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ROLE OF KUPFFER CELLS IN PEROXISOME PROLIFERATOR-INDUCED HEPATOCYTE PROLIFERATION*

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I. INTRODUCTION

Peroxisome proliferators are a group of structurally diverse compounds that cause an increase in both the number and size of peroxisomes, elevate rates of cell proliferation, and cause liver cancer in rodents [1,2]. Although the mechanism by which peroxisome proliferators cause tumors in rats and mice is not known, two hypotheses have been proposed [3–5]. One view is that oxidative stress from peroxisomal oxygen metabolism is a critical event in the carcinogenic process [5], whereas others contend that elevated and sustained cell replication is respon-

sible for the induction of tumors [4]. In contrast to mitochondrial fatty acid β-oxidation, peroxisomal fatty acid β-oxidation produces H_2O_2 [6]. Peroxisome proliferators cause a disproportionate elevation in H_2O_2 due to the large increase in peroxisomal fatty acyl CoA oxidase with minimal induction of degradative enzymes such as catalase [5]. The ''oxidative stress'' hypothesis proposed that excess H_2O_2 then diffuses out of the peroxisome and reacts with biological macromolecules such as DNA either directly or indirectly by generating several unstable oxygen species (e.g., superoxide anion and hydroxyl radical). In support of this theory, peroxisome proliferators generated 8-hydroxydeoxyguanosine (8-OH-dG) adducts after treatment with ciprofibrate, and lipofuscin accumulated following chronic exposure to phthalates [7,8]. However, background levels of 8-OH-dG were high, making interpretation difficult. Moreover, increases in 8-OH-dG adducts did not correlate well with the potency of the drug administered. For example, rats fed diets containing peroxisome proliferators regarded as potent (0.025% ciprofibrate) and weak [1.2% di(2-ethylhexyl)phthalate; DEHP] tumor promoters both increased 8-OH-dG similarly [8,9]. Furthermore, consideration of enzyme kinetics makes it very unlikely that H_2O_2 would exit the peroxisome because degradation of H_2O_2 via catalase is over five orders of magnitude faster than the rate at which it is produced [10]. The hypothesis that H_2O_2 leakage from peroxisomes does not occur was supported by the demonstration that perfluorooctanoate and ciprofibrate did not increase H_2O_2 production in whole liver [11]. The reason for this is that the rate-limiting step in H_2O_2 production in whole cells is the fatty acid supply. Therefore, H_2O_2 increased in subcellular fractions when fatty acids were added in excess and when lipid was added to whole liver [11,12]. Similar results were obtained in perfused rat liver following chronic exposure to bezafibrate [13]. Moreover, lipid peroxidation measured in animals given 20,000 ppm DEHP in the diet for 6 weeks did not differ from controls, providing further evidence against the oxidative stress hypothesis [14].

If H_2O_2 production from increased peroxisomal enzymes were responsible for the carcinogenicity of peroxisome proliferators, the degree of peroxisome induction should correlate with the tumorigenicity of the compound. However, peroxisome proliferation and hepatocarcinogenicity were not correlated in studies where rats were fed two peroxisome proliferators (DEHP and WY-14,643) for up to 365 days [4]. Both chemicals increased hepatic peroxisome number and peroxisomal enzyme activity equally, although the incidence of tumors was significantly greater in rats treated with WY-14,643 than in animals exposed to DEHP. These findings further questioned the idea that oxidative stress contributes to the mechanism by which peroxisome proliferators form tumors. Thus, alternatives were considered. Marsman et al. showed that WY-14,643 produced a 5- to 10-fold sustained increase in cell turnover, whereas the weaker tumor promoter DEHP did not [4]. It was observed that the potency of the compound as well as the dose administered was critical in determining whether a particular peroxisome

proliferator could produce either a transient or sustained increase in hepatic mitogenesis. For example, low doses of WY-14,643, nafenopin, and methylclofenaopate did not produce sustained DNA synthesis in rat hepatocytes and were not as carcinogenic as high doses of WY-14,643 [15]. Therefore, it was proposed that stimulation of cell replication is a critical component in the mechanism of hepatocarcinogenesis for peroxisome proliferators [4,5]. For example, increasing mitogenic rates can elevate the rate of conversion of DNA lesions into mutations prior to repair and increase the probability of ''spontaneous'' mutations forming from normal DNA replication [6]. Increases in cell proliferation are also important in the promotion of spontaneously initiated cells. Several investigators demonstrated the presence of numerous foci of putative preneoplastic cells in livers of older compared to younger rats following chronic exposure to WY-14,643 and nafenopin [16,17]. More recently, a link between WY-14,643-induced basophilic foci and the incidence of tumors has been proposed [18]. Basophilic foci exhibit much higher rates of cell proliferation than surrounding hepatocytes. Collectively, these studies support the hypothesis that cell proliferation is a critical factor in peroxisome proliferator-induced tumor formation.

This review describes several studies that attempt to dissect the mechanism by which peroxisome proliferators increase cell proliferation, a critical component of carcinogenesis. It has been shown recently that peroxisome proliferators activate Kupffer cells, which are a rich source of mitogenic cytokines (Fig. 1) [19]. These mitogenic factors then trigger cell growth in nearby hepatocytes [20]. Modulation of Kupffer cell activity with inhibitors such as dietary glycine and methyl palmitate have demonstrated the role of Kupffer cell-derived cytokines in peroxisome proliferator-induced cell proliferation (Fig. 1) [21,22]. Moreover, the transcription factor NFκB plays a role in Kupffer cell cytokine production (Fig. 1) [23]. Taken together, these data support the hypothesis that peroxisome proliferators first activate Kupffer cells, which produce mitogens that trigger increases in hepatocyte turnover.

II. PEROXISOME PROLIFERATORS STIMULATE PROTEIN KINASE C IN VIVO

A. Peroxisome Proliferators Activate Protein Kinase C

Protein kinase C isoforms are a component of a second messenger system which is elevated during increased cell turnover and is involved in signaling cell proliferation [24]. Its activation by tumor promoters such as phorbol esters is well documented [25]. Because peroxisome proliferators are nongenotoxic carcinogens, it was hypothesized that they may act as tumor promoters, such as

