Cell cross-talk mediates PPARα null hepatocyte proliferation after peroxisome proliferator exposure

Teresa C.Weglarz and Eric P.Sandgren[1]

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, 2015 Linden Drive, Madison, WI 53706, USA

[1]To whom correspondence should be addressed
Email: sandgren@svm.vetmed.wisc.edu

Peroxisome proliferator activated receptorα (PPARα) mediates the liver's responses to peroxisome proliferator compounds. These responses include induction of specific hepatic enzymes, peroxisome proliferation and hepatocyte proliferation. PPARα null mice, which lack receptor in all cells of the body, do not respond to peroxisome proliferators, indicating that hepatocellular proliferation and other responses require the presence of this receptor in at least some cells. To determine if PPARα is required specifically in hepatocytes for each response, we used hepatocyte transplantation to generate chimeric livers composed of PPARα null and positive hepatocytes in PPARα null or positive hosts. Upon exposure to a peroxisome proliferator, peroxisome proliferation and enzyme induction were restricted to receptor positive hepatocytes, indicating that these responses are cell autonomous with respect to hepatocyte receptor status. However, both PPARα null and positive hepatocytes in chimeric livers displayed elevated DNA synthesis regardless of host receptor status, as long as at least some hepatocytes contained receptor. These findings indicate that the mitogenic response to peroxisome proliferators does not require PPARα in all hepatocytes.

Introduction

Peroxisome proliferators are a structurally diverse group of compounds to which humans frequently are exposed [reviewed in (1,2)]. They include commonly prescribed hypolipidemic drugs such as clofibrate, used worldwide alone or in combination with HMG-CoA reductase inhibitors (statins) or bile acid sequestrants, to treat hypercholesterolemia. Peroxisome proliferators also include industrial plasticizers such as di(2-ethylhexyl)phthalate, other industrial solvents and herbicides. These compounds are termed peroxisome proliferators because they can induce proliferation of hepatocyte peroxisomes, subcellular organelles that carry out β-oxidation of fatty acids and cholesterol metabolism. Exposure to these agents also induces transactivation of peroxisomal enzymes, hepatocellular proliferation and hepatocarcinogenesis in sensitive species. Many of these pleiotropic effects are mediated by peroxisome proliferator activated receptors (PPARs),

members of the steroid receptor superfamily. There are three isoforms of PPARs, α, γ and δ. PPARα is the predominant form in the liver. Following exposure to a peroxisome proliferator, (i) PPARα is activated, (ii) PPARα heterodimerizes with retinoid X receptor, then (iii) this dimer binds to the peroxisome proliferator response element in target genes and activates transcription. Targets include genes involved in the β-oxidation of fatty acids, such as acyl CoA oxidase and peroxisomal L-bifunctional enzyme (L-PBE).

As summarized by Melnick (3) in a recent commentary, despite improved understanding of molecular responses in cells exposed to peroxisome proliferator compounds, there remains controversy regarding the mechanism by which they induce cancer. Estimation of risk is particularly problematic because the response to peroxisome proliferators is species specific. Mice and rats display enzyme induction, peroxisome and hepatocyte proliferation and hepatocarcinogenesis. Thus, peroxisome proliferators had been classified as probable human carcinogens by the United States Environmental Protection Agency. However, human hepatocytes appear to be less responsive, displaying minimal peroxisome and hepatocyte proliferation following exposure (4). Based on these mechanistic considerations, the International Agency for Research on Cancer changed recently the classification of this class of compounds to 'not classifiable as to its carcinogenicity to humans'. Nevertheless, reduced responsivity does not exclude the possibility of cancer risk in humans (3). Specifically, humans display the hypolipidemic effects of peroxisome proliferator compounds via PPAR activation, so a carcinogenic risk should not be excluded until peroxisome proliferation is identified as a necessary step for carcinogenesis, and/or epidemiologic studies conclusively indicate that human exposure to medicinal, industrial or environmental peroxisome proliferators is not associated with increased incidence of cancer. Because of this uncertainty, it is imperative to identify the precise mechanism by which peroxisome proliferators induce carcinogenicity in rodents.

