FORMATION OF 8-OXODEOXYGUANOSINE IN LIVER DNA AND HEPATIC INJURY BY PEROXISOME PROLIFERATOR CLOFIBRATE AND PERFLUORODECANOIC ACID IN RATS

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ABSTRACT — In this study, we examined whether the production of hydrogen peroxide by peroxisome proliferators causes oxidative DNA damage in the form of 8-oxodeoxyguanosine (8-oxodG) and hepatic injury, and whether it is related to their tumor-promoting or carcinogenic activities in female rats treated with the peroxisome proliferators clofibrate and perfluorodecanoic acid (PFDA). Clofibrate has tumor-promoting and carcinogenic activities, whereas PFDA does not. We also tested whether peroxisome proliferators directly induce mutagenic events in Salmonella typhimurium strains TA 98 and TA 1537. Rats were treated either by 5% clofibrate in diet or by an i.p. injection of corn oil containing 10 mg/kg body weight of PFDA every week for 2 or 8 weeks. 8-OxodG in liver DNA was analyzed by HPLC coupled with an electrochemical detector. Hepatic injury was evidenced by liver enlargement and by levels of serum enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and hepatic γ-glutamylpeptidase (γ-GT) activity. Clofibrate and PFDA increased the activity of catalase about or less than 2-fold, whereas FAO activity was increased about 6 to 7-fold by clofibrate and about 3 to 4-fold by PFDA. Neither clofibrate nor PFDA induced mutation at any dose tested. Clofibrate significantly increased the formation of 8-oxodG, but PFDA only slightly increased. Serum AST and ALT levels, and hepatic γ-GT activity were not significantly changed at both time points, whereas the ratio of liver/body weight was significantly increased by clofibrate and PFDA at 8 weeks. These data imply that the magnitude of the production of hydrogen peroxide-generated FAO is related to the induction of oxidative DNA damage by peroxisome proliferators, and their tumor-promoting or carcinogenic activities. However, the effect of hydrogen peroxide in hepatic injury is not clear.

KEY WORDS: Peroxisome proliferator, 8-oxodeoxyguanosine, Hepatic injury and hepatocarcinogenesis

INTRODUCTION

Several hypolipidemic drugs, phthalate ester, and other environmental chemicals induce hepatic peroxisome proliferation and hepatocellular carcinomas when administered to rodents (Reddy and Lalwani, 1983; Conway et al., 1989; Borges et al., 1993). The mechanism by which these agents cause hepatocellular carcinoma is not known, but is likely related to biochemical changes or other changes in gene expression induced by peroxisome proliferators, since most studies have not shown them to be directly genotoxic (Reddy et al., 1983; Gupta et al., 1985; Elliot and Elcombe, 1987). Peroxisome proliferators induce increased expression of enzymes in the peroxisomal β-oxidation pathway and the hydrogen peroxide detoxifying enzyme, catalase (Capdevila et al., 1992; Brass and Ruff, 1991; Nemali et al., 1989).

It has been hypothesized that tumorigenesis by peroxisome proliferators is related to cellular damage from hydrogen peroxide generated by fatty acyl-CoA oxidation, the first reaction in the peroxisomal β-oxidation pathway (Reddy and Lalwani, 1988). The activ-
ity of fatty acyl-CoA oxidase (FAO) has been found to be greatly increased in rats treated with peroxisome proliferators, whereas catalase activity is only slightly induced. Therefore, the relatively small increase in catalase activity is not sufficient to metabolize the hydrogen peroxide resulting in the oxidative damage within cells. Reactive oxygen species such as hydrogen peroxide have been suggested to play a role in aging and carcinogenesis (Ames, 1989), and in xenobiotic-induced hepatic toxicity (Stevenson et al., 1995).

The purpose of the present study was to examine whether peroxisome proliferator-induced hydrogen peroxide causes oxidative DNA damage and hepatic injury, which could be related to their carcinogenic and/or tumor-promoting activities. Clofibrate is an efficacious peroxisome proliferator, and has tumor-promoting and carcinogenic activities in the liver (Hess et al., 1965; Hosokawa et al., 1989; Reddy and Qureshi, 1979), whereas, unlike most other peroxisome proliferators, PFDA lacks hepatic tumor-promoting activity (Borges et al., 1993). Therefore, in this study, we compared the effects of two different peroxisome proliferators, clofibrate and PFDA, on mutagenic events, the formation of oxidative DNA damage in terms of 8-oxodeoxyguanosine (8-oxodG) in liver DNA, and hepatic injury.