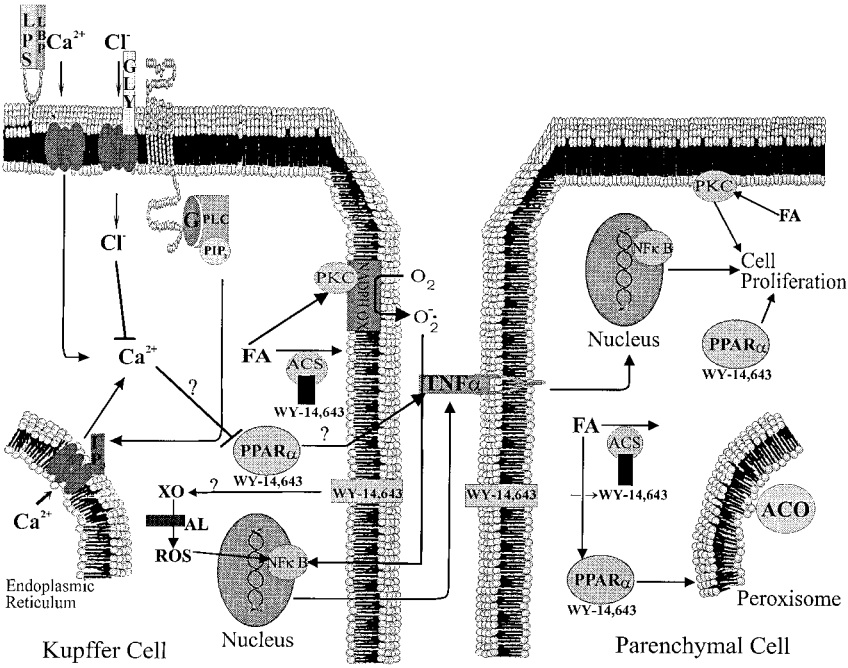


FIG. 1. Scheme depicting working hypothesis for the role of Kupffer cells in WY-14,643-induced hepatocyte proliferation. WY-14,643 activates tumor necrosis factor α (TNFα) production most likely via pathways involving nuclear factor κB (NFκB). WY-14,643-induced activation of NFκB may involve increased production of reactive oxygen species by xanthine oxidase (XO) which is inhibited by allopurinol (AL) or by superoxide (O_2^-) generated by NADPH oxidase (NADPH OX). Kupffer cell-derived TNFα is responsible for WY-14,643-induced hepatocyte proliferation because antibodies to TNFα prevented increased cell replication. TNFα activates NFκB in hepatocytes, which is known to increase hepatocyte replication. Protein kinase C (PKC) is also associated with tumor formation and is activated by WY-14,643 by unknown mechanisms. One likely possibility is that inhibition of acyl CoA synthetase (ACS) by WY-14,643 leads to a buildup of free fatty acids (FA) which are known activators of PKC. In Kupffer cells, PKC phosphorylates specific subunits of NADPH oxidase leading to its activation and production of superoxide. Kupffer cell production of TNFα and subsequent hepatocyte proliferation is prevented by inactivating Kupffer cells with methyl palmitate or dietary glycine (GLY). Dietary glycine activates a chloride channel (Cl^-) on the Kupffer cell leading to hyperpolarization of the membrane and inhibition (⊥) of calcium signaling (Ca^{2+}) which blunts (⊥) TNFα production. Interestingly, the peroxisome proliferator-activated receptor α (PPARα) has been shown to be expressed by Kupffer cells and could play a role in Kupffer cell production of TNFα stimulated by WY-14,643 in addition to its role in peroxisomal acyl CoA oxidase (ACO) induction, hepatocyte proliferation, and tumor formation.

phorbol esters and increase protein kinase C. In support of this hypothesis, we demonstrated that the potent peroxisome proliferator WY-14,643 (100 mg/kg, IG) increased protein kinase C threefold in 5 h and fivefold in 10 h (Fig. 2) at a dose comparable to that used in chronic studies which caused tumors in 100% of rats within 1 year [4]. When a wide range of peroxisome proliferators, differing

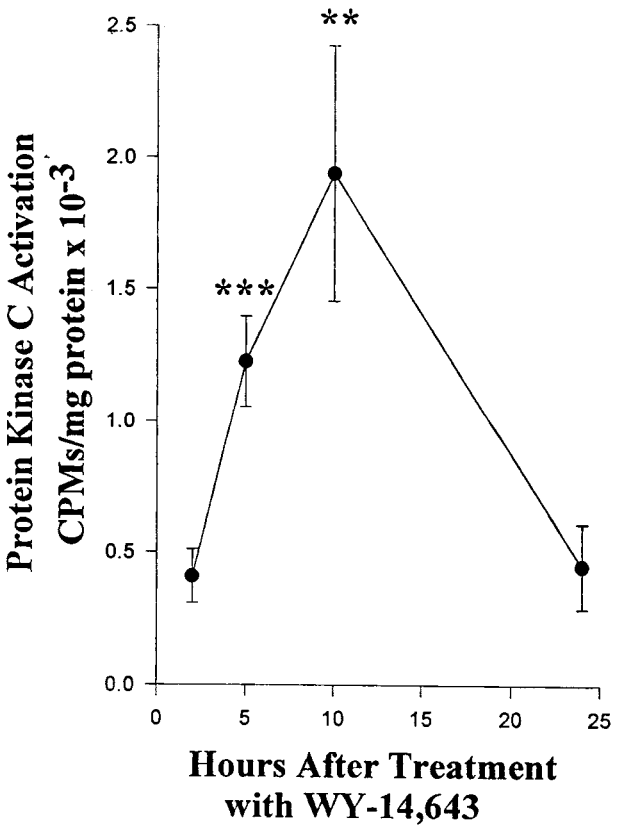


FIG. 2. Effect of WY-14,643 on protein kinase C in vivo. Male Fisher 344 rats were given 100 mg/kg WY-14,643 in olive oil (IG) while control rats received equal volumes of oil vehicle. Hepatic microsomal fractions were isolated by standard techniques of differential centrifugation after 2, 5, 10, or 24 h [26]. Protein kinase C was measured as described elsewhere [26]. The asterisks (*) denote a significant difference from control by one-way analysis of variance (ANOVA) and Dunnett's test. Data represent the mean ± SEM (n = 3–6). $^{**}p < 0.05$; $^{***}p < 0.01$.

in the degree to which they induce peroxisomes and cause tumors, were examined [26], it was shown that they elevated protein kinase C roughly in proportion to their carcinogenicity in long-term feeding studies [26]. It was proposed, therefore, that the mitogenic effect of peroxisome proliferators involved activation of protein kinase C. These findings are supported by studies by Bronfman et al., who showed that protein kinase C was activated in isolated rat brain and liver tissue by several peroxisome proliferators of the fibrate family [27].

Direct administration of peroxisome proliferators to subcellular fractions did not alter protein kinase C; therefore, it was hypothesized that an intermediate factor such as free unsaturated fatty acids was involved in the activation process [26]. Stimulation of protein kinase C by unsaturated fatty acids has been demonstrated in vitro [28], and it is likely that a similar mechanism takes place in vivo. In fact, lipid accumulation in hepatocytes shortly after peroxisome proliferator treatment is well characterized [29–32]. For example, small droplets of fat were observed following 1 day of fenofibrate and ciprofibrate exposure [30] and increases in hepatic triglycerides were noted following 2 weeks of administration of LY 171883 [33]. Moreover, elevated levels of unsaturated free fatty acids (e.g., oleate and palmitate) were demonstrated with several perfluorocarboxylic acids, including perfluorooctanoic, perfluorodecanoic, and nonadecafluoro-*n*-decanoic acids [26]. Also, long-chain fatty acids are converted into fatty acyl CoA compounds by acyl CoA synthetase, an essential step in the oxidation, elongation, and esterification of fatty acids [33]. Due to the central role this enzyme plays in lipid metabolism, it was hypothesized that inhibition of acyl CoA synthetase by peroxisome proliferators leads to elevation of free fatty acids which can activate protein kinase C (Fig. 1). Indeed, a significant correlation ($r = -0.80$) between the ability of peroxisome proliferators to block acyl CoA synthetase activity and stimulate protein kinase C was observed [26]. Furthermore, WY-14,643 inhibited acyl CoA synthetase in a competitive manner in vitro [26]. Although a direct link between inhibition of acyl CoA synthetase activity and increases in protein kinase C has not yet been demonstrated, it is likely that elevation of free fatty acids activates protein kinase C, which triggers signaling events leading to proliferation (Fig. 1).

