Role of Kupffer cells and oxidants in signaling peroxisome proliferator-induced hepatocyte proliferation

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1. Introduction

There is widespread human exposure to peroxisome proliferators, which are structurally diverse compounds. Peroxisome proliferators received their name based on their ability to induce proliferation of peroxisomes in rodent hepatocytes [1]; they include hypolipidemic drugs, phthalates, steroids, herbicides, and solvents [2]. Despite their structural diversity, these compounds induce predictable pleiotropic responses in rats and mice consisting of hepatomegaly, induction of peroxisomal enzymes of fatty acid β-oxidation, and an increase in both the number and size of peroxisomes [1]. Hepatomegaly has been attributed to hypertrophy of hepatocytes as well as hepatocellular hyperplasia [3]. Importantly, continuous exposure to these agents for 1 to 2 years leads to the development of hepatocellular carcinomas [4,5].

The mechanism by which peroxisome proliferators cause liver tumors in rodents remains unclear. One hypothesis states that elevated levels of hydrogen peroxide produced by peroxisomes causes oxidative DNA damage which leads to the development of cancer [2]. However, there are several problems, both theoretical and experimental, with this hypothesis. For example, if H2O2 production were responsible for their carcinogenicity, the extent of peroxisome proliferation should correlate with the tumorigenicity of the compound. This was not the case in a key experiment designed to test this hypothesis. Specifically, peroxisome proliferation and hepatocarcinogenicity were not correlated when rats were given DEHP, a weak carcinogen, and WY-14,643, a strong tumor promoter [6]. Both chemicals increased hepatic peroxisome number and peroxisomal enzyme activity equally although the incidence of tumors was greater in rats treated with WY-14,643 than in
animals exposed to DEHP. Since peroxisome induction did not correlate with tumorigenicity, alternatives to the oxidative stress hypothesis were considered.

Marsman et al. [6] showed that WY-14,643 produced a 5- to 10-fold increase in cell turnover while the weaker tumor promoter DEHP did not. The potency of the compound and the dose administered were critical for increased hepatic mitogenesis. For example, low doses of WY-14,643, nafenopin, and methylclofenapate did not produce sustained DNA synthesis in rat hepatocytes and were not as carcinogenic as high doses of WY-14,643 [7,8]. Therefore, increased cell replication was proposed to be key in the mechanism of peroxisome proliferators [6].

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 [9]. The promotion of spontaneously initiated cells also relies on cell turnover. This is true for peroxisome proliferators as well. For example, more foci of pre-neoplastic cells are present in livers of older than younger rats following chronic exposure to WY-14,643 and nafenopin, most likely due to the presence of more spontaneously initiated cells in older rats [10,11]. Moreover, formation of basophilic foci following WY-14,643 treatment has been linked to increased tumor incidence [12], and basophilic foci exhibit much higher rates of cell proliferation than surrounding hepatocytes. Taken together, these studies support the hypothesis that cell proliferation is critical to peroxisome proliferator-induced tumor formation.

While peroxisome proliferators increase cell proliferation nearly 10-fold in vivo, replication rates are only doubled in isolated parenchymal cells [6,13]. One hypothesis to explain this discrepancy is the possible involvement of non-parenchymal 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 mediators upon activation (e.g., tumor necrosis factor-α (TNFα), hepatocyte growth factor, and prostaglandin E₂) and therefore may be involved in growth.

![Diagram](image)

**Fig. 1.** Scheme depicting hypothesized mechanism by which peroxisome proliferators stimulate hepatocyte replication. Kupffer cells are activated directly by peroxisome proliferators (e.g., WY-14,643 and MEHP) via mechanisms involving activation of PKC. This leads to the production of superoxide (ROS) by Kupffer cells most likely from NADPH oxidase (NADPH OX). Other studies have demonstrated that oxidants, including superoxide, activate NFκB [68] and that WY-14,643 activates NFκB in Kupffer cells [24]. Further, NFκB is involved in the production of TNFα [69], which is responsible for WY-14,643-induced cell proliferation [21]. Inactivation of Kupffer cells with either dietary glycine or methyl palmitate prevents production of TNFα and subsequent cell proliferation without affecting peroxisome induction (ACO).
modulation of nearby hepatocytes [14–16]. Consistent with this hypothesis, it was shown recently that Kupffer cell activation increased significantly following treatment in vivo with either nafenopin or WY-14,643 [17]. These novel results indicate that peroxisome proliferators activate Kupffer cells in vivo by mechanisms most likely involving protein kinase C (PKC), superoxide and NFκB and suggest a possible role for Kupffer cells in the mechanism of peroxisome proliferators.