One important mechanistic consideration concerns the dependence of phenotypic response on the presence of PPARα in the target cell. Thurman and colleagues have determined that Kupffer cells (liver macrophages) are activated upon exposure to peroxisome proliferators (5) and release TNFα and other mitogenic cytokines (6–8), and inactivation of Kupffer cells in rats administered a peroxisome proliferator blocks hepatocyte proliferation and TNFα production but not peroxisome proliferation, as measured by acyl CoA oxidase activity (7). They therefore proposed that peroxisome proliferators induce hepatocyte proliferation via activation of Kupffer cells (9,10). However, Kupffer cells do not contain measurable PPARα (9), yet PPARα null mice display neither hepatocyte nor peroxisome proliferation (11), indicating the dependence of these responses on the receptor. Similarly, Hasmall *et al*. (12) using an *in vitro* system demonstrated that co-cultures of mouse non-parenchymal cells (NPC) and
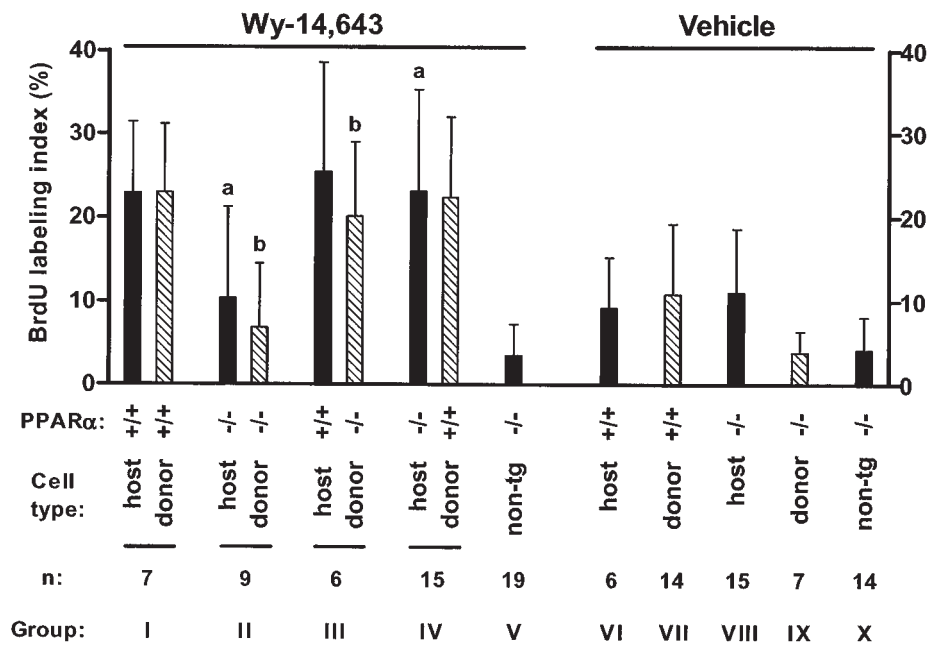
Fig. 1. Hepatocyte BrdU labeling indices in mice treated with WY-14,643 or vehicle. Mice with chimeric livers were implanted with a mini-osmotic pump that continuously released BrdU, then administered WY-14,643 or vehicle for 7 consecutive days by oral gavage. Livers then were examined to determine BrdU labeling indices for donor and host hepatocytes in each mouse examined. Data are expressed as mean ± SD. On average 1000 cells were counted for each population of hepatocytes. PPARα +/+ indicates receptor positive; PPARα −/− indicates receptor null. 'Host' indicates the hepatocyte recipient PPARα genotype, which is present in some hepatocytes and all other cells in the body; 'donor' indicates the donor hepatocyte PPARα genotype, which is present only in some hepatocytes in the chimeric liver. *n*: number of mice examined. a: Significantly different at $P = 0.017$ (host cell response in Group II versus Group IV); b: significantly different at $P = 0.012$ (donor cell response in Group II versus Group III). Each comparison used the Mann–Whitney test. For vehicle Groups VI–IX, data were pooled according to cell type (host versus donor) and PPARα receptor status.

PPARα positive (but not PPARα negative) hepatocytes supported hepatocyte DNA synthesis in response to peroxisome proliferator, regardless of whether the NPC were derived from PPARα positive or null mice, yet receptor positive hepatocytes did not proliferate following identical treatment when cultured without NPC. PPARα null hepatocytes did not proliferate with or without NPC. Collectively, these findings suggested that Kupffer cell induction of hepatocyte proliferation might require PPARα in hepatocytes.