MATERIALS AND METHODS

Materials

8-OxodG was purchased from Wako Chemicals (Tokyo, Japan). PFDA was a generous gift from Dr. Howard P. Glaubert (University of Kentucky, Lexington, KY, USA). Clofibrate, DNase, nuclease P1, proteinase K, alkaline phosphatase, Aroclor and other reagents were from Sigma Chemical Co. (St. Louis, MO, USA). Solvents used in HPLC were from Fisher Scientific (Cincinnati, OH, USA). ALT and AST assay kits were purchased from Inhwa Pharmaceutical Co. (Seoul, Korea).

Animals

100-120 g female Sprague-Dawley rats obtained from the Korea Food and Drug Administration (KFDA, Seoul, Korea) were housed three per plastic cage in a controlled environment maintained at 22°C with a 12 hr

![Fig. 1. Effect of clofibrate and perfluorodecanoic acid (PFDA) on the formation of 8-oxodeoxyguanosine (8-oxodG) in rat liver DNA. Values are mean ± standard error of 12 animals. Female rats were treated either by 5% clofibrate in diet or by an i.p. injection of corn oil containing 10 mg/kg body weight of PFDA every week. At 2 and 8 weeks after the first treatment, DNA from liver was isolated, purified and digested. 8-oxoG in liver DNA was then analyzed by HPLC coupled with an electrochemical detector. Values significantly different from control are marked * (p<0.05).](image)

<table>
<thead>
<tr>
<th>Table 1. Effect of clofibrate and PFDA on FAO and catalase activities.</th>
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<tr>
<td>FAO (nmole/mg protein/min)</td>
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<tr>
<td>2 weeks</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Clofibrate</td>
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<td>PFDA</td>
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Female rats were treated either by 5% clofibrate in diet or by an i.p. injection of corn oil containing 10 mg/kg body weight of PFDA every week. At 2 and 8 weeks after the first treatment, FAO and catalase activities were determined in liver homogenate.

Results are expressed as the means ±SE of 12 animals.

*Significantly different from control (P<0.05).
peroxisome proliferator, oxidative DNA damage, hepatic injury.

light/dark cycle. After 1 week of acclimatization, rats were treated either by 5% clofibrate in diet or by an i.p. injection of corn oil containing 10 mg/kg body weight of PFDA every week. At 2 and 8 weeks after the first treatment, serum was used for AST and ALT measurement. Livers were removed and stored at -80°C until time of assay.

Quantitation of 8-oxodG by HPLC-ECD
Isolated DNA from liver was purified and digested as described previously (Cho et al., 1993). Digested DNA was then analyzed by HPLC coupled with an electrochemical detector (ECD) system with TSK-gel 120 T column (Tosoh, Japan). The HPLC running conditions were 10% methanol (pH 5.5) containing 0.1 M sodium phosphate as mobile phase, 1.0 ml/min of flow rate, 240 nm wavelength for UV detector, and 600 mV oxidation potential for ECD.

Salmonella mutagenicity assay
The Salmonella mutagenicity test as described by Maron and Ames (1983) was used to test mutagenicity. Salmonella typhimurium strains TA98 and TA1537 were obtained from the Korea Research Institute of Chemical Technology (Taejeon, Korea). Female (200 g) rats pretreated with Aroclor 1254 (500 mg/kg) were used to obtain liver S-9 fraction. Benzo(a)pyrene and sodium azide were used as positive mutagens.

Table 2. Effect of clofibrate and PFDA on mutagenicity in Salmonella Typhimurium.

<table>
<thead>
<tr>
<th>Concentration (g/plate)</th>
<th>TA 98</th>
<th>TA 1537</th>
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<tr>
<td>Control</td>
<td>20.7±1.9</td>
<td>16.4±1.5</td>
</tr>
<tr>
<td>Clofibrate</td>
<td>0.1</td>
<td>26.3±1.1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>23.6±1.3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>27.3±3.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>26.6±1.8</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>25.2±0.9</td>
</tr>
<tr>
<td>PFDA</td>
<td>1</td>
<td>24.0±1.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>23.9±2.3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>25.6±2.1</td>
</tr>
<tr>
<td>B(α)P</td>
<td>2</td>
<td>2344.7±49.1*</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>1</td>
<td>2131.8±29.7*</td>
</tr>
</tbody>
</table>

Results are expressed as the means ± SE from 3 separated experiments.
*Significantly different from control (P <0.05).
B(α)P: Benzo(α)pyrene
Enzyme assay
Activity of fatty acyl-CoA oxidase (FAO) was determined by measuring the lauroyl CoA-dependent production of hydrogen peroxide by FAO through a horseradish peroxidase-coupled reaction as described by Poosch and Yamazaki (1986). Activity of catalase was determined by measuring the breakdown of hydrogen peroxide as described by Beer and Seizer (1953). Levels of serum enzymes (AST and ALT) were measured by the product specification (Inhwa Pharmaceutical Co, Seoul, Korea). Hepatic γ-GT activity was determined as described elsewhere (Hong et al., 1995). Protein level was determined by the Bradford method using Bio-Rad protein assay dye reagent (Bradford, 1976).

