor known as PP-activated receptor (PPAR)-α. All

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Abbreviations: PP, peroxisome proliferator; PPAR, peroxisome proliferator–activated receptor; WY, Wy-14643; DEN, diethylnitrosamine; PB, phenobarbital; GEM, gemfibrozil; DBP, di-*n*-butyl phthalate; DEHP, di(2-ethylhexyl) phthalate; AT, α-1 antitrypsin; α2U, α2-urinary globulin; AGP, α1-acid glycoprotein; PCR, polymerase chain reaction; MUP-1, major urinary protein-1; APP, acute-phase protein; TNFα, tumor necrosis factor-α; IL, interleukin.

known PP-responsive genes in rodents and humans have PPARα recognition sequences in gene regulatory regions, but so far there is no evidence for similar sequences in genes with known roles in regulating cell growth or differentiation [3]. In addition to their lack of DNA reactivity, PP-induced preneoplastic foci and neoplasms have at least two unique properties when compared with similar lesions induced by other carcinogens. First, PP-induced lesions do not express glutathione S-transferase-placental form or γ-glutamyl transpeptidase, two markers associated with foci and tumors induced by both DNA-damaging agents and some nongenotoxic carcinogens [7,8]. Moreover, in contrast to other hepatocarcinogenesis model systems, the progression of PP-induced foci to hepatocellular adenomas requires continual exposure to the compound. Treatment cessation leads to regression of the lesions by a mechanism involving both decreased proliferation and increased apoptosis [9,10]. The lack of PP-DNA reactivity, the presence of a specific xenobiotic-activated transcription factor capable of exerting broad-ranging transcriptional induction after PP exposure, and the regression of hepatic adenomas after PP withdrawal argue persuasively that PP-induced perturbations in homeostatic gene transcription play a central role in PP-induced carcinogenesis.

Only a few studies have examined alterations in gene expression associated with PP-induced tumors, but these studies also suggested that PPs induce liver tumors by a different mechanism than do other rodent hepatocarcinogens. Hepatocytes in PP-induced tumors do not carry mutations in the tumor suppressor gene *p53* [11] and express lower levels of hepatocyte growth factor mRNA than do normal hepatocytes [12]. Previously, we showed that mRNA levels for the hepatocyte growth factor receptor c-met, as well as the transcription factors c-myc and PPARα, are increased in PP-induced tumors relative to naive liver but are not significantly elevated in the surrounding non-tumorous parenchyma [13]. Moreover, mRNA levels of transforming growth factor-α and its receptor, epithelial growth factor receptor, are not changed in Wy-14643 (WY)–induced liver foci and tumors [14]. These investigations, which did not identify molecular pathways of PP-induced tumor growth, suggested that these PP-induced tumors arise through a unique genetic mechanism.

Understanding the mechanism of PP-induced tumor formation in rodents will be an integral step toward more accurately assessing the potential human health risks associated with exposure to these xenobiotics [4]. One common approach to studying mechanisms of complex biological processes is to identify differences that exist at the level of gene expression. Historically, this has been accomplished by cDNA library subtraction and differential screening. While these techniques are useful, they require large amounts of RNA and are technically difficult and laborious. The more recently developed mRNA differential display [15] overcomes these limitations by requiring very small amounts of RNA, allowing comparison of a number of different samples simultaneously, and allowing visualization of both positive and negative differences from the same set of reactions. This makes differential display an ideal method for studying changes in gene expression profiles associated with multistep biological processes such as tumor progression [16–20]. We used differential display to begin to test the hypothesis that specific and consistent PP-induced alterations in hepatic gene expression are the basis for the rodent carcinogenicity associated with long-term PP exposure. The goals of the experiments described here were to (i) identify patterns of gene expression associated with the critical transition from chronically treated, nontumor liver to reversible hepatic adenomas, (ii) determine if these changes precede and are therefore predictive of tumor formation, (iii) determine if these changes are PP-compound specific and are similar in the rat and mouse, and (iv) determine if these alterations are mediated via PPARα.

MATERIALS AND METHODS

Animals and Treatment

This study was conducted under federal guidelines for the use and care of laboratory animals and was approved by the Chemical Industry Institute of Toxicology Institutional Animal Care and Use Committee. F344 (CDF(F344)/CrlBR) rats were obtained from the Charles River Breeding Laboratories, Inc. (Raleigh, NC). Male SV129 wild-type mice were purchased from Taconic (Germantown, NY). Male SV129 *PPARα*-null mice [5] were the offspring of breeder mice obtained as a kind gift from Dr. Frank Gonzalez (National Cancer Institute, Bethesda, MD). All animals were provided with NIH-07 rodent chow (Ziegler Bros., Gardner, PA) and deionized filtered water ad libitum. Lighting was on a 12-h light and 12-h dark cycle. In the first study, male F344 rats were fed a WY (1000 ppm)–containing diet or a control NIH-07 diet ad libitum for 78 wk. In the diethylnitrosamine (DEN) phenobarbital (PB) carcinogenesis study, 11-wk-old male F344 rats were administered DEN at 150 mg/kg by intraperitoneal injection. Two weeks later, the DEN-initiated rats were exposed to PB (500 ppm) in the diet, ad libitum, for 78 wk. For the subchronic rat study, F344 rats were fed 500 ppm WY, 8000 ppm gemfibrozil (GEM) (Sigma Chemical Co., St. Louis, MO), 20 000 ppm di-*n*-butyl phthalate (DBP) (Aldrich Chemical Co., Milwaukee, WI), or a control NIH-07 diet for 13 wk. For the subchronic mouse study, SV129 wild-type and SV129 *PPARα*-

null mice were fed WY (1000 ppm), di(2-ethylhexyl) phthalate (DEHP) (6000 ppm), or a control NIH-07 diet for 3 wk. At the designated times after treatment, animals were deeply anesthetized by isofluorane inhalation or intraperitoneal pentobarbital injection and then killed by exsanguination. Their livers were removed, rinsed with isotonic saline, snap-frozen in liquid nitrogen, and stored at −70°C until analyzed.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated by modification of the guanidinium isothiocyanate method by using RNAzol B or STAT-60 according to the manufacturer's instructions (Tel-Test, Friendswood, TX). Denatured total RNA (20 μg) was separated on 1.0% formaldehyde-agarose gels and transferred to Hybond-N Nylon membranes (Amersham, Corp., Cleveland, OH) in 20% standard saline citrate. Equal loading and transfer were assessed by ethidium bromide staining of agarose gels and methylene blue staining of the nylon membranes. Attempts to use the housekeeping genes albumin, actin, and glyceraldehyde-3-phosphate dehydrogenase as loading controls in the carcinogenesis study were unsuccessful because all those genes exhibited altered expression in the tumors (data not shown). These data are in concordance with results reported by several other investigators who found that transcript levels of both albumin and actin were significantly lower and the glyceraldehyde-3-phosphate dehydrogenase levels were significantly higher in the livers of rats receiving various PP treatments for 1 mo [21,22].

The RNA was fixed by ultraviolet cross-linking with a Stratalinker ultraviolet cross-linker (Stratagene, La Jolla, CA). The membrane was prehybridized with Hybrisol I (Oncor Inc., Gaithersburg, MD) at 42°C and hybridized overnight. The probes included a 400-bp fragment of the rat α-1 antitrypsin (*AT*) obtained by differential display, a full-length rat α2-urinary globulin (α2U) isolated from a rat heart cDNA library (Corton JC, unpublished data), a rat α1-acid glycoprotein (*AGP*) oligonucleotide complementary to nt 660–689 in the rat cDNA, a rat β-fibrinogen cDNA (gift from Dr. Gerald Fuller), a rat ceruloplasmin cDNA complementary to nt 421–711 [23] (gift of Dr. Jonathan Gitlin), and a rat haptoglobin cDNA (American Type Culture Collection, Manassas, VA). The probes were labeled with [α-^{32}P]dCTP (Amersham Corp.) by using a random-primer DNA labeling kit according to the manufacturer's instructions (Stratagene). After hybridization, the membranes were washed in 1% standard saline citrate and 1% sodium dodecyl sulfate at 42°C for 30 min and then in 0.1% standard saline citrate and 1% sodium dodecyl sulfate at 42°C for 32 min. After washing, the membranes were exposed to Kodak BioMax Radiographic film (Fisher Scientific, Pittsburgh, PA) with an intensifying screen at −70°C for 12 h to 7 d. The sizes of the mRNAs were determined by comparing their mobility to that of an RNA ladder (GIBCO/BRL, Gaithersburg, MD).

Differential Display Analysis and Confirmation

Differential display was performed essentially as described by Zhu and Liang [15]. One microgram of total RNA digested with DNase I (MessageClean; GenHunter Corporation, Brookline, MA; or RQI DNAse; Promega Corp., Madison, WI) was reverse-transcribed according to the manufacturer's instructions by using SuperScript II (GIBCO/BRL) and one of three H-T$_{11}$V primers (GATCT$_{11}$A, GATCT$_{11}$C, or GATCT$_{11}$G) synthesized on an ABI 392 DNA/RNA Synthesizer (Applied Biosystems Incorporated, Norwalk, CT). The resulting cDNA was diluted fivefold with distilled H_2O, and 2 μL was amplified in the presence of the appropriate H-T$_{11}$V primer and a randomly derived 10-bp primer of defined sequence (50–60% GC) (Genosys Corp., The Woodlands, TX). The amplification conditions were as follows; an initial denaturing step of 94°C for 120 s followed by 40 cycles of 30 s at 94°C, 120 s at 40°C, and 30 s at 72°C. A final 10-min extension at 72°C was performed at the end of the amplification. The reactions were performed in the presence of 2 μM dNTPs; 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 5 μCi of [^{35}S]dATP (Amersham Corp.), and 0.5 U of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), in a total volume of 20 μL. The resulting polymerase chain reaction (PCR) products were separated on a 6% denaturing acrylamide gel that was then dried and exposed to film for 12–36 h. Bands showing putative differential expression were cut from the gel with a clean scalpel blade, eluted in 100 μL of distilled H_2O, boiled, and ethanol-precipitated in the presence of 50 μg of glycogen carrier (Boehringer Mannheim Corp., Indianapolis, IN).

For reverse northern blot analysis [24], the amplicons were reamplified by 40 cycles of PCR under the same reaction conditions, except that the dNTP concentration was increased to 20 μM, [^{35}S]dATP was omitted, and the total volume was increased to 80 μL. Aliquots (35 μL) were applied to duplicate Hybond-N nylon membranes (Amersham Corp.) by using a 96-well dot-blot vacuum apparatus (GIBCO/BRL). The membranes were dried and cross-linked with ultraviolet light (Stratagene). The blots were then probed with [α-^{32}p]dCTP (Amersham Corp.)–labeled total cDNA generated by random-primer-labeling of oligo d(T)-primed total RNA derived from hepatic adenomas or adjacent nontumorous liver. A total of six probes, each from a different animal, was used. The washing conditions were previously described [24]. PCR amplicons showing differential expression were subcloned by using either the TA Cloning vector (Invitrogen, Carlsbad, CA) or a TA-like vector created from a pBS

II vector (Stratagene) [25] and sequenced by using the Sequenase kit (United States Biochemical Corp., Cleveland, OH). The sequences were compared with sequences in the GenBank and EST databases accessed through the Entrez program at the National Center for Biotechnology Information.

Western Blot and Immunohistochemical Analyses

Whole-cell protein extracts (50–120 μg of total protein) made according to the method of Wilcke and Alexson [26] were denatured and separated by 12.5% or 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes (Stratagene) and visualized by Ponceau Red staining to confirm transfer. The membranes were washed with phosphate-buffered saline with Tween. The membranes were blocked by incubation in phosphate-buffered saline with Tween containing 5% nonfat dry milk. The blotted proteins were probed with polyclonal or monoclonal antibodies followed by anti–rabbit or anti–mouse Immunoglobulin G coupled to horseradish peroxidase and visualized by enhanced chemiluminescence (ECL, Amersham Corp.; or SuperSignal, Pierce Chemical Co., Rockford, IL). The sizes of the immunoreactive proteins were estimated with gel-fragment sizer software (DogStar Software, Bloomington, IN). The antibodies to α2U and ceruloplasmin were kind gifts from Dr. Otto Neuhaus and Dr. Johnathan Gitlin, respectively. Anti–β-fibrinogen and anti-AT antibodies were purchased from Affinity Biologicals (South Bend, IN) and Zymed Laboratories (South San Fransisco, CA), respectively. For immunohistochemical analysis, livers were fixed in 10% phosphate-buffered formalin, dehydrated, and embedded in paraffin. Sections (5 μm) were deparaffinized, and immunohistochemical staining was performed with a TechMate 1000 automated immunostainer (BioTek Solutions, Atlanta, GA) by the streptavidin-biotin method (Zymed Labs) by using anti–rat α2U and anti–mouse immunoglobulin G (Vector Labs, Burlingame, CA). The sections were lightly counterstained with hematoxylin after immunohistochemical visualization of α2U.

Densitometric and Statistical Analysis

Densitometric data were obtained by using Image-1 (Universal Imaging Corporation, West Chester, PA) and NIH Image software (developed at the National Institutes of Health and available from the Internet by anonymous FTp from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, VA, part number PB95-500195GEI). Statistical analysis was performed by using JMP software (SAS Institute, Cary, NC). Treatment groups were tested for statistical significance by using the Tukey-Kramer HSD test at a level of $P < 0.05$.

RESULTS

Identification of Differentially Expressed Transcripts in PP-Induced Hepatic Adenomas

During the multistage progression of hepatocellular carcinogenesis induced by PPs, hepatocellular replication in adenomas and carcinomas is roughly 8- and 13-fold higher, respectively, than basal levels seen in untreated rats [10]. This adenoma-carcinoma transition can be viewed as a critical event during the progression, because stopping PP exposure before this point results in complete regression of the lesions by a mechanism involving both increased apoptosis and decreased cell proliferation [9,10,27]. To identify transcriptional differences associated with this critical event, we compared mRNA transcription patterns between WY-induced hepatic adenomas and adjacent non-tumorous hepatic parenchyma by using mRNA differential display [15]. Three anchored oligo-d(T) primers were used in combination with one of 50 internal primers (for a total of 150 different primer combinations) to generate an estimated 15 000 cDNA sequence tags. Of these sequence tags, 154 appeared to exhibit altered intensities between the adenomas and surrounding tissue. Initial screening by reverse dot-blot northern analysis [24] and DNA sequencing indicated that at least two genes, *α2U* and *AT*, had altered mRNA levels in the tumors (data not shown). These results were confirmed by northern blot analysis (Figure 1). *α2U* mRNA was significantly decreased in the surrounding liver and essentially undetectable in the adenomas relative to the control liver. In contrast, the mRNA levels of *AT* were unchanged in the surrounding liver but were significantly increased (about twofold) in the adenomas.

Expression of α2U and AT Proteins in PP- and DEN/PB-Induced Adenomas

Because mRNA and protein levels can diverge as a result of translational and posttranslational mod-

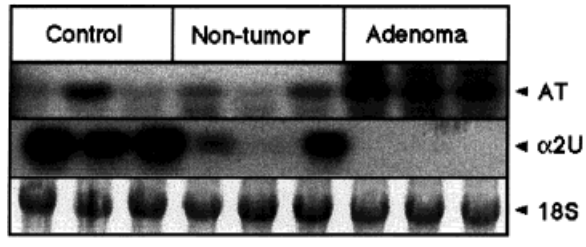

Figure 1. Differential expression of *AT* and *α2U* mRNA in WY-induced hepatocellular adenomas. Northern blot hybridizations of total RNA from control male F344 rats and rats treated for 78 wk with 1000 ppm WY in the diet (non-tumor and adenoma). Total RNA was extracted from the tissues, and 20 μg was subjected to northern blot hybridization with the ^{32}P-labeled cDNAs indicated, as described in Materials and Methods. Equal loading and transfer of RNA were confirmed by ethidium bromide (agarose gel) and methylene blue (nylon membrane) staining of the 18S ribosomal RNA band.

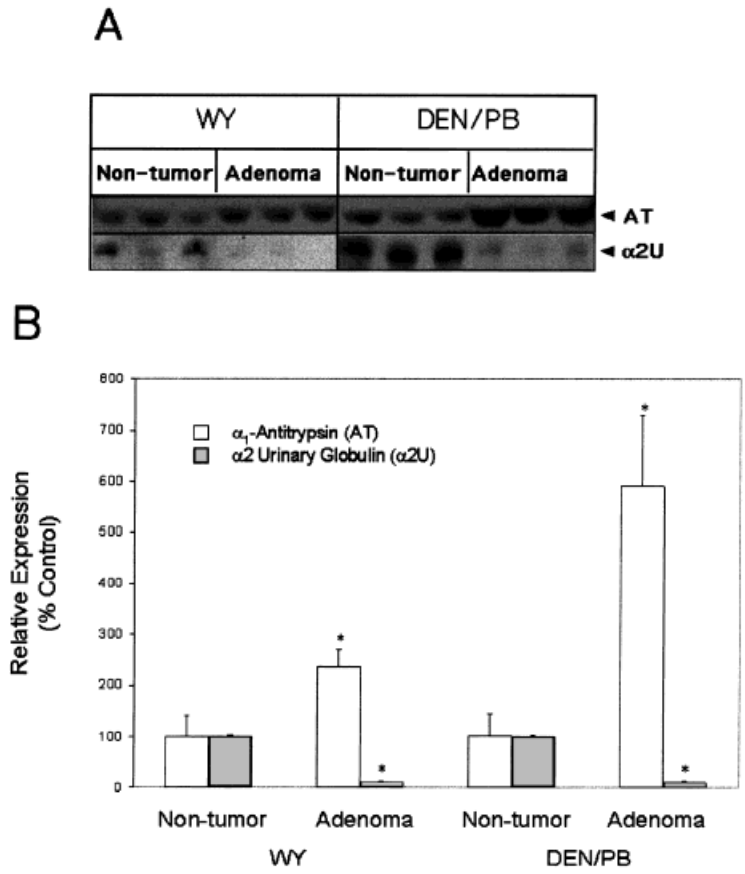

Figure 2. Differential expression of AT and α2U protein in WY- and DEN/PB-induced hepatocellular adenomas. (A) Western blot analysis of adenomas and surrounding non-tumor liver from male F344 rats initiated with DEN (150 mg/kg, intraperitoneal injection) and then treated with PB (500 ppm in the diet) or treated with WY (1000 ppm in diet) only for 78 wk. Whole-cell extracts (50–120 μg total protein) were size-fractionated by 12.5% (AT) or 15% (α2U) sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After transfer to nitrocellulose membranes, the proteins were probed with antibodies as described in Materials and Methods and were visualized by chemiluminescence. (B) Quantitation of AT and α2U protein expression in tumors. Relative signal intensity is expressed as a percentage of control. *, significantly different from control, $P < 0.05$.

ification, we sought to determine the degree of concordance in the tumors. Western blot analysis of whole-cell extracts prepared from three separate WY-induced hepatic adenomas from different animals and surrounding non-tumor liver from three of those animals revealed that protein levels paralleled mRNA expression for AT and α2U (Figure 2A). A polyclonal antibody against AT detected a 46-kDa protein showing approximately twofold increased expression in adenomas. A polyclonal antibody against α2U detected an 18-kDa protein, consistent with the size of α2U protein, that was decreased about eightfold in the adenomas relative to adjacent liver. To assess whether these changes in expression were specific to tumors induced by PPs, we measured expression of these genes in rat hepatic adenomas obtained from animals induced by DEN/PB, a well-characterized model of rat hepatocarcinogenesis not linked to peroxisome proliferation [28]. AT and α2U showed about 5-fold induction and about eightfold repression, respectively (Figure 2B), indicating that changes in expression of these genes are not unique to tumors induced by PP.

A previous study reported that expression of the mouse α2U homolog, major urinary protein-1 (MUP-1), might serve as an early tumor marker during mouse hepatocarcinogenesis. Northern blot analysis showed high levels in normal liver but 15- to 100-fold lower levels in spontaneous and p-dimethylaminoazobenzene– or urethane-induced liver tumors. Immunohistochemical staining showed marked reductions in MUP-1 within DEN-induced liver nodules [29]. To assess whether α2U could serve as a tumor marker during rat hepatocarcinogenesis induced by PPs, sections of control, non-tumor, and adenomatous liver were stained with a rat α2U–specific antibody. α2U expression was localized to centrilobular hepatocytes in the livers of untreated F344 rats, similar to previous reports for MUP-1 in the mouse [29]. Staining in these regions was of marked intensity and was relatively uniformly distributed (Figure 3A). Non-tumorous hepatic parenchyma adjacent to adenomas induced by treating rats with WY for 72 wk showed a similar lobular distribution for α2U, but expression was moderately decreased relative to

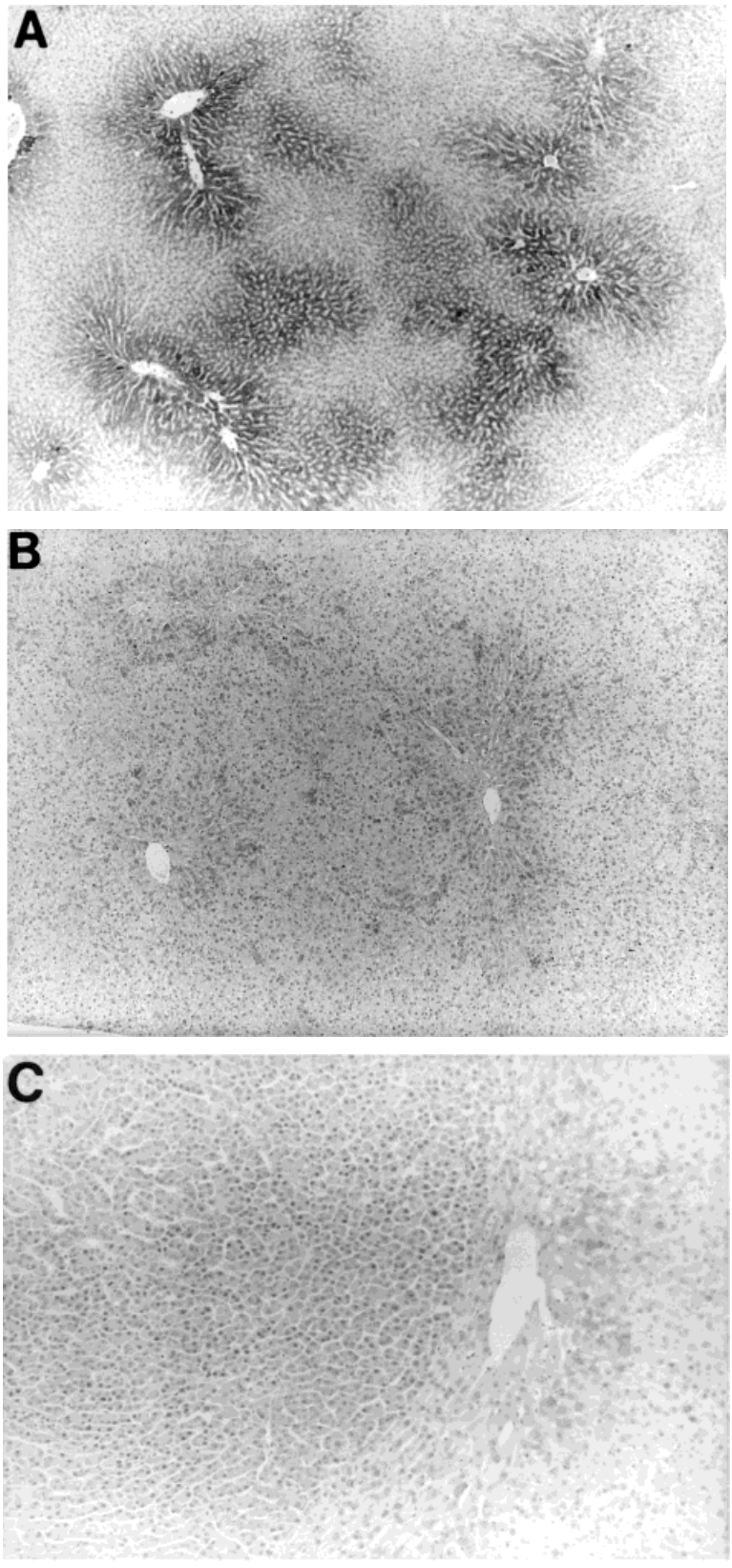

Figure 3. Distribution of hepatic α2U protein in adenomas induced by WY. Immunohistochemical staining of treatment groups was described in Materials and Methods. Note that α2U is present at high levels in the periportal hepatocytes in untreated control liver (A) but is downregulated in non-tumorous surrounding liver (B) and absent in hepatic adenomas (C), left from animals treated with WY for 78 wk.

control animals (Figure 3B). Hepatic adenomas were virtually devoid of α2U expression (Figure 3C). These results suggest that decreases in α2U, similar to MUP-1, are an early, generic event in the development of rodent liver tumors and that these genes may be useful as tumor markers in these species.

Type 1 and Type 2 Acute-Phase Gene mRNA Expression in Hepatic Adenomas

AT is a member of the acute-phase protein (APP) family, a group of hepatic proteins that function to limit the systemic response to localized trauma. Expression of APP genes is regulated primarily at the transcriptional level, when promoter and enhancer regions contain cis-acting sequences that recognize nuclear transcription factors, many of them cytokine induced, to potentiate or depress transcriptional initiation [reviewed in 30,31]. The APP genes are grouped into two types based upon cytokine inducibility. Type 1 APP genes are induced by tumor necrosis factor-α (TNFα) (and interleukin (IL)-1) activation of the transcription factors NFκB and

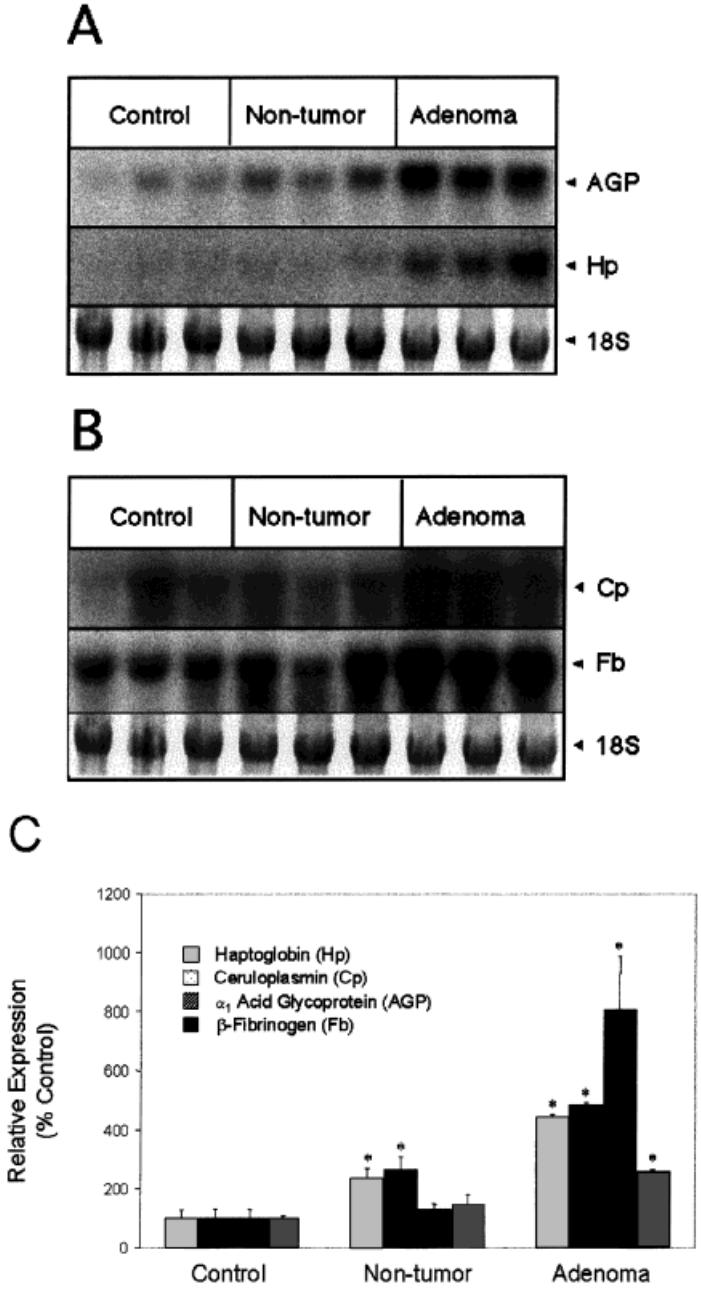

Figure 4. Expression of type 1 and type 2 *APP* gene mRNAs in WY-induced hepatic adenomas. Northern blot analysis was performed as described in Figure 1 by using ^{32}P-labeled cDNAs of type 1 (A) and type 2 (B) APP genes. (C) Quantitation of APP mRNA expression. *, significantly different from control, $P<0.05$. Hp, haptoglobin; Cp, ceruloplasmin; Fb, β-fibrinogen.

