Long-term exposure of rodents to peroxisome proliferators leads to increases in peroxisomes, hepatocellular proliferation, oxidative damage, suppressed apoptosis, and ultimately results in the development of hepatic adenomas and carcinomas. Peroxisome proliferators–activated receptor (PPAR)\(x\) was shown to be required for these pleiotropic responses; however, Kupffer cells, resident liver macrophages, were also identified as playing a role in peroxisome proliferators–induced effects, independently of PPAR\(x\). Previous studies showed that oxidants from NADPH (nicotinamide adenine dinucleotide phosphate, reduced) oxidase mediate acute effects of peroxisome proliferators. In particular, reactive oxygen species produced as a result of acute peroxisome proliferator treatment were shown to be Kupffer cell derived using NADPH (nicotinamide adenine dinucleotide phosphate, reduced) oxidase deficient (\(p47^{phox}\)-null) mice (Rusyn et al., 2005; Peters et al., 1997, 1998). Suppressed apoptosis caused by WY-14,643 was dependent on both NADPH oxidase and PPAR\(x\). Collectively, these findings suggest that involvement of Kupffer cells in WY-14,643–induced parenchymal cell proliferation and oxidative stress in rodent liver is an acute phenomenon that is not relevant to long-term exposure, but they are still involved in chronic apoptotic responses. These results provide new insight for understanding the mode of hepatocarcinogenic action of peroxisome proliferators.

Key Words: Kupffer cells; PPAR\(x\); peroxisome proliferators; carcinogenesis.

Peroxisome proliferators are a class of chemically diverse compounds such as hypolipidemic drugs, plasticizers, industrial solvents, and pesticides. Peroxisome proliferators cause a number of adverse cellular and molecular changes in rodent liver, including an increase in the number and size of peroxisomes and proliferation of hepatocytes (Marsman et al., 1988). Rodents also develop hepatocellular neoplasia as a result of chronic administration of peroxisome proliferators (Lalwani et al., 1981; Reddy et al., 1976, 1979). Most of the pleiotropic effects of these agents are nuclear receptor–mediated through peroxisome proliferators–activated receptor (PPAR)\(x\). PPAR\(x\) activation is required for peroxisome proliferators–induced growth responses and for liver carcinogenesis (Hays et al., 2005; Peters et al., 1997). However, a number of early events in liver that are independent of PPAR\(x\) have also been reported, including Kupffer cell activation, release of reactive oxygen species, and production of mitogenic cytokines (Rose et al., 1997b, 1999; Rusyn et al., 2000).

Increased oxidant generation and cell proliferation, along with suppression of apoptosis, is thought to be a key step in the mode of action of nongenotoxic liver carcinogens, including peroxisome proliferators (Bayly et al., 1994; Marsman et al., 1988; Reddy and Rao, 1989). Within hepatocytes, these responses facilitate the formation and fixation of oxidative DNA lesions and clonal expansion of mutated cells, which could predispose cells to tumor development. It is well known that PPAR\(x\) is required for sustained growth responses to peroxisome proliferators (Hays et al., 2005; Peters et al., 1997, 1998); however, the chronic PPAR\(x\)-independent mediators of cellular response are poorly understood. Previous studies demonstrate that Kupffer cells, the resident macrophages of the liver, are important mediators of acute phase responses to peroxisome proliferators. In particular, reactive oxygen species produced as a result of acute peroxisome proliferator treatment were shown to be Kupffer cell derived using NADPH (nicotinamide adenine dinucleotide phosphate, reduced) oxidase–deficient (\(p47^{phox}\)-null) mice (Rusyn et al., 2000, 2001). Furthermore, since Kupffer cells do not express PPAR\(x\) (Peters et al., 2000) it is likely that these effects are nuclear receptor independent. Peroxisome proliferators–induced production of mitogenic cytokines is also a result of Kupffer cell activation (Bojes et al., 2007).
MATERIALS AND METHODS

