Elevated 8-hydroxydeoxyguanosine in hepatic DNA of rats following exposure to peroxisome proliferators: relationship to carcinogenesis and nuclear localization

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Increased oxidative DNA damage due to increased peroxisomal generation of \( \text{H}_2\text{O}_2 \) is a potential mechanism in the carcinogenicity of chemical peroxisome proliferators (PP) in rodent liver. In order to determine the relationship between carcinogenicity and peroxisome-dependent DNA damage, levels of DNA base oxidation were examined by comparing 8-hydroxydeoxyguanosine (8-OHdG) in DNA from unfractionated liver of male F344 rats following dietary exposure to PP [WY-14,643, 0.1% or 0.005%; di(2-ethylhexyl)phthalate (DEHP), 1.2%; clofibric acid, 0.5%] or phenobarbital (0.05%). Exposure-related increases in 8-OHdG were not observed at 3 or 11 weeks for any of the compounds fed. At 22 weeks, 8-OHdG was similarly elevated (2-3x) by WY-14,643 (0.1% and 0.005%) and clofibric acid (0.5%). These equivalent increases in 8-OHdG in DNA from unfractionated liver did not parallel the divergent carcinogenicity of these different dietary exposures in the present or previous studies. The potential oxidation of nuclear DNA was examined by comparing levels of 8-OHdG in DNA isolated from purified liver nuclei and unfractionated liver. Elevated levels of 8-OHdG were not detected in DNA isolated from nuclear fractions of livers from rats fed clofibric acid for 22 weeks, indicating the dependence of PP-induced oxidative DNA damage on extranuclear components of samples for DNA isolation. The absence of a quantitative relationship between PP-induced carcinogenicity and oxidative DNA base damage (as 8-OHdG), and the failure to localize this oxidative damage to nuclear DNA, suggest two possible conclusions: (1) quantitation of 8-OHdG, a specific and sensitive indicator of oxidative DNA damage, does not accurately reflect the potential peroxisomal \( \text{H}_2\text{O}_2 \)-dependent DNA damage and carcinogenicity of PP exposure in rodents; (2) other hepatic responses may be more critical features of the mechanism of PP carcinogenicity.

Introduction

A class of chemical hepatocarcinogens has been identified by their ability to induce proliferation of peroxisomes in hepatocytes of rodents following brief exposure (1,2). Despite their carcinogenicity, these chemical peroxisome proliferators (PP*) and their metabolites lack DNA reactivity and PP-DNA adducts have not been identified (3-5). Since peroxisome proliferation results in increased activity of \( \text{H}_2\text{O}_2 \)-producing oxidases, notably fatty-acyl CoA oxidase, oxidative DNA modification due to increased \( \text{H}_2\text{O}_2 \) production has been proposed as a mechanism of PP carcinogenesis (1). This mechanism is consistent with the relatively greater increase in \( \text{H}_2\text{O}_2 \)-generating fatty-acyl CoA oxidase activity (>5-fold) than in catalase activity (<2-fold) in livers of PP-exposed rodents, since catalase is responsible for degrading \( \text{H}_2\text{O}_2 \) in the peroxisome (6). Escape of \( \text{H}_2\text{O}_2 \) from the peroxisome may be expected to yield oxidative modification of either cytoplasmic and/or nuclear constituents. Evidence for oxidative modification of peroxisomal constituents is equivocal and depends on the indicator evaluated (7-9). However, cytoplasmic accumulation of lipofuscin, a proposed product of oxidative modification of membrane lipids, is increased in hepatocytes of PP-exposed rats in a manner quantitatively predictive of carcinogenicity (9,10).