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 [18], effects not commonly seen with peroxisome proliferators, it has been demonstrated that it also promotes hepatocyte growth [19,20]. In fact, Schulte-Hermann et al. [74] have demonstrated that peroxisome proliferators are capable of suppressing apoptosis in some models, consistent with the idea that TNFα is not present in apoptotic concentrations. More importantly, the increase in cell replication caused by WY-14,643 in vivo was completely prevented by pretreatment with anti-TNFα antibodies demonstrating for the first time that TNFα is involved in the mechanism by which WY-14,643 activates cell turnover [21]. These exciting 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 action of peroxisome proliferators.

Studies were designed to determine the mechanism by which peroxisome proliferators increase hepatocyte proliferation, a critical component of carcinogenesis. PKC, a second messenger system involved in signaling increases in cell replication, has been shown to be activated following treatment with peroxisome proliferators both in vivo and in vitro. Moreover, it has been demonstrated recently that inhibiting Kupffer cell activity with dietary glycine and methyl palmitate prevents TNFα production and peroxisome proliferator-induced cell proliferation [22,23]. Moreover, the transcription factor NFκB is activated in Kupffer cells rapidly after WY-14,643 treatment in vivo, most likely by mechanisms involving Kupffer cell oxidant production [24]. Taken together, these data support the hypothesis depicted in Fig. 1 that peroxisome proliferators first activate Kupffer cells which produce mitogens that trigger increases in hepatocyte proliferation.

2. Peroxisome proliferators activate PKC in vivo

PKC is a second messenger system which is elevated during increased cell turnover and is involved in signaling cell proliferation [25]. Moreover, phorbol esters, well-known tumor promoters, are activators of PKC [26]. Because peroxisome proliferators also act as tumor promoters, it was hypothesized that they increase PKC. Indeed, WY-14,643 increased PKC 5-fold (Fig. 2) at a dose comparable to that used in vivo.

![Fig. 2. Effect of WY-14,643 on PKC in vivo. Male Fisher 344 rats were given 100 mg/kg WY-14,643 in 0.4 ml olive oil (i.g.) 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 [27]. PKC was measured as described elsewhere [27]. Asterisks (*) denote a significant difference from control by One-way ANOVA and Dunnett’s test. Data represent the means ± S.E.M. (n = 3, * p < 0.05).](image-url)
to that used in chronic studies, which caused tumors in 100% of rats within 1 year [6]. Further, various peroxisome proliferators elevated PKC roughly in proportion to their carcinogenicity in long-term feeding studies [27]. It was proposed, therefore, that the mitogenic effect of peroxisome proliferators involved activation of PKC.

3. Peroxisome proliferators activate PKC in the isolated perfused liver

Further experiments were designed using the isolated perfused liver to examine the effects of peroxisome proliferators on activation of PKC in liver, since this model allows precise delivery of known concentrations of chemicals without metabolism by the gut. Perfluorooctanoate and oleate were infused after 10 and 20 min of perfusion, respectively. Control livers were perfused with 0.06% bovine serum albumin vehicle. Oxygen uptake increased quickly after addition of perfluorooctanoate to the perfusate by nearly 30 μmol/g/h (Table 1). Upon the addition of oleic acid, respiration increased by a further 25 μmol/g/h.

