Relationship of Hepatic Peroxisome Proliferation and Replicative DNA Synthesis to the Hepatocarcinogenicity of the Peroxisome Proliferators Di(2-ethylhexyl)phthalate and [4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic Acid (Wy-14,643) in Rats

Daniel S. Marsman,1 Russell C. Cattley,2 James G. Conway,3 and James A. Popp

Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina 27709

ABSTRACT

The mechanism of hepatocarcinogenesis caused by peroxisome proliferators (PP) is poorly understood, making it difficult to predict the carcinogenicity of PP to rodents or other species. It has been suggested that the carcinogenic potential of individual PP in rodents is correlated with the degree of PP-induced hepatic peroxisome proliferation. To evaluate this possible correlation, di(2-ethylhexyl)phthalate (DEHP) at 1.2% and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643) at 0.1% were fed to male F-344 rats for up to 365 days and hepatic peroxisome proliferation and DNA replication were measured. All rats fed Wy-14,643 for 365 days had numerous grossly visible nodules in comparison to none in the livers of DEHP-fed or control rats. Despite the difference in the induction of tumors, both DEHP and Wy-14,643 increased the peroxisomal volume density 4- to 6-fold from 8 to 365 days of treatment. Peroxisomal β-oxidation enzyme activities were increased 8-fold by both DEHP and Wy-14,643 after 18 days. At later time points (77 to 365 days), these enzyme activities were about 25% increased, with the degree of PP-induced hepatic peroxisome proliferation and DNA replication being similar to the tumorigenicity studies. However, a strong correlation was observed between increases in replicative DNA synthesis in both DEHP and Wy-14,643-fed rats, with a return to control levels by 4 days. Additional rats were implanted with 7-day osmotic pumps containing tritiated thymidine. With this more extended method of labeling a 5- to 10-fold increase in replicative DNA synthesis was observed in rats receiving Wy-14,643 for 39 to 365 days as compared to DEHP-fed rats or controls. In conclusion, when performed under conditions similar to the hepatocarcinogenicity of DEHP or Wy-14,643, replicative DNA synthesis was observed to be a quantitative indicator of the carcinogenic risk of individual PP, we measured peroxisome proliferation following the feeding of DEHP or Wy-14,643 under conditions similar to the above carcinogenicity studies.

INTRODUCTION

A wide variety of structurally unrelated chemicals cause proliferation of peroxisomes in rodent liver (1-9). Chronic administration of PP results in the induction of hepatocellular carcinomas in rats and mice (10, 11). The mechanism of PP-induced carcinogenesis is poorly understood because classical genotoxicity tests have been uniformly negative (12-21) and promotion studies have shown variable results (22). In general, PP cause large increases in the activity of the peroxisomal H2O2-producing enzyme fatty acyl-CoA oxidase (23), but minimal increases in the activity of peroxisomal catalase (24-26), the enzyme responsible for H2O2 degradation. These data led Reddy et al. (27) to hypothesize that the carcinogenic mechanism of the PP may involve leakage of H2O2 from peroxisomes and subsequent oxidative damage to macromolecules (28). Recent data support the leakage of hydrogen peroxide out of peroxisomes during high rates of peroxisomal β-oxidation in livers of PP-treated rats (29).

Although PP by definition cause proliferation of peroxisomes in hepatocytes and the associated increases in the activities of peroxisomal β-oxidation enzymes, the carcinogenicities of individual PP vary widely (30-35). For example, DEHP fed at 1.2% in the diet produced a 10% incidence of male rats with hepatocellular carcinomas after 2 years (30, 36), whereas Wy-14,643 at 0.1% in the diet resulted in a 100% incidence of male rats with hepatocellular carcinomas after 60 weeks (37-39). Because of the possible importance of peroxisome proliferation as a quantitative indicator of the carcinogenic risk of individual PP, we measured peroxisome proliferation following the feeding of DEHP or Wy-14,643 under conditions similar to the above carcinogenicity studies.