Animals and diet. pPpara−/− null male mice (C57BL/6J background; Jackson et al., 1995), PPARα-null male mice (SV129 background; Lee et al., 1995), and corresponding wild-type counterparts (6–8 weeks of age at the beginning of treatment) were used in these experiments. All animals used for this study were housed in sterilized cages in a facility with a 12-h night/day cycle. Temperature and relative humidity were held at 22 ± 2°C and 50 ± 5%, respectively. The UNC Division of Laboratory Animal Medicine maintains these animal facilities, and Institutional guidelines and studies were performed according to protocols approved by the appropriate institutional review board. Prior to experiments, animals were maintained on standard lab chow diet and purified water ad libitum. WY-14,643 was obtained from Aldrich (Milwaukee, WI). NIH-07 was used as the base for the pellet diet (prepared by Harlan Teklad, Indianapolis, IN) containing either 0% (control), or 0.1% wt/wt of WY-14,643. Dietary concentration of WY-14,643 was measured by high-performance liquid chromatography after the pellets were made and determined to be ± 18% of the target concentration. Diet was administered ad libitum for 1 week, 5 weeks, or 5 months. Animals had free access to water throughout the study and the health status of the animals was monitored every other day.

Tissue collection. Body weight was recorded on a weekly basis per cage, not for individual animals. Three days prior to sacrifice mice were administered bromodeoxyuridine (BrdU)–containing water (0.2 g/l). At sacrifice, mice were anesthetized with pentobarbital (100 mg/kg) and following exsanguination, livers were removed and weighed. A section from the left lateral lobe was fixed in 10% formalin. A section of the duodenum, which is a rapidly proliferating tissue, was also collected and fixed in formalin for use as a positive control in immunohistochemical staining and to confirm that mice received BrdU. The remaining tissue was placed in an eppendorf tube and snap frozen in liquid nitrogen. These samples were stored at –80°C until assayed.

Histopathological evaluation. A quantitative method was employed to determine the severity of necrosis, steatosis, or inflammation in mouse liver. Slightly modified from the previous method (Mauton, 2001), the scoring involves overlaying a grid on a low magnification photo of the hematoxylin/eosin-stained liver section. The necrosis index was calculated as the number of points overlapping a necrotic region/total number of points overlapping the liver section. The same procedure was followed for steatosis and inflammation indices. Grid points over portal or central veins were excluded from the calculation in cases in which the entire liver section was too large to be captured in one photo, the average score was taken from photos of two or more unique fields.

BrdU immunohistochemistry. Liver tissue sections were fixed in 10% formalin for 24 h and transferred to 70% ethanol. Sections were embedded in paraffin and 5-μm slices were mounted onto Probe-On Plus (Fisher Scientific, Pittsburgh, PA) slides. Slides were rehydrated in serial solutions of xylene, 100%, 95%, 70%, 50%, and 30% ethanol and water. For BrdU staining, the tissue was hydrolyzed in 4% HCl for 20 min at 37°C and permeabilized in a 0.2% pepsin/0.1% HCl solution for 15 min at 37°C. Immunostaining was performed with a monoclonal antibody against BrdU (Dako, Carpinteria, CA) diluted 1:200 in 1% bovine serum albumin in phosphate buffered saline. Immunohistochemical detection was performed using a horseradish peroxidase (HRP)–labeled goat anti-mouse secondary antibody followed by staining with a 3,3′-diaminobenzidine chromogen solution. Slides were counterstained using hematoxylin. Image analysis was performed at a magnification of ×200 using Bioquant Nova Prime (Bioquant Image Analysis Corp., Nashville, TN) software. A labeling index was calculated as the number of positively stained nuclei/total number of nuclei counted × 100% (at least 2000 nuclei/sample).