Table 1
Effect of perfluorooctanoate and oleate on oxygen uptake in the perfused liver

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oxygen uptake (μmol/g/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>101 ± 7</td>
</tr>
<tr>
<td>Perfluorooctanoate</td>
<td>141 ± 15 *</td>
</tr>
<tr>
<td>Oleate</td>
<td>124 ± 4 *</td>
</tr>
<tr>
<td>Perfluorooctanoate + oleate</td>
<td>145 ± 16 *</td>
</tr>
</tbody>
</table>

Rats (Sprague–Dawley, female, 150–250 g) were anesthetized with sodium pentobarbital (75 mg/kg) and livers were perfused in a non-recirculating system with Krebs–Henseleit buffer (pH 7.6, 37°C) as described elsewhere [72]. Effluent perfusate was directed past a Teflon-shielded Clark-type electrode to monitor oxygen concentration. Rates of oxygen uptake were determined from influx minus effluent oxygen concentration differences, flow rate and liver wet weight. Perfluorooctanoate (0.5 mM, bound to bovine serum albumin, 0.06% final concentration) and oleate (0.3 mM, also bound to albumin) were infused after 10 and 20 min of perfusion, respectively. Control livers were perfused with 0.06% bovine serum albumin. Values represented are means ± S.D., n = 6 livers per group. The differences in basal oxygen uptake for treatment groups compared to control were significantly different (*) using ANOVA with Tukey’s post-hoc tests (* p < 0.05).

Livers were perfused as described in Table 1. Perfluorooctanoate (0.5 mM, bound to bovine serum albumin, 0.06% final concentration) and oleate (0.3 mM, also bound to albumin) were infused after 10 and 20 min of perfusion, respectively. Control livers were perfused with 0.06% bovine serum albumin. PKC activity was assessed from the binding of 3H-phorbol-12, 13-dibutyrate as described in detail elsewhere [73]. PKC activity is reported as specific binding in CPM/milligrams protein. Protein concentration was determined by the method of Lowry et al. [71]. Values represented are means ± S.D., n = 6 livers per group. The differences in PKC activity for treatment groups compared to control were significantly different (*) using ANOVA with Tukey’s post-hoc tests (* p < 0.05).

Table 2
Effect of perfluorooctanoate and oleate on PKC activity in the perfused liver

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PKC activity (CPM/mg protein × 10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Perfluorooctanoate</td>
<td>2.8 ± 0.5 *</td>
</tr>
<tr>
<td>Oleate</td>
<td>3.2 ± 0.5 *</td>
</tr>
<tr>
<td>Perfluorooctanoate + oleate</td>
<td>3.8 ± 0.8 *</td>
</tr>
</tbody>
</table>

Since unsaturated fatty acids are known activators of PKC in vitro [28], oleate was infused as an internal control in these studies. PKC activity increased 2-fold over control after infusion of oleate into the perfused liver (Table 2). Perfluorooctanoate is known to stimulate PKC in vivo [27]. To determine whether this also occurs in the perfused liver, perfluorooctanoate was infused. Interestingly, PKC activity increased to values nearly twice that of controls under these conditions (Table 2). PKC activity of livers treated with perfluorooctanoate and oleate followed the same pattern as observed in livers perfused with perfluorooctanoate alone (i.e., PKC activity was increased nearly twice over control values).

Peroxisome proliferators did not alter PKC when added directly to subcellular fractions. It was proposed, therefore, that free unsaturated fatty acids were involved in the activation process [27] since stimulation of PKC by unsaturated fatty acids has been demonstrated in vitro [28]. In addition, lipid accumulation occurs in hepatocytes shortly after fenofibrate and ciprofibrate treatment [29–32], and
hepatic triglycerides were increased following 2 weeks of administration of LY 171883 [33]. Perfluorooctanoic, perfluorodecanoic, and nonadecfluoronon-decanoic acid elevated levels of unsaturated free fatty acids (e.g., oleate and palmitate) [27]. Moreover, Reo et al. [34] demonstrated that perfluorodecanoic acid increased serum TNFα levels consistent with the hypothesis that PKC and TNFα are involved in the mechanism of action of peroxisome proliferators. Acyl CoA synthetase plays a central role in lipid metabolism [33] and, it is inhibited by peroxisome proliferators which is hypothesized to lead to an elevation of free fatty acids which can activate PKC (Fig. 2). In fact, a significant correlation between inhibition of acyl CoA synthetase activity by peroxisome proliferators and stimulation of PKC was observed [27]. Although a direct link between inhibition of acyl CoA synthetase activity and increases in PKC has not yet been demonstrated, it is likely that elevation of free fatty acids activates PKC which triggers signaling events leading ultimately to cell proliferation.

