

Formation of 8-Hydroxydeoxyguanosine in Liver DNA of Rats following Long-Term Exposure to a Peroxisome Proliferator¹

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ABSTRACT

The mechanism by which nongenotoxic peroxisome proliferators induce hepatocellular carcinomas in rats and mice remains intriguing. The available experimental evidence suggests that the proliferation of peroxisomes and induction of peroxisome-associated enzymes results in oxidative stress which then leads to tumorigenesis. However, so far no direct evidence for oxidative DNA damage in livers of peroxisome proliferator-treated animals has been established. In the present study we have examined the DNA obtained from the livers of rats treated with ciprofibrate, a potent peroxisome proliferator, for variable periods of time for 8-hydroxydeoxyguanosine (8-OH-dG), an adduct that results from the damage of DNA caused by hydroxyl radical. Administration of ciprofibrate in diet at a concentration of 0.025% for 16, 28, 36, or 40 weeks resulted in progressive increases in the levels of 8-OH-dG. At 16, 28, and 40 weeks of ciprofibrate treatment, the 8-OH-dG in the liver DNA was significantly increased as compared to controls. This increase in 8-OH-dG levels is attributed to persistent peroxisome proliferation resulting from chronic ciprofibrate treatment as no increase in 8-OH-dG was found in liver DNA of rats that received a single large dose of ciprofibrate. The results of this study clearly demonstrate, for the first time, that persistent proliferation of peroxisomes leads to specific oxidative DNA damage.

INTRODUCTION

Peroxisome proliferators are chemicals which induce hepatomegaly and peroxisome proliferation in liver cells (1-3). These include structurally diverse compounds such as the lipid lowering drugs clofibrate, ciprofibrate, and gemfibrozil, and the industrial phthalate ester plasticizer, di(2-ethylhexyl)-phthalate (3). Long term administration of these chemicals to rats and mice results in the development of hepatocellular carcinomas (3-5). However, unlike a majority of chemical carcinogens, none of the known carcinogenic peroxisome proliferators interacts with or damages DNA (5-7). Therefore, formation of carcinogen-DNA adducts does not appear to be an essential step in the carcinogenesis by this class of chemicals (4, 6, 7). It was proposed that the carcinogenicity of these agents may be mediated by oxidative DNA damage resulting from persistent peroxisome proliferation and over 20- to 30-fold increase in H₂O₂-generating peroxisomal β -oxidation enzyme system in liver (4, 8). Although increased lipid peroxidation and excessive accumulation of lipofuscin in liver parenchymal cells of rats chronically treated with peroxisome proliferators supported such a hypothesis (8-10), evidence to date of oxidative DNA damage in livers with peroxisome proliferation is lacking. In this report we show that ciprofibrate, a potent carcinogenic peroxisome proliferator, causes significant increase in the formation of 8-OH-dG³ in rat liver DNA following chronic administration but not after a single large dose. These

results suggest that 8-OH-dG formation *in vivo* in livers with proliferated peroxisomes is most likely due to H₂O₂ escaping from these organelles and provide additional support for the role of oxidative stress in carcinogenesis by peroxisome proliferators.

MATERIALS AND METHODS

Male F-344 rats weighing 80-90 g were obtained from Charles River Breeding Laboratories, Wilmington, MA and were housed in individual metal cages in an air-conditioned room with a 12-h dark/12-h light cycle. Eighteen rats were fed a diet containing (0.025% w/w) ciprofibrate (Sterling-Winthrop Research Institute, Rensselaer, NY). Rats in groups of four were killed at 16, 28, and 36 weeks after exsanguination under ether anesthesia. The remaining six rats were killed at 40 weeks under ether anesthesia without exsanguination. Nine rats that were fed a normal diet (without ciprofibrate) were sacrificed at 40 weeks (three after exsanguination and six without exsanguination). To evaluate the effect of a single dose of ciprofibrate 12 male rats weighing 80-90 g were given ciprofibrate in dimethyl sulfoxide by gavage at a dose of 250 mg/kg body weight and killed after 3, 16, and 24 h in groups of four. Three control rats were given 0.1 ml of dimethyl sulfoxide alone and sacrificed at 16 h. All these rats used for acute study were also killed after exsanguination under ether anesthesia. Livers from all animals were quickly removed and snap frozen in liquid nitrogen. The liver from each animal was divided into three parts and stored at -20°C.

