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# Review Article

# Novel Role of Oxidants in the Molecular Mechanism of Action of Peroxisome Proliferators

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## ABSTRACT

**Peroxisome proliferators are nongenotoxic rodent carcinogens that act as tumor promoters by increasing cell proliferation; however, their precise mechanism of action is not well understood. Oxidative DNA damage caused by leakage of hydrogen peroxide ($H_2O_2$) from peroxisomes was hypothesized initially as the mechanism by which these compounds cause liver tumors. It seems unlikely that oxidants of peroxisomal origin explain the mechanism of action of peroxisome proliferators because treatment with these compounds *in vivo* does not lead to increased $H_2O_2$ production. On the other hand, Kupffer cell-derived oxidants, such as superoxide, may play a role in initiating tumor nerosis factor-$\alpha$ (TNF-$\alpha$) production that leads to hepatocyte proliferation. Peroxisome proliferators have been shown to activate Kupffer cells both *in vitro* and *in vivo*, and the use of Kupffer cell inhibitors such as methyl palmitate and dietary glycine have demonstrated that Kupffer cells are responsible for hepatocyte proliferation by mechanisms involve TNF-$\alpha$. Moreover, peroxisome proliferators activate the transcription factor NF-$\kappa$B, one of the major regulators of TNF-$\alpha$ expression, in Kupffer cells. Importantly, activation of NF-$\kappa$B by peroxisome proliferators was shown to be oxidant-dependent, leading to the hypothesis that oxidants of Kupffer cell origin are involved in the mechanism of action. Many of the effects of peroxisome proliferators, including peroxisome induction and hepatomegaly, involve the peroxisome proliferator-activated receptor-$\alpha$ (PPAR$\alpha$). Recently, it was shown that peroxisome proliferator-induced cell proliferation and tumors require the PPAR$\alpha$. However, PPAR$\alpha$ is not involved in TNF-$\alpha$ production by Kupffer cells because it is not expressed in this cell type. How it is involved in liver tumor remains unclear and one possible explanation is that both Kupffer cell TNF-$\alpha$ and parenchymal cell PPAR$\alpha$ are required. Collectively, recent data are consistent with the hypothesis that oxidants play a role in signaling hepatocellular proliferation due to peroxisome proliferators via activation of NF-$\kappa$B and incrase in mitogenic cytokines such as TNF-$\alpha$.** Antiox. Redox Signal. 2, 607–621.

## PEROXISOME PROLIFERATORS ARE A CLASS OF XENOBIOTICS THAT MAY BE IMPORTANT FOR HUMAN RISK ASSESSMENT

### *General use, sources of human and animal exposure*

Many chemicals have been shown to produce similar pleiotropic responses in rats and mice consisting of hepatomegaly, induction of peroxisomal enzymes of fatty acid $\beta$-oxidation, and an increase in both the number and size of peroxisomes (Reddy and Krishnakantha, 1975). Importantly, continuous exposure of rodents to these agents for 1 to 2 years leads to the development of hepatocellular carcinomas (Reddy *et al.*, 1980; Lake, 1995). On the basis of the striking similarity of their effects they have

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been combined in one group called "peroxisome proliferators." Due to a wide range of classes of chemicals that fall into this category, these chemicals are often referred to as "structurally dissimilar," or "chemically unrelated" (Cohen and Grasso, 1981; Reddy and Lalwani, 1983). To name just a few, clinically used pharmaceuticals, plasticizers, food flavors, herbicides, and industrial solvents all have representative compounds that produce peroxisomal proliferation in rodent liver (IARC, Working Group on Peroxisome Proliferation, 1995).

One of the most important groups of peroxisome proliferators, the phthalate esters, are widely used as plasticizers to impart softness and flexibility to polyvinyl chloride (PVC) resins for fabricating flexible vinyl products. These PVC resins are used to manufacture many products, including teething rings, pacifiers, soft squeeze toys, balls, vinyl upholstery, vinyl gloves, food containers, and flexible devices for administering parenteral solutions (Huber *et al.*, 1996). Total U.S. production of dioctyl phthalates, the common name for a group of related phthalate esters such as di(2-ethylhexyl) phthalate, remained almost constant at about 300 million pounds/year in 1980s (National Toxicology Program, 1998). There is particular concern of possible health effects to children because plasticizers are used in pacifiers and other plastic children's toys. Indeed, on December 1, 1999, the European Union (EU) Commission approved an emergency EU-wide ban on use of phthalates in chewable children' toys. Another potential high-risk segment of the population consists of individuals receiving treatments via phthalate-containing tubing or containers. For instance, it was estimated that exposure levels in some patients may be up to 1–2 mg di(2-ethylhexyl) phthalate/day (Huber *et al.*, 1996), and in 3- to 12-month-old infants chewing on their toys to be about 10–100 $\mu$g/kg/day (Wilkinson and Lamb, 1999). Moreover, a substantial fraction of the U.S. population is exposed to measurable levels of phthalates via inhalation, ingestion, and dermal contact, as total releases of these chemicals to the environment are measured in millions of pounds per year, mainly from disposal of plastic products.