CCAAT enhancer binding protein, whereas type 2 APP genes are induced by IL-6 (and IL-11) induction of the transcription factors CCAAT enhancer binding protein and signal transducer and activator of transcription. Because TNFα and IL-6 both play at least facultative roles in hepatocyte proliferation after partial hepatectomy [reviewed in 32,33], and TNFα may be a central mediator of PP-induced hapatocellular replication [34,35], we were interested in whether APP gene expression in PP-induced hepatic adenomas could offer insight into upstream signaling events potentially relevant to tumorigenesis. We used northern blot analysis to examine changes in mRNA levels of additional type 1 (haptoglobin and *AGP*) (Figure 4A) and type 2 (ceruloplasmin and β-fibrinogen) (Figure 4B) APP genes during the development of PP-induced hepatic adenomas. The expression of all genes was quantified by scanning densitometry, and the results are shown in Figure 4C. Haptoglobin and ceruloplasmin, but not *AGP* or β-fibrinogen, mRNAs were significantly ($P < 0.05$) increased (about twofold to threefold) in the surrounding parenchyma relative to the control liver. All APP genes were increased from twofold to sevenfold in the adenomas relative to the control liver. These results suggest that there is a global upregulation of APP gene expression in PP-induced tumors. Because response is not restricted to a particular APP class, both cytokine signaling pathways may be perturbed in the tumors. Whether changes in these pathways precede or cause tumorigenesis is unknown.

Effect of Subchronic PP Treatments on APP Gene mRNA Abundance

The hepatocarcinogenesis induced by PPs is a multistage process consisting of hepatocellular hyperplasia and formation of altered hepatocellular foci, adenomas, and carcinomas [2]. This process is reversible, before carcinoma formation, if PP exposure is discontinued [9,10,27]. Furthermore, different PP compounds demonstrate different efficacies in inducing tumor formation [reviewed in 3,4]. To determine if increases in APP gene expression precede tumor formation and if the magnitude of these alterations is PP-compound specific, we examined mRNA expression of the type 2 APPs *AT* and ceruloplasmin and the type 1 APP haptoglobin in

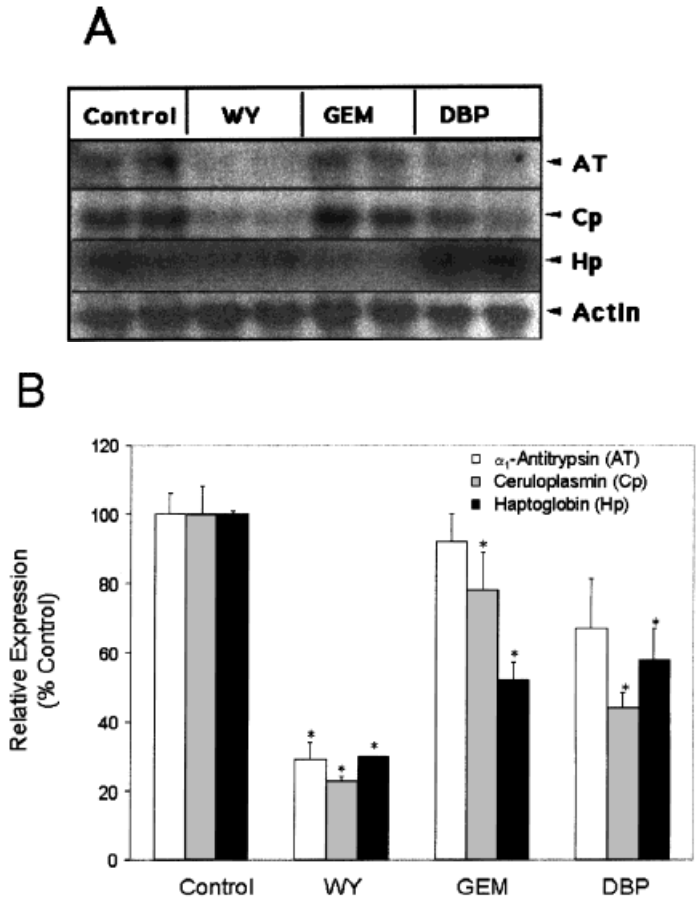

Figure 5. Hepatic mRNA expression of type 1 and type 2 APP genes in rats chronically treated with several PPs. (A) Northern blot analysis using 20 μg of total RNA isolated from male F344 rats fed an NIH-07 diet (Control), 500 ppm WY, 8000 ppm GEM, or 20 000 ppm DBP for 13 wk. AT, α_1-antitrypsin; Cp, ceruloplasmin; Hp, haptoglobin. (B) Quantitation of APP mRNA expression. *, significantly different from control, $P < 0.05$.

the livers of rats treated for 13 wk with the PPs WY, GEM, and DBP. This subchronic time-point was chosen based on previous work demonstrating that feeding WY for this period results in a 16-fold increase in hepatocellular replication over that in nontreated rats [36]. Figure 5A shows that the mRNA levels of all three genes were markedly decreased after WY treatment, and quantification (Figure 5B) revealed that they were expressed at levels approximating 30% of those of untreated controls. The levels of ceruloplasmin and haptoglobin, but not *AT*, were also moderately decreased after treatment with the less potent PPs GEM and DBP. These results, along with our previous work showing downregulation of the APPs β-fibrinogen and *AGP* [37], suggest a global downregulation of APP gene expression in the livers of subchronically treated rats that correlates with PP-compound potency and that is inversely correlated with cell proliferation.

APP mRNA Abundance in PP-Treated *PPARα*-Null Mice

PPARα is required for PP-induced peroxisomal proliferation, hepatocellular hypertrophy and hyperplasia, enzyme induction [5] and hepatocarcinogenesis [6] in mice. PPARα also plays a role in some types of inflammatory responses, as evidenced by its ability to downregulate several aspects of these responses [38,39]. To determine the role of PPARα in mediating changes in APP expression, we examined the hepatic mRNA levels of *AT*, ceruloplasmin, and haptoglobin in SV129 wild-type ($PPAR^{+/+}$) and SV129 *PPARα*-null ($PPAR^{-/-}$) mice treated for 3 wk with the potent PP WY or the less potent DEHP. Expression of cytochrome P450 4a2 was examined as a positive control (Figure 6A). As expected,

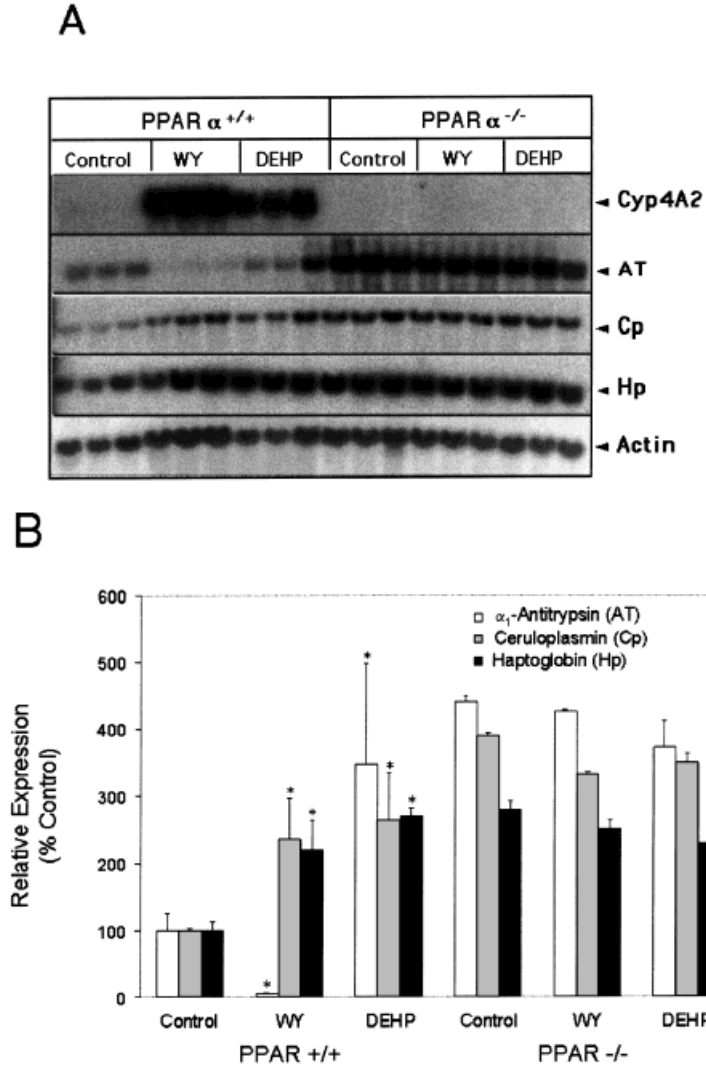

Figure 6. Hepatic expression of type 1 and type 2 APP mRNAs after PP treatment of $PPAR^{+/+}$ and $PPAR^{-/-}$ mice. (A) Northern blot analysis using 20 μg of total RNA from SV129 wild-type and SV129 *PPARα*-null mice fed 1000 ppm WY, 600 ppm DEHP, or NIH-07 diet (control) for 3 wk. AT, α_1-antitrypsin; Cp, ceruloplasmin; Hp, haptoglobin; Cyp4A2, cytochrome P450 4a2. (B) Quantitation of APP mRNA expression. *, significantly different from $PPAR^{+/+}$ control, $P < 0.05$.

cytochrome P450 4a2 was strongly induced in PP-treated wild-type mice but not in *PPARα*-null mice, confirming previous results from other investigators [5]. Quantitation of hepatic mRNA levels (Figure 6B) revealed that treating $PPAR^{+/+}$ mice for 3 wk with WY, but not with DEHP, led to a marked downregulation of *AT* (to about 10% of control levels). In contrast, treatment of these mice with either WY or DEHP resulted in a twofold to threefold induction of ceruloplasmin and haptoglobin. This lack of correlation between APP gene expression in mice and rats is not surprising because there is great variability in the APP response among humans, rabbits, rats, and mice [31]. For example, AT is reportedly an APP in rats but not in mice [40]. Interestingly, APP expression was not altered by PP treatment of $PPAR^{-/-}$ mice, but these mice had higher (3.5- to 5-fold) constitutive levels of expression of all genes examined when compared with control $PPAR^{+/+}$ mice. These results are in concordance with other work showing decreased serum and hepatic fibrinogen levels in wild-type mice treated with the PP fenofibrate and higher basal levels in *PPARα*-null mice that were refractory to PP treatment [41]. Taken together, these results and the results of others [38,39] suggest that PPARα activation not only mediates the rodent hepatocarcinogenicity associated with chronic PP exposure but also plays a role in homeostatic regulation of the inflammatory response.

DISCUSSION

The mechanism underlying rodent hepatocarcinogenesis after PP exposure is poorly understood but is of interest given the significant potential for human exposure to these agents [4]. The lack of genotoxicity after PP exposure, coupled with recent demonstrations that PPARα is mandatory for hepatic responses to PP [5], including carcinogenesis [6], strongly argues that alterations in gene expression play a central role in the rodent response. To begin to identify some of those alterations, we used mRNA differential display to compare the patterns of gene expression in tumor and non-tumor liver. In this study, we identified *α2U* and the APP gene *AT* as two genes showing altered expression in PP-induced hepatocellular adenomas in male F344 rats (Figures 1 and 2). These responses were not unique to PP-induced tumors and, in fact, were more pronounced in tumors induced by DEN/PB. *α2U* is a rodent gene induced by the male-specific pulsatile release of growth hormone but repressed by inflammation [42]. Members of this gene family appear to be regulated by PPARα, as *PPARα*-null mice do not show the expected downregulation of the orthologous murine gene *Mup-1* after PP treatment [37,43]. Our observation that *α2U* was downregulated in PP-induced adenomas extends previous work demonstrating decreased mRNA levels of this gene after subchronic PP treatment [37,43,44,45]. There is no clear association between *α2U* expression and rodent hepatic carcinogenesis, but others have suggested that expression is inversely correlated with cell proliferation [43,46].

This is the first report showing increased expression of both type 1 (haptoglobin and *AGP*) and type 2 (*AT* and ceruloplasmin) APPs in adenomas induced by administering the potent PP WY to rats (Figures 1 and 4). This could be an adaptive response to inflammation, necrosis, or compression associated with tumor growth. However, PP-induced tumors are not typically accompanied by inflammation and necrosis except in advanced stages of malignancy (Cattley RC, unpublished observations), and if compression were the cause, expression would be expected to be higher in the surrounding tissue than in the expanding tumor. Alternatively, as others have shown increased APP levels in regenerating liver after partial hepatectomy [47], increased expression may indicate a common effector mechanism for increasing APP gene expression and hepatocellular proliferation in rats. While we did not examine APP expression in liver tumors from mice, it should be pointed out that there are significant differences in hepatic APP gene expression between rats and mice during inflammation [31], and others have reported no differences in APP mRNA levels in spontaneous liver tumors and tumors induced by DEN in mice [48].

In contrast to our findings in the adenomas, hepatic expression of APP genes in rats was decreased after subchronic (13-wk) exposure, and the magnitude of suppression correlated with the reported potency of the various PP examined (e.g., WY, the most potent PP tested, elicited the strongest suppression of APP expression). This downregulation of APP expression is in concordance with recent reports that PPARα activators elicit an anti-inflammatory effect in mice [38] and humans [39], possibly through increased leukotriene B_4 catabolism [38], or inhibition of IL-1–induced production of IL-6, prostaglandin, and cyclooxygenase-2 [39]. Other APP genes may be similarly regulated by PP in other species, as demonstrated by the recent finding that hyperlipidemic human patients receiving therapeutic doses of the PP fenofibrate have decreased fibrinogen and C-reactive protein plasma concentrations [39]. There are, apparently, species differences in APP gene expression after PP exposure, as subchronic (3-wk) treatment of mice resulted in discordant changes in APP gene expression when compared with rats (Figure 5 and 6). *AT* expression was downregulated in SV129 mice, similar to the rat, but the ceruloplasmin and haptoglobin levels were increased. These changes may reflect a true species difference in responsiveness or may simply be the result of a fourfold difference in treatment duration. Surprisingly, *PPARα*-null mice were not responsive

to PP-induced increases in APP expression but exhibited higher basal expression of these genes when compared with untreated wild-type mice (Figure 6). This suggests that PPARα exerts a basal inhibitory effect on the expression of APP genes.

At present, the relationship between carcinogenesis and PPARα-mediated effects on the hepatic inflammatory response is speculative. While hepatocellular proliferation is significantly increased in both PP-induced adenomas and in hepatocytes from subchronically treated rats [10], we report that APP gene expression in the rat was upregulated in the tumors but downregulated in the livers of rats subchronically treated with PP, relative to control livers. Although a recent study suggested that increased expression of the APP genes *AT* and *AGP* may inhibit select apoptosis pathways in mouse liver [49], our results showing a lack of correlation between APP gene expression and cell proliferation in subchronically treated animals suggest that changes in APP expression probably do not influence hepatocellular proliferation. However, there is accumulating evidence that two of the cytokines important in APP regulation, IL-6 and TNFα, do have prominent roles in promoting hepatocellular growth [32,33]. Plasma IL-6 increases dramatically during liver regeneration following partial hepatectomy, and partially hepatectomized *IL-6*–null mice have a significantly suppressed rate of DNA synthesis [50]. Likewise, abrogation of TNFα signaling by administration of anti-TNFα antibodies [51] or homozygous deletion of TNFα receptor-I has similar effects that appear to be mediated through inhibition of NFκB activation [52]. Restoration of hepatocellular proliferation in partially hepatectomized TNFα receptor-I–null mice after IL-6 administration suggests that these pathways converge and that TNFα induces IL-6 secretion by hepatic Kupffer cells [52]. These findings are of interest because the acute cell proliferation after PP exposure appears to depend, at least in part, on Kupffer cell–derived TNFα [34,35,53], perhaps through activation of nuclear transcription factors such as NF-κB [54]. Because Kupffer cells [55] and hepatocytes [56] both express PPARα, either or both of these cell types may initiate PP-dependent gene expression, cell signaling, and cell proliferation. Moreover, because PP-induced hepatocellular neoplasms express PPARα [13], differential receptor activation or repression of currently unknown target genes in premalignant lesions may disrupt cell signaling networks that modulate hepatocellular growth and maintain differentiation.

In summary, we conclude that (i) mRNAs representative of both class 1 and class 2 APP genes are induced in hepatic adenomas from rats chronically treated with the strong PP WY; (ii) APP gene expression in the liver is not predictive of eventual tumor formation, because these mRNAs are repressed in the livers of rats treated subchronically with several different PPs, whereas cell proliferation is elevated in the livers from animals treated with these compounds [36]; (iii) subchronic treatment of rats with other PPs such as GEM, a hypolipidemic drug, and DBP, an industrial plasticizer, also leads to downregulation of hepatic APP genes that correlates with potency of the PP and is dependent upon functional PPARα; (iv) with one exception (i.e., AT), the mouse responds differently after subchronic PP treatment in that APP genes are induced in the liver; and (v) PPARα exerts a basal suppressive effect on hepatic APP mRNA levels in the mouse.

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Chapter 3

Manuscript II: Hepatocellular Proliferation in Response to a Peroxisome Proliferator Does Not Require *Tnfα* Signaling

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Carcinogenesis (in press)

Abstract

Rodents exposed to peroxisome proliferator xenobiotics respond with marked increases in hepatocellular replication and growth that results in tumor formation. Recently, tumor necrosis factor-α (*Tnf* □α) was proposed as the central mediator of this maladaptive response. To define the role of *Tnf*α signaling in hepatocellular growth induced by peroxisome proliferators we administered 3 daily gavage doses of the potent peroxisome proliferator, Wy-14,643, to mice nullizygous for Tnf-Receptor I (*Tnfr1*), *Tnfr2*, or both receptors. We demonstrate here that regardless of genotype the mice responded with almost identical increases in liver to body weight ratios and hepatocyte proliferation. Lacking evidence that *Tnf*α signaling mediates these effects, we then examined the possible contribution of alternative cytokine pathways. Semi-quantitative, reverse transcriptase polymerase chain reaction analysis revealed that wild type mice subacutely exposed to Wy-14,643 had increased hepatic expression of *IL1β*, *IL1r1*, *Hnf4*, and *Stat3*. Moreover, hepatic adenomas from mice chronically exposed to Wy-14,643 had increased expression of *IL1β*, *IL1r1*, *IL6*, and *Pparγ1*. Expression of *IL1β*, *Tnfα*, *Tnfr1*, *Tnfr2*, *Pparα*, or *C/ebpβ* was not altered by either acute Wy-14,643 exposure, or in adenomas induced by Wy-14643. These data suggest that the hepatic mitogenesis and carcinogenesis associated with peroxisome proliferator exposure is not mediated via *Tnfα* but instead may involve an alternative pathway requiring *IL1β* and *IL6.*

Abbreviations

APP, acute-phase proteins; *C/ebp*, CCAAT enhancer binding protein; *Hnf*, hepatic nuclear factor; *IL*, interleukin; LPS, lipopolysaccharide; *Nfκ B*, nuclear factor kappa B; PP, peroxisome proliferator; *Ppar*, Peroxisome proliferator-activated receptor; RT-PCR, reverse transcriptase-polymerase chain reaction; *Stat*, signal transducer of activated transcription; *Tgf*, tumor growth factor; *Tnf*, tumor necrosis factor; WY, Wy-14,643

Introduction

In the livers of rodents, chronic exposure to members of a diverse group of xenobiotics or endogenous fatty acids leads to increases in the quantity and size of single-membrane organelles called peroxisomes (1). Xenobiotic peroxisome proliferators (PP) include several commonly used hypolipidemic drugs, phthalate ester plasticizers, herbicides, and synthetic fatty acids. Mice and rats exposed to these compounds quickly manifest an adaptive hepatic response, consisting of hepatocellular hypertrophy and hyperplasia, that progresses to overt hepatic neoplasia (2). The carcinogenic properties of PP in rodents, coupled with relatively high exposure potential, have generated concern about potential adverse human health effects (3).

Although the mechanism of rodent carcinogenesis is largely unknown, several lines of evidence suggest that the tumors arise as a result of perturbations in homeostatic gene expression in the liver. First, PP generally lack evidence of genotoxicity as measured by classic initiation, DNA adducts, or short-term mutagenicity assays (3). Second, hepatic responses to PP exposure, including gene induction (4) and tumorigenesis (5), are mediated through ligand activation of a nuclear receptor transcription factor known as the peroxisome proliferator-activated receptor α (*Ppar*α). Third, proliferative hepatic lesions induced by PP regress by a mechanism involving decreased cell proliferation and increased apoptosis shortly after exposure is discontinued (6,7,8), a phenomenon not reported for genotoxic hepatocarcinogens. Collectively, these observations argue persuasively that PP-induced hepatocellular tumors are a maladaptive consequence of altered hepatic gene expression.

Thus far, *Pparα* binding sites have not been identified in regulatory regions of any known growth control genes, indicating that PP may induce perturbations in hepatocyte growth indirectly (9). Recent studies suggest that several cytokines with mitogenic and inflammatory potential are attractive candidate mediators of the hepatic mitogenesis that follows PP exposure. Indirect evidence that cytokine signaling is altered after PP exposure comes from our studies on a group of inflammatory response genes known as acute-phase proteins (APP). Exposing rats or mice to various PP, including Wy-14,643 (WY), gemfibrozil, di-*n*-butyl phthalate, or di-(2-ethylhexyl) phthalate, results in altered hepatic mRNA levels for APP. Subchronic exposure times ranging from 5 to 13 weeks reduces APP expression (10), while hepatic adenomas from rats exposed to PP for 78 weeks have increased APP expression compared to untreated animals (11). APP gene expression is primarily regulated at the level of transcription through interaction between *cis*-acting sequences in the regulatory regions of APP genes and *trans*-acting nuclear transcription factors (12,13). Many of these transcription factors are regulated by cytokines. For example, type 1 APP are induced by tumor necrosis factor α (*Tnfα*) (and *IL1*) activation of *NfκB* and *C/ebp* transcription factors, while type 2 APP are induced by *IL6* (and *IL11*) activation of *C/ebp* and *Stat* transcription factors.

Hepatic *Tnfα*, *IL1*, and *IL6* are important modulators of hepatocyte proliferation following partial hepatectomy (14,15) or chemical-induced necrosis (16). Three recent findings suggest that *Tnfα* mediates the effects of PP on hepatocyte growth (17,18) and thus may play a role in the events that lead to cancer. First, inactivation of Kupffer cells, which can secrete *Tnfα* and other cytokines, decreases PP-induced hepatocyte proliferation (19). Second, antibodies against *Tnfα* injected into rats before PP treatment diminishes hepatocyte proliferation (17). Third, *Tnfα* gene expression is increased in the

livers of PP-treated rats under some conditions (17,20), although not consistently (21, 22). However, it is unclear if a change in *Tnfa* expression in rodents treated with PP drives the increased hepatocellular proliferation, or if this alteration is merely a bystander event.

Here, we report experimental results designed to directly test the hypothesis that *Tnfα* is required for PP-induced hepatocyte growth. Using mice with genetically disrupted *Tnfα* signaling we show that this pathway is not required for PP to induce hepatocyte proliferation. Examination of other cytokines capable of regulating cell growth leads us to propose an alternative pathway involving *Il1β*, and perhaps *Il6*, is activated after PP exposure and is required for PP-induced hepatocyte growth in rodents.

Materials and Methods

Animals

These studies were conducted under federal guidelines for the use and care of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the CIIT Centers for Health Research (CIIT). Control and treated mice and rats were provided with NIH-07 rodent chow (Zeigler Bros.) and deionized, filtered water *ad libitum*. Lighting was on a 12-hr light-dark cycle. In Study I, 6- to 7-week-old male C57BL/6-TNFR-1 (*TNFrsfla*^{tm1Imx}), C57BL/6-TNFR-2 (*Tnfrsflb*^{tm1Imx}), or C57BL/6-TNFR-1/TNFR-2 (*Tnfrsfla*^{tm1Imx}/*Tnfrsflb*^{tm1Imx}), C57BL/6 wild-type mice (Jackson Laboratories) were acclimated for 12 days and given a single gavage dose of Wy-14,643 (50 mg/kg body weight) in methyl cellulose vehicle, or vehicle alone each day for 3 consecutive days. In Study II, B6C3F1 male mice (Charles River) 10.5 weeks old were

given a gavage dose of WY (50 mg/kg body weight) or vehicle as in Study I every day for up to 3 days. Groups of mice were euthanized at 2, 4, 6, 9, 12, 24, 48, and 72 hours after the initial dosing. In Study III, male F344 rats (Charles River) approximately 9 months of age were given a single intravenous injection of lipopolysaccharide (TCA-extracted *Salmonella typhimurium*, Sigma Chemical Co.) at 1.0 mg/kg body weight in 0.9% NaCl or an equivalent volume of 0.9% NaCl alone. The rats were euthanized 2 hours after injection. In Study IV, male wild type SV129 mice from the CIIT *Ppar*α-null mouse breeding colony were housed 2 per cage and fed the NIH-07 diet containing 0.1%WY w/w for 12 months. Mice in Studies I and II were administered BrdU as previously described (23).

Necropsy

Animals were deeply anesthetized with pentobarbital injection and killed by exsanguination. Portions of the major organs were rapidly minced and then snap-frozen in liquid nitrogen and stored at -70°C until analysis. Liver tumors (> 5 mm) were dissected away from non-lesion liver prior to freezing. Slices of livers and liver tumors were fixed in 10% neutral buffered formalin, routinely processed, and stained with hematoxylin and eosin. H&E stained liver sections were examined by light microscopy, and tumors were diagnosed as adenomas or carcinomas using standard criteria (8).

RNA isolation and quantitation

RNA was isolated as previously described (11) and DNase-treated (Promega). First-strand cDNA was generated from 5 µg of total RNA according to the manufacturer's recommendation (SuperScript II™ reverse transcriptase (RT) GIBCO/BRL) using random hexamer primers. Polymerase chain reaction (PCR) was performed on 1 µl of

the cDNA reaction using commercially available (Clontech; Ambion) or custom-designed (Primer Express™, PE BioSystems), gene-specific primers and Amplitaq (PE Biosystems) or Advantage 2 DNA Polymerase™ (Clontech), according to the manufacturer's recommendations. Glyceraldehyde phosphate dehydrogenase (*Gapdh*) (Clontech) or ribosomal *18S* (Ambion) genes were PCR amplified for endogenous controls. The optimal number of cycles required for detection of products in the linear range of amplification was determined for each of the cDNA-primer pair combinations prior to each experiment. Each target gene was amplified a minimum of three separate times. PCR products were resolved on a 1.5 % agarose gel containing ethidium bromide. Photographs of the gels were scanned using a flatbed scanner and analyzed by NIH Image as previously described (11). The mRNA transcript levels were determined by taking the ratio of the PCR product for the target gene to the PCR product of the endogenous control. Statistical test of significance was ANOVA post-hoc testing was performed using the Tukey-Kramer test using a P-value of ≤ 0.05 (JMP™, SAS Institute).

Determination of Hepatocyte Proliferation

Light microscopy was performed using a Nikon Microphot™ microscope with a Dage CCD color video camera at a magnification of 700 ×. The hepatocytes were analyzed using the object recognition system (CHRIS), (Sverdrup Medical/Life Sciences Imaging Systems). A minimum of 1000 cells were counted for each animal. Nuclei that incorporated BrdU were identified by immunohistochemistry. The labeling index was calculated by dividing the number of labeled hepatocyte nuclei by the total number of hepatocyte nuclei counted, and the results were expressed as a percentage.