Statistics
The data were analyzed by one-way analysis of variance. If significances were seen, data were further analyzed using Dunnett’s test (Gill, 1978). Differences were considered significant at p<0.05.

RESULTS
In the present study, we examined whether the production of peroxisome proliferator-induced hydrogen peroxide resulted in oxidative DNA damage and hepatic injury. We first determined the activity of FAO, the first enzyme in the peroxisomal β-oxidation pathways, as an indicator of production of hydrogen peroxide. Both clofibrate and PFDA significantly increased FAO activity at both time points (Table 1). Clofibrate increased FAO activity about 6-fold at 2 weeks and 7-fold at 8 weeks after treatment, whereas PFDA increased FAO activity about 3-fold at 2 weeks and 4-fold at 8 weeks after treatment. We next determined the activity of catalase. Clofibrate significantly increased catalase activity at both time points, whereas PFDA significantly increased it only at 8 weeks (Table 1). However, the increases were about or less than 2-fold at 2 and 8 weeks with both clofibrate and PFDA.

Next, we examined whether the magnitude of the production of peroxisome proliferator-induced hydrogen peroxide is related to the induction of oxidative DNA damage, mutagenicity and hepatic injury. We first measured the level of 8-oxodG in liver DNA as an indicator of oxidative damage. 8-OxodG level was significantly increased by clofibrate at both time points. PFDA, however, did not significantly increase its level either at 2 weeks or 8 weeks (Fig. 1). To determine whether clofibrate and PFDA induce direct DNA damage, we did a mutagenicity test. None of the clofibrate

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<th>Table 3. Effect of clofibrate and PFDA on serum AST and ALT levels.</th>
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<td>Control</td>
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<tr>
<td>Clofibrate</td>
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<td>PFDA</td>
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Female rats were treated either by 5% clofibrate in diet or by an i.p. injection of corn oil containing 10 mg/kg body weight of PFDA every week. At 8 weeks after the first treatment, AST and ALT were determined from serum. Results are expressed as the means ± SE of 12 animals.

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<th>Table 4. Effect of clofibrate and PFDA on hepatic γ-GT activity.</th>
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<tr>
<td>Control</td>
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<td>Clofibrate</td>
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Female rats were treated either by 5% clofibrate in diet or by an i.p. injection of corn oil containing 10 mg/kg body weight of PFDA every week. At 2 and 8 weeks after the first treatment, γ-GT activities were determined in liver homogenate. Results are expressed as the means ± SE of 12 animals.
or PFDA induced mutagenesis in *Salmonella* bacteria (Table 2).

The ratio of liver/body weight was significantly increased by both clofibrate and PFDA at 8 weeks and by PFDA at 2 weeks after treatments (Fig. 2). We also determined the serum AST and ALT levels, and hepatic γ-GT activity to find out whether the magnitude of the production of hydrogen peroxide results in a different hepatic injury. Neither clofibrate nor PFDA significantly changed AST and ALT levels in the serum either at 2 weeks (data not shown) or at 8 weeks (Table 3). Clofibrate and PFDA did not significantly change γ-GT activity either (Table 4).

**DISCUSSION**

In this study, we examined the ability of peroxisome proliferator-induced hydrogen peroxide to cause oxidative DNA damage and hepatic injury in rats. We found clofibrate and PFDA differently increased FAO activity, showing that the magnitude of the production of hydrogen peroxide was different, since the increase of catalase activity (about or less than 2-fold with both clofibrate and PFDA) was not different. We also found that clofibrate significantly increased the 8-oxodG level, whereas PFDA did not. Neither clofibrate nor PFDA induced mutation. Neither clofibrate nor PFDA changed serum AST and ALT levels, and hepatic γ-GT activity although the ratio of liver/body weight was significantly increased by both clofibrate and PFDA.