*Studies in laboratory animals*

In rodents, peroxisome proliferators cause liver enlargement that is due to both hyperplasia and hypertrophy of parenchymal cells. The major biochemical changes include induction of peroxisomal lipid metabolizing enzymes and the CYP4A subfamily of iso-enzymes (reviewed in Doull *et al.*, 1999). While marked effects have been observed in liver parenchyma, only insignificant increases in transcription of peroxisomal $\beta$-oxidation enzymes have been detected in other tissues such as kidney, intestine, or heart (Nemali *et al.*, 1988). Another important feature characteristic of this class of chemicals includes marked and rapid induction of cell proliferation in rodent liver *in vivo*, an effect that is relatively small in isolated hepatocytes (Marsman *et al.*, 1988). This effect is transient in nature; however, potent carcinogens from this class of chemicals can increase cell proliferation as long as they are administered leading to the development of liver tumors (Reddy and Lalwani, 1983). Moreover, peroxisome proliferators decrease apoptosis both *in vivo* (Marsman *et al.*, 1992) and *in vitro* (Gerbracht *et al.*, 1990; Bayly *et al.*, 1994). Increased cell proliferation and decreased apoptosis are two processes by which initiated cells may achieve a selective growth advantage. Indeed, the hepatocarcinogenic effects of different peroxisome proliferators have been demonstrated in rats and mice (Reddy *et al.*, 1980; National Toxicology Program, 1982).

*Human response and possible hazard*

Several studies have examined the potential human response to peroxisome proliferators. In an *in vitro* study in human hepatocytes, these chemicals had no significant effect on peroxisomal enzyme activities, number of peroxisomes, or replicative DNA synthesis (Lake, 1995). Furthermore, when several known rodent peroxisome proliferators were administered to human volunteers, no human peroxisome proliferative response was observed (Ashby *et al.*, 1994). Because some of the therapeutic hypolipidaemic agents are carcinogenic in rodents, several clinical trials have looked into their potential human carcino-

genicity. Historically, increased mortality from cancer of the lung and of the lymphatic and hematopoietic system has been linked to a low serum cholesterol, but was interpreted as a result of a confounding factor because it was restricted to certain groups within cohorts studied (Law and Thompson, 1991; Jacobs *et al.*, 1992). Also, two clinical randomized trials (WHO Clofibrate Study and Helsinki Heart Study) yielded no long-term differences in the incidence of death from malignancy in patients who received hypolipidaemic therapy with fibrates (Huttunen *et al.*, 1994). At the same time, one study reported that clinical use of these chemicals increases malignancy risk for humans (Newman and Hulley, 1996); however, this has been challenged and remains controversial (Dalen and Dalton, 1996).

## PEROXISOME PROLIFERATION: WHAT DOES IT MEAN?

### *Physiological role of peroxisomes*

Peroxisomes, the intracellular organelles that contain several hydrogen peroxide ($H_2O_2$)-producing oxidases and catalase, are present in virtually all eukaryotic cells. Morphologically, they are characterized by a fine granular matrix surrounded by a single membrane (De-Duve and Baudhuin, 1966). The average diameter of the peroxisomes in liver and kidney where they are most abundant is 0.5 $\mu$m; however, in other tissues they are two-to three-fold smaller (Novikoff *et al.*, 1973). The major function of peroxisomes is the $\beta$-oxidation of fatty acids and their derivates. In animal cells both mitochondria and peroxisome are capable of $\beta$-oxidizing fatty acids via a similar four-step mechanism: initial oxidation, hydration, second oxidation, and release of acyl-CoA from the original molecule with the latter re-entering the $\beta$-oxidation spiral (Lazarow and De-Duve, 1976). The first step of this reaction, catalyzed by a FAD-containing acyl-CoA oxidase, is rate limiting (Bronfman *et al.*, 1984). Despite the similarities in degradation of saturated and unsaturated fatty acids by mitochondria and peroxisomes, there are several important differences between the two systems. Peroxisomal $\beta$-oxidation of fatty acids is unique because it produces $H_2O_2$ instead of NADH, and the energy that is produced in the first oxidation step is lost as heat (Mannaerts *et al.*, 1979). The former is important because it was initially thought that these compounds caused oxidative stress leading to oxidized DNA bases (Lazarow, 1978) (see below); however, $H_2O_2$ is degraded by catalase, which is abundant in peroxisomes. Other important metabolic functions of peroxisomes include $\beta$-oxidation of cholesterol derivates into biliary salts, D- and L-amino acid oxidation, polyamine breakdown, and purine catabolism (Lazarow, 1987).

### *Mechanisms of induction of peroxisome proliferation*

The initiation of peroxisome proliferation in parenchymal cells was proposed to be a result of activation of an intracellular receptor, and/or substrate overload because peroxisome proliferators can serve as a substrate for peroxisomal enzymes (Reddy and Lalwani, 1983; Bentley *et al.*, 1993). Indeed, Issemann and Green (1990) cloned a peroxisome proliferator-activated receptor (PPAR) from mouse liver, and later three nuclear receptors were found to share significant sequence similarity. Of those, PPAR$\alpha$ mediates the activation of genes encoding peroxisomal enzymes through dimerization with the retinoid X receptor $\alpha$ (RXR$\alpha$) and binding to *cis*-acting regulatory elements upstream of the promoter regions in target genes (Schoonjans *et al.*, 1997). Indeed, binding of peroxisome proliferators to PPAR$\alpha$ has been demonstrated (Kliewer *et al.*, 1997). In rats and mice, PPAR$\alpha$ is expressed at high levels in the liver and kidney, the primary sites of peroxisome proliferation. Furthermore, PPAR$\alpha$-null mice lack morphologic and biochemical responses to peroxisome proliferators (Lee *et al.*, 1995). Besides lipid metabolism, PPAR$\alpha$ activation is also involved in gluconeogenesis, alteration of cytochrome P450s and acute-phase proteins, changes in fibrinogen levels, and other effects (reviewed in Torra *et al.*, 1999). Importantly, PPAR$\alpha$-dependent alterations in cell cycle regulatory proteins, which includes