Results

Liver weight and DNA synthesis in Tnfα-receptor (Tnfr)-null mice

Tnfα signaling pathways play important roles in stimulating APP gene expression (12,13) and also in promoting hepatocellular regeneration following either partial hepatectomy (14,15) or exposure to certain hepatotoxins (24,25). *Tnfα* elicits responses through binding to one of two receptors. High concentrations of *Tnfα* induce responses through the Tnf receptor 1 (*Tnfr1*, p55), which transmits signals promoting growth inhibition and cell death, while low concentrations stimulate responses through the Tnf receptor 2 (*Tnfr2*, p75), which stimulates cell proliferation (26). Paradoxically, only the *Tnfr1* is required for normal hepatic regeneration (16). Recent reports suggest that *Tnfα* is also a central mediator in the hepatic mitogenesis associated with PP (17,20). If so, mitogenesis induced by PP should be absent or greatly diminished in mice lacking one or both of these receptors. To test this prediction, we evaluated hepatomegaly and DNA synthesis following PP treatment of wild-type mice and in mice nullizygous for *Tnfr1, Tnfr2,* or both receptors (*Tnfr1*/*Tnfr2*). Livers from wild type and nullizygous mice constituted roughly 5% of the body weight in untreated mice (Figure 3.1A). Administration of WY (50 mg/kg body weight) by daily gavage for 3 days resulted in modest but significant hepatomegaly (to about 6% of body weight) regardless of *Tnfr* status. Hepatocyte proliferation assessed by BrdU-positive nuclei revealed a 3-fold increase after WY treatment in wild type, *Tnfr1*, and *Tnfr1/Tnfr2* mice (Figure 3.1B). *Tnfr2* mice administered WY had twice the number of labeled hepatocytes (~8%) as did wild type, WY-treated mice (~4%). These results demonstrate that the mitogenic effects of PP are not mediated through *Tnfα* activation of either *Tnfr1* or *Tnfr2*.

Cytokine gene expression during hepatic mitogenesis induced by WY

Several converging lines of evidence suggest that, in addition to *Tnfα*, other cytokine signaling pathways play important roles in hepatic regeneration following partial hepatectomy. Cytokines and cytokine receptors that might stimulate hepatocellular growth include *IL1α*, *IL1β*, *IL1r1*, and *IL6*. Those thought to inhibit this process, primarily through negative regulation of *Tnfα* expression, include *IL4* and *IL10* (14,15). Therefore, it seemed plausible that these pathways might also modulate the hepatic mitogenesis associated with exposure to PP or other nongenotoxic hepatocarcinogens.

To test the hypothesis that hepatic mitogenesis induced by PP is driven by alterations in signaling by cytokines other than *Tnfα*, we examined hepatic expression of several cytokines and their receptors at 7 time points following daily gavage doses of WY in B6C3F1 mice. Hepatocellular proliferation, measured by BrdU incorporation, was minimal up to 24 hours following the initial WY gavage. Thereafter, the rate of cell proliferation sharply increased at 48 hours and was maintained at the 72-hour time point (S. P. Anderson, unpublished data). We postulated that cytokines influencing the cell cycle status in the liver would exhibit altered expression preceding or coincident with the 48-hour time point. The relative mRNA abundance of each of these factors, as well as the cytokine-induced inflammatory mediators *Cox2* and *iNos*, were determined by RT-PCR because preliminary experiments revealed that many of the cytokines were not detectable using Northern blot, ribonuclease protection, or competitive RT-PCR assays (S. P. Anderson, unpublished data). Transcript abundance was normalized to ribosomal *18S* RNA expression and the results were expressed as ratios. Hepatic *IL6* and *Tnfα* mRNAs were expressed at very low levels at all time points and could only be consistently amplified after 35 (*Tnfα*) or 40 (*IL6*) cycles of amplification using a high-sensitivity, high-fidelity DNA polymerase (Advantage 2 cDNA Polymerase™, Clontech).

The paucity of transcripts for these cytokines in mouse liver is consistent with observations in human liver (27). Furthermore, expression of these cytokines was not altered throughout any of our experiments. In contrast, *IL1β* showed a statistically significant increase in expression that began at 9 hours, peaked at 12 hours (~2.5-fold increase), and was elevated at 24, 48, and 72 hours (Figure 3.2). *IL1r1*, the major receptor for both *IL1α* and *IL1β*, showed slight but significant increases at 12 and 48 hours. *Tnfr1* showed slight, but significant increases at 24 and 72 hours. The expression of *IL1α* and *Tnfr2* was not altered at any of the time points. As expected, the expression of *ApoAI*, a gene that is down regulated by PP in mouse liver (28), was decreased after PP treatment. *IL4*, *IL10*, *Cox2*, and *iNos* were undetectable at any of the time points (data not shown). As positive controls, hepatic cytokine expression was evaluated by RT-PCR in animals sacrificed 2 hours following a single intravenous dose of bacterial lipopolysaccharide (LPS). As expected, there was mild induction of *IL6*, moderate induction of *IL1β*, and marked induction of *Tnfα*, *Cox2*, and *iNos* (Figure 3.3).

Transcription factor gene expression during hepatic mitogenesis induced by WY

All mammals, including rodents and humans, exhibit a stereotypical systemic response to localized inflammatory insults termed the acute-phase response. An integral component of this response is increased hepatic synthesis of a family of serum proteins known as the APP (12,13). Previously, we found that hepatic APP expression was altered following subchronic and chronic exposure of rodents to PP of variable potency (10,11). Many of these genes have response elements for transcription factors with known or suspected roles in regulating hepatocellular growth and differentiation (29). To test the hypothesis that altered expression of hepatic acute-phase genes is an indicator of dysregulated hepatic transcription factor homeostasis, we examined the relative

mRNA abundance of several transcription factors with roles in the regulation of hepatic growth regulation as well as in inducing expression of inflammatory mediators. Hepatocyte nuclear factor-4 (*Hnf4*) was induced 2-fold at 6 hours following a single gavage dose of WY and 2.5-fold at 12 hours. Thereafter, mRNA levels gradually decreased and achieved basal levels by 72 hours (Figure 3.4). Signal transducer and activator of transcription-3 (*Stat3*) was induced 2-fold at 12 hours and 1.5-fold at 48 and 72 hours. *Stat5b* showed a slight but significant induction (1.7-fold) at 6 and 9 hours that peaked at 12 hours then and gradually declined to 1.5-fold at 48 hours. *Ppar*α, *Ppar*γ (isoform 1), and CCAAT enhancer binding protein-α (*C/ebp*α) levels were unaltered by WY treatment.

Cytokine gene expression during hepatocarcinogenesis induced by WY

Since the hepatic mitogenesis induced after acute exposure to WY was preceded by transcriptional induction of several cytokine signaling factors, it seemed plausible that the chronic mitogenesis driving the production of hepatocellular tumors was stimulated by transcriptional induction of one or more of these factors. To test this hypothesis, we examined hepatic cytokine gene expression in the WY-induced hepatocellular adenomas and the adjacent, non-tumor tissue in 4 different mice, and compared expression to age-matched control mice. Expression patterns of several genes affecting cell cycle control and proliferation during mitogenesis induced by PP were examined as positive controls (30). As expected, Cyclin D1 (*Ccnd1*), *p21*, *Tgf*β, and c*Myc*, were induced from 2- to 5-fold in non-tumor hepatic parenchyma in animals receiving chronic WY exposure compared to controls (Figure 3.5). c*Myc* was induced even higher (up to 4.5-fold) in WY-induced tumors compared to surrounding tissue. Analysis of cytokine mRNA levels revealed that *IL1*β was induced more than 2-fold in the treated, nontumor

liver, and this induction persisted in the adenomas (Figure 3.6). *IL6* was significantly induced in the adenomas but not in the nontumor tissue. Expression of *IL1r1*, *IL1α*, *Tnfα*, *Tnfr1*, and *Tnfr2* was unchanged. These results suggest that hepatocarcinogenesis induced by PP associated with increased *IL1*, and possibly *IL6*, signaling and occurs independently of *Tnfα* pathways.

Transcription factor gene expression during hepatocarcinogenesis induced by WY

We also examined tumor-bearing and control livers for changes in mRNAs encoding several transcription factors involved in regulating the expression of cytokine or PP-responsive genes (Figure 3.7). *Pparγ1* was induced ~2-fold in nontumor tissue compared to controls, and was further induced in the adenomas up to ~3-fold in relation to control liver. Expression of *Hnf4*, *Pparα*, *Stat3*, or *Stat5b* was not altered in any of the groups.

Discussion

Although the development of PP-induced liver tumors in mice depends on *Pparα*, more proximate mediators of the carcinogenesis have not been identified. However, some evidence suggests that certain cytokines may play an important role. First, compensatory liver regeneration following partial hepatectomy of rodents is associated with increased transcription of *IL1β*, *IL6*, and *Tnfα* (13,14,15,16, 31), and regeneration is defective in mice lacking the *Tnfr1* gene (16,31). Second, rodents treated with PP have altered hepatic expression of the cytokine-regulated acute-phase protein genes (10,11). *Tnfα* is a direct hepatic mitogen (18) and is reportedly induced, albeit weakly, in the liver following PP exposure (17,19). Therefore, it was suggested that the hepatic growth stimulating effects of PP in rodents are due to increased production and secretion of

Tnfα from hepatocytes, most likely following induction of *NfκB* in Kupffer cells (32). Contrary to this conjecture, preliminary studies from our laboratory revealed no difference in *Tnfα* mRNA levels in the livers of rodents treated with PP (21). To resolve this discrepancy, we sought to more definitively test the hypothesis that *Tnfα* or perhaps other cytokines mediate the rodent hepatocellular proliferation induced by PP.

TNFα is not required for WY-induced hepatocyte proliferation

Compared to wild-type mice, mice lacking *Tnfr1*, *Tnfr2*, or both genes, exhibit no difference in hepatocellular proliferation or hepatomegaly after treatment with the potent PP, WY. Consistent with these findings, we (unpublished studies) and others (33) have observed no difference in hepatocellular proliferation in *Tnfα*-null mice compared to wild-type mice after an acute PP administration. Contrary to the prevailing view, these results demonstrate that *Tnfα* signaling is not required for WY-induced hepatocellular proliferation in rodents. Possible explanations for this discrepancy are numerous but unconvincing. For example, it is possible that the developmental programs of *Tnfr*-null and *Tnfα*-null mice have been altered in a manner that compensates for defective *Tnfα* signaling. If so, one might expect the null mice to have an altered pattern of hepatic cytokine gene expression. Thus far, using the sensitive ribonuclease protection assay, we have not observed any such differences between WY-treated *Tnfr*-null mice and wild type mice (unpublished observations). Furthermore, compared to wild type mice, *Tnfr*-null mice actually have decreased rates of hepatocellular proliferation following partial hepatectomy, or treatment with carbon tetrachloride (16,31,34), or fumonisin B1 (35), suggesting that *Tnfr*-null mice have selectively compensated for WY-induced cell proliferation alone. This is unlikely since there are clear differences in WY induction of acyl-CoA oxidase protein between wild type and *Tnfr*-null mice (A. J. Stauber,

manuscript in preparation). Another possibility is that *Tnfr*-null mice do show cell proliferation rates equivalent to wild-type mice after WY treatment but that the onset of maximum replication is simply delayed. However, mice showed increased hepatocellular proliferation beginning between 24 and 48 hours that peaked between 48 and 72 hours under our experimental conditions (S. P. Anderson, manuscript in preparation). In our studies with the null mice, hepatocyte proliferation was examined less than 1 day after peak in hepatocyte proliferation. If the *Tnfα*-null mice do have delays in hepatocyte proliferation, the proliferation recovers to or exceeds wild-type levels in less than 1 day, an unlikely scenario. In contrast, *Tnfr1*-null mice exhibited significant decreases in the level of but not the time of maximum DNA synthesis after partial hepatectomy (16,34). Another possible explanation for the inconsistent *Tnfα* induction could be PP-induced cytotoxicity in the liver at higher doses. In the reports of increased hepatic *Tnfα* mRNA expression, the animals were exposed to doses of WY 2-fold higher than our conditions (17,20). Thus, increased *Tnfα* production may have been a nonspecific response to cell death. Consistent with this, LPS-treated mice express higher levels of *Tnfα* and have more extensive liver damage when pretreated with WY at this high dose (36).

Central to the current model for PP-induced hepatocellular proliferation favored by many investigators is increased hepatic expression of *Tnfα* We show here that expression of *Tnfα*, *Tnfr1*, and *Tnfr2* not only remains constant both prior to and during the period of maximum mitogenesis (48 hours), but that expression of these genes does not change in livers during acute exposure, or in liver tumors induced by chronic exposure, to WY. Furthermore, we have been unable to detect *Tnfα* protein in the livers of control and WY-treated mice with a commonly used commercial antibody specific to that protein in the mouse (unpublished data). These results confirm and extend our

previous observations that *Tnfα* levels are not altered during hepatocarcinogenesis induced by WY in rats (21). Taken together, our results and the results of others (22,24,25,33), show that PP-induced hepatic mitogenesis in rodents occurs independently of changes in *Tnfα* expression levels.

Interleukins and Stats in WY-induced hepatocarcinogenesis

Lacking evidence that *Tnfα* signaling pathways significantly contribute to hepatocellular proliferation induced by PP, we examined mRNA levels of other mitogenic pathways including cytokines, their receptors and transcription factors that mediate their downstream effects. Our data suggest a role for *IL1β*, and possibly *IL6*, in WY-induced liver cancer. *IL1β*, *IL1α*, and *IL6*, like *Tnfα*, are hepatic mitogens elaborated by Kupffer cells in response to a variety of stimuli (18,37), as well as partial hepatectomy (14,15). Here, we demonstrate that *IL6* levels are increased in WY-induced hepatic adenomas, but are unchanged at earlier timepoints. Although an earlier study found that *IL6* inhibited the growth of cultured normal and transformed rat liver cells (38), a more recent study found that co-expression of *IL6* and the soluble IL6 receptor in double-transgenic mice lead to nodular regenerative hyperplasia and adenomas of the liver (39). *IL6* is reported to stimulate the growth of several other tumor types, including human colon carcinoma cells (40), non-tumorigenic rat urothelial cells (41), normal and neoplastic human cervical epithelial cells (42), and human bladder carcinoma cells (43). Thus, although *IL6* mRNA levels do not change prior to adenoma formation in our studies, *IL6* may play a role in stimulating or maintaining WY-induced tumors.

In contrast to expression pattern of *IL6*, hepatic levels of *IL1β*mRNA significantly increase 12 hrs following WY gavage, and remain elevated throughout the period of

maximum cell replication (48 hrs) and adenoma formation. *IL1β* is an attractive candidate mediator of cell proliferation in our studies because upregulation precedes stimulation of cell cycle regulatory genes. *IL1β*, like *IL1α*, elicits responses via the *IL1r1*. This receptor,similar to the *Tnfr1* receptor, activates several mitogenic signaling pathways involving *NfκB* and *Jnk* family kinases (44). To our knowledge, PP activation of hepatic *Jnk* has not been reported, while activation of *NfκB* has (32,45) and has not (24) been observed after PP exposure. While *IL1β* usually induces hepatic APP gene expression by activating *NfκB*, *IL1β* and *IL6* also activate *Stat3* (46). Our results demonstrate that the mitogenesis induced by PP is preceded by increases in hepatic mRNA levels of *Stat3*, *Stat5b a*nd transcriptional targets of *Stat3*. *Stat3* and *Stat5b* are induced in hepatocytes by hepatocyte growth factor, epithelial growth factor, and insulin or insulin-mimetics (47,48,49,50,51). *Stat3* is also induced in regenerating liver (29), which may simply be a nonspecific, stress-induced response (52), and its activity decreases during hepatocyte differentiation (53). Ligand-dependent activation of *Stats* often leads to differentiation and growth regulation, while constitutive, or ligand-independent, activation promotes growth dysregulation. Similarly, constitutively active *Stat5b* promotes cell proliferation in certain cell lines (54). Many tumor cell lines and human cancers have activated *Stats*, most often *Stat3* (55). *Stat3* can serve as an oncogene in hepatoma cells, where constitutive activation leads to transformation (56). Why *Stat3* is oncogenic is unknown, but several genes involved in regulating the cell cycle, including the *Ccnd1*, *cMyc*, and *p21* genes examined here, are downstream targets of *Stat3* (56). Among other *Stat3* targets is the anti-apoptotic gene *Bcl-X_L*, a gene we previously reported to be overexpressed in the great majority of WY-induced hepatic adenomas in mice (57). Collectively, our results are consistent with *IL1β*- (and possibly *Il6)* dependent induction of *Stat3* playing a role in PP-induced hepatocyte proliferation

and hepatocarcinogenesis, although electrophoretic mobility shift assays will be required to confirm this.

Hepatocyte nuclear factor-4 is induced after an acute dose of WY

Hnf4, another hepatic transcription factor important in regulating APP gene expression, as well as hepatocyte growth and differentiation, is transiently induced prior to the onset of mitogenesis but not in the tumors. *Hnf4* induction is accompanied by a decrease in *ApoAI*, a gene negatively regulated by *Hnf4* (28,58). *Hnf4* and *Hnf1* are the only liver-enriched transcription factors known whose expression is strictly correlated with hepatic differentiation in cultured rat hepatoma cells (59), but whether or not this correlation is true in vivo is uncertain. One study reported that only a fourth of chemically induced liver tumors had decreased expression of *Hnf1*α and *Hnf4* (60), while other studies found expression was markedly reduced (61), or varied extensively (62). Moreover, treating rats with the PP Medica 16 or bezafibrate for 6 days leads to a downregulation of *Hnf4* mRNA, and *Hnf4* protein decreased at 3 days but not at 1 or 2 days (63). Taken as a whole, these results indicate that *Hnf1* and *Hnf4* are unlikely candidates for genes significantly affecting hepatocarcinogenesis induced by PP. In our studies, the early induction of *Hnf4* after PP exposure may simply reflect a commitment to the liver cell phenotype by rapidly replicating hepatocytes.

Role of PPARγ*in WY-induced hepatocarcinogenesis*

WY is a very potent PP and is used mainly because of its efficacy as a *Ppar*α agonist. In addition, WY also activates *Ppar*γisoforms (9). Previously, we reported that *Ppar*α mRNA is upregulated in non-tumorous hepatic parenchyma and also in hepatic adenomas from rats treated with WY (23). Here, we report that *Ppar*γ*1* is induced during

chronic exposure to WY and is further induced in hepatocellular adenomas. *Pparγ1* is modulates several critical aspects of development and homeostasis, including adipocyte differentiation, glucose metabolism, and macrophage development and function (64). A role for *Pparγ1* in PP-induced rodent liver cancer has not been previously reported, but increased expression has been reported in mouse liver tumors induced by griseofulvin (65), and in rat colon tumors induced by azomethane (66). Also, administering *Pparγ1* ligands to C57BL/6J-APC^{Min}/+ mice, a strain genetically predisposed to intestinal neoplasia, leads to a dramatic increase in tumor incidence compared to untreated C57BL/6J-APC^{Min}/+ mice (67,68). The mechanisms underlying the effects of chronic *Pparγ1* activation in PP-exposed animals are unknown, but some *Pparα* agonists are capable of activating *Pparγ1* and *Pparδ* in *Pparα*-null mice, possibly by autoregulation (69). Because *Pparγ1* is normally expressed at quite low levels in the liver, activation of this receptor may induce expression of other, normally quiescent genes controlling cell growth. For example, colons from C57BL/6J-APC^{Min}/+ mice treated with the *Pparγ* agonist, BRL 49,653, over-express β-Catenin, a protein implicated in tumorigenesis (68). Significantly, β-Catenin mutation and nuclear protein accumulation is frequently observed during several types of liver tumors in mice and in humans (70,71,72,73), but similar changes have not been observed in liver tumors induced by PP. Currently, we are examining whether WY induces cancer in rodents via *Pparγ1*-mediated dysregulation of cell growth.

Model of IL-1β induction hepatocyte proliferation

As a summary of the data presented here, we propose a molecular model for the increased hepatocellular proliferation observed in rodents following PP administration (Figure 3.8). In this model, PP stimulate hepatic Kupffer cells (or perhaps hepatocytes)

to upregulate *Nfκ B*, which leads to increased synthesis and secretion of *IL1β*. *IL1β* acts in a paracrine fashion to stimulate *IL1r1* on neighboring hepatocytes, which then upregulates *Stat3*. *Stat3* then stimulates or represses genes involved in hepatocellular proliferation or apoptosis, respectively. Several variations of this model are possible. As one example, *IL1β* also induces the production of reactive oxygen species through Nadph Oxidase-mediated activation of *Nfκ B* (74), and mice lacking the catalytic subunit of Nadph Oxidase are resistant to hepatocellular proliferation after PP exposure (32,45). Conceivably, then, *Nfκ B* activation could result in a decrease in the level of hepatocyte apoptosis typically observed during acute exposure to PP (75), setting the stage for additional events, such as increased expression of *Pparγ1*, to trigger tumor formation. Future work will be aimed at determining the components of the *IL1β* signaling pathway required for PP-induced hepatocyte growth.

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Figure Legends

Fig. 3.1. Tumor necrosis factor α receptor 1 and 2 are not required for WY-induced hepatomegaly and hepatocellular DNA synthesis. (A) Liver weights expressed as a percentage of body weight. (B) Cell proliferation was determined as described in Materials and Methods. * denotes statistical differences from control, † denotes difference between wild type and Tnfr-/- (ANOVA followed by Tukey-Kramer test, $P < 0.05$, $n = 5$ per group).

Fig. 3.2. Hepatic cytokine expression in B6C3F1 mice receiving daily gavage doses of WY (50 mg/kg body weight) for 3 days. (**A**) RT-PCR was performed as described in Materials and Methods, using total RNA from 3 individual animals per treatment group. Equal loading was assessed by expression of ribosomal *18S*. *ApoA1* expression was examined as a positive control. (B) Densitometric analysis. mRNA transcript levels are expressed as ratios of the mean cytokine band intensities ($n = 3$) to the mean *18S* band intensities ($n = 3$). Statistical significance (see text) was determined using ANOVA followed by Tukey-Kramer test ($P < 0.05$).

Fig. 3.3. Hepatic cytokine expression 2 hours following intravenous injection (1.0 mg/kg body weight) of bacterial lipopolysaccharide (LPS). Expression was determined by densitometric analysis of RT-PCR products as described for Fig. 2.

Fig. 3.4. Hepatic transcription factor expression in wild-type mice receiving daily gavage doses of WY (50 mg/kg body weight) for 3 days. Analysis was as described in Fig. 2. (A) RT-PCR. (B) Densitometric analysis.

Fig. 3.5. Hepatic cell cycle regulatory gene expression during hepatocarcinogenesis induced by feeding wild-type mice WY (1000 ppm) for 52 weeks. Analysis was as described in Fig. 2. (A) RT-PCR. (B) Densitometric analysis.

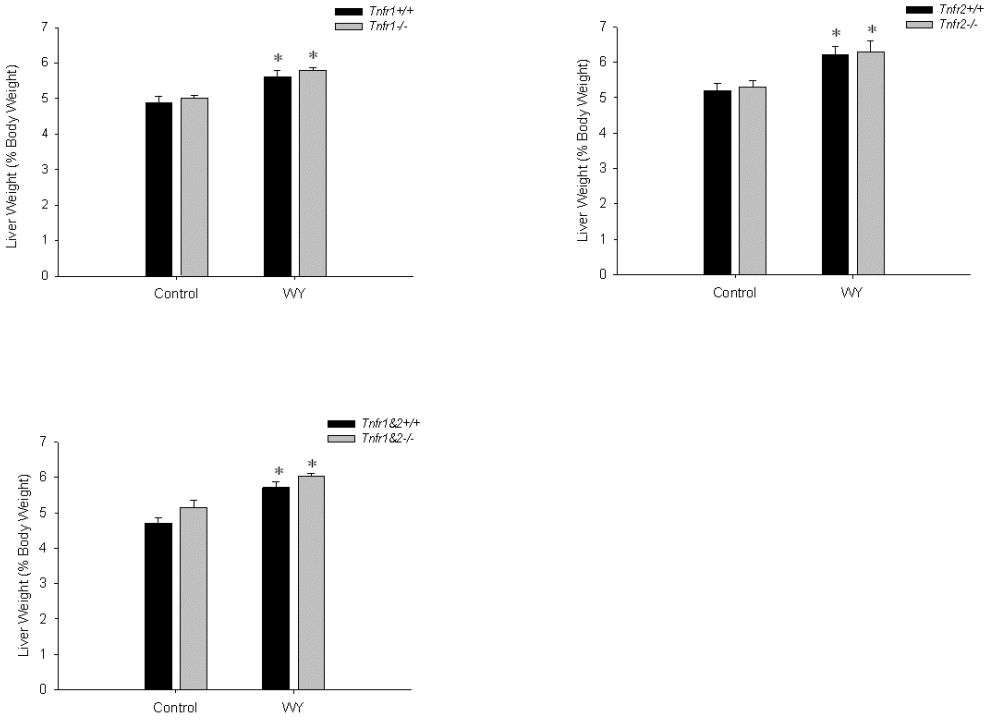
Fig. 3.6. Hepatic cytokine gene expression during hepatocarcinogenesis induced by feeding wild-type mice WY (1000 ppm) for 52 weeks. Analysis was as described in Fig. 2. (A) RT-PCR. (B) Densitometric analysis.

Fig. 3.7. Hepatic transcription factor expression during hepatocarcinogenesis induced by feeding wild-type mice WY (1000 ppm) for 52 weeks. Analysis was as described in Fig. 2. (A) RT-PCR. (B) Densitometric analysis.

Fig. 3.8. Model of PP-induced hepatocellular proliferation and hepatocarcinogenesis.