Preparation of protein extracts. Cytosolic and nuclear protein extracts were prepared by homogenizing 50 mg of liver tissue in 400 μl of buffer A, which contained 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10mM KCl, 0.1mM ethylenediaminetetraacetic acid (EDTA), 0.1mM, ethyleneglycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1mM dithiotreitol (DTT), and 0.5mM phenylmethylsulfonyl fluoride (PMSF), and the homogenate was placed on ice for 15 min. After adding 25 μl of NP-40, the homogenate was centrifuged at 4°C for 30 s at 16,000 × g. The supernatant was aliquoted as the cytosolic fraction. The remaining pellet was resuspended in 400 μl of buffer A and 25 μl of NP-40 and centrifuged at the above conditions. The supernatant was discarded and remaining cells were resuspended in 250 μl of buffer B containing 20mM HEPES, 0.4M NaCl, 10mM KCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.5mM PMSF, and 1mM Na3VO4 for 30 min on ice. After spinning the mixture at 4°C for 5 min at 16,000 × g, the supernatant was aliquoted as the nuclear fraction. Protein concentration of the cytosolic and nuclear extracts was determined using a bicinchoninic acid assay (Pierce Biotechnology, Inc., Rockford, IL) prior to storing at 80°C.

Western blot analysis. To assess protein levels of Cyclin B1, Cdk1, Cdk2, and C-myc, nuclear protein extracts were used, while cytosolic fractions were used for measuring Caspase 8 and Caspase 9. Proteins (10 μg per lane) were separated on a 4–12% Bis–Tris gel and transferred to a nitrocellulose membrane. Immunodetection was performed by incubating membranes with the primary antibody then conjugating with a HRP-labeled secondary antibody
Detection of apurinic/apyrimidinic sites. Genomic DNA was extracted by a procedure slightly modified from the method reported previously (Nakamura et al., 2000). To minimize formation of oxidative artifacts during isolation, 2,2,6,6-tetramethylpiperidinoxyl (20 mmol/l) was added to all solutions, and all procedures were performed on ice. The apurinic/apyrimidinic (AP) site assay was performed as previously described (Nakamura and Swenberg, 1999).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) (Panomics, Inc., Redwood City, CA) was performed according to manufacturer’s instructions. Briefly, 10 μg of nuclear protein extract from liver tissue was preincubated with 1 μg of poly (I-C) at room temperature. Binding buffer, 10 ng of DNA probe and water were added to the mixture and allowed to incubate for 30 min at 17°C. The mixture was resolved on a 6% polyacrylamide gel (Invitrogen Corp., Carlsbad, CA). After electrophoresis, the probe was transferred to a Biodyne B nylon membrane (Pall Corp., East Hills, NY), then dried for 1 h at 80°C. To reduce nonspecific binding, the membrane was placed in blocking buffer, followed by incubation with HRP–streptavidin conjugate (1:1000). The membrane was washed prior to adding ECL detection buffer and then exposed to film. Specificity of the nuclear factor kappa B (NFκB) probe was confirmed by competition assays using Hela cell nuclear extracts, whereby twofold excess unlabeled NFκB probe was added to the mixture prior to addition of labeled probe.

Acyl-coA oxidase activity. Acyl-coA oxidase (ACO) activity and expression were measured as indicators of peroxisomal induction (Tomaszewski et al., 1987). The activity of ACO was determined by measuring formaldehyde, which is formed from catalase-mediated oxidation of methanol by hydrogen peroxide. Liver tissue (100 mg) was homogenized in 10 volumes of 0.25M sucrose buffer. A volume of 1.4 ml of reaction mixture (see Rose et al., 1997b for details) was warmed at 37°C and mixed with 100 μl of homogenate. The reaction was terminated after 5 min by adding 40% trichloroacetic acid. Blanks were prepared in parallel, in which 40% trichloroacetic acid was added before homogenate. Samples and blanks were centrifuged to pellet protein and 1.0 ml of the supernatant was added to 0.4 ml of Nash reagent containing acetyl acetone, which reacts with formaldehyde to form diacetyl-dihydrolutidine (Nash, 1953). The concentration of diacetyl-dihydrolutidine was measured spectrophotometrically at λ = 405 nm. Protein concentration was determined using the BCA protein assay (Smith et al., 1985).