In addition to peroxisome proliferation, PP commonly cause hepatomegaly associated with a short burst in DNA replication (10, 40). Since DNA replication plays an important role in the induction of chemical carcinogenesis by other classes of chemicals (37, 41), replicative DNA synthesis was also quantitated in DEHP- and Wy-14,643-fed rats. Osmotic pumps were used to deliver tritiated thymidine to label the replicating DNA over 7 days. Compared to the traditional pulse labeling of animals with tritiated thymidine, the use of osmotic pumps increased the ability to measure low levels of DNA synthesis in liver. In the present study a strong correlation between increases in replicative DNA synthesis and hepatocarcinogenicity was observed while there was no quantitative correlation between peroxisome proliferation and hepatocarcinogenicity.

MATERIALS AND METHODS

Animal Treatment. Male Fischer 344 rats were obtained from Charles River Breeding Laboratories, Inc. (Raleigh, NC), at 6-7 weeks of age (121.3 ± 6.8 g) and were housed 5 rats per cage. Upon arrival, the animals were quarantined for 2 weeks, after which time they were found to be virus-free as determined by the standard murine antibody determination tests (Microbiological Associates, Bethesda, MD). Rats were maintained on NIH-07 chow and purified water ad libitum and housed in biologically clean rooms with filtered air and a 12-h night/day cycle. Temperature and relative humidity were held at 22 ± 2°C and 50 ± 5% (SD), respectively. Body weights and clinical observations were recorded weekly, while food consumption was recorded biweekly throughout the study. Treated animals were given the same rodent chow blended with either DEHP (Eastman Chemical Products, Kingsport, TN) or Wy-14,643 (ChemSyn Science Laboratories, Lenexa, KS) at target...
concentrations of 1.2 and 0.1%, respectively. The concentration of DEHP or Wy-14,643 in the feed was assayed after each blending with measured values of 1.208 ± 0.027 and 0.093 ± 0.003%, respectively.

Seven days prior to sacrifice on days 8, 18, 39, 77, 151, and 365, Alzet osmotic pumps (Palo Alto, CA; flow rate, 10 μl/h; 7-day delivery) containing 2 mCi of 40 Ci/mmol tritiated thymidine (Amersham, Arlington Heights, IL) were implanted s.c. in five rats per group. Two h prior to sacrifice on the mornings of days 1, 2, 4, 8, 18, and 39, five additional rats per group were given 1 μCi/g body weight tritiated thymidine (New England Nuclear, Boston, MA; specific activity, 6.7 Ci/mmol) ip.

Histopathology and Autoradiography. On the mornings of days 1, 2, 4, 8, 18, 39, 77, 151, and 365 five or ten animals per group were killed by exsanguination while under methoxyflurane anesthesia. At the time of necropsy, terminal body weights and wet liver weights were recorded.

Liver sections were made for hematoxylin and eosin staining and autoradiography. Autoradiographic sections were dipped in emulsion (Kodak NTB-2) and then allowed to develop in darkness at −20°C. Sections from the left lobe of pulse-labeled rats were developed for 8 weeks while those from pump-implanted rats were developed for 13 weeks. A section of ileum was included from each rat as a positive control of nuclear labeling. Labeled and unlabeled hepatocyte nuclei were counted per rat to calculate the percentage of labeled nuclei (labeling index).

At the 365-day time point, livers were sectioned at 1–2-mm intervals for quantification of grossly observable lesions. Frozen and formalin-fixed sections of lesions were taken randomly from each of 3 rats/group at days 8, 39, 151, and 365. Peroxisome morphometry. Additional sections of nonnodular liver were taken from the left lobe for electron microscopy and stained using a diaminobenzidine technique (42). Twelve electron micrographs were taken randomly from each of 3 rats/group at days 8, 39, 151, and 365. Peroxisomal volume density was determined from the area density of peroxisomes in cross-sections of nonlesional hepatocyte cytoplasm at a final magnification of ×20,000 (43). Peroxisomal mean volume and numerical density determinations were calculated using a minor modification of a standard stereological technique (44). Peroxisomes were assumed to be spherical, with a minimal identifiable radius of 0.1 μm.

Enzyme Assays. Liver from the left lobe was used to prepare 20% homogenates in 50 mM Tris-HCl-154 mM KCl, pH 7.2. Samples were kept on ice until frozen at −20°C. The postnuclear supernatant of the thawed liver homogenate was prepared on the day of enzyme assays by centrifugation at 2500 x g for 10 min.