DNA isolation was carried out as described before (11, 12). Briefly powdered frozen livers were homogenized (three to four strokes) in a precooled teflon homogenizer in the absence of air. Oxygen was removed from all the tubes and buffers by argon gas prior to homogenization. DNA was isolated according to the procedure described by Marmur (12) with a slight modification (11). The isolated DNA was stocked in dried state under argon at 5°C until 8-OH-dG analysis. DNA was digested to deoxynucleotides by treatment with nuclease P₁, then with *Escherichia coli* alkaline phosphatase, and analyzed by high-performance liquid chromatography-electrochemical detection system (13, 14). Two to three samples from each liver were analyzed.

RESULTS

In rats fed ciprofibrate for 40 weeks liver tumors were found as expected (5). Analysis of the DNA isolated from portions of liver that were not involved with tumor in these rats, revealed significantly ($P < 0.001$) higher levels of 8-OH-dG residues when compared to controls (Fig. 1). Higher levels of the 8-OH-dG content were also detected in liver DNA isolated from rats that were maintained on ciprofibrate containing diets for 16, 28, and 36 weeks (Fig. 2). The levels of 8-OH-dG at 16 and 28 weeks are significantly high ($P < 0.001$) when compared to 40 week controls. Although, the 8-OH-dG levels are very high in livers of rats fed ciprofibrate for 36 weeks it is not statistically significant. To determine if increases in 8-OH-dG levels in liver DNA are secondary to persistent peroxisome proliferation or occur as a result of possible ciprofibrate-radicals, we analyzed the 8-OH-dG in liver DNA of rats killed 3, 16, and 24 h after a single large dose of this chemical. No increase in 8-OH-dG residues occurred following administration of a single dose of this drug (Fig. 3). These results suggest that the compound itself does not lead to the formation of free radicals, but sus-

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³ The abbreviation used is: 8-OH-dG, 8-hydroxydeoxyguanosine.