Statistical analysis. All assays were performed using at least n = 3 samples, except PPARα wild type mice fed control diet for 5 months, for which only two samples were available. Data represented as mean values plus or minus the standard error, unless otherwise noted. Two-way analysis of variance with Tukey’s multiple comparison test was employed for statistical comparison between experimental groups. A p-value less than 0.05 was selected prior to the study to determine statistical differences between groups.

RESULTS AND DISCUSSION

Liver Toxicity and Cell Proliferation Effects of WY-14,643 in Mouse Liver

To determine if Kupffer cells are involved in chronic effects of peroxisome proliferators in liver, NADPH oxidase–deficient (p47phox-null) and C57BL/6J wild type mice were fed WY-14,643–containing diet (0.1% wt/wt) for 1 week, 5 weeks, or 5 months. In parallel, Pparα-null and SV129 wild type mice were also treated for up to 5 months. Over the course of treatment there was a significant decline in body weight (approximately 20%) in all WY-14,643–treated mice as compared to controls, except for Pparα-null mice (Fig. 1A). This effect was accompanied by significant progressive increases in absolute and relative liver weight in all WY-14,643–treated groups, except for Pparα-null mice (Fig. 1B). By 5 months of treatment, the liver enlargement was most pronounced in p47phox-null mice. The effect of WY-14,643 was much greater in C57BL/6J mice than in SV129 strain. High attrition was also observed in WY-14,643–treated mice (Fig. 1C), particularly those on the C57BL/6J background strain.

Upon necropsy and histopathological assessment, necrotic gross liver lesions (no more than 2 mm in diameter) were found in one-half of the SV129 wild type mice and all of the p47phox-null mice treated with WY-14,643 for 5 months. Overall liver necrosis and inflammation scores in these two groups were significantly higher than in control-fed mice or C57BL/6J and Pparα-null mice fed WY-14,643 (Fig. 2E and Supplemental Table 1). Activity of serum alanine aminotransferase (ALT), a serum marker of liver injury, was significantly elevated in WY-14,643–treated wild type strains and p47phox-nulls, but not in Pparα-null mice (Table 1) at all time points.

Low grade lipid accumulation in hepatocytes was found in both WY-14,643–treated wild type strains and p47phox-null mice (Fig. 2F). Livers from control or WY-14,643–treated Pparα-null mice showed moderate levels of lipid accumulation (Fig. 2C and Supplemental Table 1). Other groups of mice fed control diet displayed normal liver morphology.

The selection of the dose of WY-14,643 for this study was based on previous acute, subchronic and chronic studies in the mouse. We observed that the 5 months survival of p47phox-null and wild type C57BL/6J mice on the WY-14,643 diet was below 40% with pronounced temporal increases in serum ALT levels. Similarly, several long-term dietary feeding studies with peroxisome proliferators in rodents reported higher attrition rates (Peters et al., 1997; Torrey et al., 2005; Ward et al., 1998). This indicates that 0.1% (wt/wt) WY-14,643 is higher than a maximal tolerated dose in mice and the data in this study should be interpreted with caution.

The similarities in WY-14,643–induced liver injury across strains and the disparate survival of C57BL/6J versus SV129 mice suggest that strain variations in response to WY-14,643 may not be a result of liver toxicity alone. Several studies reported significant weight loss in rodents given peroxisome proliferators chronically (Hurt et al., 1997; Peters et al., 1997; Torrey et al., 2005; Ward et al., 1998). Excessive energy metabolism resulting in a significant reduction in fat stores is thought to be the primary contributing factor to this effect of PPARα agonists. Indeed, we also observed that WY-14,643 “responder” strains (SV129 wild type, C57BL/6J wild type...
and p47phox-nulls) lost at least 20% of the body weight. However, the rate of weight loss between strains was remarkably different. C57BL/6J mice had a much higher initial rate of weight loss, a response that could be associated with high morbidity/mortality of these mice when given WY-14,643. Given the considerable loss of body mass, it is possible that muscle wasting (Johnson et al., 2005) was the contributing factor in the premature deaths.