B. A Role for Protein Kinase C in the Mechanism of Action of Peroxisome Proliferators

It was hypothesized that peroxisome proliferators increase free fatty acids, leading to a stimulation of protein kinase C and initiation of cell proliferation (Fig. 1). The role of protein kinase C in cell proliferation and tumorigenesis is complex and poorly understood; however, a maximal increase in protein kinase C was shown to precede mitogenesis, suggesting a possible relationship between

these two events. Moreover, evidence exists that tumors develop subsequent to changes in protein kinase C-mediated signal transduction [34,35]. For example, alterations of protein kinase C activity have been associated with increases in cell proliferation [34,35]. Furthermore, abnormalities in the expression of genes that are often associated with tumors such as *c-myc* and *c-fos* can be linked with changes in protein kinase C-mediated pathways [34,35].

Although protein kinase C-mediated signal transduction has been investigated extensively, its role in cell proliferation signaling mechanisms and tumor formation remains unclear. Protein kinase C isoforms are rare or absent in the nucleus, suggesting the role of an additional transcription factor in the regulation of peroxisomal gene expression, such as the peroxisome proliferator activated receptor α (PPARα). The PPARα is a nuclear hormone receptor and has been identified as a putative mediator of peroxisome proliferator gene transcription [36–38]. Genes encoding peroxisomal enzymes involved in β-oxidation of fatty acids as well as long-chain acyl CoA synthetase and cytochrome P450 4A1 have been shown to contain PPARα-responsive elements in their promoter regions [39]. Additionally, it was demonstrated that disruption of the ligand-binding domain of the alpha isoform of PPAR in mice abolished the pleiotrophic effects of peroxisome proliferators, including peroxisome induction, increased rates of cell proliferation, and, ultimately, formation of tumors [40]. Therefore, it is possible that changes in protein kinase C may signal increases in hepatocyte proliferation via mechanisms involving PPARα.

III. TUMOR NECROSIS FACTOR α IS INVOLVED IN WY-14,643-STIMULATED HEPATOCYTE PROLIFERATION

A. Kupffer Cells Are Activated by Peroxisome Proliferators In Vivo and In Vitro

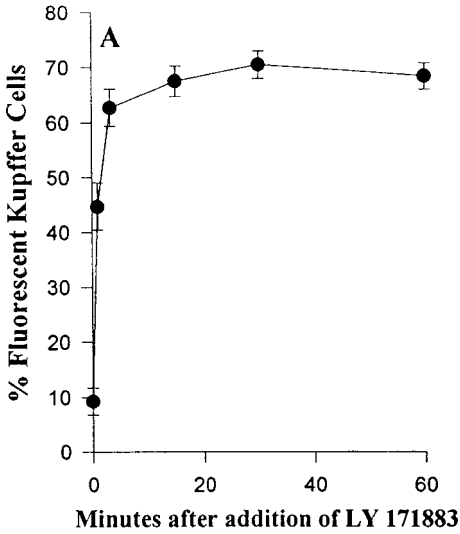
Whereas peroxisome proliferators increase cell proliferation eightfold in vivo, replication rates are only doubled in isolated parenchymal cells [4,41]. One factor that may account for this discrepancy is the involvement of nonparenchymal cell types such as the Kupffer cell in peroxisome proliferator-induced hepatocyte proliferation. Kupffer cells are the resident hepatic macrophages and are a rich source of a variety of chemotactic and mitogenic meaditors upon activation [e.g., epidermal growth factor, tumor necrosis factor α (TNFα), hepatocyte growth factor, and prostaglandin E_2] and therefore may be involved in growth modulation of nearby hepatocytes [42–44]. Consistent with this hypothesis, we showed that Kupffer cell activation, measured as rates of colloidal carbon uptake in the iso-

lated perfused liver, increased following treatment in vivo with either nafenopin or WY-14,643 about twofold over control [19]. Nafenopin also increased rates in a dose-dependent manner (half-maximal response, ~75 mg/kg). Moreover, treatment of isolated Kupffer cells in vitro with the peroxisome proliferator LY 171883 demonstrated that they take up fluorescent particles of this lipophilic compound very rapidly (Fig. 3A) and activate Kupffer cell nitric oxide production to about the same extent as lipopolysaccharide (Fig. 3B) [45]. Collectively, these results indicate that peroxisome proliferators activate Kupffer cells and strongly suggest a role for these cells in the mitogenic mechanism of peroxisome proliferators.

It is possible that peroxisome proliferators enter Kupffer cells by first being incorporated into low-density lipoproteins (LDLs) or by simply diffusing into membranes based on their lipophilic nature. Recently, the LDL receptor was identified on Kupffer cells, and cholesterol esters of oxidized LDLs were rapidly hydrolyzed by these cell types in vivo [46]. The binding of the peroxisome proliferator clofibrate to plasma proteins was also demonstrated following acute exposure [47]. Although indirect mechanisms of Kupffer cell activation by peroxisome proliferators are possible, it was demonstrated recently that WY-14,643 and monoethylhexylphthalate (MEHP) were capable of activating Kupffer cell superoxide production when isolated Kupffer cells were treated in vitro [48]. Therefore, it seems likely that peroxisome proliferators activate Kupffer cells directly (Fig. 1).