Figure 3.1

A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Tnfr1-/-
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Control
WY
Tnfr2+/+
Tnfr2-/-
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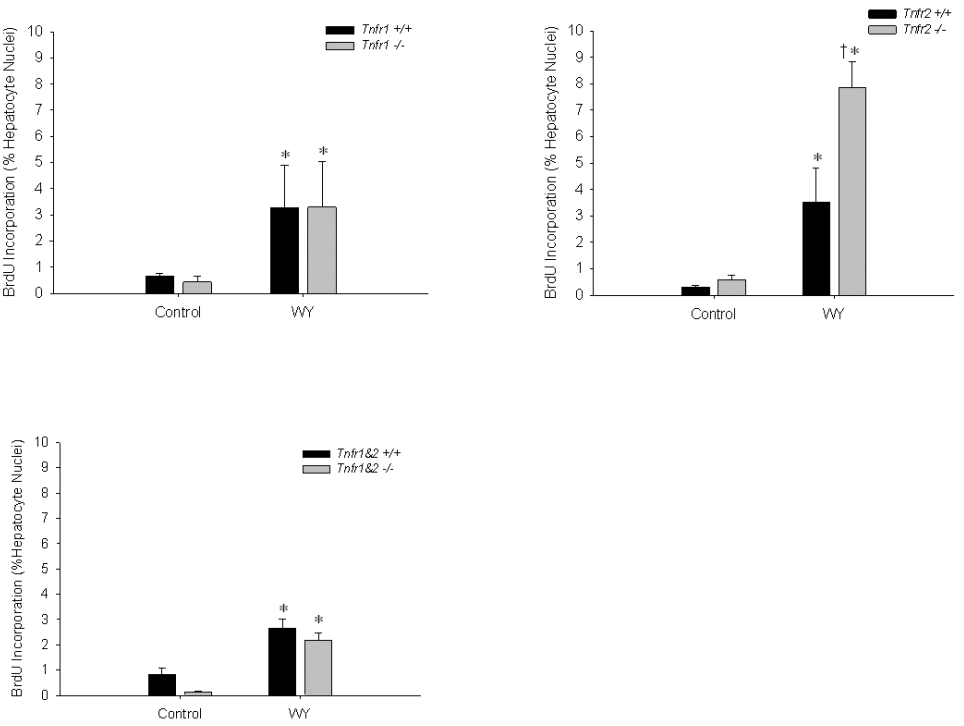
WY
Tnfr1&2+/+
Tnfr1&2-/-
Liver Weight (% Body Weight)
Control
WY

B


Tnfr1 +/+
Tnfr1 -/-
BrdU Incorporation (% Hepatocyte Nuclei)
Control
WY
Tnfr2 +/+
Tnfr2 -/-
BrdU Incorporation (% Hepatocyte Nuclei)
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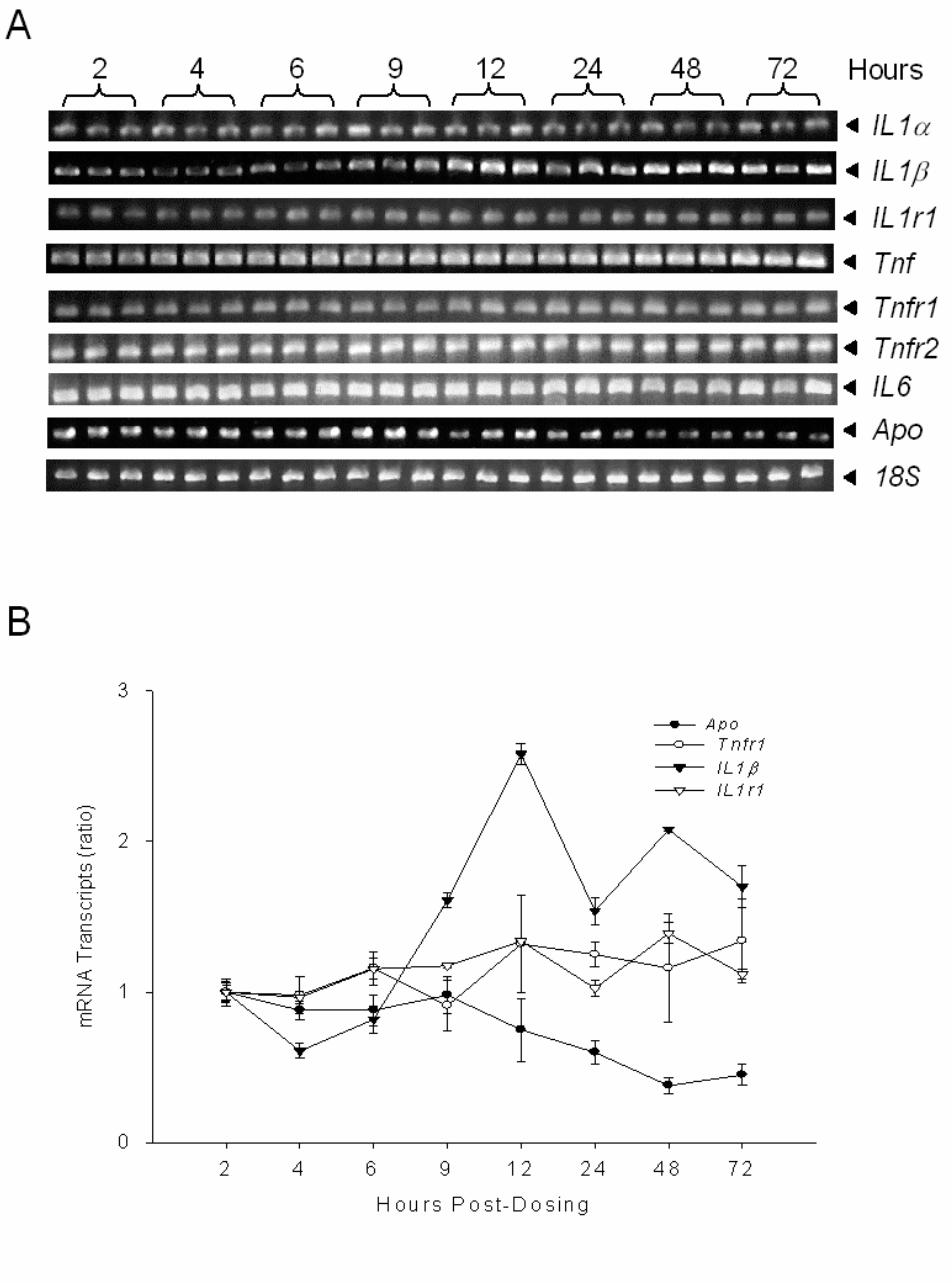
BrdU Incorporation (%Hepatocyte Nuclei)
Control
WY

Figure 3.2


A
2
4
6
9
12
24
48
72
Hours
IL1α
IL1β
IL1r1
Tnf
Tnfr1
Tnfr2
IL6
Apo
18S
B
mRNA Transcripts (ratio)
0
1
2
3
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2
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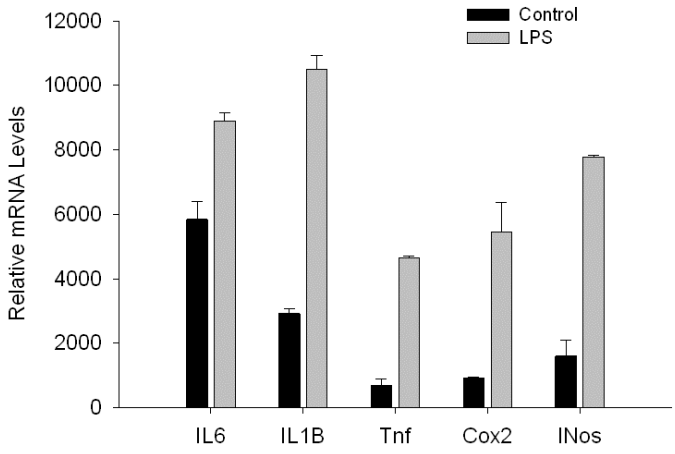
12
24
48
72
Hours Post-Dosing

Figure 3.3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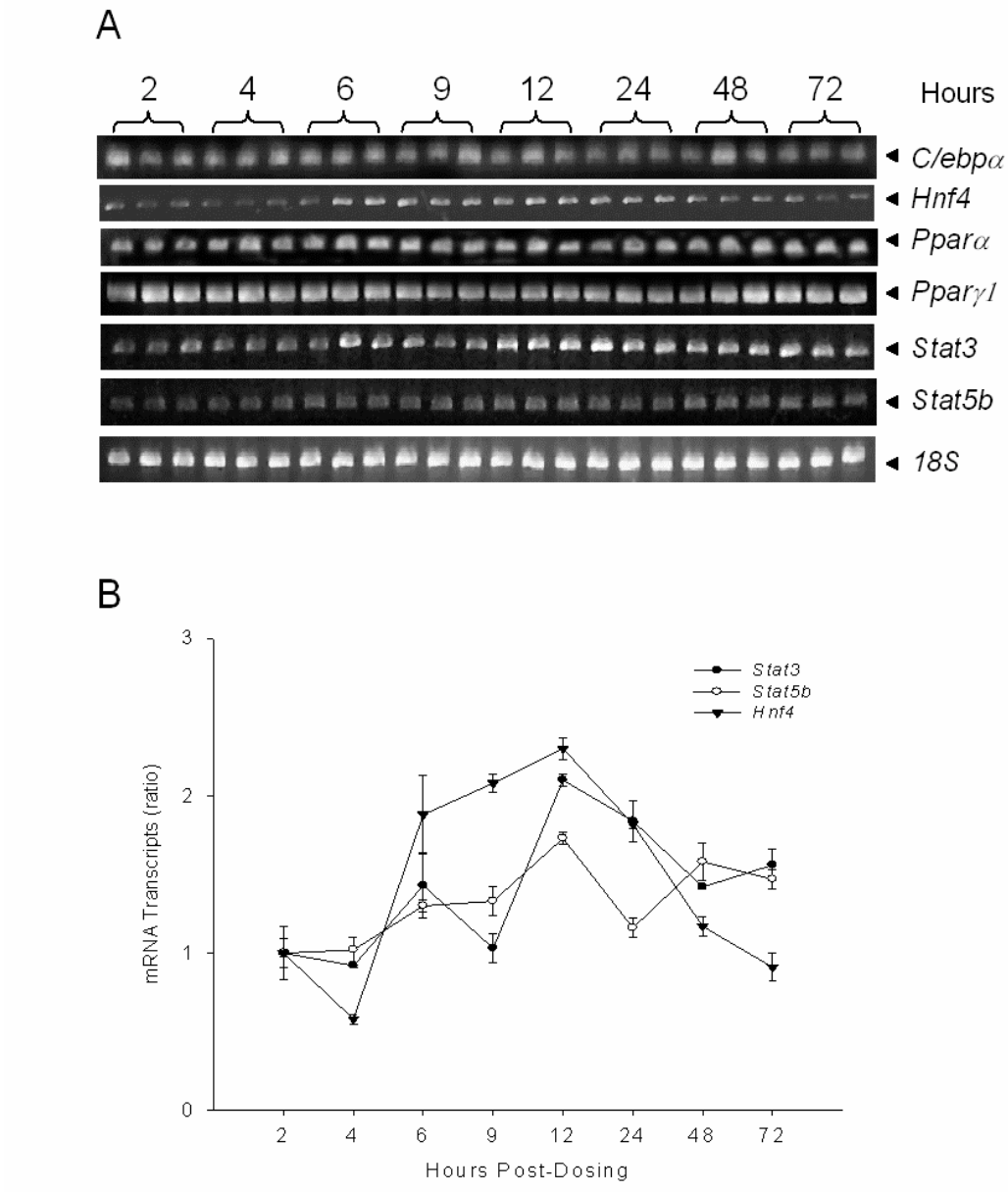
IL6
IL1B
Tnf
Cox2
INos

Figure 3.4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72
Hours
C/ebpα
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Pparα
Pparγ1
Stat3
Stat5b
18S
B
mRNA Transcripts (ratio)
0
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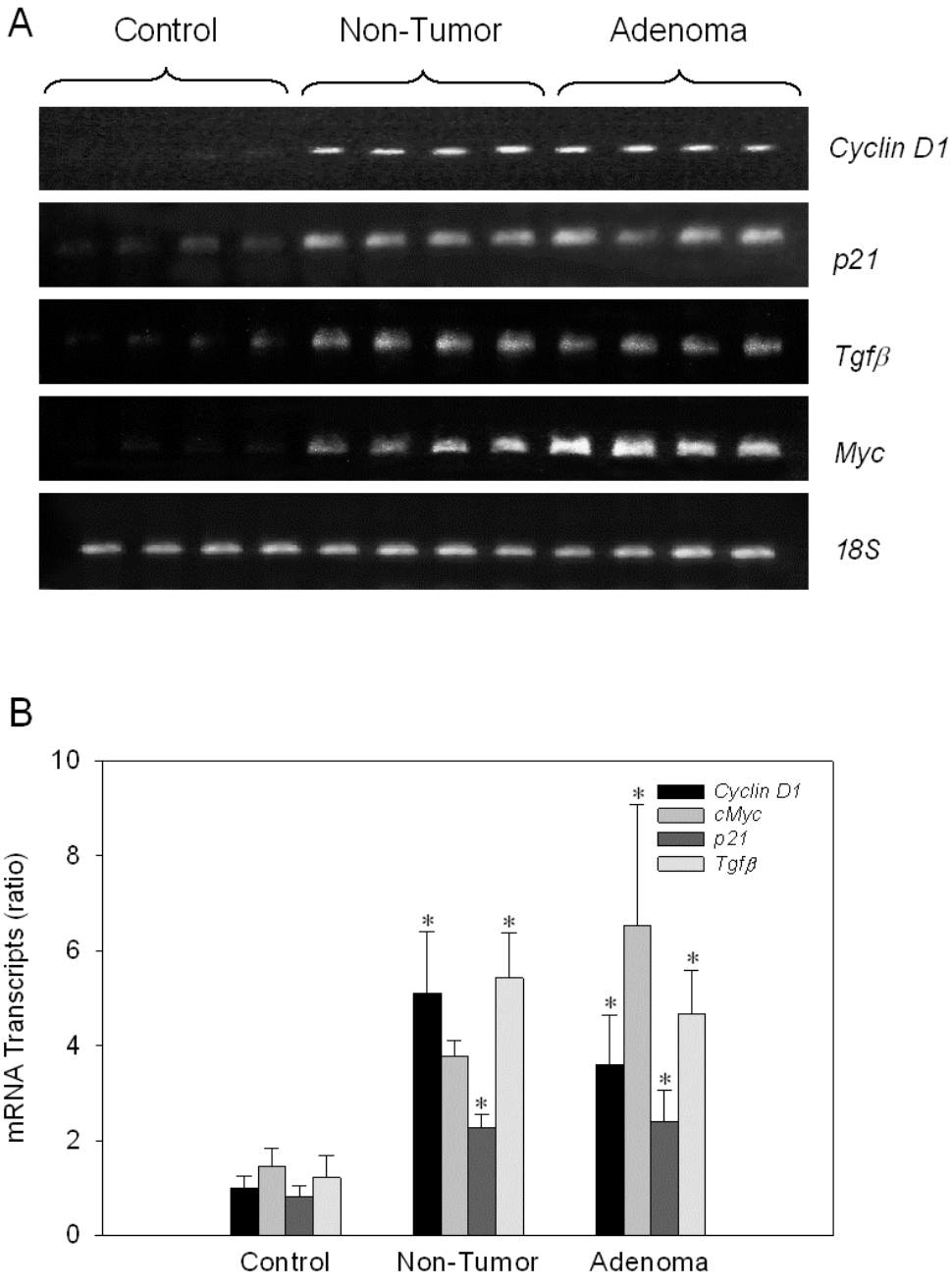
72
Hours Post-Dosing

Figure 3.5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Non-Tumor
Adenoma
Cyclin D1
p21
Tgfβ
Myc
18S
B
mRNA Transcripts (ratio)
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2
0
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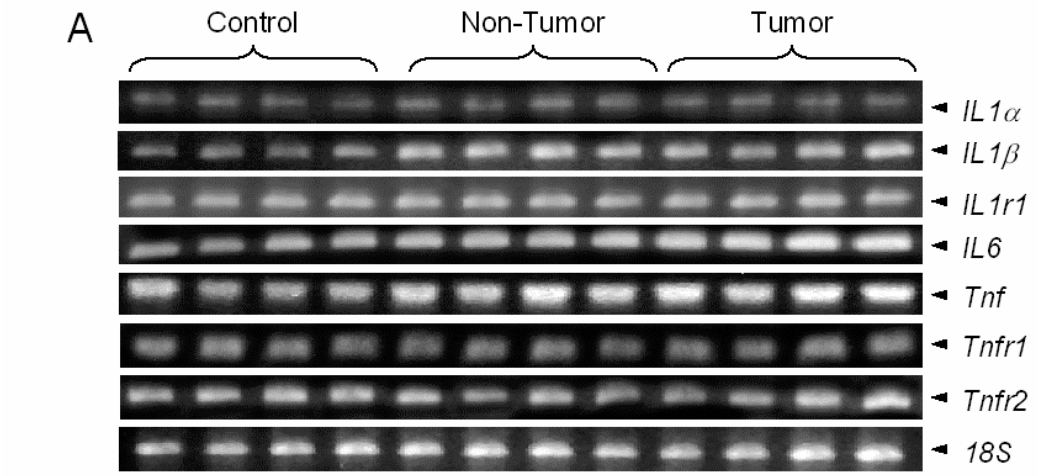
cMyc
p21
Tgfβ
Control
Non-Tumor
Adenoma

Figure 3.6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Tumor
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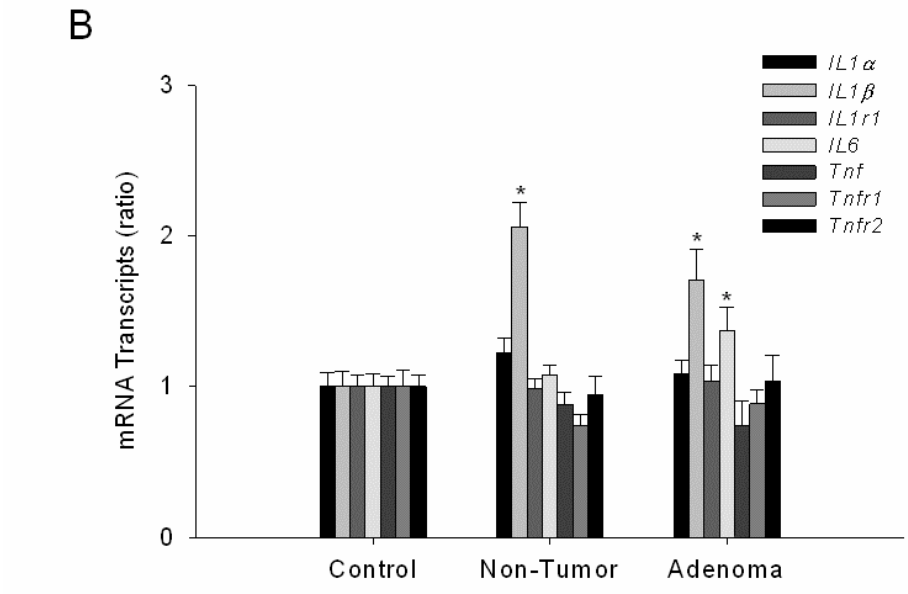
18S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mRNA Transcripts (ratio)
0
1
2
3
Control
Non-Tumor
Adenoma
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IL1β
IL1r1
IL6
Tnf
Tnfr1
Tnfr2
*
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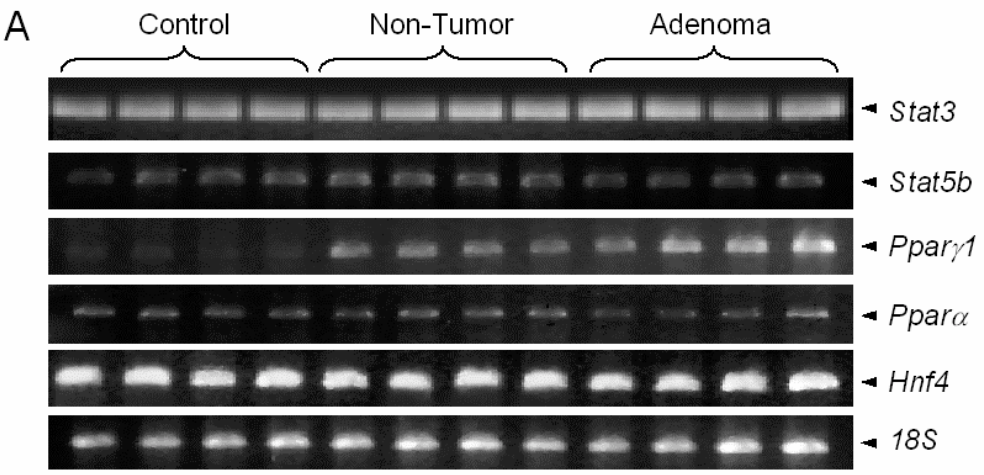
*

Figure 3.7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Control
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Adenoma
Stat3
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Hnf4
18S

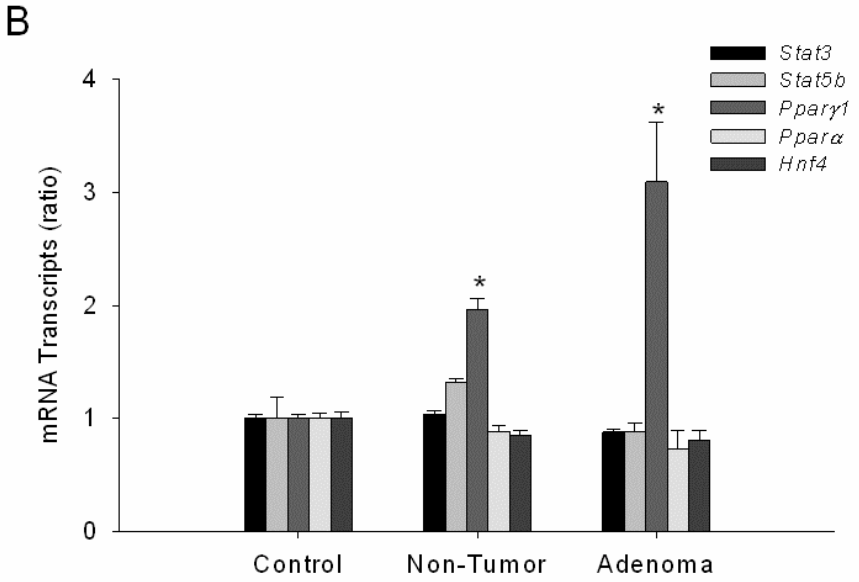
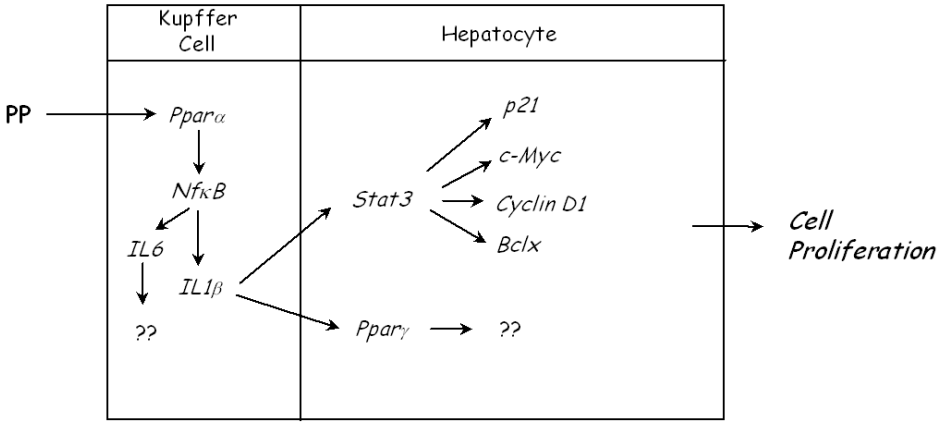

B
mRNA Transcripts (ratio)
0
1
2
3
4
Control
Non-Tumor
Adenoma
Stat3
Stat5b
Pparγ1
Pparα
Hnf4
*
*

Figure 3.8


Kupffer Cell
Hepatocyte
PP
Pparα
NfκB
IL6
IL1β
??
Stat3
p21
c-Myc
Cyclin D1
Bclx
Pparγ
??
Cell Proliferation

Chapter 4

Manuscript III: Delayed Liver Regeneration In Peroxisome Proliferator-Activated Receptor-α-Null Mice.

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Running title: Delayed liver regeneration in Pparα-null mice

Abbreviations

Aco, acyl-CoA oxidase; ANOVA, analysis of variance; Bclx, Bcl2-related gene; BrdU, 2-bromodeoxy-uridine; Ccna, Cyclin A; Ccnb, Cyclin B; Ccnd1, Cyclin D1; Ccnde: Cyclin E; Cdk, cyclin-dependent kinase; cMyc, avian myelocytomatosis viral oncogene; C/ebpβ, CCAAT/enhancer-binding protein beta; Crem, cAmp-responsive promoter element modulator; Cyp4a14, cytochrome P450 IVA14; Erk, extracellular-related kinase; Hgf, hepatocyte growth factor; iNos, inducible nitric oxide synthase; IL1, interleukin 1; IL6, interleukin 6; Mdm2, mouse double minute 2; Nf-κB, nuclear factor kappa B; PH, partial hepatectomy; PP, peroxisome proliferator; Pparα, peroxisome proliferator-activated receptor alpha; RT-PCR, reverse transcriptase-polymerase chain reaction; Stat3, signal transducer and activator of transcription 3; Tgfα, transforming growth factor-alpha; Tnfα, tumor necrosis factor-alpha; Tnfr1, tumor necrosis factor receptor 1; Tnfr2, tumor necrosis factor receptor 2; Ts53, tumor suppressor protein 53; Upa, urokinase-type plasminogen; Wy, Wy-14, 643.

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Abstract

Compared to wild type mice, peroxisome-proliferator-activated receptor α (Pparα)-null mice have a delayed onset of hepatic regeneration and a lower peak DNA synthesis following partial hepatectomy. Delayed DNA synthesis in Pparα-null mice correlated with delayed hepatic expression of the G_1/S checkpoint regulator genes *Ccnd1* and *cMyc.* The hepatocyte mitogen gene *IL1β* and the PH delayed-early gene *Bclx* exhibited increased expression after PH in Pparα-null but not wild-type mice, indicating that alternative pathways of liver growth were employed by the Pparα-null strain. The expression of two receptors involved in cytokine signaling, *IL1 receptor 1* and *IL6 receptor*, as well as *cMyc* and *Ccnd1,* was altered in wild type but not in Pparα-null mice after acute Wy-14,643 (Wy) exposure highlighting differences in hepatocyte growth pathways induced by PH or Wy. Wild type but not Pparα-null mice show a dramatic increase in the expression of the fatty acid ω-hydroxylase gene *Cyp4a14* after PH that may be involved in normalizing the levels of lipids that accumulate in the livers of Pparα-null mice. In conclusion, our results demonstrate that Pparα-null mice have transiently impaired hepatic regeneration following PH that may result from the use of alternative signaling pathways for induction of hepatocyte growth.

Introduction

Peroxisome proliferator (PP) xenobiotics are potent hepatic mitogens and carcinogens in mice and rats (1). Increases in hepatocyte replication and tumor formation after PP exposure require activation of the peroxisome-proliferator-activated receptor-α (Pparα) (2). Many transcriptional targets of Pparα have been identified, but none have direct roles in regulating cell proliferation or apoptosis. One of the most effective models for studying hepatocellular proliferation is liver regeneration following hepatocellular loss due to partial hepatectomy (PH) or chemical damage (3). In the original technique for PH described by Higgins and Anderson in 1931 (4) resection of 70% of the hepatic mass of a rat results in 95% of the remaining hepatocytes rapidly entering the cell cycle. DNA synthesis follows about 12–14 hours after resection and reaches a maximum activation at 24 hours. These events occur about 20 hours later in mice. The original mass of the liver is restored within 7 days, with most of the recovery occurring by 3 days (4). The signals that stimulate and maintain this process are not entirely clear, but include the transcriptional activation of several groups of genes in a distinct temporal order (3,5-7). First, hepatocytes must be primed, presumably by cytokines, to respond to various growth factors. After priming, transition from G_0 to G_1 phases of the cell cycle requires induction of immediate-early class genes, which begins at about 30 minutes post-PH and lasts for about 4 hours. Progression of hepatocytes *in vivo* through late G_1 phase requires growth factors such as transforming growth factor- α (*Tgf* α), hepatocyte growth factor (*Hgf*), urokinase-type plasminogen activator (*uPa*) and several protooncogenes, including *cFos*, *cJun*, and *cMyc*. The delayed-early phase begins at 4 to 8 hours, when there is upregulation of additional genes, including the primary antiapoptotic gene in the liver, *Bclx*. Induction of cell-cycle control genes such as

Ts53, *p21*, and *Mdm2* follows, beginning between 8 and 20 hours. Between 20 and 48 hours, there is a transition through late G_1, and DNA replication and mitosis genes are induced, including the cyclins *Ccna*, *Ccnb*, *Ccnd1, Ccnce*, and *Ras*. A number of genes involved in lipogenesis are also induced.

Speculation that induction of several members of each gene class might be attributed to the activation of only a limited number of transcription factors or receptors led to the generation of strains of mice with targeted deletions in those genes (Table I). Investigators subsequently assessed the regenerative capacity of the liver following PH in these knockout mice. Regeneration is faulty in at least 6 strains of knockout mice: tumor necrosis factor-α receptor 1 (*Tnfr1*), interleukin 6 (*IL6*), inducible nitric oxide synthase (*iNOS*), urokinase plasminogen activator (*Upa)*, cAMP-responsive promoter element modulator (*Crem*), and CCAAT/enhancer-binding protein β (*C/ebpβ*) (7). Because liver regeneration is an enormously complex process requiring participation of multiple, integrated, and perhaps overlapping, genetic control circuits, additional critical regulators of this process seem likely.

Among the many unexamined candidate regulators of liver regeneration is the gene encoding Pparα. Direct evidence that Pparα is critical for liver regeneration has not been reported. In this study, we test the hypothesis that Pparα is necessary for liver regeneration *in vivo* by studying hepatic regeneration after 70% PH in wild-type and Pparα-null mice. To better understand molecular differences between the two strains, we also examined the relative mRNA abundances for several genes with known or suspected correlation with the hepatocellular proliferation that follows PP exposure. We conclude that Pparα-null mice subjected to PH have an impaired ability to regenerate

hepatic mass that may result from the use of alternative signaling pathways for induction of liver growth.

Materials and methods

Partial hepatectomy and acute Wy-14,643 treatment of mice

These studies were conducted under federal guidelines for the use and care of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the CIIT Centers for Health Research. Control and treated mice (*n* = 5–6/group) were provided with NIH-07 rodent chow (Zeigler Brothers; Gardner, PA) and deionized, filtered water ad libitum. Lighting was on a 12-h light-dark cycle. All animals were acclimated for at least 10 days before initiation of the study. In the first study, 12-week-old male Pparα-null mice on a SV129 background and Sv129 wild-type mice from the Pparα mouse colony at CIIT were subjected to PH according to a slightly modified method of Higgins and Anderson (4). Left lateral and median lobes and the gallbladders were completely excised. In the second study, 12-14 week old mice were given a single gavage dose (50 mg/kg) of WY-14,643 (Wy) (ChemSyn Science Laboratories; Lenexa, KS). At the designated time after treatment, animals were deeply anesthetized with isofluorane anesthesia or pentobarbital injection and killed by exsanguination. The tissues were removed, snap-frozen in liquid nitrogen, and stored at –70°C until analysis.

Evaluation of liver regeneration

Liver mass regenerated. At the time of PH, the resected liver was weighed, and the initial liver weight for each mouse was extrapolated based on the assumption that the resected liver = 0.70 $\times$ total liver weight (8). When mice were sacrificed after PH, the

remnant livers were weighed. For each mouse, the weight of the remnant liver was divided by the initial liver weight of that animal and normalized to total body weight to derive the percentage of liver weight that had been reconstituted after PH (8). Significant differences were identified by multiple independent Student's *t*-tests at each time point, comparing wild-type to *Ppar*α-null$^-$ mice ($P < 0.05$).