As expected, we found that WY-14,643 causes remarkable hepatomegaly and induces cell proliferation in liver (Fig. 3A). However, the temporality of WY-14,643–induced liver enlargement and cell proliferation patterns observed in this study is not consistent with previous reports that peroxisome proliferators cause a rapid up-and-down surge in cell proliferation in the first week of treatment (Marsman et al., 1988). In our study, all WY-14,643 “responder” mouse strains exhibited a robust elevation in BrdU labeling in liver for as long as treatment continued, which has been demonstrated in chronic studies with other peroxisome proliferators (Ward et al., 1988; Yeldandi et al., 1989). While there are notable differences in our experiment and previous reports (e.g., rodent species, detection methods, etc.), we argue that the pathophysiological effects of peroxisome proliferators in rodent liver, including the robust proliferative response, extend beyond the time frame that was traditionally considered. In addition, our findings of significant liver injury may suggest that hepatocellular proliferation may be elevated, at least in part, due to compensatory liver regeneration.

**NADPH Oxidase Deficiency Does Not Prevent Hepatocellular Proliferation, but Affects the Decrease in Apoptosis Caused by WY-14,643**

It was suggested that activation of Kupffer cells and resultant production of oxidants and mitogenic cytokines play a role in acute cell proliferation response caused by peroxisome proliferators (Rose et al., 1997a,b). To determine if Kupffer cell NADPH oxidase is necessary for a sustained growth response caused by these agents, changes in liver morphology, BrdU incorporation, and alterations in protein markers of cell turnover were assessed.

Histological evaluation of livers from WY-14,643–treated p47phox-null revealed significantly hypertrophied hepatocytes, an increase in mitotic bodies, and presence of binucleate hepatocytes, effects identical to those observed in C57BL/6J wild type mice. Furthermore, a significant temporal increase in hepatocellular proliferation was observed at all time points with a peak at 5 weeks in both p47phox-null and wild type mice (Fig. 3A). Western blot analysis of 5 month liver tissue was conducted to determine if Kupffer cell oxidants are important...
FIG. 2. Liver histopathology in mice treated for 5 months with WY-14,643. (A) Normal liver morphology in PPARα +/- mice on the control diet. (B) Photomicrograph of a necro-inflammatory lesion in liver of PPARα +/- mice treated with WY-14,643 (0.1%). Necrotic (n) and inflamed (i) areas are indicated. (C) Steatosis (s) is observed in PPARα +/- mice fed WY-14,643 (0.1%). (D) Extensive hepatocellular hypertrophy is evident in PPARα +/- mice treated with WY-14,643 (0.1%). (E) Regions of necrosis and inflammation in liver from p47^phox +/- mice fed WY-14,643 (0.1%). (F) High magnification photomicrograph showing mitotic cells (m) in PPARα +/- mice treated with WY-14,643 (0.1%). (G) High magnification photomicrograph showing mitotic cells (m) in PPARα +/- mice on the control diet.

in cell cycle regulation of proliferation (Table 2). WY-14,643–induced increases in Cyclin B1, Cdk1, and Cdk4 expression were not affected by Kupffer cell NADPH oxidase deficiency. However, as previously reported (Peters et al., 1998), altered expression of these proteins caused by peroxisome proliferators was dependent on PPARα.

Suppression of apoptosis has been suggested as another key mechanism by which peroxisome proliferators may affect liver cell turnover and contribute to carcinogenesis. A number of studies have argued that reduced apoptosis after exposure to peroxisome proliferators is a TNF-α–mediated (i.e., Kupffer cell-dependent) response (Hasmall et al., 2000; Rolfe et al., 1997). To determine if, in fact, a sustained reduction of apoptosis is present during long-term feeding of these agents and whether it is dependent upon Kupffer cell NADPH oxidase, expression of Caspase 8 and Caspase 9 proteins was assessed. Treatment with WY-14,643 for 5 months led to a decrease in expression of Caspase 8 in both wild type strains (Table 2). Interestingly, this effect was dependent on both PPARα and Kupffer cell NADPH oxidase since no reduction in Caspase 8 expression in response to WY-14,643 was observed in either knockout strain. Levels of Caspase 9, which leads to apoptosis as a result of mitochondrial stress, remained unchanged in all WY-14,643–treated animals.