changes in cyclins and cyclin-dependent kinases, have been reported recently (Peters *et al.*, 1998). PPAR$\gamma$ is highly expressed in white and brown adipose tissues, where it is required for the differentiation of adipocytes and the tissue-specific regulation of genes (Spiegelman, 1998). Moreover, it was recently shown that PPAR$\gamma$ is not expressed in hepatocytes but is abundant in Kupffer cells (Peters *et al.*, 2000). Unlike other two isoforms, PPAR$\beta$ is more ubiquitously expressed and is most abundant in the central nervous system; however, the target genes and physiologic function of PPAR$\beta$ are not yet completely understood (Lemberger *et al.*, 1996).

Furthermore, it has been suggested that peroxisome proliferators inhibit fatty acid oxidation and displace fatty acids from cytosolic fatty acid-binding proteins, thus causing an increase in levels of fatty acids and an increase in peroxisomes due to substrate overload (Lock *et al.*, 1989; Bojes and Thurman, 1996a). However, this adaptive response to perturbations in lipid metabolism cannot be differentiated from the PPAR pathway, since fatty acids and products of their metabolism are also ligands for PPAR$\alpha$ (Devchand *et al.*, 1996; Kliewer *et al.*, 1997). Indeed, Bentley *et al.* (1993) have suggested that these two hypotheses are not mutually exclusive.

### *Species differences*

Large species differences in the sensitivity to peroxisome proliferators exist. Of many species, mice and rats are most responsive, whereas humans and nonhuman primates chronically exposed to fibrate drugs are responsive only to hypolipidemic effects, but showed little evidence of peroxisome proliferation (see above). Whether this is due to lower doses given to humans remains a subject to discussion. Rats and mice given these agents for prolonged periods of time develop hepatocellular adenomas and carcinomas; hamsters and rabbits also exhibit peroxisome proliferation, albeit to a lesser degree than rats (Watanabe *et al.*, 1989; Makowska *et al.*, 1992). In contrast, guinea pigs and marmosets are not responsive, even to potent agents such as nafenopin and WY-14,643 (Lake *et al.*, 1989, 1993). The mechanisms by which humans and some other species are resistant to peroxisome proliferation are debatable. Even though species differences in the absorption, distribution, metabolism, and excretion of peroxisome proliferators have been reported, it was suggested that they could not comprehensively explain this phenomenon. On the other hand, it is known that humans possess a functional PPAR$\alpha$, indicating that lack of receptor is not responsible for the lack of peroxisome proliferation upon treatment with these compounds (Sher *et al.*, 1993; Palmer *et al.*, 1998). However, expression of PPAR$\alpha$ mRNA in liver is an order of magnitude lower in humans than in mice. Moreover, human PPAR$\alpha$ mRNA does not encode a wholly functional PPAR$\alpha$, which results in over 10-fold lower amounts of PPAR$\alpha$ DNA-binding activity compared to the mouse (Palmer *et al.*, 1998). In addition, the human acyl-CoA oxidase gene lacks a peroxisome proliferator-responsive element (PPRE) that is required for activation of gene expression by PPAR$\alpha$, possibly explaining differences between humans and rodents (Lambe *et al.*, 1999; Woodyatt *et al.*, 1999). Finally, it was shown that when mouse PPAR$\alpha$ and its heterodimerization partner RXR$\alpha$ were introduced to guinea pig hepatocytes, where PPAR$\alpha$ expression is low, the response to peroxisome proliferators was comparable to that in mice and rats (Macdonald *et al.*, 1999). Collectively, from these data it is evident that differences in PPAR$\alpha$ might play an important role in the lack of response to peroxisome proliferators in hepatocytes from humans and other unresponsive species. This observation is of a particular importance for human risk assessment because it is known that humans have less than one-tenth the levels of PPAR$\alpha$ expression observed in mice and that reduced levels may be the result of lower transcription rates, inefficient premessenger RNA splicing, or both (Palmer *et al.*, 1998).

### *Peroxisome proliferation as a marker for carcinogenic potency*

Induction of peroxisomes has been hypothesized to play a role in the carcinogenicity of this class of compounds (Reddy and Lalwani, 1983) and it has been suggested that a direct rela-

tionship between potency of the chemical for peroxisome proliferation and potency for hepatocarcinogenesis exists. Furthermore, it was proposed that peroxisome proliferation and acyl-CoA oxidase induction, which generates $H_2O_2$, could serve as a useful biological marker for identifying the potential carcinogenicity of these compounds. However, no direct relationship was observed when a weak carcinogen (DEHP) was compared with the potent one (WY-14,643) (Marsman *et al.*, 1988). An initial burst of DNA replication occurs with both compounds within a few days after initiation of feeding of either compound; however, livers from WY-14,643-treated animals showed a persistent increase in DNA labeling over control whereas DEHP-fed rats did not. At the same time, peroxisomes were increased to the same extent by both DEHP and WY-14,643. This result shows that peroxisome proliferation alone is not associated with carcinogenicity, but suggests that persistent increased cell replication may be an important factor (see below). Indeed, it was demonstrated that after treatment with a peroxisome proliferator the extent of DNA synthesis and peroxisomal enzyme induction were independent events (Grasl-Kraupp *et al.*, 1993; Bojes and Thurman, 1996b; Rose *et al.*, 1997a). Combined, these studies show that the processes of peroxisome proliferation and mitogenesis can be functionally distinguished, thus supporting the hypothesis that peroxisomal $H_2O_2$ is not responsible for genetic damage leading to carcinogenesis. Therefore, the use of peroxisome proliferation as a short-term biological marker to identify the potential carcinogenic nature of an agent may not be useful.