B. Antibodies to TNFα Prevent Hepatocyte Proliferation Stimulated by WY-14,643

Activation of Kupffer cells was hypothesized to lead to the release of hepatocyte mitogens. Although TNFα is known primarily as an inflammatory cytokine associated with necrotic injury and the induction of apoptosis [49], effects not commonly seen with peroxisome proliferators, it has been demonstrated that it also promotes hepatocyte growth [50,51]. As shown in Fig. 4, the eightfold increase in cell replication caused by WY-14,643 in vivo was completely prevented by pretreatment with anti-TNFα antibody, demonstrating that TNFα is involved in the mechanism by which WY-14,643 activates cell turnover [20]. Additionally, anti-TNFα antibody blunted the WY-14,643-induced increase in protein kinase C, and immunohistochemical staining for TNFα localized it to sinusoidal lining cells, most likely Kupffer cells. These results indicate a role for WY-14,643 as an indirect mitogen on hepatocytes via TNFα and suggest a role for the Kupffer cell, the major hepatic source of TNFα, in the mechanism of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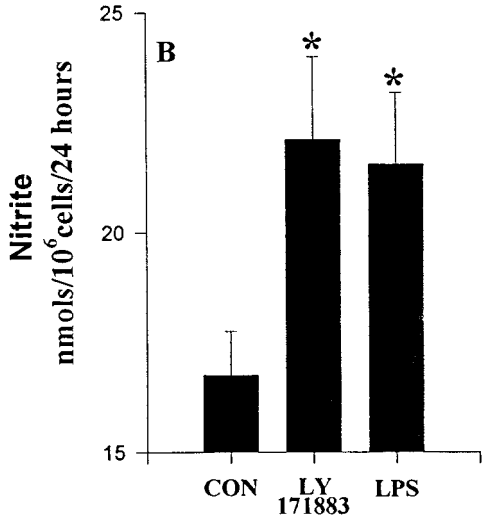
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Nitrite
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action of peroxisome proliferators (Figs. 1 and 4). Several studies support these ideas. For example, TNFα given to rats IV induces hepatocyte proliferation [52], and treatment of isolated hepatocyte cultures with TNFα in vitro demonstrated that it was a direct hepatocyte mitogen [51]. These observations have been confirmed recently by Roberts and colleagues [53]. Moreover, Akerman et al. demonstrated that antibodies to TNFα blocked liver regeneration following partial hepactectomy, further establishing its role as a mediator of cell turnover [50]. Similarly, pretreatment with endotoxin, which stimulates TNFα production by Kupffer cells, enhanced liver regeneration, whereas inactivation of endotoxin with antibiotics or binding agents impaired growth [54]. Conversely, there are reports that TNFα impairs growth directly [55]. Therefore, TNFα most likely displays biphasic effects which are concentration dependent, with low doses producing a mitogenic effect and high concentrations triggering an inflammatory response. Consistent with this hypothesis, treatment of cultures of human smooth airway muscle cells with a concentration of TNFα known to cause bronchoalveolar inflammation inhibited the proliferative effects of growth factors, whereas low concentrations stimulated DNA synthesis [56]. These data are consistent with the hypothesis that TNFα, most likely of Kupffer cell origin, is responsible for WY-14,643-stimulated hepatocyte proliferation.

FIG. 3. (A) Time course of Kupffer cell phagocytosis of the peroxisome proliferator LY 171883. Kupffer cells were isolated and cultured for 24 h, as described elsewhere [65]. LY 171883 was dissolved in ethanol and added to culture medium at a concentration of 100 μ*M* to form particles which are fluorescent when exicted at a wavelength of 380 nm with peak emission at 510 nm. Cells were incubated for the times indicated on the *x* axis, and the percentage of fluorescent Kupffer cells was assessed by counting 10 fields. Each point represents the mean ± SEM of four experiments. Only about 10% of the Kupffer cells exhibited autofluorescence in the absence of LY 171883 particles. (B) LY 171883 stimulates nitric oxide production by Kupffer cells. LY 171883 was added to the culture medium at a final concentration of 3 μ*M*. Lipopolysaccharide (1 μg/mL) stimulation of Kupffer cells was used as a positive control. Nitrite accumulation in the media was measured colorimetrically by the Griess reaction after 24 h of culture. Each point represents the mean ± SEM of four experiments; the asterisks (*) indicate statistical difference from control ($p < 0.05$) using ANOVA and Student–Newman–Keuls post hoc tests.

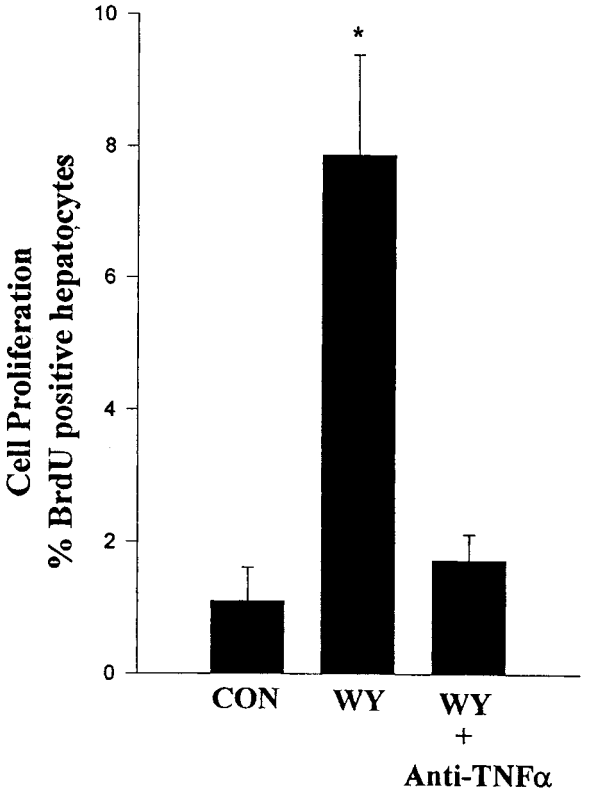

FIG. 4. Effect of anti-TNFα antibody on BrdU incorporation in replicating hepatocytes. Liver tissue was obtained from rats injected with either polyclonal antibodies to TNFα (IP) or an equal volume of control rabbit anti-rat immunoglobin G (0.5 mL). One hour later, they were given WY-14,643 in olive oil vehicle (100 mg/kg IG) with control rats receiving an equal volume of oil. After 24 h, increases in hepatocyte DNA replication were examined by staining for BrdU expression in liver sections. The asterisk (*) denotes a significant difference from control ($p < 0.05$) by Kruskal–Wallis one-way ANOVA on ranks and Dunn's method on pairwise multiple comparisons. All data points are the mean ± SEM ($n = 5–6$) [20].

IV. KUPFFER CELLS ARE CAUSALLY RESPONSIBLE FOR WY-14,643-INDUCED CELL PROLIFERATION

A. Inactivation of Kupffer Cells with Methyl Palmitate Prevents WY-14,643-Stimulated Hepatocyte Replication

Methyl palmitate is a nonmetabolizable fatty acid which supresses reticuloendothelial function and blunts uptake of colloidal carbon, a measure of Kupffer cell activation, by about 70% [57]. Inactivation of Kupffer cells with methyl palmitate has been used to demonstrate their role in the mechanism of 1,2-dichlorobenzene hepatotoxicity and in graft failure following transplantation of cold-stored livers [58,59]. Moreover, methyl palmitate inhibited hepatocyte proliferation following partial hepatectomy, proving that Kupffer cells are involved in liver regeneration [60]. Therefore, methyl palmitate was used to determine if hepatic macrophages are causally involved in hepatocyte mitogenesis stimulated by WY-14,643.