The possibility that \( \text{H}_2\text{O}_2 \) may escape from the peroxisome and result in damage of cytoplasmic constituents also raises the possibility of oxidative damage to nuclear constituents, especially DNA, in livers of PP-exposed rodents. Several studies have evaluated the effect of PP treatment on DNA by measuring levels of oxidatively modified nucleosides in livers of rodents. These oxidized nucleosides, which have been considered appropriate markers of oxidative DNA damage, include 8-hydroxydeoxyguanosine (8-OHdG) and 5-hydroxymethyl deoxyuridine (HMdU) that can result from a \( \text{H}_2\text{O}_2 \)-derived hydroxyl radical (\( \cdot \text{OH} \)) acting on guanine and thymine bases, respectively, in DNA (11,12). Elevated levels of 8-OHdG have been observed in DNA from livers of rats following oral exposure to various PP, including ciprofibrate, di[2-ethylhexyl]phthalate (DEHP), aluminum clofibrate and simfibrate (13,14), and elevated HMdU in liver DNA has been observed following oral exposure to ciprofibrate (15). These studies, therefore, suggest that a qualitative relationship between peroxisome proliferation, DNA oxidation and cancer in rodents may exist, although quantitative aspects of this mechanistic interrelationship have not been addressed. In contrast, elevations of 8-OHdG and HMdU were not observed in DNA from livers of rats following oral exposure to nafenopin (16) or WY-14,643 (17), respectively. Critical differences in compound selection, experimental design and methods may account for the apparent inconsistent elevation of oxidized nucleoside in DNA of PP-exposed rats in these studies. One difference in experimental method, which may pertain to nuclear localization of DNA damage, is noted among these studies. The method of DNA isolation in the studies in which nuclear localization of DNA damage was examined differed from one study to another (7,9).

Elucidation of the mechanism of PP-induced carcinogenicity is crucial for determining the potential cancer risk in humans exposed to chemicals that are carcinogenic PP in rodents. Previous studies have suggested that the magnitude of the increase in peroxisomal fatty-acyl CoA oxidase is not predictive of cancer risk in rodents (10,18). However, increased acyl CoA oxidase...
activity may not be the only determinant of oxidative DNA damage in livers of PP-exposed rats. The following work, therefore, examines the relationship between oxidative DNA damage potentially resulting from increased peroxisomal H₂O₂, measured as 8-OHdG in hepatic DNA, and carcinogenicity in rats exposed to PP for which relative carcinogenicity has been established (18,19). In addition, since discrepant reports of DNA oxidation by PP exposure may have resulted from non-nuclear location of the damage, the potential for PP exposure to result in oxidation of nuclear DNA was examined by comparing levels of 8-OHdG in DNA isolated from purified liver nuclei and unfractionated liver.

Materials and methods

All reagents, enzymes and isomyl alcohol were obtained from Sigma Chemical Co., St Louis, MO, except organic solvents (Fisher Scientific Co., Pittsburgh, PA) and sucrose and proteinase K (Gibco BRL, Gaithersburg, MD).

Male F344 rats, 10 weeks of age, were obtained from Charles River Breeding Laboratories, Raleigh, NC. Rats were housed in polycarbonate cages over a cellulose bedding and maintained in a mass air displacement room (Hazeltine Systems Inc, Lenoax, VA) with automatically controlled temperature (22°C), humidity (50%) and light (12 h light/12 h dark cycle). Rats were randomly assigned to six dietary exposure groups beginning at 12 weeks of age: 1.0% WY-14,643; 0.005% WY-14,643; 1.2% DEHP; 0.05% phenobarbital; 0.5% clofibric acid; control. The PP (WY-14,643, DEHP and clofibric acid) and their dietary concentrations were selected based on previously conducted studies of peroxisome proliferation and carcinogenesis in this sex and strain of rat (10,18,19). For comparison, since PP cause hepatomegaly, phenobarbital was selected as a non-PP that also causes hepatomegaly (19). All diets were mixed using NIH-07 diet (Ziegler Brothers, Gardners, PA). The compounds were obtained from the following sources: WY-14,643 (>98% purity) from ChemSyn Science Labs, Lenexa, KS; DEHP (>99.5%) from Eastman Chemical Company, Kingsport, TN; clofibric acid (>99%) and phenobarbital (>99%) from Sigma Chemical Co., St Louis, MO. Diets were analyzed for chemical after mixing and used if the actual concentration was within ±10% of target concentration. The methods used for diet analysis were as follows. WY-14,643, clofibric acid and phenobarbital were extracted with acetone/methanol containing internal standards (aceoxybenzene, p-chlorophenoxyacetate and hexobarbital, respectively). Extracts were analyzed by reverse phase high performance liquid chromatography (HPLC) with u.v. absorbance detection (280, 230 and 254 nm, respectively). DEHP was analyzed with methylene chloride containing hexacosane as the internal standard. This extract used for gas chromatography or flame ionization detection.