Although the role of PKC in cell proliferation and tumorigenesis is complex, it was increased prior to WY-14,643-induced mitogenesis supporting a possible causal relationship. Changes in PKC-mediated signal transduction also have been noted prior to tumor development [35,36]. For example, abnormalities in the expression of c-myc and c-fos were linked with changes in PKC-mediated pathways [35,36].

4. Inactivation of Kupffer cells with methyl palmitate prevents WY-14,643-stimulated hepatocyte replication

Methyl palmitate is a non-metabolizable fatty acid which suppresses Kupffer cell activation, as measured by colloidal carbon clearance, by about 70% [37]. Inactivation of Kupffer cells with methyl palmitate prevented graft failure following transplantation of cold-stored livers [38]. In addition, it inhibited cell proliferation stimulated by partial hepatectomy, sup-

![Fig. 3. (A) Effect of methyl palmitate on hepatocyte replication 24 h after treatment with WY-14,643. Hepatocyte DNA replication was examined by staining for BrdU expression in liver sections as described in detail elsewhere [22,23]. Rates of BrdU incorporation are reported as means ± S.E.M. for rats treated with methyl palmitate or saline vehicle for 4 days prior to treatment with WY-14,643 (100 mg./kg. i.g.) 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. Asterisk (*) denotes statistical difference from control, methyl palmitate, and WY-14,643 + methyl palmitate groups (p < 0.05; n = 4 for all groups). (B) Cell proliferation after 3 weeks of WY-14,643 and glycine in the diet. Proliferation rates are reported as means ± S.E.M. for the following groups: control (CON), 5% glycine diet (GLY), 0.1% WY-14,643 (WY) diet, 5% glycine diet with 0.1% 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) using the statistical tests described in Fig. 2A.
porting the idea that Kupffer cells play a key role in signaling liver regeneration [39]. Therefore, methyl palmitate was used to determine if these liver macrophages are causally involved in WY-14,643-induced hepatocyte proliferation.

When Kupffer cells were inactivated with methyl palmitate (Fig. 3A), the 8-fold increase in cell proliferation caused by WY-14,643 was prevented completely. This demonstrated clearly that Kupffer cells are responsible for the mitogenic effect of WY-14,643. While increased hepatocyte proliferation is a critical component of the carcinogenic process, the mechanism by which peroxisome proliferators increase cell replication remains unclear. The hypothesis that non-parenchymal cells, particularly Kupffer cells, are involved in stimulation of cell proliferation is supported by several studies. For example, WY-14,643 increases DNA synthesis in vivo nearly 8-fold [6] while in pure cultures of hepatocytes, it was elevated only about 2-fold [13]. It is likely that the absence of non-parenchymal cells, such as the Kupffer cell, which is a rich source of mitogenic stimuli [14] and is involved in regulation of hepatocyte growth, plays a role in these lower replication rates in vitro [39]. Since methyl palmitate inactivation of Kupffer cells completely prevented hepatocyte proliferation stimulated by WY-14,643, it was concluded that Kupffer cells were responsible for the mitogenic effect of WY-14,643 in vivo.

Originally, the carcinogenicity of this class of compounds was hypothesized to involve the induction of peroxisomes [2]. Therefore, the role of Kupffer cells in signaling peroxisome proliferation was determined by inactivating them with methyl palmitate [40]. Treatment with WY-14,643 increased acyl CoA oxidase activity about 2-fold after a single treatment. This increase was not prevented by inactivation of Kupffer cells with methyl palmitate. These data suggest that peroxisome induction does not involve activation of Kupffer cells and support the hypothesis that peroxisome induction and increased rates of hepatocyte replication occur via distinct pathways [6]. Further, unlike mitogenesis, induction of peroxisomes occurs independently of Kupffer cells.