## HEPATOCARCINOGENICITY OF PEROXISOME PROLIFERATORS IN RATS AND MICE

### *Possible mechanisms*

A number of nonexclusive hypotheses have been postulated to contribute to the mechanisms underlying hepatocarcinogenicity of peroxisome proliferators including: (i) oxidative damage to DNA or proteins resulting from increased intracellular levels of $H_2O_2$ (Fahl *et al.*, 1984); (ii) alterations in cell proliferation and cell cycle control (Marsman *et al.*, 1988; Peters *et al.*, 1998); (iii) inhibition of apoptosis (Gerbracht *et al.*, 1990; Bayly *et al.*, 1994); and (iv) a combination of these vents (see Fig. 1). A hypothesis that $H_2O_2$ generated from peroxisomal fatty acyl-CoA oxidase causes DNA damage in liver parenchymal cells (Fig. 1A) has not received overwhelming experimental support (see below). On the contrary, the role of cell proliferation in nongenotoxic carcinogenesis due to peroxisome proliferators is widely accepted (Marsman *et al.*, 1988) (Fig. 1B). This hypothesis is based on the finding that all peroxisome proliferators markedly increase cell proliferation in liver of rats and mice, at least during the first few weeks of administration (reviewed in Conway *et al.*, 1989a). However, only potent carcinogens of this class of compounds cause a sustained increase in hepatocyte replication. Collectively, a good correlation between sustained increases in replicative DNA synthesis associated with hyperplasia

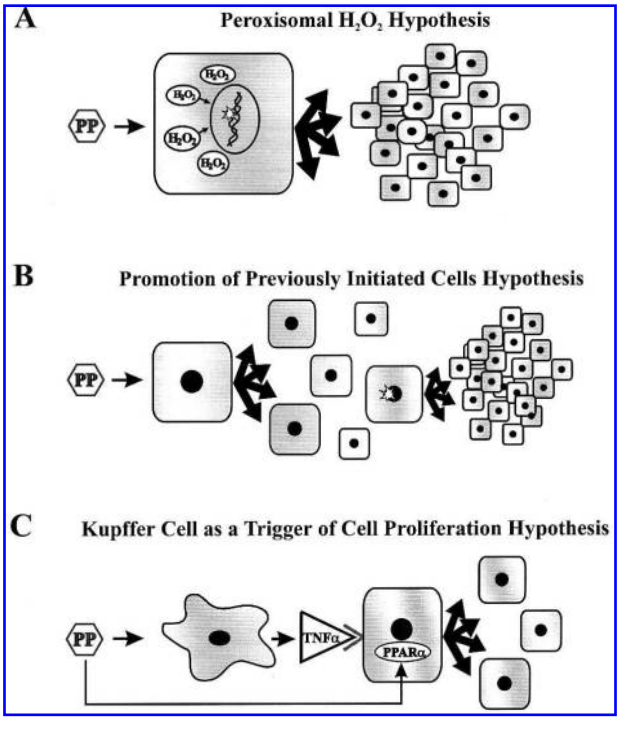


**FIG. 1. Hypotheses that have been proposed to explain peroxisome proliferator-induced carcinogenesis in rodent liver.** PP, Peroxisome proliferators; TNF-$\alpha$, tumor necrosis factor-$\alpha$; PPAR$\alpha$, peroxisome proliferator-activated receptor $\alpha$.

and the carcinogenicity of peroxisome proliferators exists (Marsman *et al.*, 1988); however, signaling events involved in stimulation of the cell cycle and its contribution to tumor formation remain unclear. Alternatively, since peroxisome proliferators inhibit apoptosis both *in vivo* (Marsman *et al.*, 1992) and *in vitro* (Gerbracht *et al.*, 1990; Bayly *et al.*, 1994), it is possible that inhibition of programmed cell death may predispose cells to ultimately form tumors (Gonzalez *et al.*, 1998).

Another piece of evidence for the above-mentioned hypothesis, that has been proposed for peroxisome proliferators, is promotion of spontaneously initiated cells (Cattley *et al.*, 1991; Grasl-Kraupp *et al.*, 1991). These studies demonstrated a higher incidence of hepatic tumors in old than in young rats after treatment with peroxisome proliferators. The fact that young rats developed tumors after administration of either weak or potent peroxisome proliferators was interpreted by some investigators as evidence that peroxisome proliferators are not simply promoters but complete carcinogens (Rao *et al.*, 1988, 1990). However, peroxisome proliferators have not been shown to exhibit initiating activity when tested in two-stage models of rodent hepatocarcinogenesis (Williams *et al.*, 1987; Popp and Cattley, 1993). Collectively, the tumor-promoting activity of peroxisome proliferators appears to be related to their effect on hepatocellular proliferation and is distinct from the biochemical effects of phenobarbital, a classic liver tumor promoter (Doull *et al.*, 1999).