To identify unequivocally the cell type within which PPARα is necessary and sufficient to induce hepatocyte proliferation in an intact animal, we generated mice that were chimeric for receptor status (+/+ versus −/−) in liver. We then exposed these mice to a potent peroxisome proliferator and correlated hepatocyte PPARα status with peroxisome proliferation, enzyme induction and hepatocyte proliferation.

Materials and methods

Animals

PPARα heterozygous null (+/−) mice in the SV/129 background were obtained from Dr Frank Gonzalez, National Cancer Institute, Bethesda, MD. These mice were backcrossed at least six generations into the C57BL/6 strain. PPARα null mice have been described (11), and are identified using PCR (11). We described recently transgenic C57BL/6 strain mice carrying a major urinary protein-urokinase-type plasminogen activator (MUP-uPA) construct (13,14). These mice are identified using PCR, as described (13). Hepatocyte-targeted expression of uPA is hepatotoxic (15). However, individual hepatocytes can delete or otherwise stably inactivate expression of the transgene, and subsequently proliferate following release from uPA-mediated toxicity, to repopulate the liver with healthy, transgene-deficient parenchyma (15). Similarly, livers in young uPA-expressing transgenic mice support extensive repopulation by transplanted donor hepatocytes (13,14,16). By 2 months post-transplant, MUP-uPA recipient mouse livers are chimeric: they contain donor

hepatocyte clones derived from the healthy transplanted hepatocytes, and endogenous hepatocyte clones derived from host hepatocytes that inactivated transgene expression (13). MUP-uPA transgenic mice of the C57BL/6 strain were used as recipients for transplanted syngeneic hepatocytes in the studies described below, and the extent of repopulation generally ranged from 20 to 80%. All donor cells were marked by a human placental alkaline phosphatase (hPAP) transgene to permit unequivocal identification (17). Transgenic mice used in these studies have been assigned the following genetic designations: MUP-uPA line 350-2, TgN(MUPPlau)1Eps; R26-hPAP line 952-1, TgN(R26ALPP)8Eps. Mice were housed and maintained in accordance with the Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison.

Liver cell isolation and transplantation

Donor hepatocytes carrying the R26-hPAP transgene (17) were isolated via two-step EDTA/collagenase perfusion as described (13). Recipient mice were 2–4 weeks of age at the time of hepatocyte transplant. We transplanted two classes of donor hepatocytes, PPARα positive and PPARα null, into spleens of two classes of recipient transgenic mice, PPARα positive and PPARα null. Using this approach, ~10^5 transplanted hepatocytes, or 0.1% of adult hepatocyte number, seed liver via the portal circulation (13). Donor hepatocytes then undergo 10–12 cell doublings during repopulation, but contaminating donor non-parenchymal cells are not subject to growth stimulus and therefore would not be amplified. All recipient mice were maintained for at least 16 weeks to allow for repopulation by and reorganization of donor hepatocytes before treatment.