Although Kupffer cells were shown to play a role in WY-14,643-induced cell proliferation, the mechanism by which they signaled the increase remained unknown. Since 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 [21], it was hypothesized that Kupffer cell production of TNFα was involved in increased hepatocyte proliferation.

Fig. 4. (A) Effect of methyl palmitate on TNFα mRNA expression 24 h after WY-14,643. TNFα messenger RNA was determined using RT-PCR and results normalized to the housekeeping gene G3PDH [70]. Results are reported as the percentage of control for the ratio of TNFα mRNA to G3PDH mRNA for each group described in Fig. 3A (means ± S.E.M., n = 4 each group). Treatment groups were compared and statistical differences noted as in Fig. 3 [22]. (B) TNFα mRNA expression after 3 weeks of WY-14,643 and glycine in the diet. TNFα messenger RNA was determined and results are reported as described above for each group (means ± S.E.M. for groups described in Fig. 3B). Asterisks denote statistical difference from control (CON), glycine (GLY) and WY + glycine groups (WY + GLY) (p < 0.05, n = 5). [23].
5. Dietary glycine prevents cell proliferation caused by WY-14,643

Determining the role of Kupffer cells in the mechanism of WY-14,643-induced liver cancer requires long-term studies which are not practical for methyl palmitate inactivation because it involves daily i.v. injections. Dietary agents, which inactivate Kupffer cells, would be the ideal way to study their role in sustained increases in hepatocyte replication caused by WY-14,643. Dietary glycine blunted increases in TNFα caused by endotoxin treatment in rats [41]. Because TNFα plays a key role in WY-14,643-stimulated cell proliferation and glycine diminishes TNFα production, it was hypothesized that dietary glycine would prevent the increase in hepatocyte replication caused by WY-14,643. WY-14,643 increased cell replication 24 h after a single dose by nearly 8-fold [23]. Basal rates of hepatocyte proliferation were unaffected by feeding a diet containing 5% glycine; however, it prevented increases in hepatocyte proliferation due to WY-14,643 [23].

Because dietary glycine prevented the initial increase in cell proliferation caused by WY-14,643, rats were fed 0.1% WY-14,643 with or without 5% glycine for 3 weeks to determine if a glycine-enriched diet could prevent the sustained increase in cell proliferation. WY-14,643 increased cell proliferation about 6-fold (Fig. 3B), and the addition of glycine to the WY-14,643 diet completely prevented the sustained hepatocyte replication. Thus, glycine clearly prevented cell proliferation due to WY-14,643 [23].

It was hypothesized that the mechanism by which glycine prevents WY-14,643-stimulated hepatocyte proliferation was inhibition of Kupffer cell TNFα production since TNFα had been shown to be involved in WY-14,643-induced cell replication [21]. In fact, TNFα mRNA was increased about 3-fold after 3 weeks of exposure to WY-14,643, and the addition of glycine to the WY-14,643 diet completely prevented that increase (Fig. 4B). Further, immunohistochemical staining for TNFα exhibited a similar pattern and localized the staining to Kupffer cells. In other studies, TNFα mRNA was increased 2-fold 24 h after a single dose of WY-14,643, and the increase in hepatocyte replication caused by WY-14,643 was prevented completely by pretreatment with antibodies to TNFα [21]. These studies support the hypothesis that Kupffer cell TNFα is responsible for WY-14,643-induced hepatic replication and that dietary glycine prevents WY-14,643-induced cell proliferation by inhibiting Kupffer cell TNFα production.

The mechanism by which dietary glycine inactivates Kupffer cell TNFα production likely involves chloride channels that are activated by glycine [42]. Activation of Kupffer cell chloride channels causes hyperpolarization of the cell membrane. This prevents increases in intracellular calcium, which are required for Kupffer cells to produce TNFα and many other signaling molecules [42–46]. Therefore, it is likely that glycine prevented WY-14,643-induced TNFα production (Fig. 4B) by inhibiting Kupffer cell calcium signaling via this mechanism. It is concluded that glycine inhibits the sustained increase in cell proliferation caused by WY-14,643 by preventing production of mitogenic levels of TNFα by Kupffer cells, which is consistent with the hypothesis that Kupffer cell TNFα plays a central role in WY-14,643-induced liver cancer.