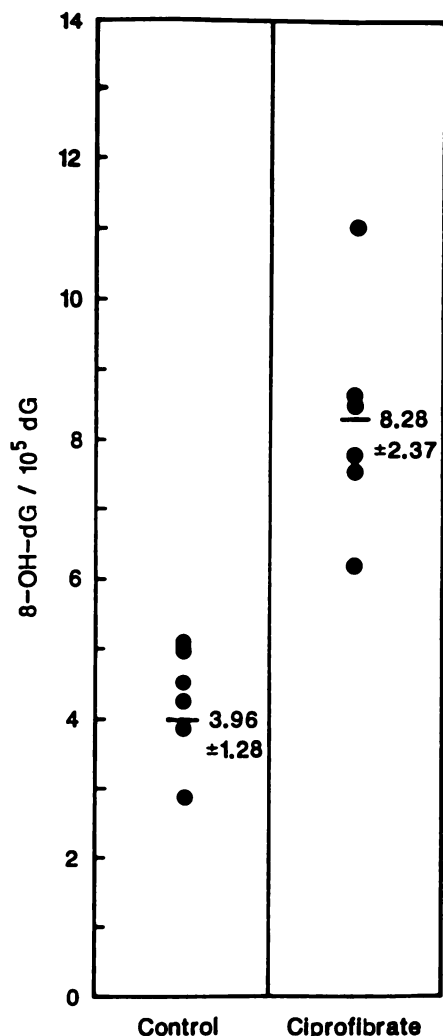


Fig. 1. Comparison of 8-OH-dG levels in liver DNA of control rats and rats treated with ciprofibrate for 40 weeks. Male F344 rats were fed a diet containing ciprofibrate (0.025% w/w in diet) for 40 weeks. Rats were killed under ether anesthesia but were not exsanguinated. The nontumorous portions of liver were quickly removed, divided into three parts, and frozen in liquid nitrogen. DNA was isolated and digested to deoxynucleosides by treatment with nuclease P₁ and then with *E. coli* alkaline phosphatase and analyzed by high-performance liquid chromatography-electrochemical detection system as described under "Materials and Methods." Digestion of DNA and high-performance liquid chromatograph analysis of 8-OH-dG were performed on the same day. Points, mean value of 8-OH-dG from two to three DNA samples from each rat. These DNA isolates were from six control and six ciprofibrate-treated rats. The numbers in the figure represent mean value \pm standard deviation. $P < 0.001$, Student's *t* test; range of 8-OH-dG in control and ciprofibrate treated group was 2.8 to 5.1 and 6.2 to 10.9, respectively.

tained increase in the number of peroxisomes is necessary for this type of oxidative DNA damage to occur.

DISCUSSION

The formation of 8-OH-dG is due to the hydroxyl radical (OH·) most likely produced from H₂O₂ generated by the many-fold increase in fatty acid β -oxidation enzyme system in the livers with persistent peroxisome proliferation (8, 15, 16). Peroxisome proliferators cause a 20- to 30-fold increase in the mRNA levels of all three β -oxidation enzyme system genes in liver due to enhanced transcription of the corresponding genes (17, 18). These agents do not appear to increase the transcriptional rates of catalase gene (18). Increases in peroxisomal steady state H₂O₂ levels and increased hydroxyl radical production in liver peroxisomal fractions of rats treated with peroxisome proliferators have been reported (19–21). Hydroxyl radi-

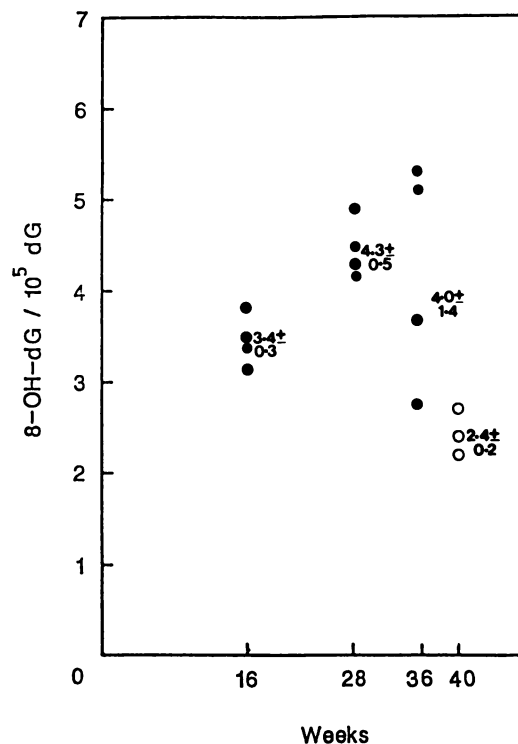


Fig. 2. Analysis of 8-OH-dG levels in liver DNA of rats during chronic treatment with ciprofibrate. Male F344 rats were fed a diet containing ciprofibrate (0.025% w/w in chow) for 16, 28, and 36 weeks. The control rats were maintained for 40 weeks on the same diet but without the drug. Rats were killed under ether anesthesia and exsanguinated. Two to three liver samples from each rat and four rats for each interval were analyzed. ○, control (three rats); ●, ciprofibrate (four rats). The numbers in the figure represent mean value \pm standard deviation. $P < 0.001$ in 16- and 28 week-groups.