Experimental design

Five experimental groups were administered peroxisome proliferator (Figure 1): (I) PPARα-positive MUP-uPA transgenic mice that received PPARα-positive donor hepatocytes; (II) PPARα null MUP-uPA transgenic mice that received PPARα null donor hepatocytes; (III) PPARα-positive MUP-uPA transgenic mice that received PPARα null donor hepatocytes; (IV) PPARα-null MUP-uPA transgenic mice that received PPARα-positive donor hepatocytes; and (V) PPARα null mice. Additional experimental groups received vehicle without peroxisome proliferator (VI–X). At 16 or more weeks post-transplant, recipient mice were implanted with a mini-osmotic pump (Alzet model 2001, Alza Pharmaceuticals, Palo Alto, CA) containing 0.2 ml of 16 mg/ml of the nucleotide analog 5-bromo-2′-deoxyuridine (BrdU), which
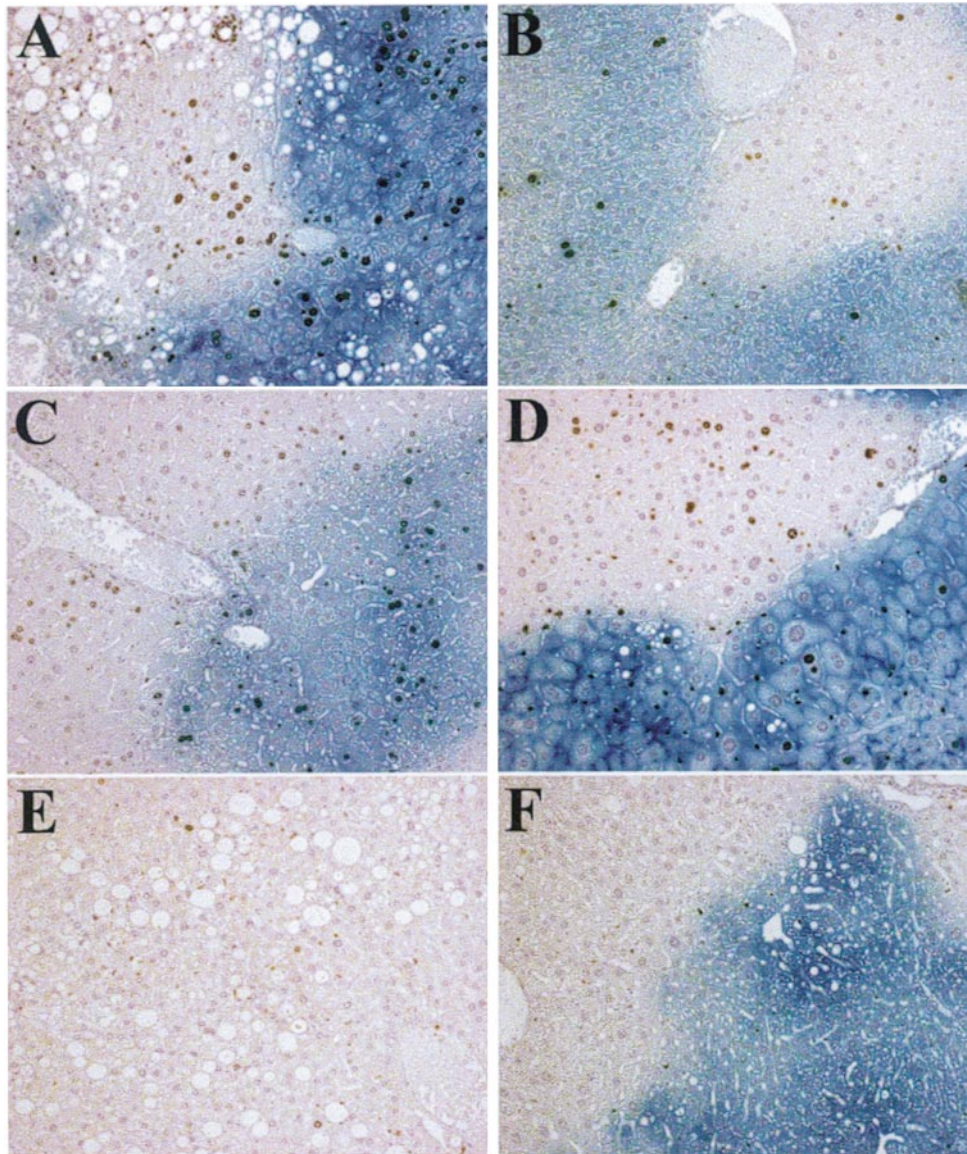
Fig. 2. Hepatocyte BrdU labeling. Livers were treated to detect hPAP activity (blue stain) to localize donor hepatocytes, then incubated with an anti-BrdU antibody to identify cells undergoing DNA synthesis (darkly-labeled nuclei). (A–E) WY-14,643-treated; (F) vehicle-treated. (**A**) PPARα positive donor and host mouse hepatocytes (Group I from Figure 1). (**B**) PPARα null donor and host hepatocytes (Group II). (**C**) PPARα null donor and positive host hepatocytes (Group III). (**D**) PPARα positive donor and null host hepatocytes (Group IV). (**E**) PPARα null non-transgenic mouse liver (Group V). (**F**) Vehicle-treated PPARα positive donor and host hepatocytes, which do not display the elevated BrdU labeling present in (A). Original magnification 200×.

is incorporated into DNA of cells undergoing DNA synthesis (14). All were administered either 50 mg/kg of the peroxisome proliferator WY-14,643 [4-chloro-6-(2,3-xylidino)pyrimidinylthio-acetic acid; ChemSyn Laboratories, Lenexa, KS] in 2% methylcellulose vehicle (Sigma) or methylcellulose only by oral gavage for 7 consecutive days.