6. NFκB is activated rapidly in Kupffer cells following treatment with WY-14,643 in vivo

NFκB is a transcription factor that is involved in regulation of genes important in inflammatory responses, immune function, and control of cell growth and differentiation [47]. The peroxisome proliferator, ciprofibrate, caused increases in NFκB activity in...
Fig. 5. WY-14,643 activates NFκB in Kupffer cells. Nuclear extracts (8 μg of total protein in each lane) from Kupffer cells of naive rats (CON), rats killed 2 h after treatment with a single dose of WY-14,643 i.g (WY) or with olive oil vehicle (VEH) were incubated with 32P-labeled double-stranded oligonucleotide encompassing the κB motif. Data are means ± S.E.M. (n = 4 for all groups). Density of the NFκB complex images of non-treated rats (CON) was set to 100%. Asterisk denotes statistical difference from control group (p < 0.05 by One-way ANOVA using Tukey’s post-hoc tests.

whole liver after 3 days of treatment [48]. Since a burst in cell replication occurs within hours after treatment with peroxisome proliferators, it is possible that earlier events may occur [6]. Indeed, NFκB activity in the non-parenchymal cell fraction was elevated about 3-fold 2 to 8 h after WY-14,643 treatment [24]. Hepatocytes, on the other hand, exhibited maximal activity at 8 h, although it was about 6-fold less than the activity in the non-parenchymal cell fraction [24]. Importantly, the active form of NF-κB was localized in Kupffer cells 2 h after treatment with WY-14,643 (Fig. 5). Because NFκB is involved in signaling TNFα production, these findings are consistent with the hypothesis that activation of NFκB in Kupffer cells leads to TNFα production and plays a role in WY-14,643-induced hepatocyte proliferation.

7. WY-14,643 directly activates oxidant production by Kupffer cells

Although Kupffer cells were known to be activated by peroxisome proliferator treatment in vivo [17], the mechanism by which they were stimulated was unclear. Therefore, to determine if peroxisome proliferators activate Kupffer cells directly, cells were isolated and cultured, and superoxide production was assessed. Activation of NADPH oxidase results in superoxide production which is a characteristic of stimulated Kupffer cells [14]. WY-14,643 increased superoxide production in a dose-dependent manner with half-maximal stimulation at 2–3 μM (Fig. 6A). WY-14,643 (10 μM) increased superoxide production maximally nearly 7-fold, indicating that it activates Kupffer cells directly. Since both WY-14,643 and diethylhexylphthalate (DEHP) elevate hepatocyte replication within the first few days of treatment, the effect of DEHP on superoxide production was also assessed. Neither DEHP nor ethylhexanol stimulated superoxide production, even at concentrations 50 times greater than WY-14,643 [49]. However, monoethylhexylphthalate (MEHP), a key lipophilic metabolite of DEHP, activated superoxide production nearly as well as WY-14,643 (half-maximal effect = 5 μM MEHP; Fig. 6B).

Since dietary glycine inhibited WY-14,643-stimulated cell proliferation and TNFα production (Figs. 3B and 4B), Kupffer cells were incubated with glycine prior to treatment with WY-14,643 to test the hypothesis that glycine inhibits superoxide production. Indeed, glycine prevented WY-14,643-stimulated superoxide production by Kupffer cells [49]. Superoxide production was completely prevented with 1.0 mM glycine, and inhibition was half-maximal at about 0.05 mM [49].

PKC is involved, at least in part, in LPS-stimulated superoxide production by Kupffer cells via phosphorylation of specific subunits of NADPH oxidase, which leads to the assembly of the active complex and the production of superoxide [50,51]. Therefore, cells were pretreated with staurosporine, a PKC inhibitor, to test the hypothesis that PKC is involved in WY-14,643-stimulated superoxide production. Indeed, staurosporine inhibited WY-14,643-stimulated superoxide production in a dose-dependent manner with an IC50 of about 0.05 pM [49].