Following 3, 11 or 22 weeks of ad libitum feeding, rats were killed by exsanguination following deep isoflurane anesthesia. Livers were removed and weighed and any nodular lesions were counted, removed and discarded from further analysis. The remaining liver was snap frozen in polypropylene vials by immersion in liquid N₂ and subsequently stored at −70°C until DNA was extracted.

Liver DNA was extracted using a previously reported method (20) with slight modifications. High purity distilled phenol was used to prevent artificial formation of 8-OHdG (21). Liver tissue was homogenized in 1% sodium dodecyl sulfate (SDS) + 5 mM EDTA, minced, homogenized using 5 strokes of a glass pestle in a glass tube and digested with proteinase K (0.5 mg/ml, 37°C, 30 min). DNA was then isolated as described (20), except that phenol was omitted from the organic extraction that followed RNase treatment. Following isolation, DNA was digested to nucleosides using P1 nuclelease and bacterial alkaline phosphatase (22). Digested DNA was analyzed by HPLC using conditions described by Kasi et al. (22). For 8-OHdG, the HPLC system consisted of a Waters 510 pump, SSI pulse dampener, Rhenyone 7125 injector, EGG&G Princeton Applied Research Model 400 electrochemical detector and Waters 440 u.v. detector. For deoxyguanosine (dG), the HPLC system consisted of an ABI Kratos 400 pump, Hewlett-Packard 1050 autosampling injector and ABI 756 A u.v. detector. 8-OHdG and dG were measured using electrochemical (oxidation potential 550 mV) and absorbance (254 nm) detection, respectively (21,22), and levels of oxidation were expressed as 8-OHdG/dG.

Fractionation of nuclei for DNA isolation was performed as described by Lynch et al. (23). Briefly, liver tissue was thawed, gently homogenized in 0.3 M sucrose/4 mM CaCl₂ and separated by centrifugation at 1500 g and 4°C. The crude nuclear pellet was resuspended in 2.0 M sucrose/1 mM CaCl₂ and centrifuged over a layer of 2.2 M sucrose at 74 000 g for 1 h at 4°C. The nuclear pellet was then resuspended in 0.1% SDS and digested with proteinase K (0.2 mg/ml, 45 min, on ice). Subsequent steps for DNA isolation and analysis were identical to those described above.

Analysis of data for body weights, liver weights and 8-OHdG/dG at each exposure interval utilized one-way analysis of variance (ANOVA) and, if ANOVA indicated that groups were likely to have different means, Dunnett’s or Student–Newman–Keuls comparison tests were performed (R/S1; BBN Software Products Corp, Cambridge, MA). Data were log transformed prior to analysis to stabilize variance.

Results

Effects of feeding the various PP (WY-14,643, clofibric acid and DEHP) and phenobarbital on body weight and liver weight are summarized in Table I. Body weight depression was evident in rats fed 0.1% WY-14,643 (3, 11 and 22 weeks), 0.005% WY-14,643 (11 and 22 weeks), 0.5% clofibric acid (3, 11 and 22 weeks) and 1.2% DEHP (22 weeks). Phenobarbital (0.05%) feeding did not affect body weight at 3, 11 or 22 weeks. Feeding rats diets containing the various PP (WY-14,643, clofibric acid and DEHP) or phenobarbital resulted in elevations in absolute liver weights at 3, 11 and 22 weeks. At 22 weeks, small nodules (<4 mm in diameter), typical of large foci and adenomas previously observed in PP-fed rats (2), were observed in some livers of WY-14,643-fed rats. The multiplicity of these lesions is summarized in Table I and was increased only in rats fed 0.1% WY-14,643.