To further assess the mechanism by which WY-14,643 perturbs normal cell turnover in mouse liver, NFκB binding activity was measured. This transcription factor is thought to be intimately involved in apoptosis and cell proliferation (Guttridge et al., 1999). Exposure to a single dose of WY-14,643 leads to rapid activation of NFκB in Kupffer cells and then in hepatocytes (Rusyn et al., 1998), an effect that was shown to be dependent upon NADPH oxidase (Rusyn et al., 2000). While some studies show that NFκB activation by peroxisome proliferators in rodent liver is a sustained response (Li et al., 1996) and may be important for tumor promotion by these agents (Glaueatt et al., 2006), others failed to observe any induction in DNA-binding activity of NFκB by peroxisome proliferators (Menegazzi et al., 1997; Ohmura et al., 1996). Our data show that subchronic treatment with WY-14,643 leads to a sustained elevation in NFκB activity in liver (Fig. 3B). Furthermore, NFκB activation appears to be dependent on PPARα and is unaffected by the lack of NADPH oxidase, further supporting a temporal shift from Kupffer cell– to hepatocyte-centric effects of peroxisome proliferators. Given that NFκB activation by peroxisome proliferators is thought to be, in part, due to oxidative stress (Calfee-Mason et al., 2004), it is possible that PPARα-dependent oxidant production in hepatocytes as a result of long-term WY-14,643 treatment is responsible for the continued induction of NFκB binding observed here.

Collectively, while several studies suggested that the acute effects of WY-14,643 in rodent liver are mediated through PPARα–dependent activation of the Kupffer cell, this report shows that this component of the mode of action is short lived and not sustained. Indeed, p47^phox-/- mice responded to subchronic treatment with WY-14,643 with the most pronounced hepatomegaly, increase in liver cell proliferation, and exhibited upregulation of cell cycle proteins, while Pparx-null mice showed no response to treatment, as expected (Peters et al., 1997; Ward et al., 1998). The lack of a sustained effect of the Kupffer cell–mediated events suggests that activation of PPARα is the primary event responsible not only for the induction of peroxisomes, but also for cell proliferation in liver. While there is no evidence that PPARα has a direct affect on...
transcription of cell cycle–regulating genes (Peters et al., 1998), there is strong evidence for transcriptional regulation by NFκB (Guttridge et al., 1999), further supporting the idea that WY-14,643–induced cell proliferation may be mediated by oxidant-dependent activation of NFκB that follows PPARα activation and induction of oxidant-generating enzymes in hepatocytes. In addition, a strong PPARα- and NADPH oxidase–dependent suppression of caspase 8, an initiator caspase of the death receptor pathway leading to apoptosis (Varfolomeev et al., 1998), was observed. This finding suggests that altered regulation of apoptosis by peroxisome proliferators is mediated by death receptor pathways that may include multiple signals from the Kupffer cells and from within the hepatocyte through a PPARα-mediated pathway. In addition, our data suggest that suppression of apoptosis may not be as biologically relevant as increased proliferation and induction of oxidant-generating peroxisomes, considering that even in absence of reduced apoptosis, NADPH oxidase–deficient mice still exhibit significant WY-14,643–induced changes in hepatocellular proliferation and liver enlargement.