cals can be easily generated from H₂O₂ in the presence of metal ion-bound DNA and may attack guanine residue to produce 8-hydroxyguanine (22–24). 8-OH-dG is one of DNA damages induced by oxygen free radicals (23–25). Formation of 8-OH-dG in cellular DNA *in vivo* was previously observed after treatment with H₂O₂ or irradiation with γ -rays. It has been shown that the levels of 8-OH-dG in the DNA of livers of mice is dependent on the dose of ionizing radiation (13). The amount of 8-OH-dG observed in the present experiment, after chronic treatment for 16, 28, 36, or 40 weeks is higher than that observed in the mouse liver DNA after exposure to 173 krad of γ -radiation. DNA damage caused by chemical and physical carcinogens can be repaired immediately and may not result in any deleterious effects (26). Kasai *et al.* have shown that the levels of 8-OH-dG in the livers of mice was gradually decreased over a period of 90 min after exposure to 80 krad of γ -rays (13). Such a repair is possible in rats given ciprofibrate for a brief period of time. However, in rats and mice chronic administration of peroxisome proliferators results in sustained oxidative stress which may overwhelm the repair process and lead to mutagenic and carcinogenic changes. In rats given a single oral dose of potassium bromate, a renal carcinogen, significant increase of 8-OH-dG was observed specifically in the target organ (kidney) but not in nontarget tissue (liver) (11). The demonstration in the present study of higher levels of 8-OH-dG residues in liver DNA of rats fed a peroxisome proliferator is the first example of specific oxidative DNA damage *in vivo* produced by chronic administration of a chemical at a low dose used for carcinogenicity testing. The formation of 8-OH-dG in DNA may cause mutagenesis and carcinogenesis as a result of misreading of bases during DNA replication (27). Additional studies are necessary to elucidate the mechanism by which free

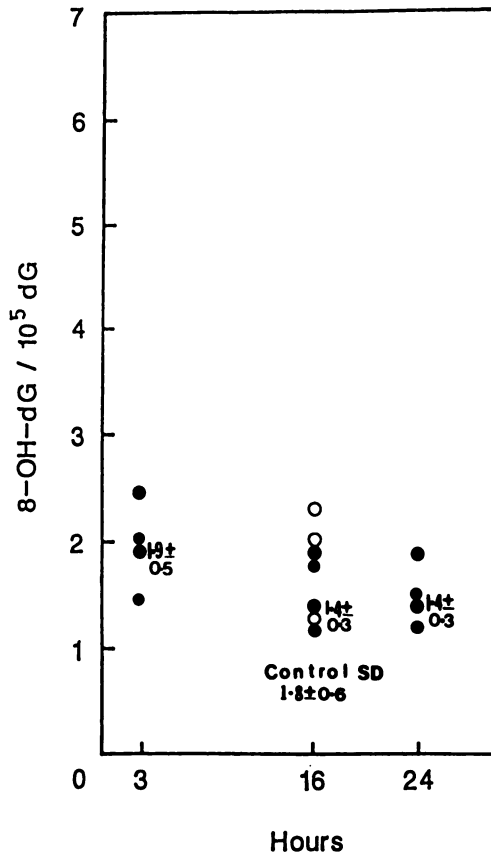


Fig. 3. Levels of 8-OH-dG in the liver DNA of rats after a single intragastric dose of ciprofibrate (●). Male F344 rats were given a single dose of ciprofibrate (250 mg/kg body weight) by gavage and killed after 3, 16, and 24 h in groups of four. Three control rats (○) received 0.1 ml of the solvent dimethyl sulfoxide. The rats were exsanguinated under ether anesthesia and killed. Note that 8-OH-dG levels in liver DNA isolated from nonexsanguinated control rats ($3.9 \pm 1.3/10^5$ dG) (Fig. 1) was higher than that from other control rats whose blood has been completely drained by decapitation after ether anesthetization ($1.1-2.7/10^5$ dG) (Figs. 2 and 3). Therefore, a small quantity of 8-OH-dG residues may be produced as artifacts during DNA isolation (23) even if a careful method to prevent oxidation is used as in this study. The numbers in the figure represent mean value \pm standard deviation.

radical generated 8-OH-dG residues play a role in tumor initiation and promotion (28, 29). The liver tumors induced by peroxisome proliferators in rats appear to exhibit certain unique phenotypic features (*i.e.*, the lack of expression of marker enzymes γ -glutamyltranspeptidase and placental form of glutathione *S*-transferase) when compared to tumors induced by mutagenic hepatocarcinogens (30, 31). This raises the possibility that oxidative DNA damage-related initiation of carcinogenesis may differ from that initiated by mutagenic carcinogens.

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