The levels of 8-OHdG/dG in DNA isolated from unfractionated liver are depicted in Figure 1. Levels of 8-OHdG/dG in livers of untreated controls were well within the range of values previously reported for rat liver (13,21). Feeding any of the various PP (WY-14,643, clofibric acid or DEHP) or phenobarbital did not affect 8-OHdG/dG following 3 or 11 weeks of feeding. At 22 weeks, levels of 8-OHdG/dG were increased in DNA from livers of rats fed either 0.1% WY-14,643, 0.005% WY-14,643 or 0.5% clofibric acid compared to controls. Levels of 8-OHdG/dG were not affected by feeding 1.2% DEHP or 0.05% phenobarbital for 22 weeks.

Livers from rats fed 0.5% clofibric acid for 22 weeks and matching control rats were selected (based on the greatest difference between exposed and control group means) for comparison of levels of 8-OHdG/dG in DNA isolated from unfractionated liver versus purified liver nuclei. To minimize variability, portions of liver tissue from each rat were used for isolation of DNA by both methods on the same day using the same extraction procedure. Results of analysis of DNA from these livers are depicted in Figure 2. In DNA isolated from unfractionated liver, 0.5% clofibric acid feeding resulted in increased 8-OHdG/dG, as was previously observed. In contrast, levels of 8-OHdG/dG in DNA isolated from purified nuclei were not affected by feeding 0.5% clofibric acid.

Discussion

This study is the first attempt to relate increased levels of oxidative DNA damage, measured as 8-OHdG, to the relative carcinogenicity and peroxisomal enzyme induction by a variety of PP under standardized experimental conditions. Previous studies with male F344 rats in this laboratory have demonstrated that a similar magnitude of peroxisomal fatty-acyl CoA oxidase induction is achieved with 0.1% WY-14,643, 0.005% WY-14,643, 0.5% clofibric acid and 1.2% DEHP-containing diets fed for 11 and 22 weeks (10,18). Feeding these diets for 22 or 52 weeks resulted in divergent carcinogenicities, based on tumor incidence and multiplicity data (10,18,19, present study). Using multiplicity of hepatocellular neoplasms at 52 weeks, the carcinogenicity of 0.1% WY-14,643 exceeded that of 0.005% WY-14,643, which in turn exceeded that of 0.5% clofibric acid.

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Elevated levels of 8-OHdG in livers of PP-fed rats (13,15, present study) have been interpreted as an indication that oxygen radical-dependent promutagenic events may be responsible for PP-induced hepatocellular neoplasia. The level of 8-OHdG is interpreted as a marker for a variety of OH’-mediated base modifications, many of which are promutagenic. The presence of 8-OHdG itself in DNA can result in G:C—T:A transversions due to mispairing at replication (25,26). Elevation of 8-OHdG similarly to the higher (0.1%) concentration, but did not increase multiplicity of hepatic nodules.

Table I. Effect of feeding WY-14,643, clofibric acid, DEHP or phenobarbital on body wts, liver wts and multiplicity of nodular hepatic lesions in male F344 rats

<table>
<thead>
<tr>
<th>Diet</th>
<th>3 weeks</th>
<th></th>
<th>11 weeks</th>
<th></th>
<th>22 weeks</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Body wt (g)</td>
<td>Liver wt</td>
<td>Body wt (g)</td>
<td>Liver wt</td>
<td>Body wt (g)</td>
<td>Liver wt</td>
</tr>
<tr>
<td>Control</td>
<td>293±6</td>
<td>10.6</td>
<td>344±3</td>
<td>11.8</td>
<td>398±2</td>
<td>13.0</td>
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<tr>
<td>WY-14,643</td>
<td>264±5</td>
<td>20.8±b</td>
<td>257±3</td>
<td>19.7±b</td>
<td>269±2</td>
<td>21.4±b</td>
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<tr>
<td>0.1%</td>
<td>278±5</td>
<td>21.0</td>
<td>314±5</td>
<td>24.8±b</td>
<td>335±5</td>
<td>26.1±b</td>
</tr>
<tr>
<td>WY-14,643</td>
<td>0.005%</td>
<td>(±6)</td>
<td>(±9)</td>
<td>(±0.8)</td>
<td>(±11)</td>
<td>(±1.5)</td>
</tr>
<tr>
<td>Clofibric acid</td>
<td>248±5</td>
<td>16.0±b</td>
<td>289±5</td>
<td>17.6±b</td>
<td>320±5</td>
<td>17.3±b</td>
</tr>
<tr>
<td>0.5%</td>
<td>278±5</td>
<td>17.3±b</td>
<td>319±5</td>
<td>19.4±b</td>
<td>351±5</td>
<td>20.2±b</td>
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<tr>
<td>DEHP</td>
<td>300±5</td>
<td>14.7</td>
<td>350±5</td>
<td>16.1±b</td>
<td>396±5</td>
<td>17.5±b</td>
</tr>
<tr>
<td>0.05%</td>
<td>(±6)</td>
<td>(±0.2)</td>
<td>(±5)</td>
<td>(±0.4)</td>
<td>(±4)</td>
<td>(±0.2)</td>
</tr>
</tbody>
</table>