### *Role of Kupffer cells in hepatocarcinogenesis of peroxisome proliferators*

Peroxisome proliferators increase proliferation of liver parenchymal cells both *in vivo* and *in vitro*; however, the *in vitro* effect is much less robust and persistent (*i.e.*, eight- to 10-fold increases *in vivo* versus only up to two-fold increases *in vitro*), regardless of the dose of the compound used (Marsman *et al.*, 1988, 1993). The possible explanation of this difference is the involvement of mitogenic cytokine(s) from nonparenchymal cell(s) in the proliferative response (Bojes *et al.*, 1997; Rose *et al.*, 1999a). Indeed, it is well known that Kupffer cells, the resident hepatic macrophages, are the predominant source of mitogens and comitogens in liver (Decker, 1990). For example, Kupffer cells are a major source of TNF-$\alpha$ as well as eicosanoids (Weinhold *et al.*, 1991), and TNF-$\alpha$ is mitogenic to hepatocytes (Beyer and Theologides, 1993) (see Fig. 2). To address the possibility that Kupffer cells participate in the mechanism of action of peroxisome proliferators, we performed several studies. First, it was shown that LY-171883, a peroxisome proliferator, was taken up by Kupffer cells via phagocytosis leading to activation (Rose *et al.*, 1999a). Next, interactions between Kupffer and parenchymal cells were studied in the intact liver. Using liver perfusion, it was demonstrated that nafenopin and WY-14,643 doubled uptake of particulate colloidal carbon, reflecting activation of Kupffer cells (Bojes and Thurman, 1996b). Furthermore, the increase in cell proliferation due to WY-14,643 was blocked by an antibody to TNF-$\alpha$ (Bojes *et al.*, 1995). Indeed, increases in whole-liver TNF-$\alpha$ mRNA (Rose *et al.*, 1997a) and serum TNF-$\alpha$ levels following treatment with peroxisme proliferators have been reported (Adinehzadeh and Reo, 1998). Collectively, these data led to the new

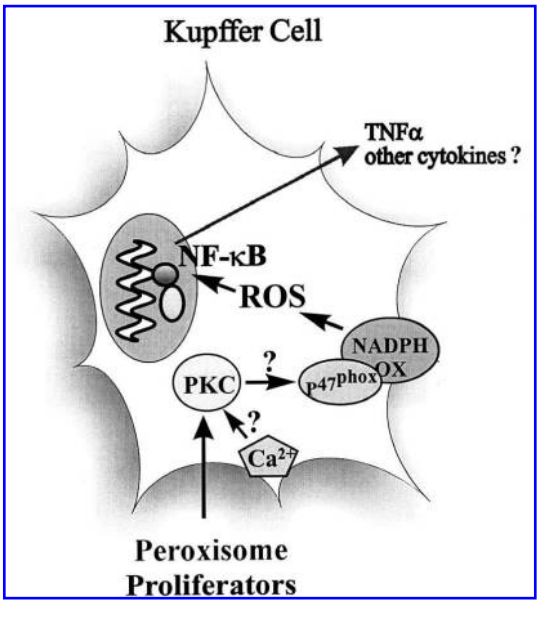


**FIG. 2. Signaling pathways induced in Kupffer cells by peroxisome proliferators.** PKC, Protein kinase C; NADPH OX, NADPH oxidase; ROS, reactive oxygen species; NF-$\kappa$B, nuclear factor $\kappa$B; TNF-$\alpha$, tumor necrosis factor-$\alpha$.

hypothesis that TNF-$\alpha$ produced by Kupffer cells is responsible for increased cell proliferation due to peroxisome proliferators (Fig. 1C). Implicit in this idea is that TNF-$\alpha$ is produced by Kupffer cells at low levels, which are mitogenic (Ankerman *et al.*, 1992), since higher levels induce apoptosis (Heller and Kronke, 1994; Schulte-Hermann *et al.*, 1995) and cause inflammation (Decker, 1990).

The hypothesis that Kupffer cells are involved in the mechanism of action of peroxisome proliferators was further supported by experiments with methyl palmitate and glycine, agents that inhibit Kupffer cells *in vivo*. Recently, it was discovered that Kupffer cells contain a glycine-gated chloride channel similar to that described earlier in the central nervous system (Wheeler *et al.*, 1999). Glycine activates a $Cl^-$ channel leading to hyperpolarization of the cell membrane thereby blunting increases in intracellular $Ca^{2+}$ and blocking signaling. Activation of this channel in Kupffer cells by feeding glycine *in vivo* reduced TNF-$\alpha$ and increased survival in rats given a lethal dose of endotoxin (Ikejima *et al.*, 1996). Furthermore, dietary glycine prevented the increases in both TNF-$\alpha$ mRNA and protein and cell proliferation due to the peroxisome proliferator WY-14,643 (Rose *et al.*, 1997b). Methyl palmitate is a nonmetabolizable fatty acid that suppresses reticuloendothelial function, and it blunts uptake of colloidal carbon, a measure of Kupffer cell activation (Cowper *et al.*, 1990). It also completely prevented the stimulation of TNF-$\alpha$ production and cell proliferation by WY-14,643 (Rose *et al.*, 1997a). From these data, it was concluded that TNF-$\alpha$ of Kupffer cell origin was causally responsible for the mitogenic effect of peroxisome proliferators.