On day 7, mice were killed. Liver was fixed in Carnoy's fixative for 30–60 min at 4°C, transferred to 70% EtOH, and embedded in paraffin, then 6 μm liver sections were cut. Liver sections were (i) treated histochemically to detect hPAP, which marked donor cells, and/or (ii) treated immunohistochemically to detect BrdU, using a rat monoclonal anti-BrdU antibody (Accurate Scientific) (14), or to detect L-PBE, using a rabbit anti-L-PBE antiserum (generously provided by Dr J.Reddy) (18). Separate pieces of liver were frozen in Histoprep compound (Fisher), sectioned at 10 μm, then treated histochemically to detect hPAP or to detect peroxisomes using a catalase reaction (18). The BrdU labeling index of both endogenous and donor hepatocytes was determined by examining approximately 1000 hepatocyte nuclei per slide for each category of hepatocyte, and expressed as the percentage of BrdU-labeled hepatocyte nuclei. Hepatocytes immediately adjacent to a donor/host boundary were not included in the counting. The Mann–Whitney test was used to compare different populations of cells and treatment groups.

Results

Control chimeric mice with identical receptor status in both host and donor cells responded as expected to a peroxisome proliferator (19). WY-14,643 administration increased proliferation (as measured by the % hepatocyte nuclei that incorporated BrdU) in both host and donor PPARα positive hepatocytes in chimeric mice (Figure 1, Group I and Figure 2A, +/+ host and +/+ donor) relative to the comparable cell type in vehicle-treated chimeric mice (see Figure 2F, Groups VI and VII). When both host and donor cells were PPARα null, proliferation was not increased by WY-14,643 treatment (Figure 2B, Group II versus Groups VIII and IX). We did observe increased proliferation of WY-14,643-treated endogenous null hepatocytes in chimeric transgenic mice versus null hepatocytes in non-transgenic mice (Figure 2B and E, respectively, Group II versus Group V; $P = 0.02$), mimicking

the increase in BrdU labeling in unmanipulated adult MUP-uPA versus adult non-transgenic mouse liver following a single injection of BrdU ($P = 0.02$). The data point to a transgene effect and indicate that appropriate comparisons must be between chimeric mouse hepatocytes.

In striking contrast, the response was different in chimeric mice discordant for receptor status (Figure 1, Groups III and IV; Figure 2C and D, respectively). Note that in experimental Group III some hepatocytes are PPARα null but remaining hepatocytes and all other cells in the body are PPARα positive,