Fatty acyl-CoA oxidase activity was assayed in the postnuclear supernatant of 5 or 10 rats/group by a measurement of hydrogen peroxide production in the presence of 25 μM palmitoyl-CoA (45). Cyanide-insensitive NADH reduction activity was measured using the same supernatants (46). Enzyme activity was normalized per g of protein using a commercial kit (Abbott Labs, Irving, TX). Since no difference in enzyme activity was noted between liver homogenates from pulse-labeled and pump-implanted animals, the data from these two groups were combined for statistical analysis.

Statistical Analysis. Data from the groups were analyzed for homogeneity of variance. Statistical significance was assessed with the t test, using a Bonferroni correction factor for multiple comparisons. All data points are reported as means ± SEM for samples of size n = 5 or 10.

RESULTS

Effects on Feed Consumption, Body Weight, Liver Weight, and Hepatocarcinogenesis. Feed consumption (w/w) fell by about 10% in Wy-14,643-fed rats initially but returned to control levels by day 18 (data not shown). Body weight gains of both DEHP- and Wy-14,643-fed rats were lower than controls, with terminal body weights of Wy-14,643-fed rats being statistically significantly lower than those of rats fed DEHP for 18 to 365 days (Fig. 1). Both compounds increased absolute liver weights between 18 and 365 days, with liver weights of 15 to 17 g, compared to control weights of 10 to 12 g (data not shown). The relative liver weight of Wy-14,643-fed rats was higher than DEHP-fed rats or controls from 18 to 365 days (Fig. 2), reflecting the lower body weights of the Wy-14,643-fed rats.

Macroscopic lesions were not observed in rats fed the control or DEHP-containing diet for 365 days (Table 1). In contrast, livers of rats fed Wy-14,643 for 365 days contained numerous macroscopic (Table 1) and microscopic lesions (data not shown) not present in DEHP or control animals. In hematoxylin and eosin-stained sections the Wy-14,643-induced significant hepatic lesions over controls, P < 0.05 for all lesion size classes.
nization with distinct borders and were compressing adjacent tissue.

Peroxisome Proliferation. Peroxisomal fatty acyl-CoA oxidase activity increased after 1 or 2 days of treatment with Wy-14,643 or DEHP, respectively (Fig. 3A). Peak activity was observed in both groups by 18 days with values 8-fold higher than controls. From 39 to 365 days of treatment the activity in livers from DEHP-fed rats was 70 to 80% of that in Wy-14,643-fed rats.

Peroxisomal β-oxidation activity as measured by cyanide-insensitive NAD⁺ reduction activity increased after 2 or 4 days of Wy-14,643 or DEHP feeding, respectively (Fig. 3B). Again, peak activity was observed in both treatment groups at 18 days with values 8-fold higher than those of controls. Only at later time points were differences noted between Wy-14,643- and DEHP-fed rats. The activity in livers from DEHP-fed rats was about 35% lower than from Wy-14,643-fed rats after 1 year.

Quantitative evaluation of electron micrographs indicated that both Wy-14,643 and DEHP induced the proliferation of numerous catalase-positive peroxisomes (Table 2). Increases in the peroxisomal volume density was similar in magnitude to the observed increases in the activity of the peroxisomal β-oxidation system with maximal values about 6-fold higher than controls at all time points studied. The peroxisomal mean volume from livers of treated and control rats increased over time, with a concomitant decrease in the numerical density. The increase in peroxisomal volume density associated with DEHP and Wy-14,643 treatment was due to an increase in both the numerical density and the mean volume of the peroxisomes.

**Table 2. Morphometry of hepatic peroxisomes from rats fed control, DEHP, or Wy-14,643 diets**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days of treatment</th>
<th>(V_v)</th>
<th>(N_v)</th>
<th>Mean volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>365</td>
<td>0.12±0.03</td>
<td>12.0±5.7</td>
<td>0.09±0.02</td>
</tr>
<tr>
<td>DEHP</td>
<td>365</td>
<td>0.17±0.02</td>
<td>12.0±5.7</td>
<td>0.28±0.09</td>
</tr>
<tr>
<td>Wy-14,643</td>
<td>365</td>
<td>0.17±0.02</td>
<td>12.0±5.7</td>
<td>0.71±0.31</td>
</tr>
</tbody>
</table>

\(V_v\), volume density (percentage of cytoplasm occupied by peroxisomes); \(N_v\), numerical density (number of peroxisomes/\(\mu\text{m}^3\) of cytoplasm; mean volume, average volume of a peroxisome in \(\mu\text{m}\).