WY-14,643 increased hepatocyte proliferation about eightfold over basal rates, an effect which was prevented completely when Kupffer cells were inactivated with methyl palmitate (Fig. 5A), clearly demonstrating that Kupffer cells are responsible for the mitogenic effect of WY-14,643. Although increased hepatocyte replication is a critical component of the carcinogenic process, the mechanism by which peroxisome proliferators increase DNA synthesis remains unknown. Several findings support the hypothesis that nonparenchymal cells are involved in the stimulation of hepatocyte DNA synthesis by WY-14,643. For example, rates of BrdU incorporation in vivo were increased nearly eightfold by WY-14,643 [4], whereas DNA synthesis in pure cultures of hepatocytes was elevated only about twofold [41]. One explanation for lower rates of hepatocyte DNA synthesis in vitro compared to in vivo is the absence of nonparenchymal cells such as the Kupffer cell. Kupffer cells are a rich source of mitogenic stimuli [61] and are involved in the regulation of hepatocyte growth [60]. Because inactivation of Kupffer cells with methyl palmitate completely prevented the WY-14,643-induced increase in hepatocyte proliferation, it was concluded that Kupffer cells were responsible for the in vivo mitogenic action of this nongenotoxic carcinogen.

Induction of peroxisomes also has been hypothesized to play a role in the carcinogenicity of this class of compounds [2]. However, recent evidence suggests that the two phenomena are not linked because the weak tumor promoter DEHP induced peroxisomes to the same extent as WY-14,643 but was not as tumorigenic even at 12 times the dose of WY-14,643 [4]. Therefore, the effect of methyl palmitate on peroxisome proliferation was determined [62]. Acyl CoA oxidase, the rate-limiting enzyme of peroxisomal β-oxidation which is under transcriptional control of PPARα, was measured as a marker of peroxisome induction

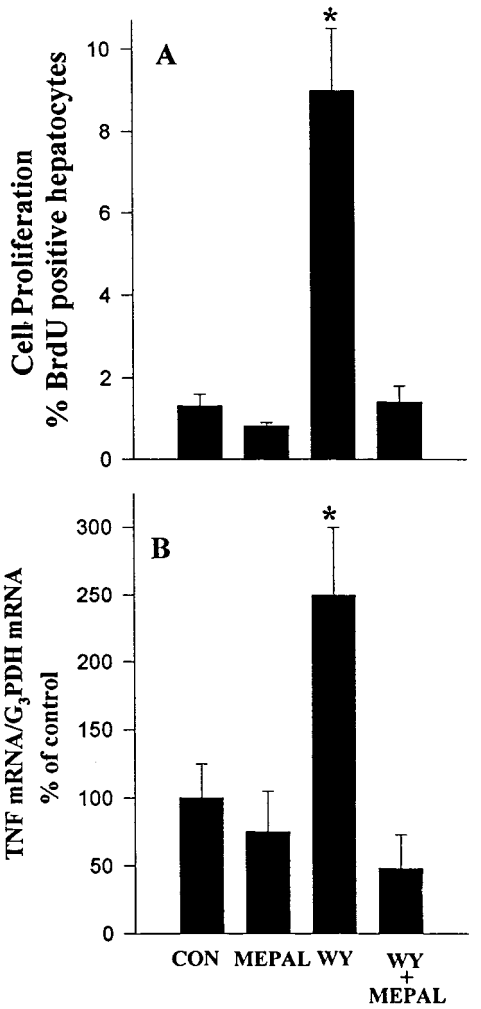

FIG. 5. (A) Effect of methyl palmitate on hepatocyte DNA synthesis 24 h after treatment with WY-14,643. Hepatocytes undergoing DNA synthesis were identified and quantitated as described in Fig. 4. Rates of BrdU incorporation are reported as means ± SEM for rats treated with methyl palmitate or saline vehicle for 4 days prior to treatment with WY-14,643 (100 mg/kg, IG) or olive oil vehicle: control (CON), methyl palmitate (MEPAL), WY-14,643 (WY), WY-14,643 + methyl palmitate (WY + MEPAL). Treatment groups were compared using one-way ANOVA and Student–Newman–Keuls post hoc tests. $p < 0.05$ was selected to define statistical differences between groups. The asterisk (*) denotes

[2,62]. Twenty-four hours after a single treatment with WY-14,643, acyl CoA oxidase activity increased about twofold. This increase was not prevented by pretreatment with methyl palmitate, suggesting that Kupffer cell activation by WY-14,643 does not play a role in peroxisome induction. These data are consistent with the hypothesis that peroxisome proliferation and stimulation of hepatocyte DNA synthesis occur via distinct pathways [4] and that induction of peroxisomes, unlike mitogenesis, does not involve stimuli from Kupffer cells.

B. Kupffer Cell Inactivation Blocks WY-14,643-Induced Increases in TNFα mRNA

Because TNFα has been shown to be involved in WY-14,643-induced cell proliferation and Kupffer cells are a major source of TNFα in liver, the effect of methyl palmitate on TNFα mRNA expression was determined [20]. Changes in TNFα mRNA transcripts were measured using reverse transcription–polymerase chain reaction (RT–PCR) [63]. WY-14,643 increased TNFα mRNA about twofold 24 h after a single dose (Fig. 5B). Very little TNFα expression was detected in livers from control and methyl palmitate-treated rats compared to livers from WY-14,643-treated animals. This transcriptional activation was prevented completely by inactivating Kupffer cells with methyl palmitate.

These studies demonstrated clearly that inactivation of Kupffer cells prevented elevation of TNFα mRNA and blocked increases in hepatocyte replication. From these data, it was concluded that TNFα of Kupffer cell origin was causally responsible for the mitogenic effect of WY-14,643. It follows that Kupffer cells are involved in the mechanism by which peroxisome proliferators cause liver tumors in rodents.

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statistical difference from control, methyl palmitate, and WY-14,643 + MEPAL groups ($p < 0.05$, $n = 4$ for all groups). (B) Effect of methyl palmitate on TNFα mRNA expression 24 h after WY-14,643. TNFα messenger RNA was determined using reverse transcription–polymerase chain reaction and results normalized to the housekeeping gene G_3PDH [20]. Results are reported as percent of control for the ratio of TNFα mRNA to G_3PDH mRNA for each group described in (A) (means ± SEM, $n = 4$ each group). Treatment groups were compared and statistical differences noted as in Fig. 4 [21].