whereas in experimental Group IV the converse is true. In each group of these mice, proliferation of both PPARα positive and null hepatocytes increased in response to WY-14,643. The increase was equivalent to that in Group I mice that were PPARα positive in all cells, and proliferation of PPARα null hepatocytes in Groups III and IV were significantly elevated relative to the respective Group II control hepatocytes ($P < 0.017$). Thus, hepatocyte proliferation in response to a peroxisome proliferator does not require the presence of the receptor in proliferating cells. Additionally, although PPARα must be
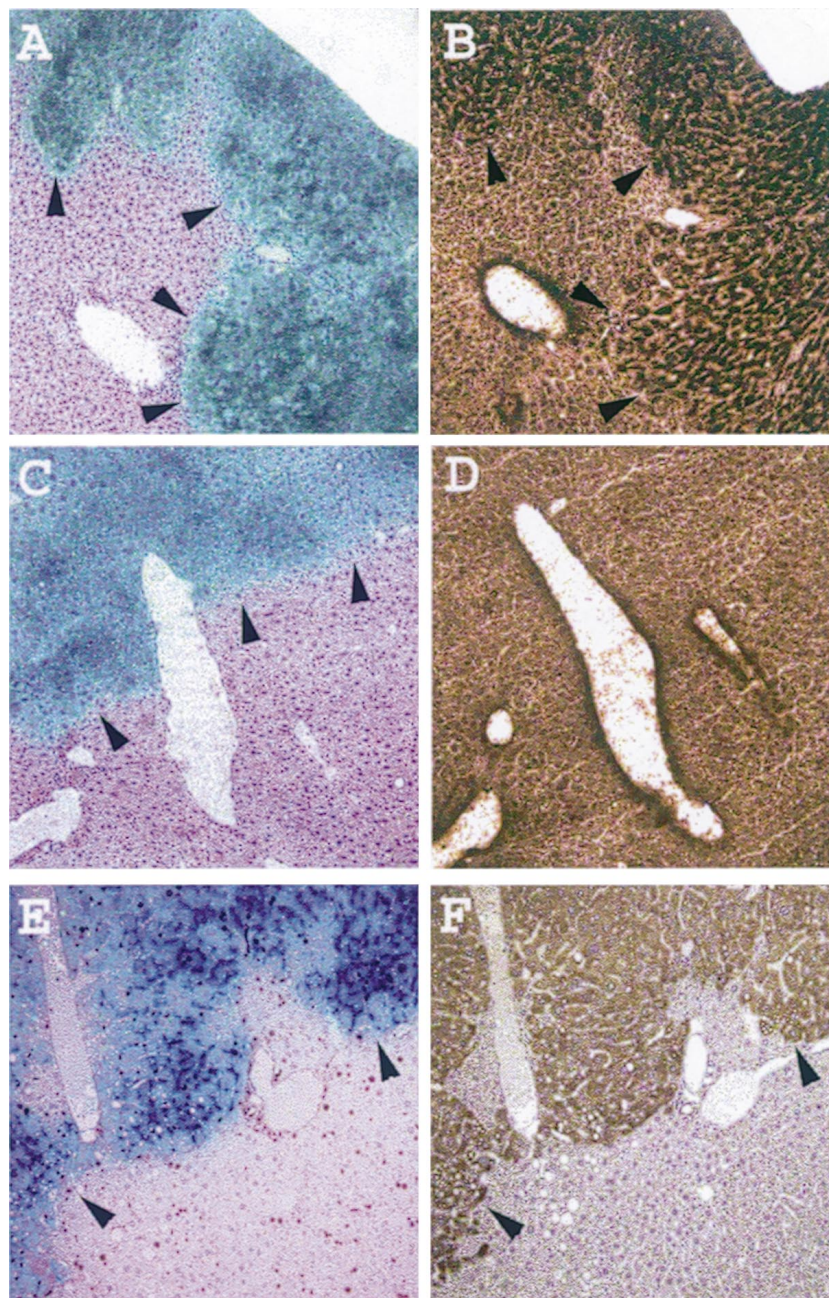

Fig. 3. Localization of peroxisomal catalase and L-PBE in chimeric mouse liver. (**A**, C and E) Livers were treated to detect hPAP activity, indicated by blue stain, to localize donor hepatocytes. (B and D) Adjacent sections were stained to localize catalase activity (dark brown). In (**B**), only PPARα positive donor hepatocytes in a PPARα null recipient mouse (Group IV) display increased catalase staining (dark brown). The boundary between donor (blue) and host hepatocytes is demarcated clearly by staining intensity in both (A) (hPAP) and (B) (arrowheads). (**C** and **D**) Neither receptor null donor (blue) nor receptor null recipient hepatocytes (Group II) display increased catalase staining (boundary indicated by arrowheads). (**E** and **F**) Adjacent sections in Group IV liver stained for hPAP (E) and L-PBE (F). Receptor positive donor hepatocytes stain strongly, indicating induction of L-PBE, while receptor negative recipient hepatocytes do not (boundary indicated by arrowheads). BrdU-labeled hepatocyte nuclei also are visible throughout receptor null endogenous parenchyma in (E). Original magnification 100×.

present in at least some hepatocytes to induce proliferation, as expected it was not required in NPC (Group IV). Because of inter-animal variability we were not able to determine if the magnitude of any response correlated with the extent of hepatic repopulation. However, our data suggest that induction of proliferation in receptor negative hepatocytes can occur when as few as 20% of hepatocytes possess receptor.

We also evaluated the relationship between PPARα status and both peroxisome proliferation and induction of peroxisome proliferator-responsive genes. We measured the former by histochemical detection of catalase activity, and the latter by immunohistochemical detection of L-PBE. In contrast to hepatocyte proliferation, there was a precise correspondence between catalase activity or L-PBE induction and hepatocyte PPARα status (Figure 3 and data not shown) in livers from all WY-14,643-treated chimeric mice (Figure 1, Groups I–IV). The data indicate that peroxisome proliferation and enzyme induction *in vivo* require the presence of receptor in the responding hepatocyte.