**DISCUSSION**

Proliferation of peroxisomes occurs in rats and mice after the administration of hypolipidemic agents, plasticizers, halogenated solvents, and herbicides (47). Following the observation that many of the chemicals that induce hepatic peroxisomes are also hepatocarcinogenic, Reddy et al. (27) hypothesized a causal relationship between peroxisome proliferation and hepatocarcinogenesis. Despite the data supporting an association between peroxisome proliferation and hepatocarcinogenesis, a causal relationship has not been established. Comparing PP with widely different carcinogenic activities could help define the relationship between peroxisomal induction and the subsequent formation of hepatic tumors. The current feeding study was designed to examine the possible correlations between peroxisome proliferation, replicative DNA synthesis, and the hepatocarcinogenicity of DEHP and Wy-14,643 under conditions of the original feeding studies (30, 36, 39).
Autoradiograms were prepared and labeling index was quantitated as described in "Materials and Methods." Pump-infused animals were implanted s.c. with an osmotic pump (10 μCi/h) containing 40 Ci/mmol tritiated thymidine 7 days prior to sacrifice.

### Table 3  Hepatic nuclear labeling indices in rats fed control, DEHP, or Wy-14,643 diets

<table>
<thead>
<tr>
<th>Days of treatment</th>
<th>Labeling index for pulse-labeled rats</th>
<th>Labeling index for pump-infused rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>DEHP</td>
</tr>
<tr>
<td>1</td>
<td>2.0 ± 0.3*</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>2.0 ± 0.5</td>
<td>5.6 ± 0.9*</td>
</tr>
<tr>
<td>4</td>
<td>1.3 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>8</td>
<td>2.0 ± 0.6</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>18</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>39</td>
<td>0.3 ± 0.0</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>77</td>
<td>151</td>
<td>365</td>
</tr>
<tr>
<td>365</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SEM.
† Nuclear labeling not measured.
‡ Significantly higher than control values, P < 0.05.
§ Significantly higher than control values, P < 0.01.

These measurements do not test the "leakiness" of peroxisomes or the intracellular detoxification of the active oxygen species (29). However, the marginal quantitative differences noted in peroxisome proliferation suggest that other factors are important in modulating the ultimate carcinogenic response. The lack of a quantitative correlation between the degree of peroxisome proliferation to hepatocarcinogenicity in the present study suggests that measurements of PP-induced peroxisome proliferation are not reliable predictors of the carcinogenic potential of these chemicals.

Replicative DNA Synthesis. As expected (47), persistent hepatomegaly was observed following the start of DEHP- or Wy-14,643-feeding. The burst of replicative DNA synthesis noted during the first several days of DEHP- or Wy-14,643 treatment could facilitate the fixation of carcinogen-induced genetic alterations. Enhanced cell replication profoundly enhances the tumorigenicity of an initiator (37, 48), and is believed to be important at multiple stages in chemically induced hepatocarcinogenesis (41). An elevation in replicative DNA synthesis was detected on Day 1 in Wy-14,643-treated compared to Day 2 for DEHP-treated rats, corresponding to a 1-day-earlier induction of hepatic peroxisomal enzymes in Wy-14,643-treated rats. The earlier induction of both parameters suggests a different rate of delivery for two chemically divergent hypolipidemic agents. One interesting observation was the higher replicative DNA synthesis in the control rats during the first 8 days of the study compared to later time points, even though the age and weights of the rats used in the present study were selected so that they were at least as old as those in the original reports for DEHP (36) and Wy-14,643 (39). What effect the early elevation in background cell proliferation due to normal growth may have had on the eventual carcinogenic response in these studies is unknown but it has been shown in numerous systems that there are sometimes striking differences in the response to xenobiotics in the immature animal (49).