V. DIETARY GLYCINE PREVENTS CELL PROLIFERATION CAUSED BY WY-14,643

A. Dietary Glycine Inhibits the Initial Burst and the Sustained Increase in Cell Replication Caused by WY-14,643

Because inactivation of Kupffer cells with methyl palmitate requires daily IV injections, long-term studies on the role of Kupffer cells in the mechanism of WY-14,643-induced liver cancer were not practical. Therefore, modulation of Kupffer cell activity with dietary agents that limit or prevent cytokine production in response to mitogenic stimuli would provide an ideal way to study the role of Kupffer cell-derived TNFα in sustained increases in hepatocyte proliferation caused by WY-14,643. Increases in TNFα in the serum of rats treated with endotoxin was both delayed and blunted in rats fed dietary glycine [64] (Fig. 1). Because TNFα, which is derived largely from Kupffer cells in liver, is involved in the stimulation of cell proliferation by WY-14,643 and glycine diminishes TNFα production, experiments were designed to test the hypothesis that a glycine-enriched diet would prevent the increase in hepatocyte replication caused by the liver carcinogen WY-14,643. Twenty-four hours following a single treatment with WY-14,643, cell proliferation increased about eightfold from 0.7 ± 0.3% to 5.1 ± 0.3% replicating hepatocytes (Fig. 6A). Although feeding a diet containing 5% glycine for 3 days did not affect basal rates of hepatocyte proliferation under these conditions (Fig. 6A), it largely prevented increases in cell replication due to WY-14,643, with values only reaching 1.9 ± 0.4% (WY + gly, Fig. 6A). This value was significantly less than the sevenfold increase in hepatocyte replication characteristically caused by WY-14,643 and did not differ from controls [22].

To determine if a glycine-enriched diet could prevent the sustained increase in cell proliferation caused by chronic feeding of WY-14,643, rats were fed 0.1% WY-14,643 with or without 5% glycine for 3 weeks. Basal rates of cell proliferation in rats fed a control diet averaged 1.1%, whereas WY-14,643 increased hepatocyte replication about sixfold (Fig. 6B). A glycine-enriched diet alone tended to reduce basal rates of cell proliferation (0.6 ± 0.2%; $p = 0.08$). Importantly, the addition of glycine to the WY-14,643 diet completely prevented the increase in hepatocyte replication caused by 3 weeks of exposure to WY-14,643 alone. Thus, glycine clearly prevented both early and sustained increases in cell replication due to WY-14,643 (Fig. 6).

Because inhibition of apoptosis could contribute to the increase in liver size due to exposure to WY-14,643, rates of apoptosis were also determined. The percentage of apoptotic cells ranged from 0.20% to 0.35% and was not affected by either glycine or WY-14,643. Therefore, it is concluded that changes in apoptosis do not contribute to changes in liver size due to WY-14,643, at least at 3 weeks [22].

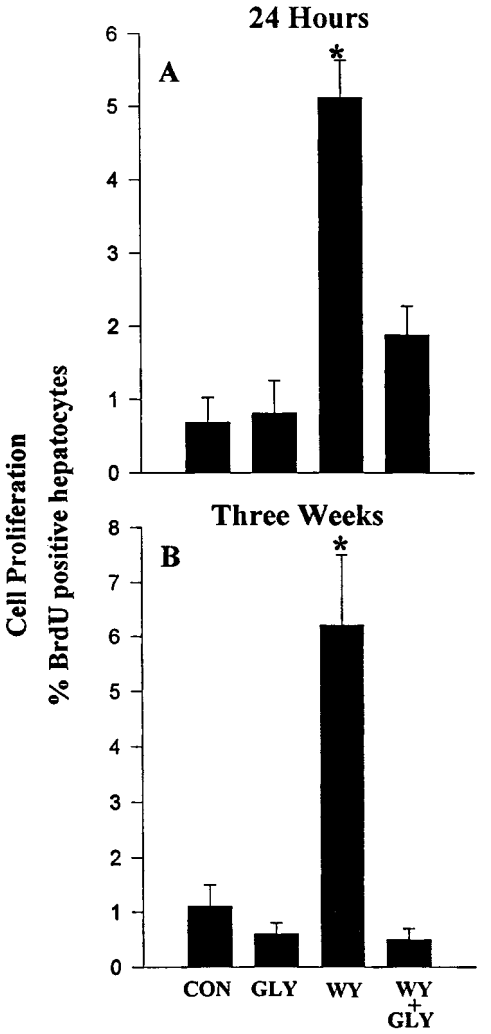

FIG. 6. (A) Hepatocyte proliferation 24 h after treatment with WY-14,643. Proliferating cells were identified and quantitated as described in Fig. 4. Proliferation rates are reported as means ± SEM for the following groups: control (CON), glycine diet (GLY), WY-14,643 (WY), and glycine diet with WY-14,643 treatment (WY + GLY). Asterisks denote statistical differences from the control, glycine, and WY + glycine groups ($p < 0.05$; $n = 4$ all groups). (B) Cell proliferation after 3 weeks of WY-14,643 and glycine in the diet. Cell proliferation was assessed as described in Fig. 4. Results are reported and significance denoted as described in (A) [22].

Twenty-four hours after treatment with WY-14,643, acyl CoA oxidase, a marker of peroxisome induction, was increased 2.5-fold. The glycine-enriched diet had no effect on basal acyl CoA oxidase activity and did not prevent the increase due to WY-14,643, similar to results obtained using methyl palmitate to inactivate Kupffer cells. Peroxisome-specific enzyme activity after 3 weeks of treatment with WY-14,643 increased even more extensively. WY-14,643 caused a sixfold increase in acyl CoA oxidase activity, which also was not prevented by glycine [22].

B. Dietary Glycine Blocks Increases in TNFα mRNA Caused by Exposure to WY-14,643 for 3 Weeks

The mechanism by which glycine prevents the stimulation in hepatocyte proliferation due to WY-14,643 is not entirely clear; however, TNFα has been shown to be involved in WY-14,643-induced cell proliferation [20]. To determine if glycine prevented TNFα production in response to WY-14,643, RT–PCR for TNFα mRNA and immunohistochemical staining for TNFα were performed. TNFα mRNA was similar in control and glycine-treated animals at 3 weeks, whereas WY-14,643 increased TNFα mRNA expression threefold (Fig. 7). Importantly, glycine added to the WY-14,643 diet completely prevented the increase in TNFα mRNA measured after 3 weeks. Immunohistochemical staining for TNFα in sinusoidal lining cells exhibited a similar pattern. Feeding WY-14,643 in the diet for 3 weeks increased staining for TNFα twofold, an increase also prevented by dietary glycine [22].

It is likely that modulation of Kupffer cell activity by glycine is pivotal in the mechanism by which dietary glycine prevents WY-14,643-stimulated cell proliferation because TNFα has been shown to be largely responsible for the WY-14,643-induced increase in cell proliferation [20]. TNFα mRNA transcripts were increased twofold 24 h after WY-14,643 treatment, and the increase in hepatocyte replication caused by WY-14,643 was prevented completely by pretreatment with antibodies to TNFα [20] (Fig. 4). Moreover, inactivation of Kupffer cells with methyl palmitate prevented both WY-14,643-induced cell proliferation and the threefold increase in TNFα mRNA [21] (Fig. 5). Taken together, these data are consistent with the hypothesis that Kupffer cell-derived TNFα is responsible for the early burst of hepatocyte proliferation caused by WY-14,643 (Fig. 1).