BrdU labeling

To evaluate hepatocyte DNA synthesis, 2-bromodeoxy-uridine (BrdU) (30 μg/g body weight) was administered via an intraperitoneal injection 2 hours before sacrifice. Remnant liver was harvested at 1, 2, 3, and 7 days post-PH, fixed, sectioned, and stained with antibodies to BrdU. The hepatocytes were analyzed using the object recognition system (CHRIS), (Sverdrup Medical/Life Sciences Imaging Systems, Fort Walton Beach, Florida) using a Nikon Microphot™ microscope with a Dage CCD color video camera at a magnification of 700 ×. At least 1000 cells were counted for each animal. The labeling index was calculated by dividing the number of labeled hepatocyte nuclei by the total number of hepatocyte nuclei counted, and the results were expressed as a percentage. Significant differences were identified using a one-way analysis of variance (ANOVA), followed by post-hoc testing using the Tukey-Kramer procedure ($P < 0.05$).

Gene expression analysis

Relative quantitation of mRNA levels for the genes listed in Table II was assessed via real-time quantitative polymerase chain reaction (RT-PCR) (TaqMan™) according to manufacturer's recommendations using AmpliTaq Gold™ and the ABI PRISM® 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Briefly, following DNAse I (Ambion, Inc., Austin, TX) treatment and Ribogreen™ (Molecular Probes, Inc., Eugene, OR) quantification, 50 ng of total RNA was transcribed

to first-strand cDNA and then amplified by polymerase chain reaction in the same tube using oligonucleotides (Table 4.2) selected by the ABI PrimerExpress™ software and manufactured by Biosource International (Foster City, CA). Hepatic RNA from 3 separate animals in each group was analyzed at least 3 times. After optimization for each target gene, expression (by the comparative method) was normalized by subtracting the corresponding C_T value of the ribosomal 18S gene. These differences were averaged in each treatment group and the fold-change, relative to control mice, was calculated as recommended (9).

Results

Assessment of regeneration and morphology in Pparα-null mice following partial hepatectomy

To test the hypothesis that *Pparα* is required for liver regeneration *in vivo*, we induced regeneration by performing 70% PH on male wild-type and *Pparα*-null mice. Groups of mice were killed 1, 2, 3, and 7 days after hepatectomy, and sham-operated mice were killed 2 and 7 days after laparotomy. Evaluation of hematoxylin and eosin-stained liver sections provided morphologic data. Regeneration was assessed by ratios of liver weight to body weight and DNA synthesis, as measured by BrdU incorporation into hepatocyte nuclei.

Prior to PH, *Pparα*-null mice had ~20% larger livers than did wild-type mice. At 1 day post-PH, the livers of wild-type mice had regenerated 87% (±4.0) of the original weight before PH, while the livers of *Pparα*-null mice had only attained 70% (±4.2) of their original weight (Figure 4.1A). Two days after PH, wild-type mice showed no increase in hepatic mass (85% ±2.9), but the livers of the *Pparα*-null mice increased 10% (79%

±3.3). By 3 days post-PH, wild-type mice had totally regained their original hepatic mass (102.0% ±5.4), while *Pparα*-null mice at this time point showed no gain in liver weight (80% ±3.4). *Pparα*-null mice regained their original hepatic mass by 7 days post-PH (104.0% ±0.9). Sham operation did not significantly affect relative liver mass in either genotype (Figure 4.1A, inset). In both genotypes, liver mass values exceeding 100% are attributed to post-surgical inappetance resulting in decreased body weights.

DNA synthesis was significantly increased at 2 and 3 days post-PH for both wild-type (9.38±1.51 and 5.20±0.74, respectively) and *Pparα*-null mice (5.62±1.37 and 6.29±0.72, respectively) (Figure 4.1B) when compared to their respective controls, but DNA synthesis at 2 days in *Pparα*-null mice was significantly lower (~40% reduction) than in wild-type mice. Maximum DNA synthesis occurred in Pparα-null mice between days 2 and 3 post-PH. Sham PH at 2 or 7 days did not influence DNA synthesis. These results suggest that in *Pparα*-null mice there is a lag in DNA synthesis following PH. Taken as a whole, these results demonstrate that Pparα-null mice are impeded in the timing of full liver regeneration following PH.

Morphologic assessment of the livers of both wild-type and *Pparα*-null mice revealed that vacuolation of hepatocytes, presumably due to lipid accumulation, was present in non-operated and sham-operated *Pparα*-null mice, but not in control and sham-operated wild-type mice (Figure 2). *Pparα*-null mice had increased vacuolation at 1 and 2 days post-PH that returned to levels of the non-operated group by 3 days post-PH. In wild-type mice, increased vacuolation was noted at 1, 2, and 3 days post-PH but returned to levels of the non-operated group by 7 days post-PH. However, the vacuolation in wild-type mice was never higher than the baseline levels in the *Pparα*-null mice. Discrete,

clear foci of hepatocellular cytoplasm, presumably glycogen, were present in non-operated mice.

Expression of genes involved in G_0 to G_1 transition

Several cytokine signaling networks stimulate resting (G_0) hepatocytes to reenter the cell cycle (G_1 phase) following loss of hepatic mass (6). Efficient priming apparently requires induction or activation of several genes, including tumor necrosis factor-α (*Tnf* α), interleukin 6 (*IL6*), interleukin 1 (*IL1*), signal transducer and activator of transcription 3 (*Stat3*), CCAAT/enhancer-binding protein beta (*C/ebpβ*), and nuclear factor kappa-B (*Nf-κB*). We examined mRNA levels of representative genes from these pathways in our studies. Because we were particularly interested in identifying a subset of these genes whose expression is influenced by *Pparα* and linked to PP-induced hepatocyte replication, we also examined hepatic expression in wild-type and *Pparα*-null mice 12 hours after a single gavage dose (50 mg/kg) of the potent Pparα agonist Wy-14,643 (Wy). Using validated oligonucleotides (Jay Strum, personal communication) we were unable to detect *Tnfα* mRNA in either wild-type or *Pparα*-null mice at any of the time points following PH or Wy gavage (data not shown). Prior to PH, *Pparα*-null mice had lower levels of *IL1β* (60% decrease), *IL6r* (25% decrease), and *Stat3* (25% decrease) compared to wild-type mice (Figure 4.3). *IL6r* and *Stat3* were also expressed at lower levels in *Pparα*-null mice prior to Wy gavage (right panels) whereas *IL1β* expression was not significantly different. Differences in the baseline levels of cytokine pathway components between the studies could be partially due to the gavage dosing regimen in the Wy study that likely induces stress responses and alters cytokine signaling pathways (Corton et al., manuscript in preparation). Following PH, *IL1β* expression did not change in wild-type mice but was increased in *Pparα*-null mice at day

1 (~3-fold) and day 2 (~4-fold) post-PH. *IL1r1* was increased ~3.5-fold at day 1 post-PH and remained elevated at day 2 in both genotypes of mice. In the acute Wy study, *IL1β* levels were not affected in either genotype after Wy gavage. *IL1r1* increased in wild-type but not *Pparα*-null mice in response to Wy.

At 1 day post-PH, *IL6* increased ~2.3- to 3-fold in both genotypes of mice and continued to increase at day 2 post-PH. Comparable increases in *IL6* were also induced by sham PH. *IL6* was not altered after Wy exposure in either strain of mice. Both strains exhibited a decline in expression of *IL6r* at 1 and 2 days post-PH. *IL6r* was also decreased after a sham PH in wild-type but not *Pparα*-null mice. Wy decreased *IL6r* expression in wild-type mice by ~75% whereas expression was not significantly affected by Wy in the *Pparα*-null mice.

Stat3 was mildly induced (~1.4- to 2-fold) at days 1 and 2 after PH in both strains of mice. *Stat3* was down-regulated in wild-type but not Pparα-null mice after sham PH. *Stat3* was decreased (50–60%) after Wy treatment in both wild-type and Pparα-null mice. Taken together, our results indicate that the basal and post-PH levels of a number of factors important in priming hepatocyte replication are different between wild-type and *Pparα*-null mice. In addition, there were clear differences between the cytokine signaling genes altered by PH and by the mitogen Wy that likely reflect differences in pathways utilized for hepatocyte growth.

Expression of genes involved in progression through G_1

The lag in DNA synthesis in Pparα-null mice suggested possible alterations in the G_1/S checkpoint of the cell cycle. Therefore, we examined the relative mRNA levels of

several genes that are typically induced prior to and during this phase. *Ccnd1*, which was recently identified as the most reliable marker for cell cycle (G_1 phase) progression in hepatocytes (10) was induced ~2.5-fold at 1 day post-PH and ~4.5-fold at 2 days relative to expression in animals that were not hepatectomized (Figure 4.4). Compared to wild-type mice, *Pparα*-null mice had a ~4-fold reduction in *Ccnd1* before PH, ~3.3-fold less at 24 hours, and no reduction at 48 hours. In the acute Wy study, untreated Pparα-null mice had a ~50% reduction in *Ccnd1* compared to untreated wild-type mice. Hepatic *Ccnd1* was increased in wild-type, but not Pparα-null mice after Wy gavage. *Bclx,* which is a delayed-early gene expressed post-PH (11) and also a target for PP during carcinogenesis (12), exhibited decreased expression in control Pparα-null mice relative to wild-type mice. *Bclx* was induced 1 day after PH in *Pparα*-null mice only. Sham PH decreased the level of *Bclx* in wild-type but not Pparα-null mice. *cMyc* levels were similar in wild-type and *Pparα*-null mice prior to PH, and PH induced expression in both genotypes. However, the peak of expression in wild-type mice was at day 1 post-PH, while in *Pparα*-null mice the peak occurred at day 2. Furthermore, Wy treatment induced *cMyc* in wild-type (~2.5-fold), but not *Pparα*-null mice. Consistent with the results of other investigators (10,13,14), we did not observe any change in expression of $p27^{kip1}$ (the main inhibitor of G_1/S progression in the liver) in either genotype at any time point following PH or Wy treatment (data not shown). Expression of *Pparα*, hepatocyte growth factor (*Hgf*), and *uPa* was unchanged at all time points post-PH (data not shown). These data suggest that hepatic mRNA expression of *Ccnd1* and *cMyc,* important stimulators of DNA synthesis and G_1/S transition in the liver is regulated in part by Pparα .

Expression of marker genes for peroxisome proliferator chemicals

As positive controls for the Wy-treated animals, we examined the expression of two genes commonly used as markers for peroxisome proliferator exposure (1). Prior to PH or Wy treatment, Pparα-null mice showed greatly reduced (*Aco*, 50% reduction) or no expression (*Cyp4a14*) of these genes (Figure 4.5). After PH, *Aco* levels did not change significantly in either genotype of mouse, but *Cyp4a14* was greatly induced at day 1 (~8.5-fold) and day 2 (~4.5-fold) in wild-type mice. In contrast, *Cyp4a14* was not detected in the Pparα-null mice at any time point. As expected in the acute Wy study, *Aco* and *Cyp4a14* were markedly upregulated in wild-type, but not *Pparα*-null mice. These results indicate that a subset of *Pparα*-regulated genes involved in fatty acid metabolism exhibit altered expression after PH.

Discussion

Pparα–null mice are transiently impaired in liver regeneration following PH. The impairment is moderate, and is accompanied by a delayed onset of peak DNA synthesis and several changes in gene transcription. Other genetically modified mice, including those carrying homozygous deletions for *uPa* (16), *iNos* (8), *Crem* (17), *Tnfr1* (18), *IL6* (20), and *C/ebpβ* (21), have been previously tested for hepatic regenerative capacity. In these models, DNA synthesis was decreased in mice lacking *IL6*, *uPa*, *C/ebpβ*, *Tnfr1*, *iNos*, and *Crem*, but not in mice lacking*Tnfr2*. Regeneration, measured by relative liver mass restitution, was decreased in *Tnfr1*- and *IL6*-null mice, but not in mice lacking *C/ebpβ*, *Tnfr2*, or *iNos*. This index was not measured in mice lacking *Crem*. Thus, mice nullizygous for *IL6*, *Tnfr1*, and *Pparα* are the only models that have both decreased restitution of mass and decreased or delayed DNA synthesis.

The mechanism underlying the impaired regeneration in *Pparα*-null mice is unknown. Altered transcription of genes stimulating hepatocytes to enter G_1 in other nullizygous mice subjected to PH has been reported (Table I). In our investigations, we observed increased (*IL1β*, *IL1r1, IL6,* and *Stat3*) or decreased (*IL6r*) expression after PH that was not appreciably different between wild-type and Pparα-null strains. In contrast, *IL1β* expression increased after PH in Pparα-null mice only, raising the possibility that these mice use an alternate pathway to prime hepatocytes for cell cycle entry. *IL1β*, along with *IL1α* and the IL1-receptor accessory protein (*IL1ra*), elicit their effects through the *IL1r1*. In hepatocytes, the consequence of IL1r1 activation is contradictory. West *et al* found that IL1β protein directly induced DNA synthesis and suppressed apoptosis in cultured mouse primary hepatocytes, and that DNA synthesis in response to PP was completely abrogated after exposing the cells to an antibody specific for IL1r1 (22). However, other investigators found that IL1α and IL1β have antiproliferative effects on rat hepatocytes *in vitro* (23). Furthermore, homozygous deletion of *IL1β* has no apparent effect on normal hepatic growth in mice (24). Resolving of the role of IL1 in PP-induced hepatocyte proliferation will require assessing cell proliferation in *IL1r1*-null mice in response to PH or PP exposure.

The G_1/S transition in hepatocytes is associated with induction of several cyclins, cyclin-dependent kinases (Cdks), and protooncogenes, but the critical drivers appear to be *Ccnd1* and *cMyc*. *Ccnd1* activates several S-phase genes, including *cMyc*. *cMyc*, in turn, serves as part of a negative feedback loop that functions to inhibit *Ccnd1* expression (10). Signals initiated by estrogen, epidermal growth factor and thyrotropin all converge on and activate Ccnd1/Cdk (3,5,6), thereby stimulating proliferation of the

target cell (25). We found that maximum expression of *Ccnd1* and *cMyc* in *Pparα*-null mice exhibited a ~1 day delay compared to wild-type mice. Similar to the reports of other investigators (26), we observed no difference in mRNA levels of the primary inhibitor of G_1/S transition in liver, $p27^{kip1}$, between wild-type and Pparα-null mice. Also consistent with previous reports is our finding that *Ccnd1* and *cMyc* (26), but not $p27^{kip1}$ (not previously reported), are regulated at least in part by *Pparα*, since both genes are induced in wild-type, but not *Pparα*-null mice after Wy exposure. These findings suggest that defects in Pparα-dependent signaling can lead to changes in the expression of G_1/S transition regulators that correlate with defects in hepatocyte proliferation.

Although *Pparα* is a common factor whose functional status affects hepatocyte proliferation induced by PH or a PP, the gene network requirements for hepatocyte proliferation induced by these manipulations are distinctly different. Some of the same nullizygous mice listed in Table 4.1 have been useful in elucidating target genes required for hepatocyte proliferation following PH. Neither IL6 (27), Tnf (28), Tnfr1, Tnfr2, nor a strain nullizygous for both Tnf receptors (Anderson *et al*., in press), exhibit significant differences in PP-induced hepatocyte proliferation compared to wild-type mice. Currently, only mice nullizygous for NADPH oxidase (29) or transgenic mice overexpressing catalase (30) are resistant to cell proliferation after PP exposure. These latter studies provide evidence that the hepatocyte proliferation induced by PP may result from activation of Nf-κB-regulated mitogenic cytokines in response to increased local concentration of reactive oxygen species (ROS)(29). Indeed, suppressing proinflammatory cytokines by pretreating wild-type mice with dexamethasone abolishes PP-induced hepatocyte proliferation (31).

In a broader sense, *Pparα*-null mice respond adversely to a number of inducers of hepatocyte damage and compensatory growth. For example, they are more sensitive to the liver toxicants acetaminophen (32), trichloroethylene (Corton *et al*., unpublished) and carbon tetrachloride (Corton *et al*., manuscript in preparation). Moreover, wild-type mice pretreated with a PP are resistant to several other hepatotoxicants (summarized in ref 33). As toxic liver damage is determined by both the extent of cellular injury, and also the degree of repair by regenerating hepatocytes (34), the molecular basis for the differences in sensitivity could be due to defects in signals initiating hepatocyte regeneration. We report that basal expression of key regulators of hepatocyte proliferation or cell fate including *IL1β, IL6r, Stat3, Ccnd1* and *Bclx* was down-regulated in Pparα-null mice compared to wild-type mice in the absence of any treatment. Expression of several of these genes (*IL6r, Stat3,* and *Ccnd1*), along with *IL1r1* and *cMyc*, was altered after exposure to Wy. With the exception of *Stat3*, altered expression of these genes was dependent on *Pparα*. Taken together, our results indicate that *Pparα* plays a role in regulating genes important in hepatocyte proliferation induced by multiple stimuli. Moreover, the livers of *Pparα*-null mice appear more sensitive to hepatotoxicants due to defects in pathways important for hepatocyte growth.

Hepatic expression of the peroxisome proliferator marker genes *Aco* and *Cyp4a14* was strongly induced in wild-type but not in Pparα mice by Wy. Surprisingly, *Cyp4a14*, but not *Aco*, was also strongly induced after PH of wild-type mice only. There have been reports of induction of lipogenic enzymes in the liver after PH (35), but to our knowledge this is the first report of induction of a gene involved in the oxidation of fatty acids. Cyp4a isoforms are increased in rodent livers and kidneys by peroxisome proliferators, physiologic states such as diabetes and starvation, and inflammation and infection

(36,37). In addition to their roles in the metabolism of fatty acids and prostaglandins, Cyp4a enzymes catalyze the synthesis of eicosanoids. Most notably, Cyp4a enzymes catalyze the ω- and ω1- hydroxylation of fatty acids, including arachadonic acid, as well as arachadonic acid epoxidations. Although fatty acid ω-hydroxylation is normally a minor pathway of hepatic fatty acid metabolism relative to mitochondrial β-oxidation, it becomes increasingly important during periods of increased delivery of fatty acids to the liver, such as in uncontrolled diabetes mellitus, starvation, or in clinical conditions such as Reye's syndrome and alcoholic liver disease where mitochondrial β-oxidation is severely impaired (38-40). Hepatocytes often accumulate small amounts intracytoplasmic prior to the major peak of DNA synthesis during liver regeneration (35). Therefore, it is reasonable to expect that, in addition to *Cyp4a14,* there may be altered regulation of other lipid metabolizing genes by Pparα. The goal of this strategy might be to normalize the lipid accumulation in hepatocytes before commencing DNA synthesis.

In summary, we provide evidence that liver regeneration in *Pparα*-null mice is transiently impaired following PH. Furthermore, differences in the timing or level of expression of genes involved in priming the hepatocytes for entry into the cell cycle or that regulate the G_1/S checkpoint may explain differences in growth responses between wild-type and Pparα-null mice.

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Figure Legends

FIGURE 4.1. — Liver regeneration is inhibited in *Ppar*α-null mice. (A) Liver weights normalized to body weights and calibrated to control liver weights were used as a measure of liver regeneration. Results shown are the means ± S.E. of data from 3–5 different mice/group per time point. Significant differences (†) between wild-type and *Ppar*α-null mice at each time point were ascertained using multiple independent t-tests ($P < 0.05$). (B) The average number of BrdU-labeled hepatocytes was determined for each mouse by counting the number of positive hepatocytes in 10 different fields. Samples from 3–5 different mice/group were evaluated at each time point. Results shown are the means ± S.E. of the averages from 3–5 mice/group per time point and are expressed as percentage nuclei labeled. Significant differences (*) between mice of the same strain before PH and after PH or sham operation were obtained using one-way ANOVA and posthoc testing by the method of Tukey-Kramer ($P < 0.05$). Significant differences (†) between wild-type and *Ppar*α-null mice at each time point were ascertained using multiple independent t-tests ($P < 0.05$).

FIGURE 4. 2.— Morphologic assessment of livers from wild-type and *Ppar*α-null mice 2 days after partial hepatectomy. Photomicrographs of hematoxylin and eosin stained paraffin sections of the liver are shown. Arrows point to vacuoles, presumably lipid, present within hepatocytes. Magnification bars = 50 μm. (A) *Ppar*α-null, 2 days after partial hepatectomy. (B) *Ppar*α-null, 2 days after sham operation. (C) Wild-type, 2 days after partial hepatectomy. (D) Wild-type, 2 days after sham operation.

FIGURE 4.3.— Expression of genes involved in regulating the G_0 to G_1 transition in hepatocytes. Relative quantification of mRNA was assessed via real-time quantitative polymerase chain reaction (RT-PCR) (TaqMan™). DNAse I-treated hepatic total RNA from 3 separate animals in each group was analyzed at least 3 times. After optimization for each target gene, expression was normalized by adjusting for the expression of the ribosomal 18S gene. Fold-change reported is the mean percentage control ± S.E. Post-hoc testing for significance ($P < 0.05$) was by Dunnett's test using the untreated, wild-type animals as controls. Wild-type mice are represented by black bars; *Ppar*α-null mice by gray bars. Left panels: hepatic gene expression at multiple time points following partial hepatectomy or laparotomy (sham) of wild-type and *Ppar*α-null mice. Right panels: hepatic gene expression 12 hours after a single gavage dose of Wy (50 mg/kg body weight) to wild-type and *Ppar*α-null mice. Significant differences between wild-type and *Ppar*α-null mice (†) or between mice before PH and after PH or sham operation within the same strain (*) are shown.

Figure 4.4. — Expression of genes involved in passage through late G_1 in hepatocytes. Gene expression was assessed as in FIGURE 4.3.

FIGURE 4.5.— Expression of marker genes for peroxisome proliferator xenobiotics. Gene expression was assessed as in FIGURE 4.3.

Figure 4.1

A

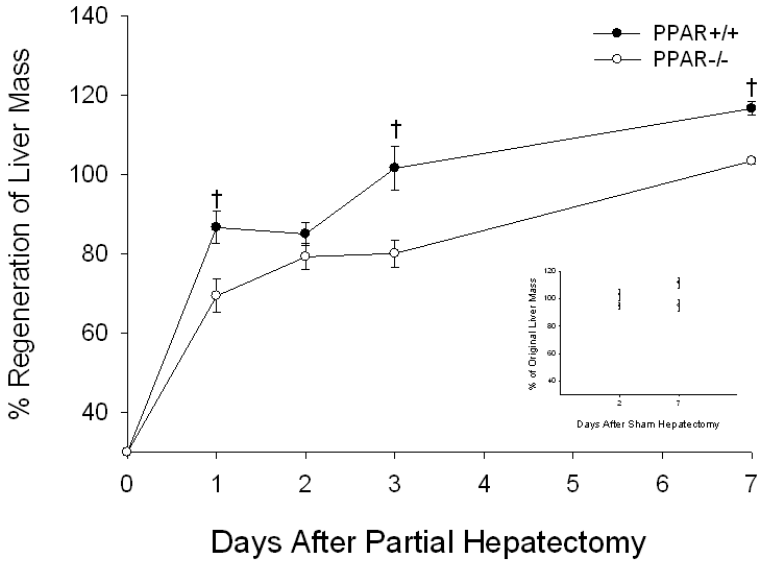
% Regeneration of Liver Mass
140
120
100
80
60
40
0
1
2
3
4
5
6
7
Days After Partial Hepatectomy
PPAR+/+
PPAR-/-
†
% of Original Liver Mass
Days After Sham Hepatectomy

Figure 4.1(cont.)

B

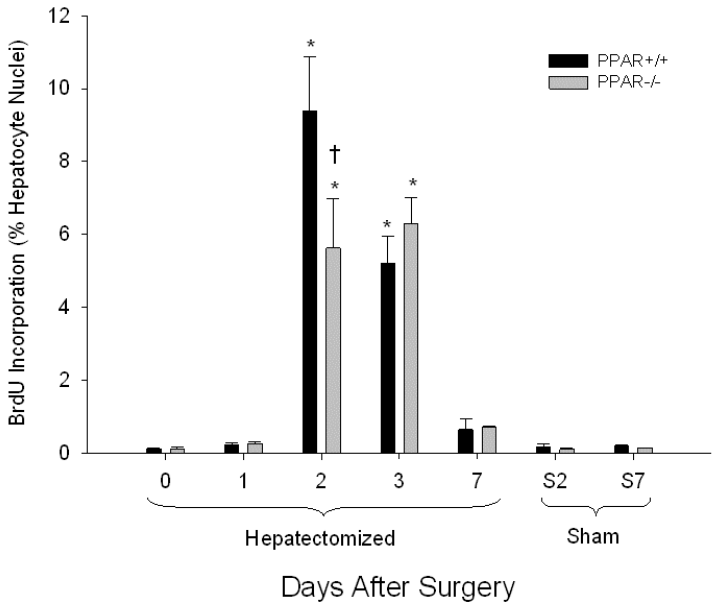

Figure 4.2

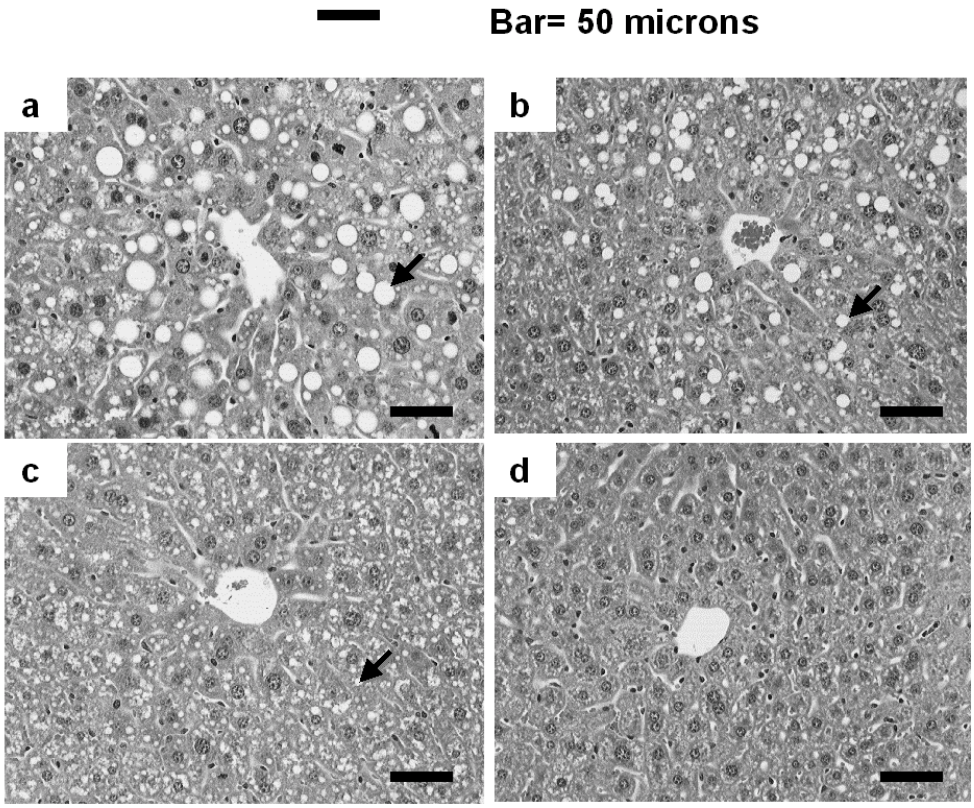

Figure 4.3

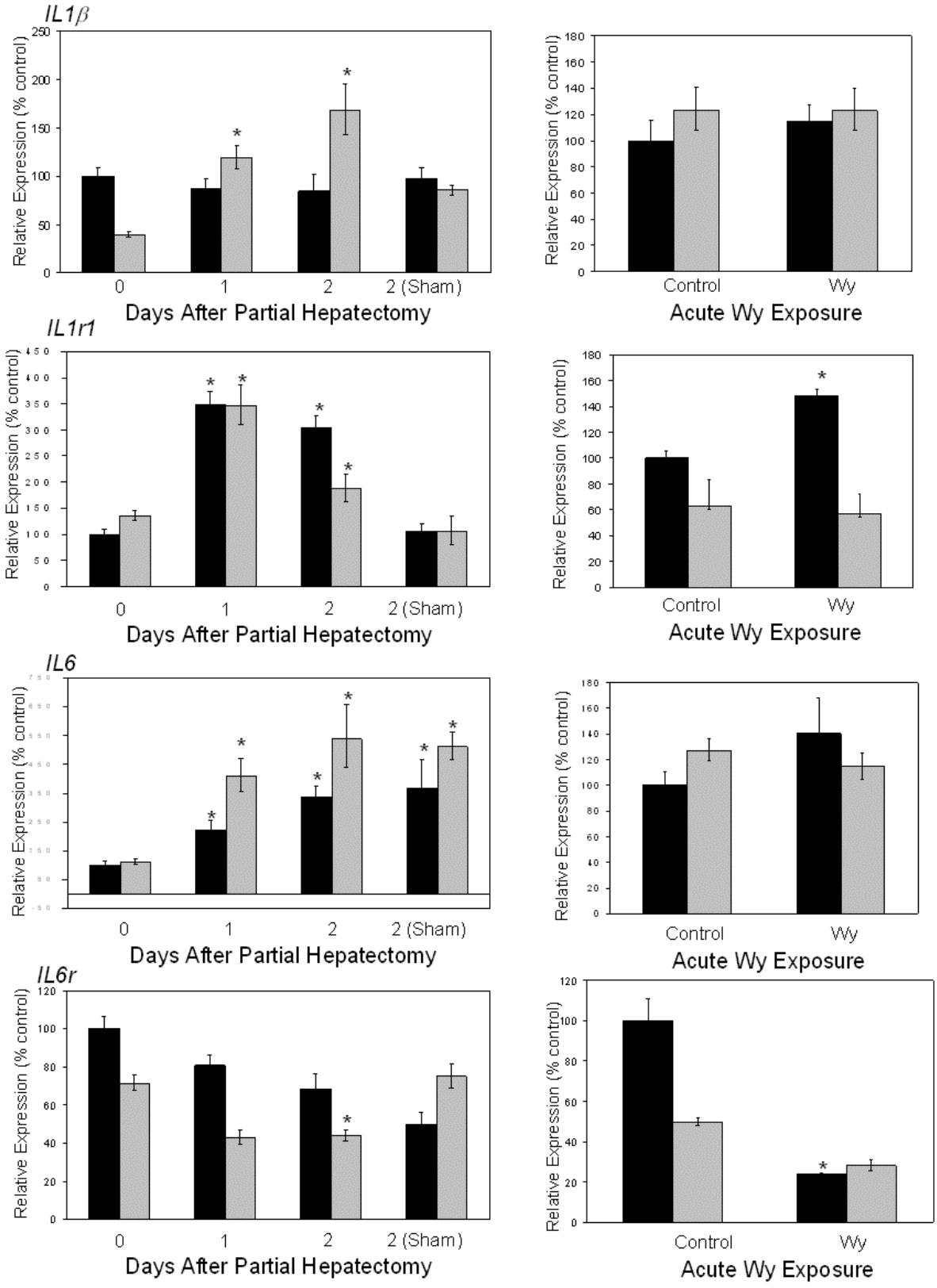
IL1β
Relative Expression (% control)
0
50
100
150
200
250
0
1
2
2 (Sham)
Days After Partial Hepatectomy
Relative Expression (% control)
0
20
40
60
80
100
120
140
160
180
Control
Wy
Acute Wy Exposure
IL1r1
Relative Expression (% control)
0
50
100
150
200
250
300
350
400
450
0
1
2
2 (Sham)
Days After Partial Hepatectomy
Relative Expression (% control)
0
20
40
60
80
100
120
140
160
180
Control
Wy
Acute Wy Exposure
IL6
Relative Expression (% control)
0
1
2
2 (Sham)
Days After Partial Hepatectomy
Relative Expression (% control)
0
20
40
60
80
100
120
140
160
180
Control
Wy
Acute Wy Exposure
IL6r
Relative Expression (% control)
0
20
40
60
80
100
120
0
1
2
2 (Sham)
Days After Partial Hepatectomy
Relative Expression (% control)
0
20
40
60
80
100
120
Control
Wy
Acute Wy Exposure

Figure 4.3(cont.)