Discussion

Our findings demonstrate the dependence of several peroxisome proliferator-induced hepatic phenotypes on the presence of PPARα in specific cell types. PPARα null mice, which lack receptor in all cells of the body, do not respond to peroxisome proliferators, indicating that hepatocellular proliferation and other responses require the presence of this receptor in at least some cells (11). PPARα null hepatocytes in chimeric livers lack peroxisome proliferation and transcriptional activation of a peroxisome proliferator responsive gene, indicating that these responses require PPARα in the responding cell (Figure 3). These events therefore are cell autonomous with respect to receptor status. In contrast, induction of hepatocyte proliferation is not cell autonomous. This response merely requires PPARα in some hepatocytes (Figure 1, Groups III and IV). Interestingly, PPARα null hepatocyte proliferation is normal when induced by the hepatomitogen TCPOBOP (20) or by partial hepatectomy (21), indicating that these stimuli activate different growth signaling pathways than peroxisome proliferators.

Our observations support a model for peroxisome proliferator-mediated induction of hepatocyte proliferation *in vivo* that is consistent with findings of Thurman and colleagues (5,7,10,22) and Hasmall *et al.* (12). Previous reports by these groups and others indicated that both Kupffer cells and PPARα-positive hepatocytes were necessary to support hepatocyte proliferation; neither alone was sufficient. Peroxisome proliferators can activate Kupffer cells directly, possibly via stimulation of oxidant production (23), even in PPARα null mice (5). The significance of Kupffer cell activation is emphasized by the finding that conditioned medium from WY-14,643-treated Kupffer cells produced a slight (2-fold) increase in rat hepatocyte DNA synthesis *in vitro* (24). Whether DNA synthesis in this experimental system requires PPARα in the responding hepatocytes was not addressed. However, direct Kupffer cell activation (and corresponding cytokine release) cannot be sufficient to induce hepatocyte replication *in vivo*, since hepatocytes in peroxisome proliferator-treated PPARα null mice do not proliferate (11).

The data still do not explain the mechanism underlying the dependence of hepatocyte proliferation on cross talk between hepatocytes and Kupffer cells. In one model, Kupffer cell and hepatocyte activation by peroxisome proliferators would be independent processes. Kupffer cells would be stimulated to release a pro-mitogenic substance like TNFα or IL-1β via a PPARα-independent mechanism, while hepatocytes would undergo PPARα-dependent replication priming and become competent to respond to the mitogenic cytokine. Interestingly, TNFα is not necessary for this response, as hepatocyte replication is observed in TNFα null and TNFα receptor null mice following peroxisome proliferation administration (8,25). In a second model, Kupffer cell and hepatocyte activation remain separate processes, but receptor positive hepatocytes produce a signal (secreted or passed via intercellular junctional complexes) that renders all hepatocytes responsive to mitogens. In a third model, signaling would be linear: activation of receptor positive hepatocytes produces a signal that instructs Kupffer cells to release key mitogenic substances (of course, receptor-independent Kupffer cell activation may be required to render Kupffer cells competent to respond to this signal). Our findings argue against the first model for regulation of peroxisome proliferator-mediated hepatocyte replication. In a PPARα null host, the presence of some PPARα positive hepatocytes is sufficient (and necessary) to induce replication in all hepatocytes, regardless of receptor status, in response to a peroxisome proliferator. Thus, hepatocyte replication is non-cell autonomous with respect to hepatocyte PPARα status. Future investigations must continue to address the hepatocyte- and Kupffer cell-derived signals that are active in this pathway.

This model has several implications. If carcinogenicity depends on peroxisome proliferation and/or transcriptional activation of PPARα-responsive genes, chimeric mice chronically exposed to a peroxisome proliferator will develop only PPARα-positive liver tumors. If hepatocyte proliferation alone induces cancer, both PPARα-positive and null tumors should develop in chimeric mice. As noted by Melnick, this information would be important for evaluating the carcinogenic risk of human exposure (3). In addition, intervention strategies to reduce cancer risk in exposed individuals would vary considerably depending on the mechanism of peroxisome proliferator-associated carcinogenesis.

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