Kupffer cells have been shown recently to contain chloride channels that are activated by glycine [65] (Fig. 1). This causes membrane hyperpolarization and prevents increases in intracellular calcium, which are required for Kupffer cells to produce many chemical mediators, including TNFα [65–69] (Fig. 1). In fact, glycine reduced TNFα production by lipopolysaccharide (LPS) stimulated Kupf-

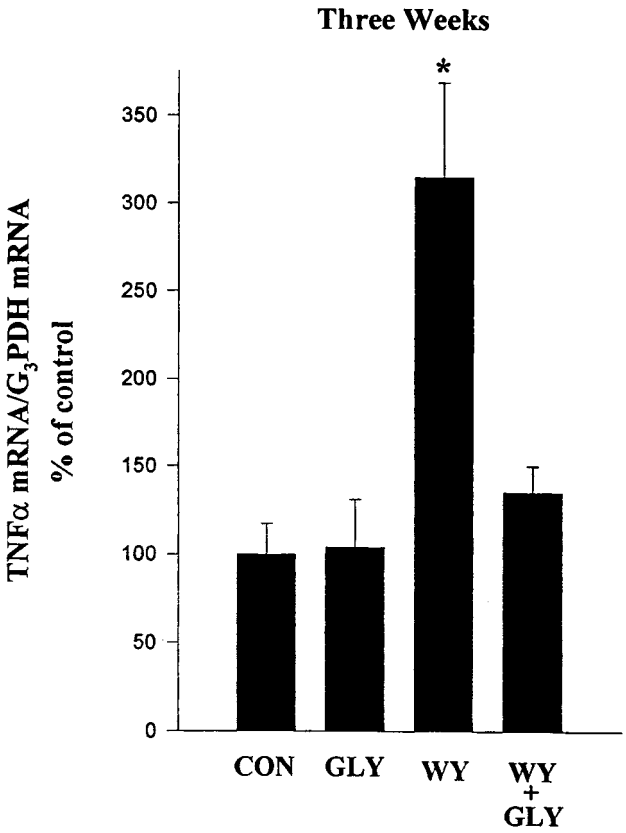

FIG. 7. TNFα mRNA expression after 3 weeks of WY-14,643 and glycine in the diet. TNFα messenger RNA was determined as described in Fig. 5B and results normalized to the housekeeping gene G_3PDH. Results are reported as percent of control for the ratio of TNFα mRNA to G_3PDH mRNA for each group (means ± SEM for groups described in Fig. 6A). Asterisk denotes statistical difference from control (CON), glycine (GLY), and WY + glycine groups (WY + GLY) ($p < 0.05$, $n = 5$) [22].

fer cells in vitro by about 50%, and a diet containing 5% glycine both slowed and blunted the increase in serum TNFα following injection of LPS in vivo [64,65]. Similarly, glycine prevented WY-14,643-induced TNFα production (Fig. 7), most likely due to inhibition of Kupffer cell calcium signaling by activating glycine-gated chloride channels. Therefore, it was concluded that glycine prevents WY-14,643-induced cell proliferation by preventing the production of mi-

togenic levels of TNFα by Kupffer cells (Fig. 1). These data are consistent with the hypothesis that production of TNFα by Kupffer cells plays a central role in the development of WY-14,643-induced liver cancer and raises the possibility that Kupffer cells may also be important in the development of cancer caused by other peroxisome proliferators. The prevention of WY-14,643-induced cell proliferation and the 50% reduction in basal levels of hepatocyte replication with a diet containing glycine predicts that it may be an effective dietary tool for the prevention of cancer caused by peroxisome proliferators.

VI. NUCLEAR FACTOR κB IS ACTIVATED BY WY-14,643

A. NFκB Is Rapidly Activated in Whole Liver Following Treatment with WY-14,643 In Vivo

The transcription factor NFκB (nuclear factor κB) 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 [70]. Dietary feeding of ciprofibrate increased NFκB activity in rat whole liver nuclear extracts after 3 days [71], whereas nafenopin or BR-931 had no effect after a single dose [72,73]. It is possible, however, that much earlier events occur, as a burst in cell replication in liver occurs within hours after treatment with peroxisome proliferators [4]. Because Kupffer cells are implicated in increased cell proliferation via mechanisms involving TNFα, experiments were designed to test the hypothesis that peroxisome proliferators stimulate proliferation of hepatocytes via early activation of the transcription factor NFκB in Kupffer cells [74].

Female Sprague–Dawley rats were treated by gavage with WY-14,643 (100 mg/kg) or vehicle. Activation of NFκB in both whole liver, nonparenchymal cells, Kupffer cells, and hepatocytes was assessed for up to 36 h using an electrophoretic mobility shift assay. In whole liver, WY-14,643 transiently increased NFκB binding maximally 3.5-fold in 2–8 h, followed by a steady decline to near control levels at 36 h (Fig. 8).

B. WY-14,643 Activates NFκB in Kupffer Cells Prior to Hepatocytes

The NFκB activity was elevated about threefold 2–8 h after WY-14,643 treatment in the nonparenchymal cell fraction and then declined toward basal values, similar to what occurred in whole liver. In contrast, hepatocytes exhibited maxi-

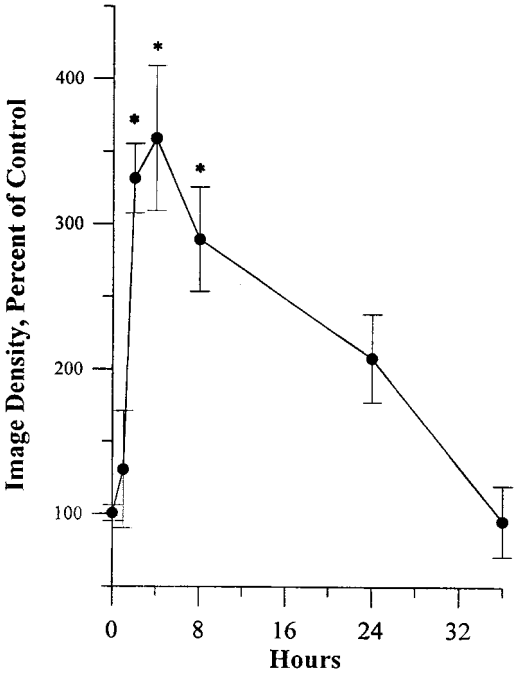

FIG. 8. Time-dependent effect of treatment with WY-14,643 on the activity of NFκB in whole rat liver. Animals were treated with a single dose of WY-14,643 (100 mg/kg, IG) or olive oil vehicle and sacrificed 1, 2, 4, 8, 24, or 36 h after treatment. Nuclear extracts (40 μg of total protein) were prepared from frozen livers, incubated with ^{32}P-labeled double-stranded oligonucleotide encompassing the κB motif, and run on 6% polyacrylamide native gel to detect NFκB DNA binding activity. Density of the NFκB/DNA complex image in liver of rats sacrificed immediately after treatment with WY-14,643 was set to 100%. Data are reported as mean ± SEM for $n = 3–5$. The asterisks (*) denote statistical differences from control ($p < 0.05$) by Kruskal–Wallis one-way ANOVA on ranks followed by Dunn's post hoc test.

mal activity at 8 h, which was about sixfold less than that in the nonparenchymal cell fraction. Importantly, 2 h after WY-14,643 treatment, the active form of NFκB was localized almost exclusively in nonparenchymal cells with values 20–25 times greater than in hepatocytes (Fig. 9). Indeed, the active form of NFκB was localized exclusively in Kupffer cells 2 h after treatment with WY-14,643 [74].