**PPARα, not NADPH Oxidase, Mediates Chronic Oxidative DNA Damage by WY-14,643**

Peroxisome proliferators induce reactive oxygen species, which are known to cause oxidative damage to cellular macromolecules (Conway et al., 1989; Deutsch et al., 2001; Reddy and Rao, 1989). Previous studies have demonstrated that early increases in reactive oxygen species by peroxisome proliferators require Kupffer cell NADPH oxidase (Rusyn et al., 2001). To determine if Kupffer cell NADPH oxidase is involved in oxidative DNA damage resulting from long-term peroxisome proliferator administration, AP sites were measured in DNA from liver of mice fed control or WY-14,643–containing diet for 5 months. Chronic treatment with WY-14,643 caused a twofold increase in AP sites, a response which required PPARα, but not NADPH oxidase (Fig. 4A). To determine if peroxisomal oxidases are a potential source of prolonged oxidant production contributing to peroxisome proliferators–induced oxidative DNA damage, ACO activity was measured in liver homogenate following 5 months of WY-14,643 treatment. Activity of this peroxisome proliferation-marker protein was increased in a PPARα-dependent manner similar to that observed in peroxisome proliferators–induced DNA damage (Fig. 4B). These findings corroborate previous reports that peroxisome proliferators cause oxidative damage (Conway et al., 1989; Rusyn et al., 2004; Tomaszewski et al., 1990) through a mechanism that involves oxidants from parenchymal cells.

Along with altered cell turnover, oxidative DNA damage is another mode of action that is considered to be important to peroxisome proliferators–induced carcinogenesis. Overproduction of reactive oxygen species from a number of cellular sources can lead to oxidative damage of macromolecules in absence of scavenging proteins (Reddy and Rao, 1989; Yeldandi et al., 2000). Induction of microsomal and peroxisomal oxidases in hepatocytes and activation of NADPH oxidase in Kupffer cells are two potential molecular sources of peroxisome proliferators–induced oxidant production, respectively. It was previously shown that dietary administration of WY-14,643 for up to 1 month led to increased expression

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time course</th>
<th>N</th>
<th>ALT* (U/l)</th>
<th>Triglycerides* (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα +/-</td>
<td>Control</td>
<td>7</td>
<td>25.1 ± 4.9</td>
<td>101.4 ± 12.3</td>
</tr>
<tr>
<td></td>
<td>WY-14,643 (0.1%)</td>
<td>3</td>
<td>109.7 ± 34.9*</td>
<td>55.0 ± 3.2*</td>
</tr>
<tr>
<td></td>
<td>5 weeks</td>
<td>6</td>
<td>572.3 ± 79.7**</td>
<td>55.0 ± 0.0*</td>
</tr>
<tr>
<td></td>
<td>5 months</td>
<td>6</td>
<td>760.7 ± 215.3**</td>
<td>29.8 ± 6.0**</td>
</tr>
<tr>
<td>PPARα -/-</td>
<td>Control</td>
<td>6</td>
<td>21.1 ± 4.1</td>
<td>33.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>WY-14,643 (0.1%)</td>
<td>3</td>
<td>28.6 ± 16.5</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>5 weeks</td>
<td>4</td>
<td>12.0 ± 1.7</td>
<td>131.7 ± 24.1*</td>
</tr>
<tr>
<td></td>
<td>5 months</td>
<td>5</td>
<td>36.6 ± 6.2</td>
<td>201.8 ± 17.6**</td>
</tr>
<tr>
<td>p47phox +/-</td>
<td>Control</td>
<td>7</td>
<td>42.5 ± 3.0</td>
<td>69.7 ± 6.6</td>
</tr>
<tr>
<td></td>
<td>WY-14,643 (0.1%)</td>
<td>12</td>
<td>105.1 ± 13.5*</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>5 weeks</td>
<td>7</td>
<td>697.5 ± 89.3**</td>
<td>52.4 ± 10.3</td>
</tr>
<tr>
<td></td>
<td>5 months</td>
<td>3</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>p47phox -/-</td>
<td>Control</td>
<td>6</td>
<td>27.8 ± 6.7</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>WY-14,643 (0.1%)</td>
<td>3</td>
<td>182.2 ± 51.5*</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>5 weeks</td>
<td>5</td>
<td>1454.0 ± 170.8**</td>
<td>30.5 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>5 months</td>
<td>3</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Note. Asterisks denote statistical significance from control (*), 1 week (**), or 5 weeks (***) at a level of p < 0.05.