The involvement of PKC in Kupffer cell superoxide production stimulated by WY-14,643 was evaluated further by measuring PKC activity in Kupffer cells. Kupffer cells were treated for 20 min with 10
Fig. 6. Effect of (A) WY-14,643 and (B) MEHP on Kupffer cell superoxide production. Kupffer cells were harvested and cultured for 24 h as described in detail elsewhere [49]. WY-14,643, MEHP, or an equal volume of ethanol vehicle (0.1%) were added at the concentrations indicated for 30 min, and superoxide production was measured as described in Methods. Data shown are means ± S.E.M. Asterisks (*) denote statistical differences from control ($p < 0.05$, ANOVA with Bonferroni post-hoc tests, $n = 5$–6 in each group).

µM WY-14,643, a dose which caused maximal stimulation of superoxide production, and PKC activity was determined using incorporation of $\gamma^{32}$P-ATP into a specific PKC substrate, the MARCKS peptide [52]. WY-14,643 increased calcium-dependent PKC activity in Kupffer cells nearly 3-fold (Fig. 7). Moreover, this activation was blocked by pretreatment of Kupffer cells with 1.0 mM glycine, a concentration which also prevented superoxide production (Fig. 7).

Activation of Kupffer cell superoxide production involves activation of PKC [50,51]. PKC activity, which is calcium-dependent, increased following Wy-14,643 treatment of isolated Kupffer cells (Fig. 7). Therefore, it is concluded that PKC is required for WY-14,643-stimulated superoxide production by Kupffer cells.

Pretreatment of Kupffer cells with glycine prevented both WY-14,643-stimulated PKC activity and superoxide production (Figs. 1 and 7) [49]. In addition, dietary glycine prevented WY-14,643-induced hepatocyte proliferation by inhibiting production of TNFα by Kupffer cells [23]. Glycine inhibits Kupffer cell TNFα production via activation of a glycine-gated chloride channel, which hyperpolarizes the cell membrane and blunts intracellular calcium signaling [42]. Since PKC activity is dependent on

Fig. 7. Effect of WY-14,643 on PKC activity in Kupffer cells. Kupffer cells were harvested and cultured as described in Fig. 6. Cells were pretreated with 1.0 mM glycine or water vehicle (1%) for 15 min prior to stimulation with 10 µM WY-14,643 (WY) or an equal volume of ethanol vehicle (0.1%, CON) for 20 min. PKC activity was assessed as calcium- and lipid-dependent incorporation of $\gamma^{32}$P-ATP into the PKC-specific MARCKS peptide [52]. Results were expressed per milligram protein in the assay [71], and data are means ± S.E.M. Asterisk (*) denotes statistical difference from control ($p < 0.05$, ANOVA with Bonferroni post-hoc tests, $n = 5$ in each group).
calcium and superoxide production requires PKC
[49], it is possible that glycine prevents WY-14,643-
stimulated superoxide production by hyperpolarizing
the Kupffer cell membrane, inhibiting calcium sig-
naling, and preventing activation of PKC (Fig. 7). These data are consistent with the hypothesis de-
picted in Fig. 1 that peroxisome proliferators directly
stimulate production of oxidants by Kupffer cells
which activate the transcription factor NFκB. This
leads to TNFα production and elevated rates of cell
proliferation via mechanisms involving PKC.

8. Kupffer cell superoxide production is increased
by treatment with long-term dietary exposure to
WY-14,643

Both WY-14,643 and DEHP elevate hepatocyte
replication during the first few days of treatment;
however, only WY-14,643 sustains rates of prolifera-
tion following 21 days of treatment [6]. Consistent
with a role for oxidants in signaling the initial burst
in cell proliferation, both WY-14,643 and MEHP,
the active metabolite of DEHP, increased superoxide
production following acute treatment (Fig. 6). To
determine if oxidants such as superoxide are in-
volved in signaling sustained 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 and basal rates of superoxide production were
measured. Kupffer cells isolated from rats fed WY-
14,643 generated superoxide at rates 2-fold greater
than cells from controls (Fig. 8); however, superox-
ide production was not stimulated by feeding DEHP
for 3 weeks. Oxidants, including superoxide, are
important signaling molecules that activate the tran-
scription factor NFκB [53], an essential factor in the
production of TNFα [54]. Therefore, Kupffer cell
TNFα production and increased hepatocyte prolifera-
tion may be signaled via activation of NFκB by
oxidants (e.g., superoxide).