Because the transcription factor NF-$\kappa$B plays an essential role in the regulation of a variety of genes involved in inflammatory responses, immune function, and control of cell growth and differentiation including production of TNF-$\alpha$ (Baldwin, 1996), it was suggested that it may be involved in signaling induced by peroxisome proliferators in Kupffer cells. Indeed, NF-$\kappa$B activity was elevated about three-fold early after WY-14,643 treatment in the hepatic nonparenchymal cell fraction and then declined toward basal values (Rusyn *et al.*, 1998a). Importantly, 2 hr after WY-14,643 treatment, the active form of NF-$\kappa$B was localized almost exclusively in nonparenchymal cells with values 20 to 25 times greater than in parenchymal cells (Rusyn *et al.*, 1998a). In contrast, parenchymal cells exhibited maximal activity only after 8 hr. These findings are consistent with the hypothesis that activation of NF-$\kappa$B in Kupffer cells leads to increases in TNF-$\alpha$ production, which are ultimately responsible for WY-14,643-induced hepatocyte replication (see Fig. 2 for scheme). Furthermore, dietary feeding of ciprofibrate increased NF-$\kappa$B activity in rat whole liver nuclear extracts after 3 days (Li *et al.*, 1996).

Because peroxisome proliferators are highly lipophilic compounds, it was hypothesized that they act in a manner similar to phorbol esters, also nongenotoxic carcinogens and increase protein kinase C (PKC). PKC is a second-messenger system that is elevated during increased cell turnover and is involved in the signaling cascade leading to increased cell proliferation (Nishizuka, 1984, 1988). In support of this hypothesis, it was demonstrated that a wide range of peroxisome proliferators elevated PKC in whole liver roughly in proportion to their carcinogenicity in long-term feeding studies (Bojes and Thurman, 1994). It was proposed, therefore, that the mitogenic effect of peroxisome proliferators involved activation of PKC. Recently, changes in PKC $\alpha$, $\beta$, $\delta$, and $\zeta$ isoforms were detected after exposure to WY-14,643 (Corton *et al.*, 1999). On the basis of these results, it was concluded that alterations in PKC isoforms could play an important role in perpetuating the high cell proliferative rate in peroxisome proliferator-induced hepatocellular adenomas. Finally, WY-14,643 increased PKC activity about three-fold in isolated Kupffer cells and pretreatment of Kupffer cells with the amino acid glycine, which blunts calcium signaling (see above), inhibited WY-14,643-stimulated increases in PKC activity completely (Rose *et al.*, 1999b). These data are consistent with the hypothesis that peroxisome proliferators directly activate Kupffer cells and TNF-$\alpha$ production via mechanisms involving PKC. A possible link between PKC and activation of NF-$\kappa$B also exists because activation of NF-$\kappa$B by peroxisome proliferators is sensitive

to reactive oxygen species (ROS) (Rusyn *et al.*, 1998a; see below) and PKC activates oxidant production (Majumdar *et al.*, 1993).

*Role of PPARα in the mechanism of peroxisome proliferators*

Recent studies in PPARα knockout mice provided unequivocal evidence for a role for this intracellular receptor in both hepatocellular proliferation and tumors due to peroxisome proliferators (Peters *et al.*, 1997). Specifically, the effect of the prototypical peroxisome proliferator WY-14,643 on replicative DNA synthesis and carcinogenesis in wild-type and PPARα-null mice was evaluated. Mice of both (+/+) and (−/−) genotypes were fed either a control diet or one containing WY-14,643 for 1 week, 5 weeks, or 11 months. Wild-type, but not knockout mice, fed the WY-14,643 diet for 1 or 5 weeks showed increased hepatocellular proliferation. After 11 months, 100% of the (+/+) mice fed the WY-14,643 diet had multiple hepatocellular neoplasms, including adenomas and carcinomas, while the (−/−) mice were unaffected. This work demonstrates clearly that the *in vivo* effects of WY-14,643 on replicative DNA synthesis and hepatocarcinogenesis involved PPARα.

A contributing role for Kupffer cells and cytokines in hepatocyte proliferation has been proposed (see above); however, whether PPARα is expressed in Kupffer cells or parenchymal cells was not known. To address this important gap in our knowledge, the expression of PPAR isoforms in rat Kupffer and parenchymal cells was examined (Peters *et al.*, 2000). Kupffer cells and hepatocytes of greater than 99% purity were isolated from rats fed either a control diet or one containing 0.1% WY-14,643 for 1 week. Protein and RNA were obtained, and PPAR expression was analyzed using Northern and Western blotting. PPARα, PPARβ, and PPARγ mRNA was detected in purified hepatocytes. In Kupffer cells, mRNA encoding PPARγ was present whereas transcripts for PPARα and PPARβ were not detected. Immunoblots were consistent with the results from Northern analysis. Combined, these results show that PPARα is expressed in rat parenchymal cells but not in Kupffer cells. These data are consistent with the hypothesis that parenchymal cells respond to Kupffer cell-derived TNF-α via mechanisms dependent on PPARα in parenchymal cells (see Fig. 3); however, how PPARα is involved in signaling proliferation of parenchymal cells remains to be determined.