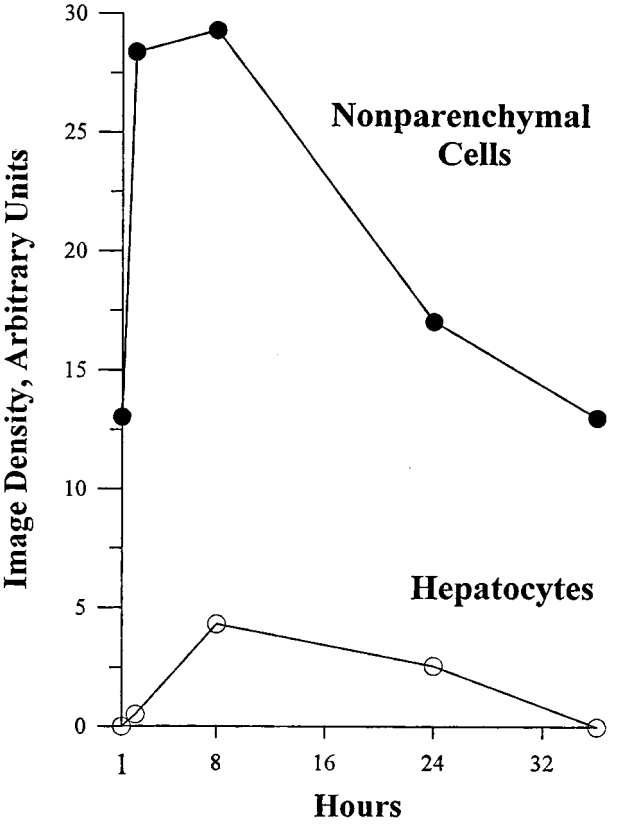

FIG. 9. The active form of NFκB is localized exclusively in Kupffer cells early after treatment with WY-14,643. Rats were treated with a single dose of WY-14,643 (100 mg/kg) and killed at the time points shown. Nonparenchymal cells (●) and hepatocytes (○) were isolated and nuclear extracts prepared. Equal amounts (20 μg) of nuclear extracts were incubated with ^{32}P-labeled double-stranded oligonucleotide encompassing the κB motif as described in the legend to Fig. 8. Densitometry analysis of the NFκB/DNA complex images is described elsewhere [74].

It is possible that activation of NFκB involves protein kinase C, which has been demonstrated to be activated in whole liver by WY-14,643 (Fig. 2). Protein kinase C (PKC) is involved in NFκB activation via two separate pathways. It is known that the ζ isoform of PKC can phosphorylate the inhibitor of κB (IκB) directly in vitro [75]. Further, protein kinase C can increase production of oxi-

dants (e.g., superoxide anion), which are redox modulators of NFκB activity, indirectly by phosphorylation of NADPH oxidase (reviewed in Ref. 76) (Fig. 1). In support of this hypothesis, treatment of Kupffer cells in vitro with WY-14,643 activated superoxide production [48]. Other reactive oxygen species generating systems could be involved also. It is known that Kupffer cells contain significant amounts of xanthine oxidase, which responds much faster to stress than this enzyme in hepatocytes [77]. Indeed, pretreatment with the xanthine oxidase inhibitor and singlet oxygen scavenger allopurinol blocked activation of NFκB by WY-14,643 (Fig. 1) [74]. Because nearly all of the increase in active NFκB is due to Kupffer cells at these early times, it is concluded that Kupffer cell-derived oxidants are involved in WY-14,643-induced activation of NFκB. Later activation of NFκB in hepatocytes occurs most likely as a consequence of TNFα production following activation of Kupffer cells. Binding of TNFα to its receptor and induction of a chain of intracellular events can indeed activate NFκB in hepatocytes [78]. This, in turn, is known to stimulate cell proliferation [79].

VII. CONCLUSION

Peroxisome proliferators have been shown to activate Kupffer cells both in vitro and in vivo [19,45,48], and the use of Kupffer cell inhibitors such as methyl palmitate and dietary glycine have demonstrated that Kupffer cells are responsible for WY-14,643-induced hepatocyte proliferation via mechanisms involving TNFα [21,22]. Moreover, WY-14,643 activated the transcription factor NFκB in Kupffer cells very rapidly after treatment [23], leading to the hypothesis that oxidants of Kupffer cell origin such as superoxide, which are known activators of NFκB, are involved in the mechanism of action of peroxisome proliferators (Fig. 1). Interestingly, oxidant stress caused by leakage of hydrogen peroxide from peroxisomes was hypothesized initially as the mechanism by which these compounds cause liver tumors in rodents [5]. Although it seems unlikely that oxidants of peroxisomal origin explain the mechanism of action of peroxisome proliferators, recent evidence suggests that Kupffer cell-derived oxidants play a key role in initiating TNFα production, which leads to hepatocyte proliferation.

Many of the effects of peroxisome proliferators, including peroxisome induction and hepatomegaly, involve PPARα [41]. PPARα is a member of the nuclear receptor superfamily which dimerizes with the retinoid X receptor (RXR) and binds to peroxisome proliferator response elements (PPREs) to activate gene expression [36]. Acyl CoA oxidase and CYP4A1 increase following treatment with peroxisome proliferators in wild-type mice but not in PPARα-null mice, demonstrating that it activates transcription of these genes [40]. Moreover, exciting new work demonstrated that WY-14,643-stimulated hepatocyte proliferation and, ulti-

mately, the development of tumors requires PPARα [80]. Mice lacking this receptor did not respond to WY-14,643 with an increase in cell proliferation or the development of tumors following feeding 0.1% WY-14,643 in the diet for 1 year [80].

The role of PPARα in Kupffer cell activation and TNFα production caused by WY-14,643 is not known, although it has been shown to be expressed in Kupffer cells [81]. It is possible, therefore, that PPARα is required for TNFα production by Kupffer cells and/or hepatocyte response to TNFα. The role of PPARα in the activation of Kupffer cells and the production of TNFα caused by WY-14,643 remains an important gap in our knowledge to understand the mechanism by which peroxisome proliferators cause liver cancer in rodents. Moreover, the involvement of Kupffer cells in the long-term formation of liver tumors is unknown. Preventing WY-14,643-induced tumors by inactivating Kupffer cells would provide a causal link between Kupffer cell activation by peroxisome proliferators and the development of tumors. In summary, the data presented here demonstrate that peroxisome proliferators activate Kupffer cells and that they are responsible for increased rates of hepatocyte proliferation, most likely via activation of the transcription factor NFκB leading to TNFα production.

ACKNOWLEDGMENT

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