9. Summary and conclusion

Oxidant stress caused by leakage of hydrogen
peroxide from peroxisomes was hypothesized ini-
tially as the mechanism by which these compounds
cause liver tumors [55]. It seems unlikely that oxi-
dants of peroxisomal origin explain the mechanism
of action of peroxisome proliferators since treatment
with these compounds in vivo does not lead to
increased hydrogen peroxide production [56]. More-
over, consideration of enzyme kinetics makes it very
unlikely that hydrogen peroxide could diffuse out of
the peroxisome since degradation via catalase is over
five orders of magnitude faster than the rate at which
it is produced [57]. Further, formation of oxidized
DNA bases following treatment with peroxisome
proliferators remains controversial raising questions
about the role of peroxisomal hydrogen peroxide in
the mechanism by which these compounds cause
liver cancer in rodents [58,59]. Interestingly, catalase
is induced about 2-fold in liver following treatment
with peroxisome proliferators [2]; however, measure-
ment of catalase activity in liver cell fractions
demonstrated that it is expressed only in hepatocytes
and not in Kupffer cells (3265.8 ± 777.3 U/g protein
in hepatocytes vs. 11.6 ± 4.2 U/g protein in
Kupffer cells) [49]. Therefore, Kupffer cell-derived
oxidants, including superoxide, may play a key role
in initiating TNFα production, which leads to hepatocyte proliferation, since mechanisms for detoxifying reactive oxygen species may be deficient in these important hepatic macrophages.

Peroxisome proliferators have been shown to activate Kupffer cells both in vitro and in vivo [17,49,60], 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 by mechanisms involving TNFα (Figs. 3 and 4). Moreover, WY-14,643 activated the transcription factor NFκB in Kupffer cells very rapidly after treatment (Fig. 5) 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).

The peroxisome proliferator activated receptor α (PPARα) plays a key role in many of the effects of peroxisome proliferators [41]. PPARα is a member of the nuclear receptor super family, which dimerizes with the retinoid X receptor (RXR). This heterodimer activates gene expression by binding to peroxisome proliferator response elements (PPREs) in responsive genes [61]. For example, acyl CoA oxidase and CYP 4A1 increase following treatment with peroxisome proliferators in wild type mice but treatment of PPARα-null mice has no effect on these enzymes demonstrating that PPARα activates transcription of these genes [62]. In addition to induction of peroxisomal enzymes, WY-14,643-stimulated hepatocyte proliferation and ultimately the development of tumors requires the PPARα [63]. Increased cell proliferation and development of tumors did not occur in mice lacking this receptor following feeding 0.1% WY-14,643 in the diet for 1 year [63]. Whether PPARα plays a role in WY-14,643-stimulated Kupffer cell activation and TNFα production is not known. It remains unclear if PPARα is expressed in Kupffer cells [64,65]; however, it is possible that the hepatocyte response to TNFα may involve the PPARα. 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. Moreover, the involvement of Kupffer cells in the long-term formation of liver tumors is unknown. Interesting new studies demonstrated that dietary glycine inhibits the development of liver tumors caused by dietary exposure to WY-14,643 [66]. While the mechanism remains unclear, it was hypothesized that glycine inhibits angiogenesis [67].

Taken together, these data are consistent with the hypothesis depicted in Fig. 1 that PKC is involved in WY-14,643-stimulated superoxide production by Kupffer cells. Oxidants, including superoxide, lead to activation of NFκB and increased TNFα production by Kupffer cells, which stimulates cell proliferation characteristic of this class of non-genotoxic hepatocarcinogens.

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