In contrast to the early effects of PP on hepatic cell replication, both the present study and past studies using pulse labeling with tritiated thymidine have not detected increases in replicative DNA synthesis in rat liver at later time points. Limited data in mice (50) have suggested a minor enhancement of cell replication with chronic PP treatment. Because of the low rate of replicative DNA synthesis in hepatocytes of normal adult rats, we decided to quantitate replicative DNA synthesis using osmotic pump infusion for 7 continuous days.

Nuclear labeling of hepatocytes in rats implanted with the 7-day osmotic pumps clearly showed that Wy-14,643 but not...
PEROXISOME PROLIFERATION AND DNA SYNTHESIS

DEHP treatment enhanced hepatocellular DNA replication at later time points (i.e., 39 to 365 days). Unpublished work from this laboratory suggests that foci and nodules of Wy-14,643-fed rats can be reliably identified by both hematoxylin and eosin, as well as the accepted ATPase histochemistry. By eliminating all visible preneoplastic lesions identified by hematoxylin and eosin staining prior to counting, the elevated level of cell replication in the Wy-14,643-fed animals indicates a general phenomenon of hepatocytes. The predominantly periportal cell replication in the Wy-14,643-fed animals indicates a genomic elevations in serum enzymes as a measure of hepatotoxicity that the increased rate of DNA synthesis is a generalized hepatic response and not merely a reflection of enhanced replication within foci or nodules. The rates of replicative DNA synthesis induced by Wy-14,643 as well as DEHP at 365 days correlate well with their carcinogenic activities. This increase in DNA synthesis noted in the Wy-14,643-fed rats may be a direct mitogenic effect on hepatocytes, starting initially with the massive burst in DNA synthesis in the first days of treatment and continuing at a low level over time. However, since the liver weights of rats fed either DEHP or Wy-14,643 were remarkably similar at all time points, it seems likely that Wy-14,643-fed rats lost hepatocytes through toxicity. Only marginal elevations in serum enzymes as a measure of hepatotoxicity have been demonstrated, suggesting that hepatocellular toxicity, if present, may be at or below the current capabilities of detection (51). Although an increase of hepatocellular ploidy cannot be excluded (52), it is difficult to explain the magnitude of the Wy-14,643-induced hepatocellular replication that the proliferation rate returns to control levels within a few days, even when the administration of the inducing substance continues (55). However, it is now evident from this study that the factor which makes the livers of Wy-14,643-fed rats quantitatively different from those of DEHP-fed rats is not the early, short burst of cell replication but the elevated, persistent level of cell replication that has been described here at all later time points. These results further suggest that the dependency in numerous studies upon pulse administration of labeled thymidine for detection of cell proliferation may have failed to address potential effects of chemicals on forced cell replication. While it is clear that the cumulative magnitude of the Wy-14,643-induced hepatocellular replication is great, what role it may play in chemically induced carcinogenesis is unknown. Additional studies are necessary to determine the effect of this elevated replication upon spontaneous mutations, exogenous initiating events, promotion, and possible alterations in the regulation of hepatocyte proliferation.

In conclusion, the lack of a quantitative correlation between the degree of peroxisome proliferation and carcinogenicity of DEHP and Wy-14,643 suggests that peroxisome proliferation alone is insufficient to explain the carcinogenicity induced by PP. The difference in replicative DNA synthesis after chronic administration of DEHP or Wy-14,643 suggests that induction of cell replication may be important in the carcinogenic mechanism of PP. The persistent elevation of DNA replication in Wy-14,643- as opposed to DEHP-fed rats represents the identification of a biological end point that is both qualitatively and quantitatively correlated with carcinogenesis.

ACKNOWLEDGMENTS

The authors wish to extend thanks to Linda K. Garvey, Susan R. Dietze, Otis Lyght, Joseph T. Martin, Douglas A. Neptun, Donald F. Deyo, and Nancy L. Youtsey for technical assistance with sacrifices, preparation and analysis of tissues, and feed analysis.

REFERENCES


Relationship of Hepatic Peroxisome Proliferation and Replicative DNA Synthesis to the Hepatocarcinogenicity of the Peroxisome Proliferators Di(2-ethylhexyl)phthalate and [4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic Acid (Wy-14,643) in Rats


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/48/23/6739

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/48/23/6739. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.