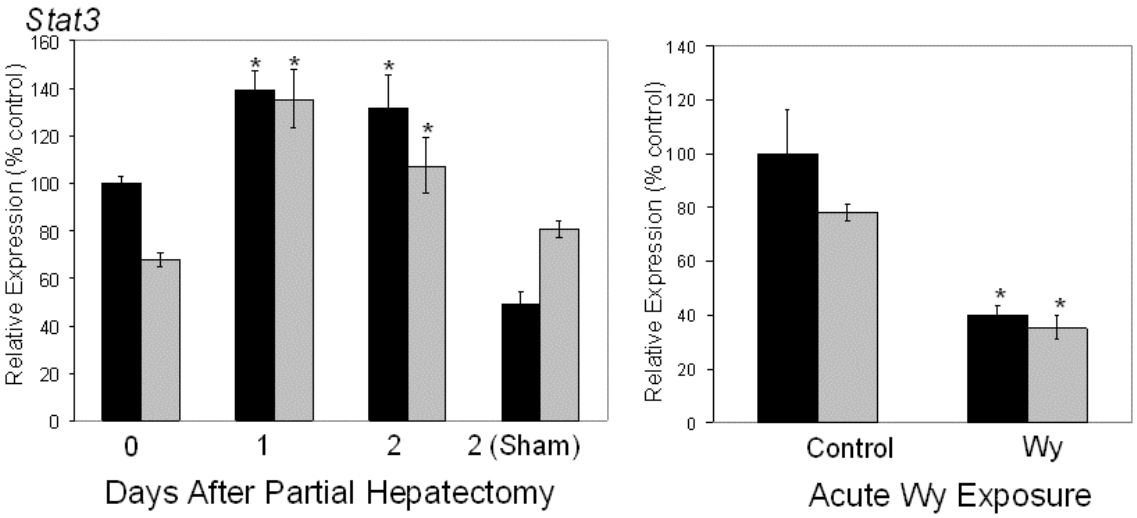

Figure 4.4

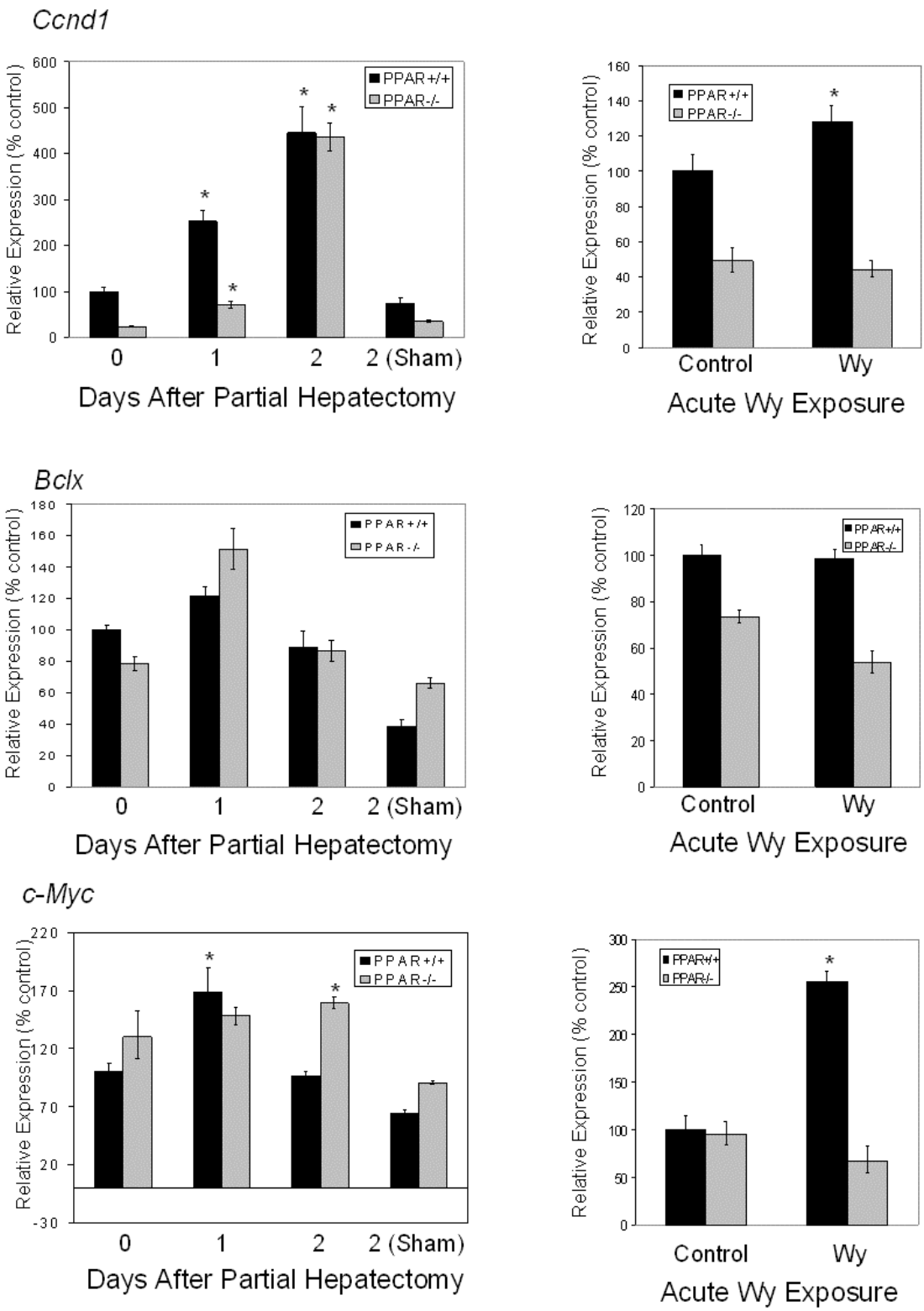
Ccnd1
Relative Expression (% control)
PPAR+/+
PPAR-/-
0
1
2
2 (Sham)
Days After Partial Hepatectomy
Control
Wy
Acute Wy Exposure
Bclx
c-Myc

Figure 4.5

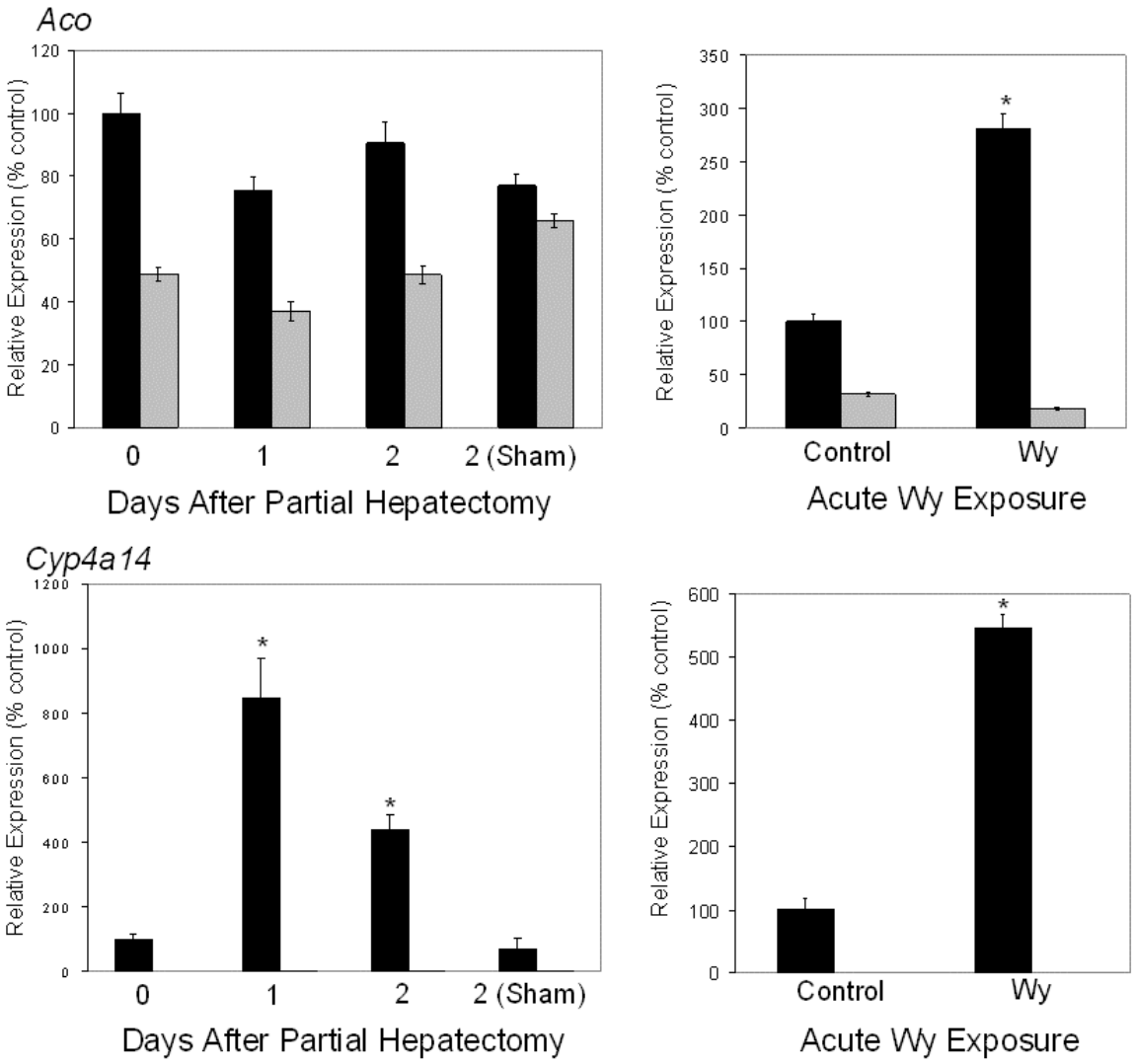
Aco
Relative Expression (% control)
120
100
80
60
40
20
0
0
1
2
2 (Sham)
Days After Partial Hepatectomy
Relative Expression (% control)
350
300
250
200
150
100
50
0
*
Control
Wy
Acute Wy Exposure
Cyp4a14
Relative Expression (% control)
1200
1000
800
600
400
200
0
*
*
0
1
2
2 (Sham)
Days After Partial Hepatectomy
Relative Expression (% control)
600
500
400
300
200
100
0
*
Control
Wy
Acute Wy Exposure

Table 4.1

Knockout Mice Showing Defective Liver Regeneration

Knockout	Decreased Activity	No Change	Reference
C/EBPβ	Ccnb, Ccne, Egr1, Hrs, Mkp1	Ccna, Ccnd1	21
Crem	Ccna, Ccnb, Ccnd1, Cdc2	IL6, Tnf	17
IL6	Ap1, Ccnd1, cMyc, Stat3	Hgf	20
Tnfr1	IL6	Ap1, NfkB, Stat3	18
Tnfr2	Ap1, C/ebp, cJun, cMyc	IL6, NfkB, Stat3	19
iNos	Caspase 3	IL6, NfkB, Stat3, Tnf	8
uPa	N.D.	N.D.	16
PPARα	Ccnd1, cMyc, Cyp4a14	Tnf, IL6, p27, Hgf, Stat3	

Table 4.2

Gene	Forward Primer (5'→ 3')	Probe (5'→ 3')	Reverse Primer (5'→ 3')	Accession No.
Ccnd1	CGTGGCCTCTAAGATGAAGGA	FAM-CCATTCCCTTGACTGCCGAGAAGTTG-TAMRA	CGGGCCGGATAGAGTTGT	S78355
cMyc	AGGCCCCCAAGGTAGTGATC	FAM-TCAAAAAAGCCACCGCCTACATCCTG-TAMRA	GTGCTCGTCTGCTTGAATGG	NM010849
Bclx	CAGACACTGACCGTCCACTCA	FAM-CTCTCACCTCCCACCCTGCCCC-TAMRA	GCAATGGTGGCTGAAGAGAGA	U51278
Stat3	TGGGCATCAATCCTGTGGTA	FAM-TGCTGACCAATAACCCCAAGAACGTGAA-TAMRA	GGTCCCAGGTTCCAATTGG	U06922
Cyp4a14	AACCTCTGTCACATGGGACCA	FAM-TGCCCTACACCACCATGTGCATCAA-TAMRA	GGATAGAGCCTCAGGGCCTC	NM007822
Aco	AATTGGCACCTACGCCCAG	FAM-ATGAGTTCCGTGGCCCATCTCCG-TAMRA	AGTGGTTTCCAAGCCTCGAA	NM015729
p27	TCCGCCTGCAGAAATCTCTT	FAM-CCCGGTCAATCATGAAGAACTAACCCG-BHQ1	CGGCAGTGCTTCTCCAAGTC	U10440
IL1β	TGGTGTGTGACGTTCCCATT	FAM-CAGCTGCACTACAGGCTCCGAGATGA-TAMRA	CAGCACGAGGCTTTTTTGTTG	NM008361
IL1r1	GCTGGGCCAGTCATCTGAAG	FAM-AGCCATATACAATGCTCTCATCCAGGAAGGA-BHQ1	TCCAACTCAAGCAGGACGATT	NM008362
IL6r	GGTGGCCCAGTACCAATGC	FAM-CATCCATGATGCCTTGCGAGGAGTG-TAMRA	GGACCTGGACCACGTGCT	NM010559
IL6	CCCAATTTCCAATGCTCTCC	FAM-AGCCACTCCTTCTGTGACTCCAGCTTATCTG-BHQ1	TGAATTGGATGGTCTTGGTCC	NM031168
Tnfα	ACAGAAAGCATGATCCGCG	FAM-CGTGGAACTGGCAGAAGAGGCACTC-TAMRA	GCCCCCCATCTTTTGGG	NM013693
uPa	CGATTCTGGAGGACCGCTTA	FAM-TGTAACATCGAAGGCCGCCCAACT-BHQ1	CCAGCTCACAATCCCACTCA	X02389
Hgf	CTGACCCAAACATCCGAGTTG	FAM-TACTGCTCTCAAATTCCCAAGTGTGACGTG-TAMRA	TTCCCATTGCCACGATAACA	D10212

Chapter 5

Summary

This dissertation consists of a series of studies aimed at identifying some of the critical events responsible for the hepatic mitogenesis and carcinogenesis in rodents exposed to peroxisome proliferators. All of the studies centered on differential gene expression (i.e., relative mRNA abundance), since peroxisome proliferator-mediated increases of peroxisome marker enzymes (e.g., *Aco* and *Cyp4a*) and mitogenesis require a functional Pparα protein.

Using an 'open' differential gene expression platform, we identified *α_2U* and *AT* as genes differentially expressed in rat hepatic adenomas induced by the potent *Pparα* agonist Wy-14, 643. Because the gene regulatory regions of many acute-phase protein (APP) genes, including *AT*, have recognition sites for several cytokine-induced transcription factors with demonstrated roles in liver growth and development, we examined whether the APPs as a class might serve as genetic markers for peroxisome proliferator-induced tumors in rodents. Moreover, we were hopeful that differential APP expression might offer insight into the mechanism driving the increased hepatocellular proliferation, primarily because of differential control of the two APP classes. Type 1 APP genes are induced by pathways involving *Tnfα*, *IL1β*, and *NfκB*, while type 2 APP are induced by *IL6*, *IL11*, and the *Stats*. C/ebp transcription factors are involved in both pathways. We found that both classes of APP genes are induced in liver tumors in rats exposed to peroxisome proliferators. Furthermore, this was not a specific response, since tumors induced by DEN reacted similarly. Mice treated subchronically exposed to several different peroxisome proliferators showed disparate hepatic expression of APP

genes: α_1AT was decreased, but ceruloplasmin and haptoglobin were increased in the livers from these animals. *Pparα* appears to have a dampening effect of APP expression, since mice *Pparα*-null mice had increased basal levels of APP gene expression that were not altered by peroxisome proliferator expression. These results indicate that *Pparα* activation by several different peroxisome proliferators leads to dysregulation of hepatic APP gene expression in rats and mice. Furthermore, this dysregulation may affect cell proliferation seen following peroxisome exposure.

One of the main drivers of APP gene expression in the liver is *Tnfα*. Because *Tnfα* has been proposed as the major mediator of hepatocellular proliferation following peroxisome proliferator exposure, we examined the role of *Tnfα* in peroxisome proliferator-induced hepatic mitogenesis using several strains of genetically engineered mice. Mice lacking *Tnfα*, or one or both of the *Tnf* receptors (*Tnfr1* & *Tnfr2*), showed no difference, compared to wild type mice, in hepatocellular proliferation after peroxisome proliferator exposure. Lacking evidence for a significant role for *Tnf* signaling in peroxisome proliferator induced mitogenesis; we examined other cytokine signaling pathways. Subchronic treatment with Wy led to increased expression of *IL1β*, its receptor *IL1r1*, and the *Stat3* and *Hnf4* transcription factors. *Tnfα* was not detected in either the control or treated animals at any time. Hepatic adenomas induced by Wy had increased expression of *IL1β*, *IL1r1*, *IL6*, and *Pparγ*. These data indicate that the hepatic mitogenesis induced by *Pparα* agonists in rodent liver is not mediated by *Tnfα*, but instead may involve *IL1*, *IL6*, or *Pparγ* signaling pathways.

While we were carrying out these experiments, *Pparα* was conclusively linked with the hepatic mitogenesis and carcinogenesis in rodents exposed to peroxisome

proliferators. Therefore, we were interested in the role of *Pparα* in normal liver growth, as typified by hepatocellular regeneration following partial hepatectomy. Our studies indicate that there is an approximately 1-day delay in regeneration in the *Pparα*-null mice. Moreover, this lag in growth is associated with a delay in expression of two of the main mediators of the G_1/S cell cycle progression, *Ccnd1* and *cMyc*. The hepatectomy studies were compared with another study examining hepatic gene expression in mice acutely exposed to Wy. *Ccnd1* and *cMyc* were not induced in *Pparα*-null mice, showing that Ppar regulates these genes, at least in part. An additional novel finding was a marked induction of *Cyp4a14* in the regenerating livers from mice subjected to partial hepatectomy.

The results of our studies could potentially influence our ability to predict human health risk associated with peroxisome proliferator exposure in several ways. First, activation of *Pparα* decreases hepatic expression of the APP genes. This response is consistent with other reports suggesting that *Pparα* activation suppresses inflammatory responses, and indicates that chronic exposure to PP may have immunosuppressive effects in people. While this would be an adverse effect in healthy people, it could be beneficial in patients with chronic inflammatory diseases such as arthritis. Second, demonstration that the hepatic mitogenesis in rodents exposed to PP is not mediated *via Tnfα* lessens enthusiasm for pursuing this signaling pathway in humans. In addition, correlation of the *IL1β* signaling pathway with the mitogenesis and tumorigenesis in mice may represent a novel mechanism of hepatocarcinogenesis in the rodent that may also apply to humans. Third, efficient hepatic regeneration requires *Pparα*, and *Pparα* may contribute to this process by direct or indirect stimulation of the main regulators of the G_1/S cell cycle checkpoint in hepatocytes, *Ccnd1* and *cMyc*. Thus, it is plausible that

Pparα agonists may have therapeutic utility in treating certain chronic liver diseases. Experiments using human cells and tissues will be necessary for resolution of these issues.

Appendix I

Down-Regulation of Cytochrome P450 2C Family Members and Positive Acute-Phase Response Gene Expression by Peroxisome Proliferator Chemicals

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ABSTRACT

In this study, we show that peroxisome proliferator chemical (PPC) exposure leads to alterations in the expression of genes in rat liver regulated by the sex-specific growth hormone secretory pattern and induced during inflammation. Expression of the male-specific *cytochrome P450* (P450) *2C11* and α2 urinary globulin (*α2u*) genes and the female-specific *P450 2C12* gene was down-regulated by some PPC. Expression of *P450 2C13*, also under control by the sex-specific growth hormone secretory pattern, was not altered by PPC treatment, indicating that regulation of *CYP2C* family members does not involve perturbation of the growth hormone secretory pattern. In contrast to the increases in expression observed when inflammation was induced in male rats, two positive acute-phase response genes, α_1-acid glycoprotein and β-fibrinogen, were decreased by PPC exposure. The down-regulation of the *P450 2C11* by WY-14,643 could be reproduced in cultured rat hepatocytes, indicating the down-regulation is a direct effect. Experiments in wild-type mice and mice that lacked a functional *peroxisome proliferator-activated receptor-α* gene showed that down-regulation by WY of *α_1-acid glycoprotein, β-fibrinogen*, and a mouse homologue of *α2u* was dependent on *peroxisome proliferator-activated receptor-α* expression. Our results demonstrate that PPC exposure leads to down-regulation of diverse liver-specific genes, including *CYP2C* family members important in hormonal homeostasis and acute-phase response genes important in inflammatory responses.

Peroxisomes are ubiquitous eukaryotic organelles that play a key role in regulating lipid homeostasis in mammals. An ever-increasing number of economically significant pharmaceutical, industrial, agricultural, and laboratory chemicals have been found to induce the proliferation of hepatocellular peroxisomes in test animals. These PPCs are a large group of >100 structurally diverse xenobiotics, including hypolipidemic drugs, herbicides, perfluorinated fluids, chlorinated solvents, and plasticizers (Ashby *et al.*, 1994). Despite the structural dissimilarities and pharmacokinetic properties of these PPCs, chronic administration to rodents leads to a remarkable stereotypical and temporal hepatic response consisting of hepatomegaly, formation of hepatic adenomas, and hepatocellular carcinomas that is concomitant with the peroxisome proliferation (Lapinskas and Corton, 1998). The carcinogenicity of some of these chemicals in rodents, coupled with widespread human exposure, raises concern that PPCs may contribute to human cancer risk (Reddy and Lalwai, 1983). However, the scientific validity of this assumption needs to be established.

Many facets of the molecular mechanism of enzyme induction by PPCs are known. PPCs, as well as long-chain unsaturated fatty acids and a number of eicosanoids, have been shown to activate members of the nuclear receptor superfamily called PPARs (reviewed in Lapinskas and Corton, 1998). Three distinct isoforms of PPAR encoded by separate genes have been isolated and termed PPARα, PPARδ (also known as Nucl and PPARβ), and PPARγ. In the presence of inducers, PPARα, the principal isoform expressed in the liver, activates expression of many genes whose products are involved in the metabolism of fatty acids; these include genes involved in fatty acid β-oxidation (*ACO*), fatty acid ω-oxidation (*P450 4A* family members), ketogenesis (*3-hydroxy-3-methylglutaryl-CoA synthetase*), lipogenesis (*malic enzyme*),

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ABBREVIATIONS: PPC, peroxisome proliferator chemical; AGP, α_1-acid glycoprotein; α2u, α_2 urinary-globulin; ACO, acyl-coenzyme A oxidase; DBP, di-*n*-butyl phthalate; DEHP, di-(2-ethylhexyl)phthalate; Fib, fibrinogen; GEM, gemfibrozil; GH, growth hormone; HNF, hepatocyte nuclear factor; MUP, major urinary protein; P450, cytochrome P450; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; tNA, total nucleic acids; WY, WY-14,643.

fatty acid transport (*fatty acid binding protein*), glycerolneogenesis (*phosphoenolpyruvate carboxykinase*), and serum transport proteins (*transthyretin, transferrin, and apolipoproteins*). All of these genes have within their regulatory regions two or more copies of the sequence TG(A/T)CCT in the direct repeat orientation separated by one base pair called PPREs. These genes are all likely activated by a heterodimer of PPARα and the receptor for 9-*cis* retinoic acid (retinoid X receptor), which recognizes PPRE in the presence of PPCs, fatty acids, or eicosanoid activators. PPARα was shown to be a global regulator of genes involved in peroxisomal β-oxidation of fatty acids in the liver because inducibility of these genes by PPCs is abolished in mice that lack a functional *PPARα* gene (Lee *et al.*, 1995).

To better understand the molecular mechanisms of the pleiotropic responses induced by exposure to PPCs, we made a concerted effort to clone and characterize genes that are modulated in the rat liver after exposure to a PPC. The α2 urinary globulin (*α2u*) was recently identified by Alvares *et al.* (1996) and by ourselves (Corton and Gustafsson, 1997) as a gene down-regulated by PPCs. Besides regulation by PPCs, this male-specific gene is controlled by the male-specific pulsatile release of GH (Roy *et al.*, 1983) and negatively controlled by cytokines released during inflammation (Birch and Schreiber, 1986). To begin to understand how PPCs down-regulate *α2u*, we determined whether other genes regulated by the sex-specific GH secretory pattern or genes induced during inflammation also were regulated by PPC. We report here that PPCs down-regulate the expression of many liver-specific genes, including those in the *CYP2C* family of steroid hydroxylases and positive acute-phase response genes induced during inflammation.

Experimental Procedures

Materials. Collagenase was purchased from Worthington Biochemicals (Freehold, NJ). Matrigel and media for culturing rat primary hepatocytes were obtained from Collaborative Biochem (Bedford, MA). Proteinase K was obtained from Merck (Darmstadt, Germany). RNase A and RNase T1 were from Boehringer-Mannheim (Mannheim, Germany). Restriction endonucleases, ligase, plasmid vectors, and reagents for *in vitro* transcription of cRNA probes were supplied by Promega Biotech (Madison, WI). [^{35}S]UTP (>1000 Ci/mmol) and [^{32}P]UTP (400 Ci/mmol) were from Amersham International (Buckinghamshire, UK).