## ROS AND THE MECHANISM OF PEROXISOME PROLIFERATORS

*Role of oxidants and DNA damage in the carcinogenesis of peroxisome proliferators*

The hypothesis that the hepatocarcinogenicity of peroxisome proliferators is due to oxidative stress relies on several sets of experimental evidence. These include disproportionate increases in the activities of enzymes that generate and degrade $H_2O_2$ in these cells, and increases in 8-HO-dG and lipid peroxidation. Increased amounts of lipofuscin and high levels of conjugated dienes have been reported in livers of rats treated for prolonged periods with peroxisome proliferators (Goel *et al.*, 1986; Lake *et al.*, 1987; Conway *et al.*, 1989b). Furthermore, livers with chronic peroxisome proliferation show a two- to four-fold increase in the amount of 8-HO-dG in DNA (Kasai *et al.*, 1989; Qu *et al.*, 1999) and DNA damage has been induced *in vitro* in SV40 DNA by peroxisomes isolated from the livers of rats exposed to a peroxisome proliferator (Fahl *et al.*, 1984). More recent evidence comes from experiments where the enzyme fatty acyl-CoA oxidase was overexpressed in mammalian cells (Chu *et al.*, 1995). In a cell line that stably express rat peroxiso-

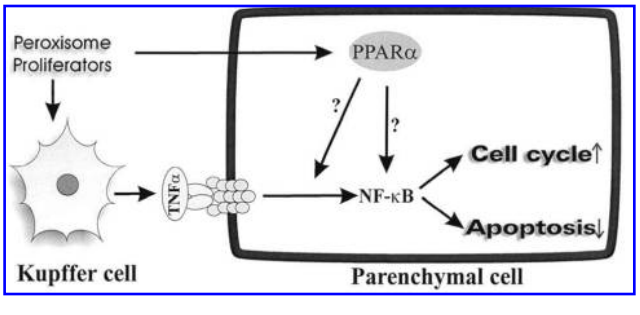


**FIG. 3. Proposed role for Kupffer cell TNF-α in proliferation of parenchymal cells induced by peroxisome proliferators.** TNF-α, Tumor necrosis factor-α; NF-κB, nuclear factor κB; PPARα; peroxisome proliferator-activated receptor α.

mal fatty acyl-CoA oxidase and which was exposed to a fatty acid for 2 weeks, a growth advantage and transformed phenotype were observed. The growth of these cells in soft agar and the formation of tumors when the transformed cells were transplanted into nude mice were also reported. Although this is an important observation, it does not establish carcinogenicity of $H_2O_2$ in an intact animal model. Finally, when ciprofibrate was fed for 21 days to transgenic mice, which overexpress catalase in the liver, the increase in cell proliferation observed in wild-type mice was abolished (Nilakantan *et al.*, 1998).

Importantly, several studies presented evidence that does not support the hypothesis of oxidative DNA damage due to peroxisome proliferators. It was reported that chronic di(2-ethylhexyl)phthalate (DEHP) treatment does not result in increased 8-hydroxydeoxyguanosine (8-OH-dG) levels in liver (Cattley and Glover, 1993). Moreover, it was suggested that such increases may be due to oxidation of mitochondrial, rather then nuclear DNA (Sausen *et al.*, 1995). Furthermore, given the extremely high rate at which peroxisomal catalase converts $H_2O_2$ into $H_2O$ and $O_2$, it should not escape peroxisomes (Nicholls and Schonbaum, 1963), and it was shown that treatment with peroxisome proliferators increased $H_2O_2$ *in vitro*, but not in the perfused liver because fatty acid supply is rate limiting for $H_2O_2$ production in intact cells (Handler and Thurman, 1988; Handler *et al.*, 1992). Moreover, spontaneous liver tumors occur in mice lacking peroxisomal fatty acyl-CoA oxidase (Fan *et al.*, 1998). Collectively, the role of oxidants in the mechanism of action of this important class of chemicals is far from clear and taking oxidant production in specific liver cell types into consideration may provide important insights (see above).

### *Evidence for increased oxidant production in Kupffer cells due to peroxisome proliferators*

Stimulation of Kupffer cells results in activation of NADPH oxidase and production of superoxide anion (Decker, 1990). Because Kupffer cells are known to be activated by peroxisome proliferator treatment *in vivo* (Bojes and Thurman, 1996b), it was hypothesized that such activation would result in increased production of oxidants. To address this hypothesis, Kupffer cells were isolated from untreated rats and cultured in the presence of peroxisome proliferators, and then superoxide production was assessed. Indeed, WY-14,643 and monoethylhexylphthalate (MEHP), a key lipophilic metabolite of DEHP, increased superoxide production in isolated Kupffer cells in a dose-dependent manner, indicating that they can affect Kupffer cells directly (Rose *et al.*, 1999b). Furthermore, WY-14,643-stimulated superoxide production by isolated Kupffer cells was prevented by glycine, consistent with the hypothesis that $Ca^{2+}$-dependent signaling (*e.g.*, via PKC, see above) is involved in activation of Kupffer cells by peroxisome proliferators. The involvement of PKC in Kupffer cell superoxide production stimulated by WY-14,643 was evaluated further by measuring PKC activity in Kupffer cells. Indeed, WY-14,643 increased calcium-dependent PKC activity in Kupffer cells nearly three-fold, an effect also blocked by glycine (Rose *et al.*, 1999b).

As mentioned above, both WY-14,643 and DEHP elevate hepatocyte replication during the first few days of treatment; however, only WY-14,643 sustains rates of proliferation with long-term treatment (Marsman *et al.*, 1988). To determine if oxidants such as superoxide are involved in sustained cell replication *in vivo*, Kupffer cells were isolated from rats treated with 0.1% WY-14,643 or 1.2% DEHP in the diet for 21 days, a time when hepatocellular proliferation is elevated only in WY-14,643 but not DEHP-treated animals, and basal rates of superoxide production were measured (Rose *et al.*, 1999b). Kupffer cells isolated from rats fed WY-14,643 generated superoxide at rates significantly greater than cells from controls; however, superoxide production was not stimulated by feeding DEHP for 3 weeks. Therefore, these data are consistent with the hypothesis that oxidants play a role in signaling a sustained increase in cell proliferation in rodent liver.