It has been hypothesized that tumorigenesis by peroxisome proliferators is related to cellular damage from hydrogen peroxide (Reddy and Lalwani, 1988). This hypothesis is based on the possibility that the relatively small increase of catalase is not sufficient to detoxify the greatly increased amount of hydrogen peroxide. It might then diffuse from peroxisome and damage the cellular component including DNA. Therefore, we first examined whether the magnitude of the production of hydrogen peroxide by peroxisome proliferators clofibrate and PFDA causes different levels of oxidative DNA damage in the form of 8-oxodG, and is related to their tumor-promoting or carcinogenic activities. 8-OxodG itself causes misrepair at replication due to T:A→A transversion, and it also induces mutations in *vivo* (Kuchino *et al*., 1987; Shibutani *et al*., 1991; Wood *et al*., 1992). Elevated levels of oxidative DNA damage including 8-oxodG by peroxisome proliferator have also been suggested to be important in peroxisome proliferator-induced hepatocarcinogenesis (Kasai *et al*., 1989). In this study, clofibrate, which increased FAO activity about 6 to 7-fold, significantly elevated the 8-oxodG level at 2 and 8 weeks after treatment. Similar to this finding, it was also reported that formation of 8-oxodG was significantly increased in liver DNA of rats treated by aluminum clofibrate (Takagi *et al*., 1990). However, PFDA did not elevate the 8-oxodG level, even though it increased FAO activity about 3 to 4-fold. The threshold of hydrogen peroxide in the cell inducing oxidative DNA damage is not clear; however, this study shows that the magnitude of hydrogen peroxide production does correlate with the formation of 8-oxodG. The increased level of 8-oxodG by clofibrate and PFDA in this study also correlates with their tumor-promoting or/and carcinogenic activities, which is consistent to other carcinogen, such as potassium bromate in the kidney (Cho *et al*., 1993). Clofibrate, as a classic hypolipidemic drug, is a potent peroxisome proliferator (Hess *et al*., 1965), and has tumor-promoting and carcinogenic activity (Hess *et al*., 1965; Hosokawa *et al*., 1989; Reddy and Qureshi, 1979). However, PFDA has not shown tumor promoting activity (Borges *et al*., 1993). It is important to notice that the treatment of FAO-overexpressed CV-1 cells (derived from African green monkey kidney cells) with linoleic acid, a substrate of the enzyme, resulted in increased hydrogen peroxide production and transformed foci. The transformed cells grew in soft agar and formed tumors when transplanted into immunodeficient (nude) mice (Chu *et al*., 1996). In a mutagenicity study using *Salmonella* bacteria, none of the doses of clofibrate and PFDA induced mutation, suggesting that while the peroxisome proliferators tested here could induce an indirect oxidative DNA damage, they did not appear to directly induce DNA damage.

We next studied whether hydrogen peroxide causes liver injury, since liver injury has been thought to be an important factor in nongenotoxic carcinogenesis (Ward *et al*., 1996). However, clofibrate and PFDA did not significantly change AST and ALT levels, indicators of hepatic injury. In line with our finding, a study showed that neither PFDA nor ciprofibrate, a derivative of clofibrate, induced hepatic toxicity as measured by loss of [3H]-thymidine uptake into hepatic DNA (Chen *et al*., 1994). In *Mutant* and Big Blue transgenic mice, AST and ALT levels were not affected after exposure for 9 days by the peroxisome proliferator methylclofenapate (Lefevre *et al*., 1994). We also found that clofibrate and PFDA did not significantly change γ-GT activity. γ-GT is another enzyme reflecting nonspecific hepatic injury, and has been
widely used for identifying putative preneoplastic lesions induced by hepatocarcinogens (Pitot and Sirica, 1980). However, hepatic foci induced by peroxisome proliferators showed a low frequency of $\gamma$-GT (Borges et al., 1993). Similar to our results in the liver, $\gamma$-GT activity was not changed in cultured hepatocytes (Edwards and Lucas, 1985). In another study, $\gamma$-GT was even decreased by peroxisome proliferators in cultured hepatocytes (Hong et al., 1995). Therefore, the significant increase in the ratio of liver/body weight (hepatomegaly) by both clofibrate and PFDA in this study may not come from hepatic injury. It was postulated that the hepatomegaly induced by the peroxisome proliferator was due to both hypertrophy and hyperplasia of hepatocytes. However, only hepatic hypertrophy is accompanied by hepatic toxicity. Thus, the mechanisms of the increase of relative liver weight are not clear. However, the hepatomegaly could be due to other types of cell hypertrophy such as Kupffer and Ito cells and/or hyperplasia of hepatocytes. In fact, it was observed that peroxisome proliferators activate Kupffer cells in vivo (Bojes and Thurman, 1996).

In summary, we have found that the magnitude of the production of hydrogen peroxide by the peroxisome proliferator clofibrate and PFDA is associated with the formation of 8-oxoG, and is related to their tumor-promoting or carcinogenic activity. However, the induction of hepatomegaly may not come from hepatic injury. Therefore, it is possible that oxidative damage of DNA by the induction of hydrogen peroxide as a by-product of the peroxisomal $\beta$-oxidation pathway may play at least a role in hepatocarcinogenesis by peroxisome proliferators. However, the role of hydrogen peroxide in hepatic injury is not clear.

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REFERENCES