Animals. This study was conducted under federal guidelines for the use and care of laboratory animals and was approved by the Chemical Industry Institute of Toxicology Institutional Animal Care and Use Committee. In the first five experiments, F344 [CDF(F344)/CrlBR] and Sprague-Dawley (Harlan Sprague-Dawley derived) rats were obtained from Charles River Breeding Laboratories (Raleigh, NC). In the sixth experiment, male Harlan Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN). In the seventh experiment, male SV129 wild-type mice were purchased from Taconic (Germantown, NY), and male SV129 PPARα null mice (Lee *et al.*, 1995) were a kind gift from Frank Gonzalez (National Cancer Institute, Bethesda, MD). Control and treated rats and mice were provided with NIH-07 rodent chow (Ziegler Brothers, Gardner, PA) and deionized, filtered water *ad libitum*. Lighting was on a 12-hr light/dark cycle. In the first experiment, F344 rats were given a single gavage dose of WY-14,643 (ChemSyn Science Laboratories, Lenexa, KS) at 50 mg/kg body weight and killed 1, 3, 6, 12, or 24 hr after treatment. In the second experiment, male and female F344 rats and male Harlan Sprague-Dawley rats were fed 500 ppm WY, 8000 ppm GEM (Sigma Chemical, St. Louis, MO), or 20,000 ppm DBP (Aldrich Chemical, Milwaukee, WI) for 13 weeks. In the third experiment, male and female F344 rats were given gavage doses of either vehicle (methylcellulose), WY (50 mg/kg body weight), or DEHP (2000 mg/kg body weight) in methylcellulose each day for 3 days. In the fourth experiment, F344 rats were given an intraperitoneal injection of killed *Corynebacterium parvum* whole cell (RIBA Immunochemical, Hamilton, MT) at 28 mg/kg body weight and killed 7 days after injection. In the fifth experiment (conducted by the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC), male Harlan Sprague-Dawley rats were fed the indicated doses of WY (ChemSyn Science Labs), GEM (Sigma Chemical), or DBP (ChemCentral) for 1, 5, or 13 weeks. In the sixth experiment, male SV129 wild-type and SV129 PPARα null mice were fed WY (0.1%), DEHP (0.6%), or a control diet for 3 weeks. At the designated time after treatment, animals were deeply anesthetized by isofluorane anesthesia or pentobarbital injection and killed by exsanguination. The livers were removed, rinsed with isotonic saline, snap-frozen in liquid nitrogen, and stored at −70° until analysis.

Hepatocyte isolation and cell culture. For preparation of primary hepatocytes, adult male Fischer F344 rats, weight ~200–250 g, were obtained from Charles River (Raleigh, NC). The animals were maintained under conditions of constant temperature and humidity and allowed chow and water *ad libitum*. A thin coat of Matrigel was applied to 100-mm-diameter plastic culture dishes and allowed to gel at room temperature. Hepatocytes were isolated by nonrecirculating collagenase perfusion through the portal vein of pentobarbital-anesthetized rats according to the method of Kedderis *et al.* (1988). Cells (1.5×10^6 per 100-mm plate with viability 85–95% as determined by trypan blue exclusion) were plated in 3 ml of modified Waymouth medium containing insulin (0.2 μg/ml) as the only hormone. Cultures were maintained in an incubator at 37° in an atmosphere containing 5% CO_2. Medium was replaced daily, commencing 24 hr after the cells were plated. Cells were cultured for 5 days before treatment to optimize for P450 2C11 expression (Chen *et al.*, 1995). DMSO or WY was added at the indicated concentrations, and the cells were incubated for 48 hr. Medium was aspirated from culture dishes and replaced with 2 ml of ice-cold, phosphate-buffered saline, with 5 mM EDTA, pH 7.4. The cells and Matrigel were transferred to 15-ml capped plastic tubes and then allowed to stand on ice for 45 min to dissolve the Matrigel. Cells were then collected by centrifugation at $750 \times g$ for 5 min and lysed in lysis buffer. Extracts were used for analysis of protein expression. Experiments were repeated twice with similar results.

Solution hybridization. The solution hybridization experiments were carried out essentially as described earlier (Tollet *et al.*, 1990). tNA were prepared by digestion of liver with proteinase K followed by phenol-chloroform extraction. The concentration of tNA in the samples was determined spectrophotometrically, and the DNA concentration was quantified using a specific fluorometric method. Abundance of the respective mRNA for P450 2C11, P450 2C12, and P450 2C13 was determined using ^{35}S-UTP-labeled cRNA probes transcribed *in vitro* from cDNA templates. The cDNA templates corresponding to the full-length cDNA base pairs were P450 2C11, 1580–1884; P450 2C12, 681–731; and P450 2C13, 1537–1720. Temperature and formamide concentration were optimized for each assay: 20% formamide and 75° for P450 2C11 and P450 2C13; 20% formamide and 65° for P450 2C12. Quantification of the mRNA species was achieved by comparison with standard curves obtained from hybridizations to liver tNA calibrated to known amounts of *in vitro* synthesized mRNA. All samples were analyzed in triplicate.

Northern blot hybridization. Total RNA was isolated by modification of the guanidinium isothiocyanate method using RNazol according to manufacturer's instructions (Tel-Test, Friendswood, TX). Denatured total RNA was separated on 1.0% formaldehyde-agarose gels and transferred to Hybond-N Nylon membranes in 20× standard saline citrate. Hybridization and washing conditions were described previously (Fan *et al.*, 1998). The probes include full-length

rat α2u-globulin cDNA isolated from a rat heart cDNA library (Corton J. C., unpublished data), an oligonucleotide specific for mouse major urinary protein-I (5′-AGGGAATAGGATTGTCTG-3′) (GenBank Accession no. M16355) (Shahan *et al.*, 1987), rat α_1-acid glycoprotein oligonucleotide (5′-TCCCCGGAGTTCAGAGAGCTGAGTTCATGC-3′) complementary to nucleotides 660–689 in the rat cDNA (Ricca *et al.*, 1981) or 645–674 in the mouse cDNA (5′-GAGTTCAGAGAGCTGAGTTCATGCCTGGCC-3′) (Lee *et al.*, 1989), and β-Fib cDNA (kindly provided by Gerald Fuller, University of Alabama at Birmingham). The 700-kb *Pst*I cDNA fragment of the ACO cDNA (kindly provided by Dr. Hilde Nebb-Sörensen, Institute of Medicine and Biochemistry, University of Oslo, Oslo, Norway) was used as a positive control. β-Actin was used as a loading control. The probes for Northern analysis were labeled with α-^{32}P-dCTP (Amersham, Cleveland, OH) using the random-primer DNA labeling kit according to the manufacturer's instructions (Stratagene, La Jolla, CA).

Western blot analysis. Whole-cell protein extracts (50–120 μg of total protein) made according to Corton *et al.* (1996) were denatured and size-separated by 12.5% or 15% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and visualized by Ponceau Red to confirm transfer. The blotted proteins were probed with polyclonal or monoclonal antibodies followed by anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase and visualized by enhanced chemiluminescence (ECL Amersham; or SuperSignal, Pierce Chemical, Rockford, IL). The anti-α2u-globulin antibodies were a kind gift from Otto Neuhaus (University of South Dakota, Vermillion, SD). Levels of the mouse homologue of rat α2u called mouse urinary protein-I could not be measured because the antibodies against the rat α2u did not cross-react with the mouse MUP proteins despite up to 50% amino acid identity shared with α2u. The anti-P450 2C11 (Morgan *et al.*, 1985) or anti-P450 2C12 (Morgan *et al.*, 1987) antibodies have been described. The anti-ACO antibody was a kind gift from Stefan Alexson (Huddinge University Hospital, Huddinge, Sweden). The anti-fibrinogen antibodies were purchased from Affinity Biologicals, South Bend, IN. Antibodies to the rat fibrinogen chains were raised starting with the three purified fibrinogen polypeptides, which migrated at 68–70, 55, and 48kDa (personal communication, Hugh Hugodorn, Affinity Biologicals, South Bend, IN) corresponding to the published molecular weights of α-, β-, and γ-fibrinogen. In our experiments, using both rat and mouse liver extracts we detect proteins of 65 and 55kDa, which correspond in size to the α and β chains of fibrinogen. Antibodies against the rat male-specific P450 2C13 (GenTest, Woburn, MA) cross-reacted equally with P450 proteins in male and female rats (data not shown) and thus were not suitable for Western blot analysis. Autoradiograms for Northern and Western blots were densitometrically scanned using Image-1 image analysis system (Universal Imaging Corporation, West Chester, PA) and NIH Image 1.54 software.

Results

Altered expression of *CYP2C* family members by PPC. To gain insight into the mechanism of *α2u* down-regulation by PPC, we examined the expression of other genes that are regulated in a manner similar to *α2u*, including members of the *P450 2C* family, known to be important in hydroxylating steroids. Like *α2u*, members of the *P450 2C* family have been shown to exhibit sex-specific expression (Legraverend *et al.*, 1992) and to be negatively regulated by bacterial lipopolysaccharide (Chen *et al.*, 1995). Expression of male-specific *CYP2C11* and *CYP2C13* and female-specific *CYP2C12* genes was assessed in the livers of male and female rats treated with PPC by solution hybridization. Male and female F344 rats were fed 500 ppm WY, 8000 ppm GEM, or 20,000 ppm DBP in the diet for 13 weeks. As shown in Table 1, WY exposure almost completely abolished expression of *CYP2C11* in male rat liver to 0.4% of control levels. GEM and DBP decreased expression to 26% and 40% of control levels, respectively. As expected, *CYP2C11* expression in untreated female livers was undetectable. Exposure of female rats to PPC did not appreciably affect expression of *CYP2C11*. Under these conditions, ACO was strongly induced by each PPC in female livers (Corton *et al.*, 1996). In female rats, GEM decreased P450 2C12 mRNA levels to 37% of control levels (Table 1). WY and DBP exposure did not result in statistically significant changes in P450 2C12 mRNA levels. In male rats, there were no significant changes in P450 2C12 expression in liver. In contrast to P450 2C11, P450 2C13 levels in male rats were not appreciably affected by exposure to any PPC. P450 2C13 expression in female rat liver was not detectable and increased after PPC treatment, although induced levels were far below those in male rat livers. Duplicate experiments with male Sprague-Dawley rats treated identically with PPC demonstrated that the two rat strains exhibited qualitatively similar changes in P450 2C gene expression (data not shown).

We next determined whether expression of the P450 2C proteins decreased in parallel with mRNA levels. In these experiments, we compared the expression of P450 2C family members with that of α2u-globulin. α2u-Globulin, P450 2C11, and P450 2C12 protein levels were determined by Western analysis of whole-cell liver extracts. Using a polyclonal antibody against α2u, a protein of the correct size (~18 kDa) was highly expressed in untreated male rat livers but not detectably expressed in female rat livers (Fig. 1A). A protein of ~65 kDa found in both male and female livers also was detected. The origin of this protein is not known. Exposure to WY or GEM severely decreased expression of α2u (2% and 17% of control, respectively) (Fig. 1D). Exposure to DBP did not alter expression of α2u. Exposure to PPC did not detectably affect expression of α2u in female rats. Few, if any, changes were detected in the expression of the ~65-kDa protein after PPC exposure.

Exposure to some PPCs resulted in changes in P450 2C11 protein levels similar to that seen with α2u (Fig. 1B). Using a monoclonal antibody against P450 2C11, one major band of ~50 kDa was detectable in untreated male, but not untreated female, rat livers. Under these conditions of maximal sensitivity (i.e., 100 μg of protein/lane and long exposure times), a number of smaller immunoreactive proteins also

TABLE 1

Expression of P450 2C family members after PPC exposure

Male and female F344 rats were fed control diets or diets containing the indicated levels of PPC for 13 weeks. P450 2C mRNA expression was determined by solution hybridization. Percentage values and statistical significance for males treated (2C11 and 2C13) and females treated (2C12) are presented relative to expression in control male (2C11 and 2C13) or control female (2C12) livers, respectively.

	2C11	2C12	2C13
		%	
Male			
Control	100	0.5	100
WY (500 ppm)	0.4[a]	2	139
GEM (8,000 ppm)	26[a]	2	131
DBP (20,000 ppm)	40[a]	2	210
Female			
Control	0	100	0
WY (500 ppm)	0	68	2
GEM (8,000 ppm)	0	37[a]	2
DBP (20,000 ppm)	0.3	99	1.5

[a] Significant differences ($p < 0.05$) from control values.

were observed that may have been proteolytic products of P450 2C11. After WY exposure, expression of P450 2C11 was abolished (0% of control) (Fig. 1D). Very little expression was detectable after GEM exposure (9.6% of control). DBP exposure did not result in a significant change in P450 2C11 expression.

Exposure of female rats to WY led to decreases in P450 2C12 protein (9% of control). GEM and DBP treatment did not lead to statistically significant changes in P450 2C12. As expected, little, if any, P450 2C12 expression was detected in male rats fed a control diet or diets containing WY or DBP. Surprisingly, GEM exposure led to a dramatic increase in a protein in male rats that was indistinguishable in size from the female P450 2C12 protein. Because the *CYP2C12* gene is not induced in GEM-treated male rats (Table 1), this GEM-inducible protein is unlikely to be P450 2C12 but may be an uncharacterized member of the P450 2C family selectively induced by GEM but not the other PPC.

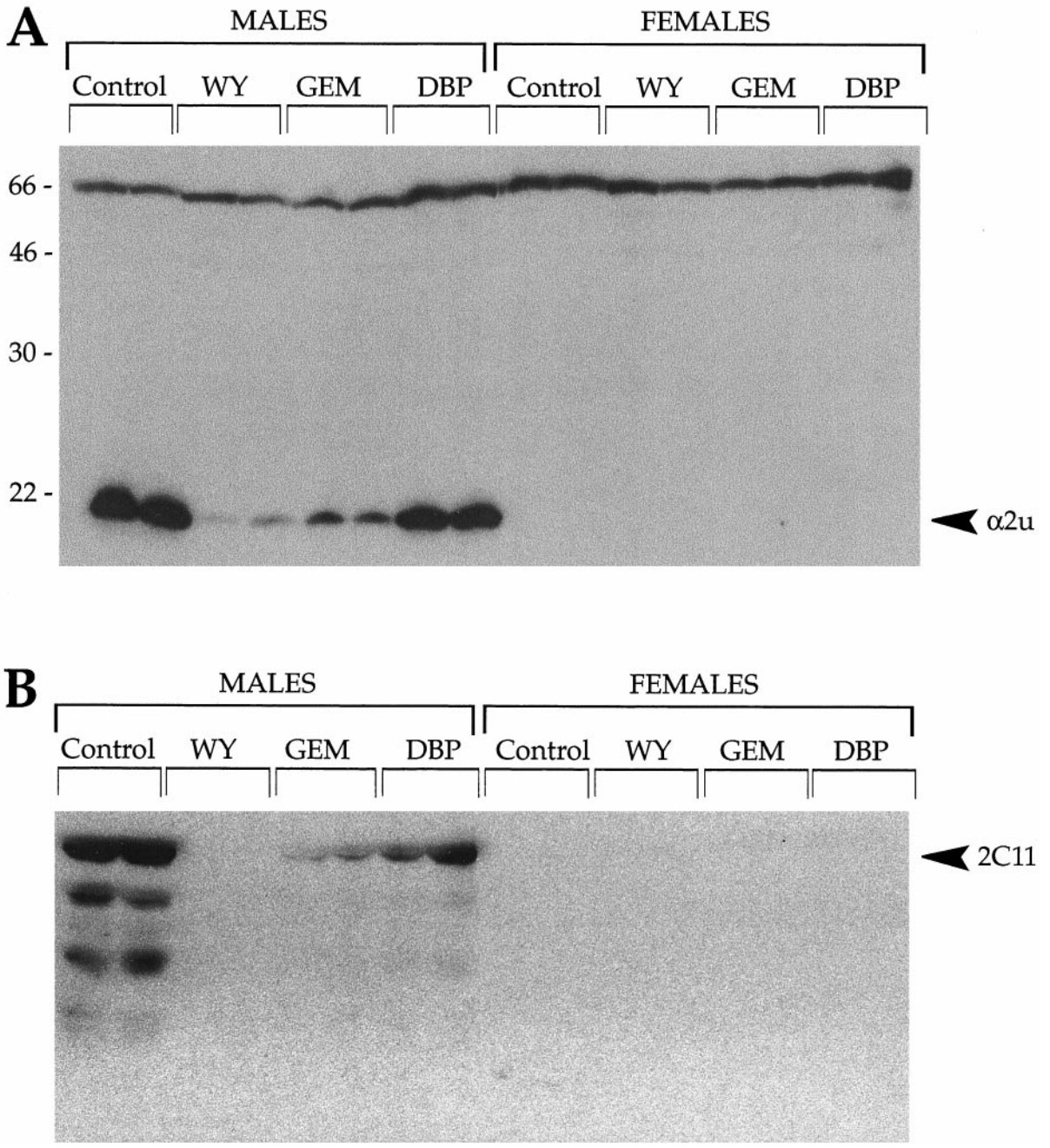

Fig. 1. Decreased expression of α2u, P450 2C11, and P450 2C12 proteins after PPC exposure. Whole-cell liver extracts from male and female F344 rats given a control diet (*Control*) or a diet of WY (500 ppm), GEM (8000 ppm), or DBP (20,000 ppm) for 13 weeks were separated by 12.5% or 15% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against α2u (A), P450 2C11 (B), or P450 2C12 (C). A, *Numbers on left*, position of molecular mass markers (in kDa). D, Quantification of protein expression. The Western data were densitometrically scanned, and protein expression was quantified. Expression of α2u and P450 2C11 in male rats and P450 2C12 in female rats was compared with treated male rats or female rats, respectively. *Histograms*, mean expression (mean ± standard deviation) from four different samples. *, Significantly different from control ($p < 0.05$). E, Expression of α2u and P450 2C11 proteins after *C. parvum* treatment. Male rats were treated with *C. parvum* for 7 days, and whole-cell liver extracts were analyzed for protein expression.

We also examined the expression of α2u and P450 2C11 proteins in male rats treated for 7 days with the classic inflammatory inducer, killed *C parvum* bacteria. As expected treatment with *C. parvum* led to parallel decreases in the expression of α2u and P450 2C11 (Fig. 1E).

Altered regulation of positive acute-phase response genes by PPC. Because PPC exposure seemed to be mimicking the down-regulation of α2u and P450 2C11 after inflammation, we examined the expression of the positive acute-phase response genes AGP, a positive class I acute-phase gene regulated by interleukin-1 and tumor necrosis factor-α, and *β-Fib*, a positive class II acute-phase gene regulated by interleukin-6. Expression of β-Fib and AGP mRNAs was shown to be similarly down-regulated in the livers of male rats after exposure to PPCs. β-Fib mRNA levels were down-regulated by WY, GEM, and DBP to 29%, 40%, and 62% of control, respectively (Fig. 2A). AGP mRNA levels were down-regulated to 20%, 36%, and 49% of control, respectively.

In parallel with the decreases in β-Fib mRNA levels, the levels of the Fib protein decreased after PPC exposure in male rat livers (Fig. 2B). Polyclonal antibodies to rat Fib raised to the three fibrinogen polypeptides α, β, and γ reacted with two proteins in control rat liver of ~66 and ~56 kDa, corresponding to the α and β forms of fibrinogen, respectively. The expression of α-Fib was almost completely abolished by treatment with all of the PPC (13%, 3%, and 8% of control for WY, GEM, and DBP, respectively). The expression of β-Fib was decreased after treatment with GEM (13% of control). β-Fib was not significantly decreased by WY or DBP. Similar changes in α- or β-Fib were observed in the livers of female rats treated identically with PPCs (data not shown).

To compare with the effects of PPC treatment, we examined the expression of Fib proteins in rats treated with *C. parvum* as described above. In contrast to the down-regulation of α- and β-Fib observed after PPC treatment, *C. parvum* treatment resulted in the elevation of both α- and β-Fib proteins (Fig. 2C). Thus, PPCs regulate the expression of the positive acute-phase genes by a mechanism different from the one induced by inflammation.

Expression of α2u, P450 2C11, and β-Fib after different times and doses of exposure to PPC. To determine whether protein expression is coordinately regulated by

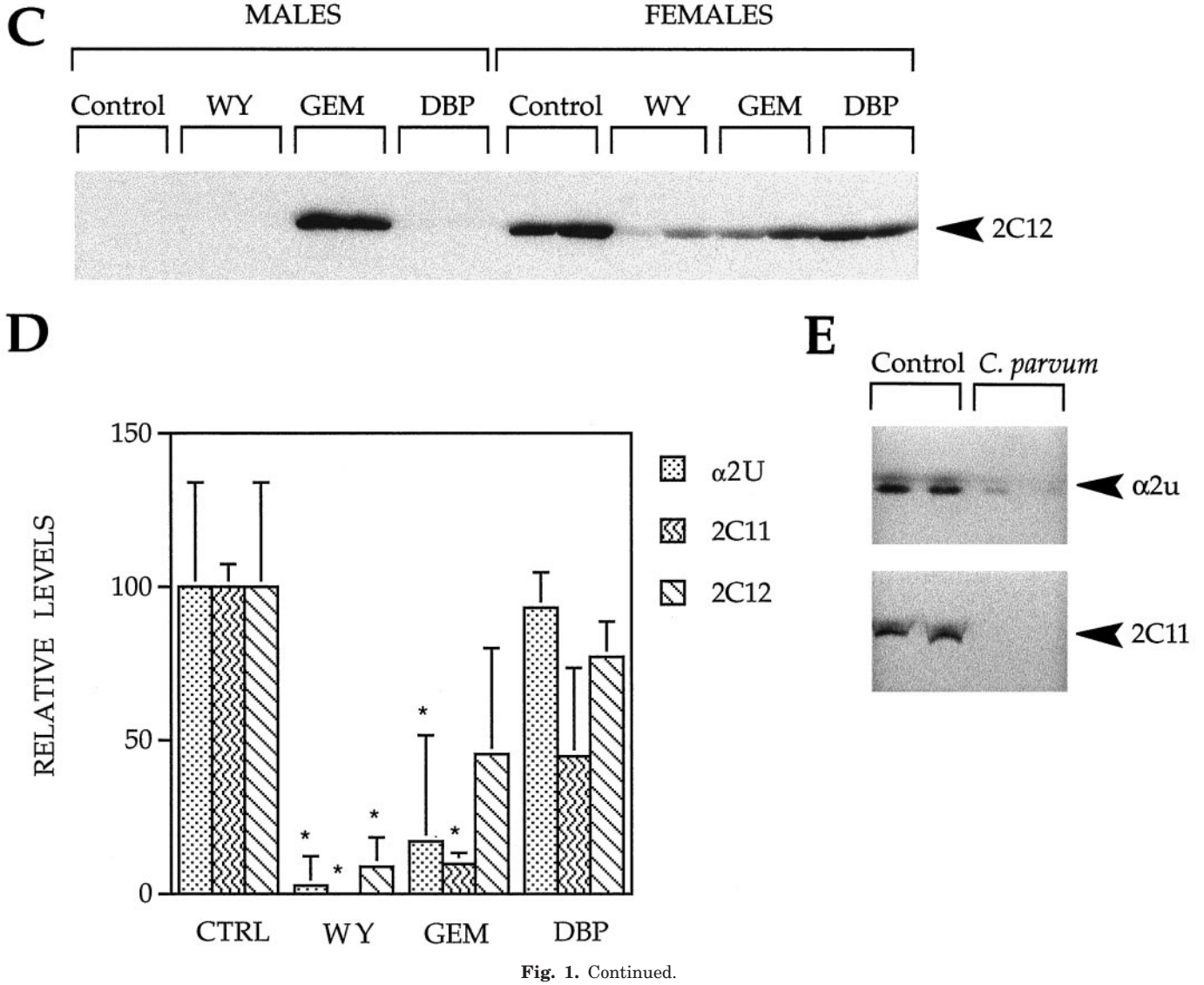

Fig. 1. Continued.

PPCs, we examined the pattern of expression of the two male-specific proteins α2u and P450 2C11 at 1, 5, and 13 weeks after initiating the feeding study using the same dietary concentrations of WY, GEM, and DBP used in Table 1. In this and the dose-response study discussed below, we used male Sprague-Dawley rats. No gross strain-specific differences in expression of α2u, P450 2C11, and ACO proteins between the Fisher and Sprague-Dawley rats after exposure to the three PPC were evident (data not shown). No differences in the expression of α2u and P450 2C11 exist over the time of the experiment in the untreated control animals (Fig. 3). The kinetics of the decreases for α2u and P450 2C11 by all three PPC were very similar. Both α2u and P450 2C11 protein levels were severely decreased after 1, 5, and 13 weeks of exposure to WY (Fig. 3). After GEM exposure, maximum decreases in P450 2C11 expression occurred at 1 week. Expression of P450 2C11 was also decreased at 5 and 13 weeks, although the decreases were less than those after a 1-week exposure. The α2u expression was not significantly changed after exposure to GEM at all time points. After DBP exposure, neither α2u nor P450 2C11 expression changed at any of the time points.

The response of α2u and P450 2C11 to different dietary concentrations of WY, GEM, and DBP after 5 weeks of exposure also was assessed in livers of male Sprague-Dawley rats. Fig. 4 shows that decreases of both proteins were observed at 5 ppm WY, the lowest dose tested. The levels of the proteins continued to decrease with increasing doses of WY. Parallel decreases in the two proteins occurred after GEM and DBP exposure. Changes due to GEM exposure were observed at doses as low as 8000 ppm, with maximal effects at 16,000 ppm. Only at the highest dose of DBP (20,000 ppm) were decreases observed for P450 2C11 but not for α2u.

To determine how rapidly protein expression changes after exposure, α2u, P450 2C11, and β-Fib expressions were compared with that of ACO, which is induced early by PPARα activation after exposures to PPC. The expression first was examined at up to 24 hr after a single gavage dose of WY. Expression of P450 2C11 protein was decreased only after 24 hr of exposure (Fig. 5A) (63% of control; $p < 0.05$). No consistent alterations in protein abundance for α2u and α- or β-Fib could be detected at any of the time points (data not shown). Increases in the three polypeptide components of ACO, ACO-A (72 kDa) (data not shown), ACO-B (52 kDa) (Fig. 5A), and ACO-C (22 kDa) (data not shown) could be detected at 24 hr but not earlier.

α2u, P450 2C11, and Fib protein expressions also were examined in the livers of male and female rats given a gavage dose of WY or DEHP each day for 3 days. As shown in Fig.

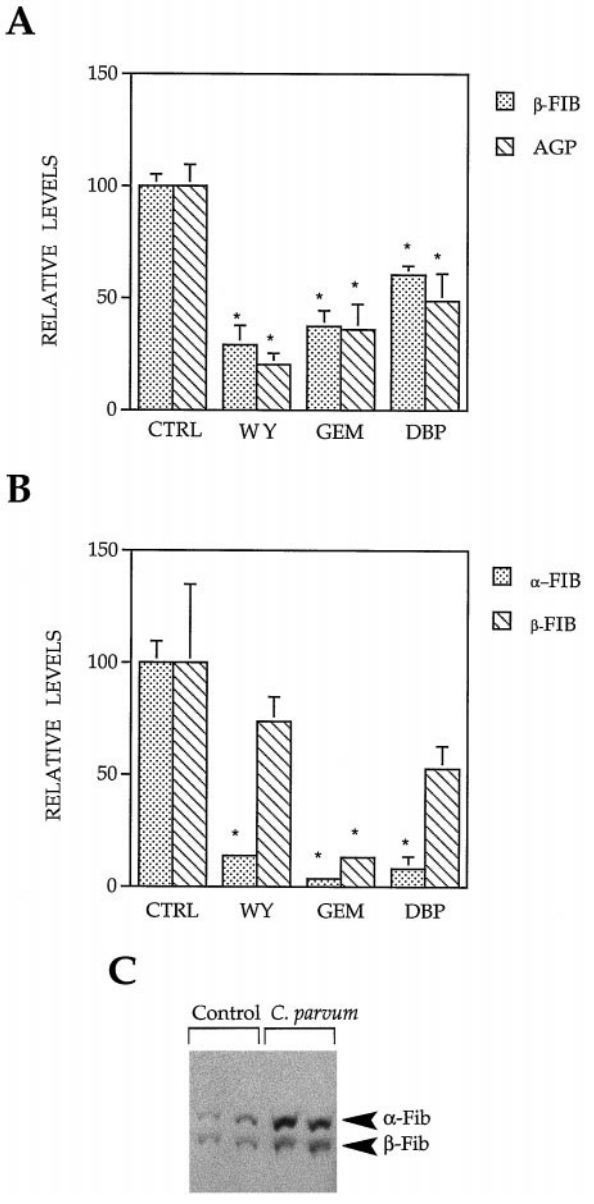

Fig. 2. Down-regulation of positive acute-phase response genes by PPC. A, Quantification of mRNA expression of β-Fib and AGP after PPC exposure. Male F344 rats were treated as in Fig. 1, and their RNA was analyzed for expression of β-Fib, AGP, and β-actin. The autoradiograms were densitometrically scanned, and expression was normalized to β-actin expression. *Histograms*, mean expression (mean ± standard deviation) from two different samples. *, Significantly different from control ($p < 0.05$). B, Analysis of α- and β-Fib protein expression after PPC exposure. Whole-cell liver extracts from male F344 rats treated as in Fig. 1 were separated by 12.5% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against Fib. Protein expression was quantified as detailed in Fig. 1D. C, Increased expression of α- and β-Fib after *C. parvum* treatment. Male rats were treated with *C. parvum* for 7 days, and whole-cell liver extracts were analyzed for Fib protein.