Because hard evidence for increased oxidant production *in vivo* after treatment with peroxisome proliferators is lacking, the spin-trapping technique and electron spin resonance (ESR) spectroscopy were employed (Rusyn *et*

*al.*, 1999). Specifically, when rats were given DEHP acutely for 2 hr, an ESR-detectable radical adduct signal was detected. Furthermore, DEHP given with [$^{13}C_2$]DMSO produced a 12-line spectrum providing solid evidence in support of the hypothesis that DEHP stimulates $HO^{\cdot}$ formation *in vivo*. Also, when rats were pretreated with dietary glycine, a treatment known to inactivate Kupffer cells, no increase in POBN ($\alpha$-[4-Pyridyl-1-oxide] N-tert-bytylnitrone)/radical signal due to DEHP was observed. This observation is important because it is the first evidence of rapid phthalate-induced free radical production *in vivo*. Moreover, this occurs long before $H_2O_2$ generating enzymes are induced in peroxisomes by phthalates.

*Oxidants as signaling molecules in hepatocellular proliferation*

Recently, it was suggested that low levels of oxidants may play a role in signaling increases in cell proliferation caused by peroxisome proliferators via a Kupffer cell-mediated mechanism involving TNF-$\alpha$ and NF-$\kappa$B (Rose *et al.*, 1999a). Collective evidence presented above supports the concept that oxidants play a significant role in the peroxisome proliferator-induced proliferative response by participating in signaling in Kupffer cells.

Because NADPH oxidase is a major superoxide-producing enzyme in macrophages, it was hypothesized that it is involved in oxidant-dependent activation of NF-$\kappa$B by the peroxisome proliferator WY-14,643 in Kupffer cells (Rusyn *et al.*, 1998b). Indeed, both activation of NF-$\kappa$B and increases in cell proliferation due to a single dose of WY-14,643 were prevented completely when rats were pretreated with diphenyleneiodonium, an inhibitor of NADPH oxidase. p47$^{phox}$ is a critical subunit of NADPH oxidase; therefore, p47$^{phox}$ knockout mice were used to address specifically the hypothesis of involvement of NADPH oxidase in the mechanism of peroxisome proliferator-induced hepatocellular proliferation. In livers of wild-type mice, WY-14,643 activated NF-$\kappa$B, which was followed by an increase in mRNA for TNF-$\alpha$. Importantly, these changes did not occur in p47$^{phox}$ knockouts. Moreover, when Kupffer cells were treated with WY-14,643 *in vitro*, superoxide production was induced in cells from wild type but not p47$^{phox}$-null mice. Finally, when mice were fed a WY-14,643 containing diet for 7 days, the increase in liver weight and cell proliferation caused by WY-14,643 in wild-type mice did not occur in p47$^{phox}$-null mice. Combined, these results provide strong evidence for a key role of NADPH oxidase in Kupffer cells as a source of oxidants increased by treatment with the peroxisome proliferator WY-14,643. Furthermore, it is suggested that reactive oxygen species play a novel signaling role in the mechanism of action of this class of tumor promoters. Therefore, these data further support a role of Kupffer cells in the mechanism of increased cell proliferation due to this important class of toxic chemicals.

## CONCLUDING REMARKS

The issue of human risk associated with exposure to chemicals that belong to the peroxisome proliferator group remains controversial. Despite several major advances in understanding the mechanisms of how these chemicals cause liver tumors in rodents, the issue of toxicity to human population is far from being resolved. Recent experimental evidence suggests that ROS, at least early after treatment with peroxisome proliferators, are derived from NADPH oxidase in Kupffer cells. Furthermore, it was hypothesized that ROS play a novel signaling role in the mechanism of action of this class of nongenotoxic liver carcinogens. Specifically, it was shown that peroxisome proliferators (i) cause formation of free radicals *in vivo*, and (ii) increase activity of the redox-sensitive transcription factor NF-$\kappa$B via oxidants produced from NADPH oxidase. Therefore, oxidants act as important second messengers and might be involved in signaling of Kupffer cells mitogen production resulting in increased proliferation of parenchymal cells.

On the other hand, PPAR$\alpha$ is required for both increases in cell proliferation and tumors due to peroxisome proliferators in rodents (Peters *et al.*, 1997), but it is not present in Kupffer cells (Peters *et al.*, 2000). Combined with evidence for the role of Kupffer cells presented above, these results suggest that the effects of

peroxisome proliferators on two separate cell types are both necessary for the maximal increase in hepatocellular proliferation. Collectively, these interesting new findings will be useful in determining the exact mechanism by which peroxisome proliferators cause liver tumors in rodents so that the potential risk of human exposure to these compounds can be better predicted.

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## ABBREVIATIONS

DEHP, Di(2-ethylhexyl)phthalate; ESR, electron spin resonance; 8-HO-dG, 8-hydroxydeoxyguanosine; NF-$\kappa$B, nuclear factor $\kappa$B; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; RXR$\alpha$, retinoid X receptor $\alpha$; TNF-$\alpha$, tumor necrosis factor-$\alpha$; WY-14,643, [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]-acetic acid.

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