*All values depicted represent mean ± SEM, n = 6.

**Significantly different from age-matched control using Dunnett’s test.

*Significantly greater than 0 by one-tailed t-test.

Fig. 1. Effect of 3, 11 or 22 weeks of dietary exposure to peroxisome proliferators or phenobarbital on levels of 8-OHdG in hepatic DNA in rats. WY, WY-14,643; PHB, phenobarbital; DEHP, di(2-ethylhexyl)phthalate; CLOF, clofibric acid (% of diet as indicated). Error bars depict SEM, n = 6. *Significantly different from all other means by Student–Newman–Keuls test, P<0.05.

Fig. 2. Effect of isolating DNA from nuclear fraction ('Nuclei') versus unfractionated liver ('Unfractionated') on clofibric acid-induced increase in 8-OHdG. Error bars depict SEM, n = 6. *Significantly different from all other means by Student–Newman–Keuls test, P<0.05.

(19). In the present study, multiplicity of grossly visible hepatic nodules, consistent with large hepatocellular foci and adenomas (24), was only increased in rats fed 0.1% WY-14,643. It is, therefore, important to note that similar levels of 8-OHdG were measured in livers of rats fed 0.1% WY-14,643 and 0.5% clofibric acid, even though relative carcinogenicity of 0.5% clofibric acid is significantly lower. Furthermore, exposure to a 20-fold lower concentration of WY-14,643 (0.005%) caused elevation of 8-OHdG similarly to the higher (0.1%) concentration, but did not increase multiplicity of hepatic nodules. Since the present study comprised, at most, 22 weeks of exposure, the possibility that more prolonged exposures might have altered 8-OHdG levels cannot be ruled out. However, the present results indicate that it is not possible to quantitatively relate magnitude of oxidative DNA damage, measured as hepatic 8-OHdG, to carcinogenicity in rat liver following PP exposure.

Previous studies have indicated that maximal induction of peroxisomal acyl CoA oxidase activity is achieved upon 2–3 weeks of dietary exposure to PP (10,18). In contrast, levels of 8-OHdG increase later during the course of continuous dietary exposure. In the present study, levels of 8-OHdG were increased by 22 weeks of feeding PP, but not by 3 or 11 weeks. Other studies have similarly suggested that PP-induced increases in 8-OHdG occur relatively later during the course of feeding. With ciprofibrate, an extremely potent PP, levels of 8-OHdG were not increased before 40 weeks of feeding (13). A similar lag between onset of PP feeding or onset of elevated peroxisomal fatty-acyl CoA oxidase activities and increases in 8-OHdG was observed for other PP, including DEHP, aluminum clofibrate and simfibrate (14). The temporal dissociation of peroxisomal enzyme induction from elevation of 8-OHdG in PP-fed rats suggests that peroxisomal H2O2 may not be responsible for the observed increase in oxidative DNA damage. Furthermore, in rats fed 0.1% WY-14,643 in the present study, elevated levels of 8-OHdG were not observed prior to the appearance of hepatocellular neoplasia.