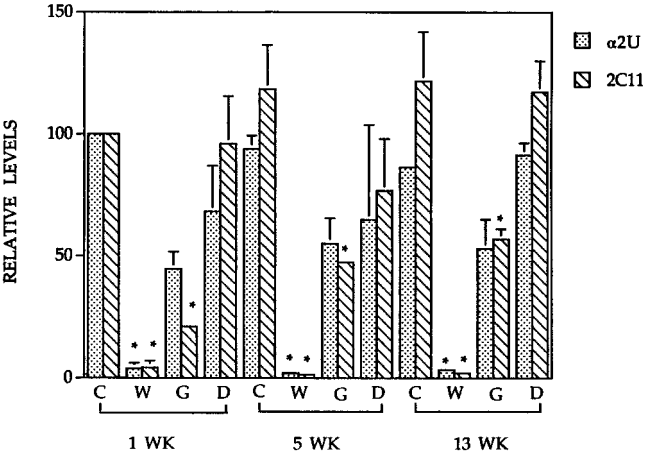

Fig. 3. Time course of α2u and P450 2C11 protein level changes. Whole-cell liver extracts from male Sprague-Dawley rats fed a control diet (*Control*) or a diet containing WY (500 ppm), GEM (8,000 ppm), or DBP (20,000 ppm) for 1, 5, or 13 weeks were separated by 12.5% or 15% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to α2u or P450 2C11. Expression was quantified as in Fig. 1D.

5B, exposure to either PPC resulted in increased abundance of ACO protein, with WY eliciting a greater effect. Exposure to either WY or DEHP had no effect on either α2u or α- and β-Fib protein expression in livers from male or female rats. In contrast, the P450 2C11 protein was severely decreased by exposure to either WY (9% of control; $p < 0.05$) or DEHP (24% of control; $p < 0.05$).

To determine whether the PPC-induced down-regulation of expression could be reconstituted *in vitro*, we examined the expression of α2u, P450 2C11, and Fib in rat primary hepatocytes after a 48-hr treatment of WY. As shown in Fig. 6, expression of ACO protein was increased after doses of 100, 200, and 400 μM WY. P450 2C11 exhibited decreased expression after WY exposure in a dose-dependent manner with a maximal decrease at 200 μM WY. α- or β-Fib and α2u did not exhibit changes in protein expression under the conditions of the experiment, possibly because of the greater stability of these proteins (data not shown). These data indicate that PPCs act directly on hepatocytes to regulate expression of P450 2C11.

In summary, 1) P450 2C11 and ACO protein levels were altered within 24 hr of PPC exposure, 2) α2u and Fib protein levels did not exhibit changes after 3 days of exposure, 3) α2u and P450 2C11 proteins exhibit similar kinetics of regulation by PPC after 1 week or more of exposure, and 4) WY acted directly on hepatocytes to down-regulate P450 2C11.

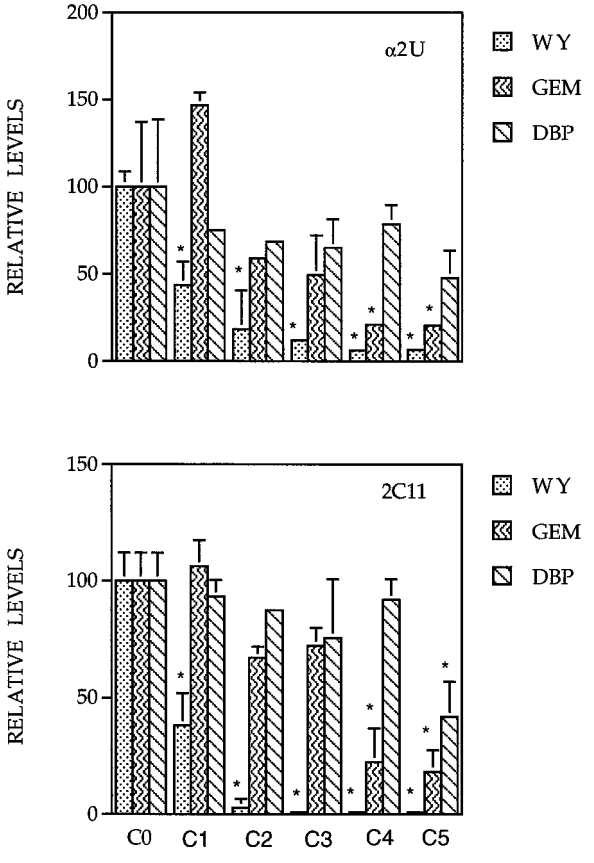

Fig. 4. Alteration of α2u and P450 2C11 protein levels after different dietary concentrations of PPC. Whole-cell liver extracts from male Sprague-Dawley rats fed a control diet or a diet containing five different concentrations of WY, GEM, or DBP for 5 weeks were separated by 12.5% (P450 2C11) or 15% (α2u) SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to α2u or P450 2C11. Expression was quantified as in Fig. 1D. Concentrations used in the study: C0, control; WY-C1, 5 ppm; WY-C2, 10 ppm; WY-C3, 50 ppm; WY-C4, 100 ppm; WY-C5, 500 ppm; GEM-C1, 10 ppm; GEM-C2, 100 ppm; GEM-C3, 1,000 ppm; GEM-C4, 8,000 ppm; GEM-C5, 16,000 ppm; DBP-C1, 1,500 ppm; DBP-C2, 2,500 ppm; DBP-C3, 5,000 ppm; DBP-C4, 10,000 ppm; DBP-C5, 20,000 ppm.

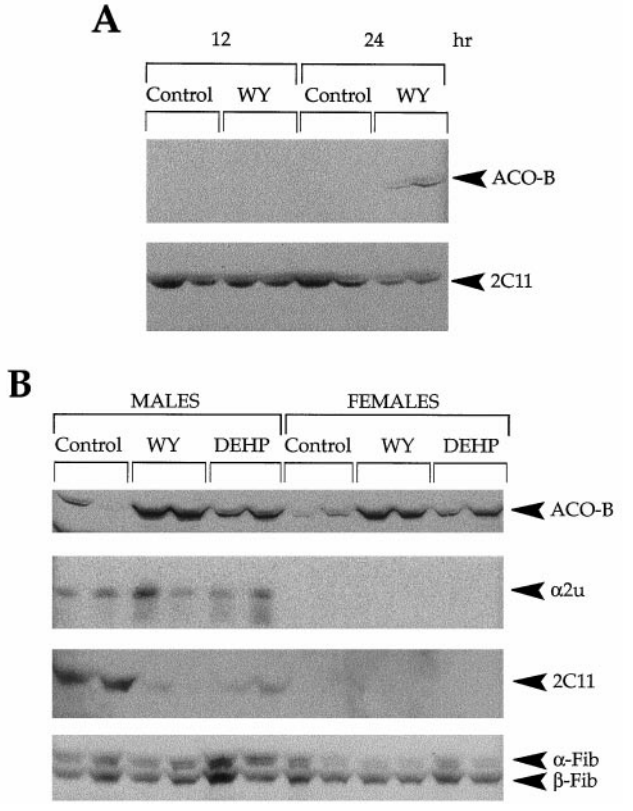

Fig. 5. Early changes in P450 2C11 protein expression after PPC exposure. A, Decreased expression of P450 2C11 protein after a single gavage dose of WY. Whole-cell extracts from male Fisher F344 rats treated with a single gavage dose of WY (50 mg/kg) or control methyl cellulose vehicle for 12 and 24 hr were size-separated on 12.5% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against ACO or P450 2C11. B, Down-regulation of P450 2C11 protein expression after 3-day exposure to WY or DEHP. Whole-cell liver extracts from male and female Fisher F344 rats given a gavage dose of WY (50 mg/kg), DEHP (2000 mg/kg), or methyl cellulose (*Control*) each day for 3 days were separated by 12.5% or 15% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to ACO, α2u, P450 2C11, or Fib.

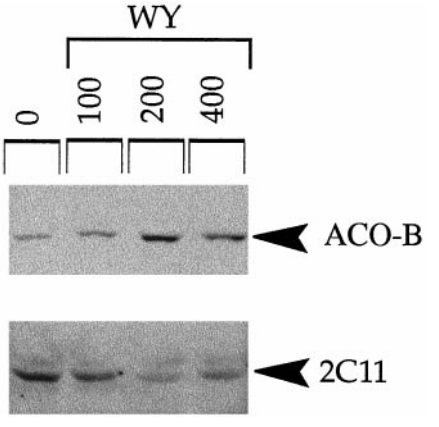

Fig. 6. Down-regulation of P450 2C11 after WY treatment in cultured rat primary hepatocytes. Primary rat hepatocytes were prepared as described in the text and treated with the indicated concentrations of WY or DMSO (0) for 48 hr. Cell extracts were used to examine protein expression by Western analysis.

Requirement for PPARα in PPC-regulation of a mouse homologue of α2u and positive acute-phase gene expression. Because PPARα has been shown to mediate many PPC-inducible responses in the liver, we examined the dependence of PPC-induced decreases in α2u and positive acute-phase gene regulation on PPARα expression. We fed wild-type mice and mice that lacked a functional form of PPARα (PPARα-null mice; Lee *et al.*, 1995) a control diet or one that contained WY (0.1%) or DEHP (0.6%) for 3 weeks. The RNA isolated from the mouse livers was used to assess gene expression. We first examined the expression of a mouse homologue of rat α2u. Mice possess a family of genes closely related to the rat α2u that are called MUPs. These proteins share conserved features of regulation, including regulation by GH, testosterone, estrogen, and inflammatory cytokines (Johnson *et al.*, 1995 and references therein). After feeding WY or DEHP, ACO mRNA levels rose dramatically in wild-type but not PPARα-null mice as expected (data not shown). When wild-type mice were fed WY, there was a decrease in the expression of MUP-I mRNA (14% of control) (Fig. 7A). In contrast, treatment with DEHP did not result in significant changes in the levels of MUP-I RNA. Treatment of PPARα-null mice with WY or DEHP resulted in no changes in MUP-I expression. Similarly, β-Fib and AGP mRNAs decreased after WY (26% and 19% of control, respectively), but not DEHP, feeding in wild-type mice. In PPARα-null mice, WY exposure did not alter the expression of β-Fib or AGP. DEHP exposure increased β-Fib levels (~4-fold), whereas AGP levels did not change.

We also examined the protein levels of ACO and Fib in the same livers of mice. ACO protein levels increased dramatically in wild-type, but not PPARα-null, mice after exposure to WY or DEHP, as expected (Fig. 7B). The levels of α- and β-Fib proteins decreased in wild-type (9% and 10% of control, respectively; $p < 0.05$), but not PPARα-null, mice after WY feeding. There was little, if any, decrease in Fib protein levels after exposure to DEHP in either strain of mice. These data demonstrate that PPARα is required for WY to down-regulate MUP-I and positive acute-phase gene expression.

Discussion

PPCs induce a broad spectrum of responses in the rodent liver, including peroxisome proliferation, cell proliferation, decreases in apoptosis, alteration of estradiol levels, increases in the metabolism of fatty acids and eicosanoids, and hepatocarcinogenesis (reviewed in Lapinskas and Corton, 1998). Most, if not all, of the effects of PPC exposure in the liver depend on the expression of PPARα (Lee *et al.*, 1995), the receptor for PPC, unsaturated long-chain fatty acids, and certain eicosanoids. Interaction with these ligands results in heterodimerization with the receptor for 9-*cis* retinoic acid RXR. The PPAR-RXR heterodimer binds to PPRE and regulates the expression of many genes involved in fatty acid metabolism, including the fatty acid β-oxidation genes and members of the *P450 4A* group of enzymes. In an effort to clone genes that are important in mediating the diverse effects of PPC, we (Corton and Gustafsson, 1997) and others (Alvares *et al.*, 1996) identified the male-specific pheromone carrier *α2 urinary globulin* as a down-regulated gene. This gene is under positive control by the male-specific GH secretory pattern and is negatively regulated under conditions of inflammation (Roy *et al.*, 1983; Birch and Schreiber, 1986). To determine whether other genes were coordinately regulated after PPC treatment, we examined the expression of genes that are regulated by sex-specific GH secretory pattern or that are induced under inflammatory conditions.

We show in this study that in addition to the *P4504A* family, PPC regulate members of the *P450 2C* family important in the metabolism of steroids. In contrast to the inducibility of *CYP4A* genes by PPCs, the male-specific *CYP2C11*

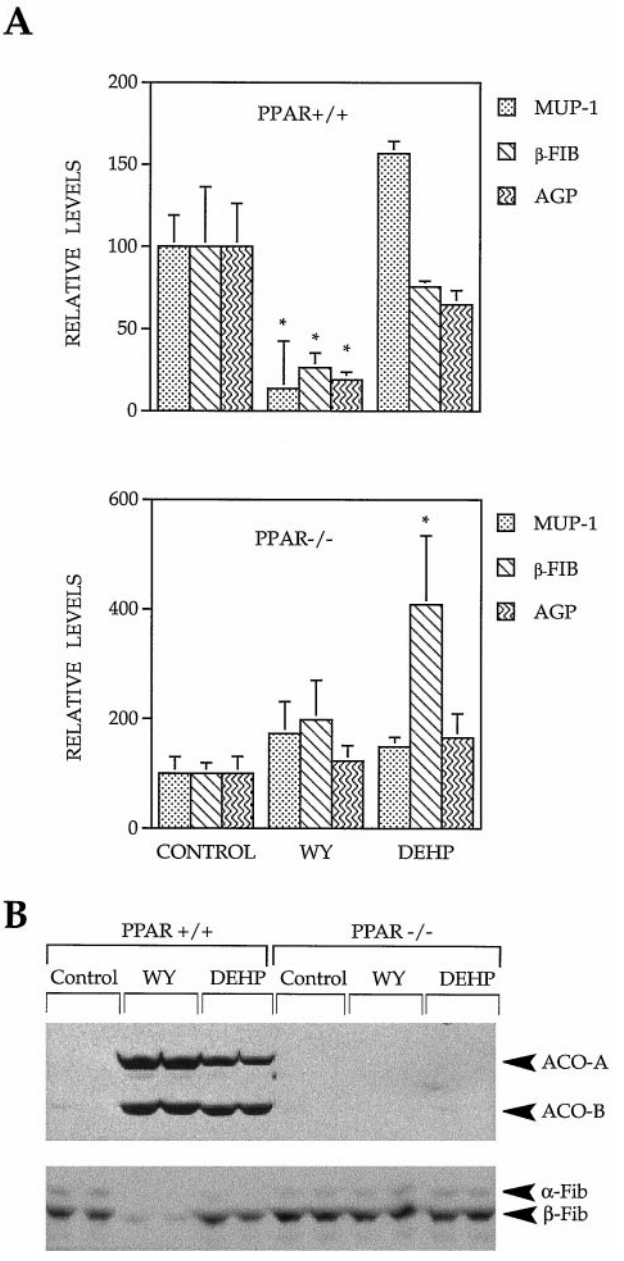

Fig. 7. Down-regulation of acute-phase response gene expression by WY is dependent on PPARα. Wild-type SV129 mice (+/+) or SV129 mice that lack PPARα (−/−) were fed a control diet (*Control*) or a diet containing WY (0.1%) or DEHP (0.6%) for 3 weeks. Total RNA or total protein isolated from the livers were used to analyze expression of the indicated mRNAs by Northern blot (A) or the indicated proteins by Western blot (B). The Northern autoradiograms were densitometrically scanned, and expression was normalized to β-actin expression. *Histograms*, mean expression (mean ± standard deviation) from three different samples. *, Significantly different from control ($p < 0.05$). Expression of Fib proteins was quantified as in Fig. 1D.

and the female-specific *CYP2C12* genes were down-regulated by WY and GEM. These *CYP2C* genes encode constitutive hepatic P450 enzymes that are regulated at the transcriptional level by GH (Legraverend *et al.*, 1992; Waxman, 1992). In our experiments, (1) P450 2C11 was down-regulated by WY, GEM, and DBP, and P450 2C12 was down-regulated by WY and GEM; (2) the levels of P450 2C11 and α2u proteins were coordinately regulated between 1 to 13 weeks of exposure to five different doses of WY or GEM; (3) under acute exposure conditions, the protein products of P450 2C11 and ACO, a PPARα-regulated gene, were altered within 24 hr of a gavage dose of WY, indicating that PPARα is important in the regulation of P450 2C11; and (4) not all members of the P450 2C family were coordinately down-regulated because the male-specific *CYP2C13* gene was not down-regulated by PPC treatment. In addition to the P4504A family members whose induction of expression is normally associated with PPC exposure, our results demonstrated that the P450 2C family, including 2C7 (Corton JC, Fan L-Q, and Brown S, unpublished observations), 2C11, and 2C12, are coordinately down-regulated by exposure to some PPCs. Thus, in addition to changes in the ω-oxidation of fatty acids and eicosanoids catalyzed by P450 4A members, exposure to certain PPCs would be expected to lead to changes in steroid metabolism.

Changes in estradiol metabolism have been observed in male and female rats after PPC exposure. Increases in estradiol levels in male rats after exposure may be partly attributed to decreases in P450 2C11. In male rats, exposure to the PPC ammonium perfluorooctanoate, DEHP, clofibrate, and WY (Eagon *et al.*, 1994; Hurtt *et al.*, 1997, and references therein) have been shown to increase serum E_2 levels. These increases have been hypothesized to contribute to Leydig cell hyperplasia and subsequent Leydig cell adenomas after long term exposure to PPC (Liu *et al.*, 1996). The experiments presented here indicate that the reactions catalyzed by P450 2C11, hydroxylations at the 2 and 16α positions of E_2 (summarized in Martucci and Fishman, 1993), would decrease in parallel with the down-regulation in P450 2C11. A decrease in the activity of 2α-hydroxylation of E_2 and male E_2 binding protein has been observed after treatment with DEHP (Eagon *et al.*, 1994). Elevation in the activity of aromatase observed in the male rat liver after treatment with ammonium perfluorooctanoate (Liu *et al.*, 1996) could also contribute to increases in E_2. Thus, it is possible that PPC exposure leads to modulation of estrogen receptor activation through alteration of E_2 levels.

Treatment of male rats with some PPCs also leads to changes in testosterone metabolism, which may be attributed to decreased levels of P450 2C11 protein. Decreases in the appearance of the 2α and 16α metabolites of testosterone, the products of P450 2C11-catalyzed hydroxylation of testosterone (reviewed in Waxman, 1991), were observed in microsomes from male rats treated for 2 weeks with clofibrate or DEHP (Lake *et al.*, 1984). Only minor increases in 6β-testosterone were observed after PPC exposure (Lake *et al.*, 1984), consistent with the minor changes we observed in the levels of P450 2C13, a 6β-testosterone hydroxylase (Waxman, 1991). With a decrease in P450 2C11 and associated testosterone metabolism in the male liver, the serum testosterone levels would be predicted to rise. In experiments that examined serum levels of testosterone, no changes were observed after treatment by PPC (Biegel *et al.*, 1992). Even though PPC treatment can lead to decreases in testosterone hydroxylation, the lack of increase in serum testosterone may be partly offset by increases in testosterone conversion to estradiol by aromatase after PPC treatment (Biegel *et al.*, 1995).

In addition to the *CYP2C* genes, we show that PPC exposure leads to down-regulation of two acute-phase response genes. The term acute-phase response refers to changes in the concentrations of a large number of plasma proteins reflecting reorchestration of the pattern of gene expression of secretory proteins in hepatocytes after a wide variety of adverse stimuli, including bacterial and viral infections, neoplasms, burns, and tissue infarction (reviewed in Moshage, 1997). These proteins are thought to increase the chances of survival of the patient and are divided into two groups: the positive acute-phase proteins such as β-Fib and AGP and negative acute-phase proteins such as transferrin and transthyretin. In our experiments, we demonstrated that β-Fib and AGP are down-regulated by PPC exposure. Decreases in expression of both β-Fib mRNA and AGP mRNA were observed at 13 weeks of exposure. Although WY, GEM, and DBP at the doses used seemed to be effective at down-regulating mRNA levels, β-Fib protein levels were decreased by GEM but not significantly by DBP or WY. This indicates that PPC may differentially affect post-transcriptional processes important in β-Fib expression. Differential post-transcriptional control by different PPC also has been shown to be important for expression of 17β-hydroxysteroid dehydrogenase IV (Corton *et al.*, 1996). As discussed below, the down-regulation of β-Fib and AGP is mediated by PPARα because down-regulation by WY is no longer observed in mice that lack a functional PPARα. Our results indicate that through PPARα, PPCs coordinately down-regulate many acute-phase response genes, including the positive acute-phase response genes β-Fib and AGP discussed here, as well as additional genes, α_1-antitrypsin, and ceruloplasmin (Anderson SA, Cattley RC, and Corton JC, manuscript submitted for publication). To our knowledge, our results represent the first example of down-regulation of basal expression of positive acute-phase response genes by pharmacological agents.

Fibrinogen has been recognized as a primary risk factor for the development of acute cardiovascular disease (Stone and Thorp, 1984). Fibrinogen, encoded by three separate genes in humans and rodents (α, β, and γ), is cleaved during clot formation by thrombin, yielding monomers that polymerize into fibrin and can act as a cofactor in platelet aggregation. Fibrinogen can contribute to cardiovascular disease through its promotion of red cell and platelet aggregation, increased rheologic stasis, and amplification of the coagulative cascade at higher concentrations. The down-regulation of β-Fib mRNA and α- and β-Fib proteins by PPCs that we observe may be the molecular basis for a number of reports that demonstrate a linkage between PPC exposure and changes in coagulation or platelet aggregation. First, in patients on hypolipidemic PPC therapy, total fibrinogen levels have been shown to be reduced (Monk and Todd, 1987, and references therein). Second, clofibrate enhanced anticoagulation when administered in conjunction with coumarins, requiring some reduction in the dosage of the anticoagulant, and a similar effect has been reported with benzafibrate in hyperlipoproteinemic patients requiring anticoagulant therapy (Vinazzer and Farine, 1983, and references therein). Last, rats treated

chronically with WY revealed hemorrhages at several sites and prolonged prothrombin and thrombin clot times indicative of defects in coagulation (Hurtt *et al.*, 1997). The use of hypolipidemic PPCs that decrease circulating fibrinogen, as well as cholesterol and saturated fatty acids, should provide maximum benefit to patients at risk of heart disease by depressing the processes in which fibrinogen is involved, including platelet aggregation, coagulation cascade, and blood viscosity.

PPARα has been shown recently to be important in down-regulating the inflammatory response. A major mediator of the inflammatory response, leukotriene B_4, was shown to bind and activate PPARα. Mice that lack PPARα have a prolonged inflammatory response time after exposure to inflammatory mediators compared with wild-type mice (Devchand *et al.*, 1996). Some nonsteroidal anti-inflammatory drugs, such as fenoprofen, ibuprofen, and indomethacin, also bind and activate PPARα (Lehmann *et al.*, 1997). These results indicate that PPARα is a negative regulator of the inflammatory response, possibly by increasing the inactivation of lipid mediators of inflammation through fatty acid β- and ω-oxidation. Our results support this hypothesis in that classic acute-phase response genes are down-regulated by PPC in the liver through a PPARα-dependent mechanism. We predict that PPARα-deficient mice are hypersensitive to inflammatory stimuli that affect expression of these positive acute-phase response genes in the liver.

How does PPC exposure lead to down-regulation of *CYP2C11* and *CYP2C12* gene expression? Expression of these and other members of the *CYP2C* family seem to be dependent on the sex-specific GH secretory pattern. It is possible, then, that exposure to PPC leads to changes in GH secretion and to *CYP2C* expression. The pattern of changes in *CYP2C11*, and *CYP2C12* expression by at least WY exposure has some of the characteristics of expression in rats that have undergone a hypophysectomy (i.e., severe drops in P450 2C11 levels in males and severe drops in P450 2C12 levels in females). Feminization of the GH secretory pattern by estradiol treatment of male rats similar to the increase in circulating estradiol levels observed after PPC treatment (discussed above) has been shown to lead to decreases in *CYP2C11* expression (Mode *et al.*, 1982). Despite these correlations, two observations argue that disruption of GH signaling is not the primary determinant of PPC-induced down-regulation of α2u and *CYP2C* family members. First, we can demonstrate down-regulation by WY of P450 2C11 expression in primary hepatocytes from male rats, indicating that the effect of PPC on expression is direct and is not mediated *in vivo* indirectly by perturbation of GH secretion. Second, the lack of changes in expression of *CYP2C13* is not consistent with PPC-induced effects on GH secretory pattern. Hypophysectomy leads to a decrease in the expression of *CYP2C13* in male rats to ~70% of wild-type levels and an increase in expression in hypophysectomized female rats to ~50% of wild-type male rats (Legraverend *et al.*, 1992). After PPC exposure, *CYP2C13* levels did not decrease in males, nor were there significant increases in females. The evidence indicates that WY is down-regulating expression of *CYP2C11* and *CYP2C12* by a mechanism that does not involve perturbation of GH signaling.

Our data and those of others indicate that exposure to some PPC leads to coordinate down-regulation of a number of genes predominately expressed in the liver. The down-regulated genes include *CYP2C11*, *CYP2C12*, *β-Fib*, *AGP* (our experiments), *α2u*, *transferrin*, *apolipoprotein cIII*, *apolipoprotein* E, *transthyretin*, and *BiP/GRP78* (summarized in Lapinskas and Corton, 1998) and a number of unique genes with no homology to gene bank databases (Corton and Gustafsson, 1997). There are a number of examples of members of the nuclear receptor superfamily down-regulating PPC-activated genes through either competition with PPAR for limited amounts of RXR heterodimerization partner (e.g., thyroid hormone receptor) or competition with PPAR for binding to PPRE (e.g., COUP-TF) (reviewed in Lapinskas and Corton, 1998). In these cases, however, PPC-induced but not basal gene expression is down-regulated, making it less likely that these mechanisms play an important role in down-regulation of liver-specific gene expression by PPC. The widespread effects of PPC exposure on hepatocyte gene regulation indicate that down-regulation could result from PPARα-dependent interference in liver-specific transcription factors that control the liver phenotype. Activated PPAR has been shown to disrupt and down-regulate HNF-4-mediated regulation of *apolipoprotein cIII* (Hertz *et al.*, 1995) and *transferrin* (Hertz *et al.*, 1996) genes by interfering with the ability of HNF-4 to activate at a PPRE-like element in the promoter regions of these genes as well as the *HNF-4* gene itself. Although the down-regulation of HNF-4 expression or activity by PPAR may be important for expression of some liver-specific genes, the ability of HNF-4 to positively regulate expression of P450 2C family members does not correlate with their down-regulation by PPC. Overexpression of HNF-4 protein had little effect on the expression of *CYP2C11* or *CYP2C12* genes but resulted in positive regulation of the expression of *CYP2C13* (Ström *et al.*, 1995), a gene not regulated by PPC. It is likely that PPCs down-regulate liver-specific gene expression through multiple pathways.

In summary, the current study demonstrates that PPC exposure results in down-regulation of P450 2C family members as well as acute-phase response genes in a manner that requires PPARα. Exposure to some PPCs is likely to alter the metabolism of steroids and DNA-damaging agents that are substrates for P450 2C members.

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Appendix II

Altered bcl-2 Family Expression During Non-Genotoxic Hepatocarcinogenesis In Mice

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Appendix III

Apoptosis, Mitosis, and Cyclophilin-40 Expression in Regressing Peroxisome Proliferator-Induced Adenomas

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Carcinogenesis 21: 647–652 (2000)