*Values represented are mean ± SEM.

**All control values represent average control across all time points.

*N/A, data are not available.
of base excision DNA repair genes, a marker of oxidative DNA damage in vivo (Rusyn et al., 2004). This effect was shown to be dependent on PPARα, not Kupffer cell NADPH oxidase. Chronic feeding studies in mice with WY-14,643 and the less potent peroxisome proliferator bezafibrate for up to a year also show PPARα dependence of peroxisome proliferators induced DNA repair and hepatocarcinogenesis (Hays et al., 2005; Peters et al., 1997). In the present study we assessed oxidative DNA damage at 5 months of continuous treatment with WY-14,643 and report that oxidative DNA damage persists and continues to be dependent on PPARα, not NADPH oxidase. Taken together with the fact that the activity of ACO, a PPARα target gene, was also observed to be elevated following

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Cyclin B1a</th>
<th>Cdk1a</th>
<th>Cdk2a</th>
<th>Cdk4a</th>
<th>Caspase 8a</th>
<th>Caspase 9a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PPARα +/-</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.4</td>
<td>1.0 ± 0.8</td>
<td>1.0 ± 0.4</td>
<td>1.0 ± 0.7</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>WY-14,643</td>
<td>3</td>
<td>32.5 ± 7.9*</td>
<td>10.4 ± 1.4*</td>
<td>0.6 ± 0.1</td>
<td>4.7 ± 0.5*</td>
<td>0.0 ± 0.0*</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td><strong>PPARα --/--</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Control</td>
<td>3</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.7</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>WY-14,643</td>
<td>3</td>
<td>1.4 ± 0.2</td>
<td>0.9 ± 0.5</td>
<td>0.9 ± 0.6</td>
<td>1.8 ± 0.6</td>
<td>1.1 ± 0.8</td>
<td>0.9 ± 0.1</td>
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<td><strong>p47phox +/+</strong></td>
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<tr>
<td>Control</td>
<td>3</td>
<td>1.0 ± 0.5</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.7</td>
<td>1.0 ± 0.6</td>
<td>1.0 ± 0.6</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>WY-14,643</td>
<td>3</td>
<td>36.7 ± 6.4*</td>
<td>3.1 ± 0.6*</td>
<td>1.2 ± 0.6</td>
<td>5.7 ± 1.3*</td>
<td>0.1 ± 0.0*</td>
<td>0.7 ± 0.3</td>
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<td><strong>p47phox --/--</strong></td>
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</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 1.0</td>
<td>1.0 ± 0.8</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.3</td>
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<tr>
<td>WY-14,643</td>
<td>3</td>
<td>19.3 ± 2.7*</td>
<td>4.1 ± 2.1*</td>
<td>2.3 ± 2.6</td>
<td>4.3 ± 0.2*</td>
<td>1.1 ± 0.5</td>
<td>0.6 ± 0.1</td>
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</table>

*Note. Asterisk (*) denotes statistical significance from control at a level of *p < 0.05.

Values represented are mean ± SEM and are normalized to strain-matched control values.
5-month-long WY-14,643 treatment, these data suggest that peroxisomal enzymes, not activated Kupffer cells are the likely source of reactive oxygen species that contribute to oxidative DNA damage in the mode of action of peroxisome proliferators.

In conclusion, this long-term feeding study with a model peroxisome proliferator compound WY-14,643 demonstrates that PPARα, not Kupffer cell–derived oxidants are important for the key steps critical for carcinogenesis—cell proliferation and oxidative DNA damage. While activated Kupffer cells mediate acute effects of these agents on cell proliferation and production of oxidants in liver, this pathway appears to not be sustained and may play a limited, if any, role in long-term effects of peroxisome proliferators such as hepatocarcinogenesis.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