Elevated levels of 8-OHdG in livers of PP-fed rats (13,15, present study) have been interpreted as an indication that oxygen radical-dependent promutagenic events may be responsible for PP-induced hepatocellular neoplasia. The level of 8-OHdG is
in nuclear DNA could therefore increase the likelihood of mutation in genes critical in cancer development. However, the present study demonstrates that PP-induced elevations in 8-OHdG detectable in DNA of unfractionated liver are not present in nuclear DNA, even in the same livers. This observation is consistent with previously reported elevation of 8-OHdG following PP feeding in DNA of unfractionated liver (13,14), but not from liver nuclei (16), although none of these reported studies examined both methods of isolation. A finding similar to the present study (differential elevation of 8-OHdG in DNA of unfractionated liver versus liver nuclei) was also observed following feeding of 0.1% WY-14,643 to rats for 5 months in a previous study, although isolations of DNA were not performed concurrently (unpublished data).

A reason for the dependence of PP exposure-related elevation of 8-OHdG on evaluation of unfractionated liver is unclear. One possibility is that increases in 8-OHdG in mitochondrial DNA may result in measurable increases in 8-OHdG in DNA from unfractionated liver. This possibility is supported by the observation of higher (~16X) levels of 8-OHdG in mitochondrial DNA than in nuclear DNA from normal livers (27), indicating that mitochondrial DNA would be more sensitive to oxidative damage that might result from increased peroxisomal H2O2. Another possibility is that elevation of 8-OHdG might be produced when DNA is released from nuclei during homogenization and lysis of proteins as liver samples of PP-fed rats are prepared for DNA extraction. This latter possibility is supported by the relative resistance of DNA in intact nuclei to OH'-mediated damage as compared to DNA in solution (17,22,28), where it is less compact, lacks histones and is more readily available to diffusable pro-oxidants. While an exact source of elevated pro-oxidants that could damage naked DNA in liver homogenates from PP-fed rats is not clear, cytoplasmic sources such as peroxisomes and lipofuscin-containing lysosomes, both elevated in the cytoplasm of PP-fed rats, are possible contributors.

The lack of oxidative DNA damage in nuclear DNA following PP exposure indicates that the proposed role of oxidative DNA damage in PP-induced carcinogenesis will require further clarification. The presumed sensitivity of mitochondrial DNA to OH' has led to speculation that various types of oxygen radical-induced mitochondrial DNA damage may be involved in the aging process or various diseases of aging (29,30). The possibility that PP-induced carcinogenicity may in some way be related to mitochondrial injury is intriguing, as effects of in vivo exposure to PP on mitochondrial energy metabolism in intact rat liver have recently been described (31). In the present study, evaluation of mitochondrial DNA for 8-OHdG was not possible, because freezing of samples precluded purification of mitochondria (32). However, evaluation of 8-OHdG or other indicators of DNA damage in liver mitochondria of PP-exposed rodents in future studies may prove relevant to the mechanism of PP carcinogenicity.

The evaluation of model PP in two-stage protocols of hepato-carcinogenesis suggests that many PP act as tumor promoters, but none act as tumor initiators (reviewed in 33). As most chemical exposures that result in promutagenic DNA modification have initiating activity, lack of initiating activity by PP in these studies fails to confirm a role for promutagenic oxidative DNA damage in carcinogenicity of PP. In contrast to the lack of initiating activity, several PP have demonstrable promoting activity in rat liver, however, the mode of promoting activity of the PP WY-14,643 was different from that of a non-PP, phenobarbital, with respect to lesion phenotype and growth (34). Phenobarbital was therefore selected as a non-PP inducer of hepatomegaly and liver tumor promoter for comparison with WY-14,643 in the present study, and did not cause any measurable increase in 8-OHdG.

In summary, levels of oxidative base damage, measured as 8-OHdG, were elevated in DNA isolated from unfractionated livers following PP exposure, although not in a manner predictive of cancer risk. Furthermore, this elevated level of oxidative base damage was not present in nuclear DNA, the presumptive location of damage relevant to carcinogenicity. It is possible that other forms of hepatic oxidative DNA damage, less studied and unrelated to 8-OHdG, may result from PP exposure in rodents. However, the present findings suggest that: (1) quantitation of 8-OHdG, a specific and sensitive marker of oxidative DNA damage, does not accurately reflect potential peroxisomal H2O2-dependent DNA damage and carcinogenesis; (2) peroxisome-dependent hepatic responses may be critical factors in the mechanism of carcinogenicity of PP in rodents.

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References


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