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DNA damage related to increased hydrogen peroxide generation by hypolipidemic drug-induced liver peroxisomes

(fatty acid β-oxidation/DNA strand breaks)

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ABSTRACT **Several hypolipidemic drugs and certain industrial plasticizers induce proliferation of peroxisomes, enhance the activity of peroxisome-associated β-oxidation of fatty acids, and produce hepatocellular carcinomas in the livers of rodents. Because these chemicals themselves are not mutagens and do not covalently modify DNA, unlike the majority of chemical carcinogens, we proposed that the persistent proliferation of peroxisomes, and the induction of associated peroxisomal oxidases, caused a sustained increase in intracellular H_2O_2 or other reduced oxygen species, which would then introduce mutagenic DNA damage. In the present study, we investigated the ability of peroxisomes purified from the livers of normal and hypolipidemic drug-treated rats to induce DNA strand scission *in vitro*. Gradient-purified peroxisomes from livers of hypolipidemic drug-treated rats produced a 30- to 70-fold increase in H_2O_2 generation when compared to controls. The levels of H_2O_2 generated in incubations containing control or hypolipidemic drug-induced peroxisomes correlated well with the induction of single strand breaks in supercoiled simian virus 40 DNA molecules that were included in these reconstituted peroxisome incubations. Addition of excess catalase to peroxisome incubations failed to prevent strand breaks, suggesting that other reduced oxygen species may be rapidly generated from H_2O_2. These experimental results are consistent with a mechanism of hepatocarcinogenesis in which hepatocellular genetic damage is introduced by the by-products of peroxisomal fatty acid β-oxidation, an oxidative pathway that is dramatically increased in hypolipidemic drug-treated livers.**

Several drugs, including clofibrate, are now used for the treatment of hyperlipidemias in humans. Clofibrate, as well as other potent hypolipidemic agents, causes massive hepatomegaly when administered to rodents, chickens, and monkeys (1–4). This hepatomegaly is characteristically associated with a marked increase in the numerical, as well as volume, densities of peroxisomes in the liver cells of several species tested (1–4). Peroxisomes contain catalase, carnitine acetyltransferase, and five hydrogen peroxide (H_2O_2)-generating enzymes, including the enzyme system involved in the β-oxidation of long-chain fatty acids (5, 6). The activities of these enzymes in liver, including their messenger RNA species, are increased in association with peroxisome proliferation (5–9). Several studies have now established that peroxisome proliferators [i.e., certain hypolipidemic drugs such as clofibrate, Wy-14643 ([4-chloro-6-(2,3-xylidino)2-pyrimidinylthio]acetic acid), nafenopin, BR-931, methyl clofenapate, ciprofibrate, and the industrial plasticizer di(2-ethylhexyl)phthalate] induce hepatocellular tumors in both mice and rats when chronically administered in the diet (6, 10). None of these carcinogenic peroxisome proliferators has been shown to be mutagenic in bacterial assays (11, 12); furthermore, they displayed no capacity to covalently modify cellular DNA either *in vivo* or *in vitro* (13). The lack of mutagenicity of these agents, combined with the consistent coupling of drug-induced proliferation of H_2O_2-generating peroxisomes and formation of hepatocellular tumors, led to the hypothesis that the induction of hepatocellular tumors in hypolipidemic drug-treated rats is related to biologically active products of the proliferated peroxisome population rather than a direct drug effect (10). In the experiments reported here, we show that the level of H_2O_2 production was markedly increased in the livers of rats exposed to peroxisome proliferating agents, and that the increased H_2O_2 production was relatable to the increased levels of fatty acid oxidation catalyzed by peroxisomal enzymes. Addition of supercoiled viral DNA to the reconstituted peroxisome incubations also showed that the by-products of peroxisomal fatty acid oxidation were clearly capable of inducing potentially harmful single strand breaks in DNA.

METHODS

Peroxisome Purification. Pooled livers (20 g) from control or hypolipidemic drug-treated rats (100 g; male, F-344 rats were fed 0.1% Wy-14643 added to the normal Purina rat chow diet for 4 weeks) were homogenized, and the peroxisome-containing light mitochondrial fractions were prepared as described by Baudhuin *et al.* (14). The light mitochondrial fractions (1 ml) were layered on top of metrizamide gradients (40 ml; 20%–50%) and subsequently centrifuged (60 min; 132,000 × *g*) in a VTi50 rotor (15). The tubes were punctured, and 2-ml fractions were collected; peroxisome-containing fractions were identified by enzyme assay (palmitoyl CoA oxidation) and electron microscope analysis.

^{3}H-Labeled Simian Virus 40 (SV40) Peroxisome Incubations. Supercoiled SV40 DNA was purified from infected monolayers of CV-1 African green monkey kidney cells by the Hirt lysate method (16). ^{3}H-labeled SV40 DNA was prepared from dishes exposed to 25 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine per ml of culture medium for 4 hr. To study DNA damage induced by the products of peroxisomal fatty acid oxidation, 500 ng of purified supercoiled SV40 DNA was incubated in a 30-μl reaction mixture containing: 10 mM Tris·HCl, pH 8.0/0.2 mM NAD/0.1 mM CoA/0.01 mM FAD/0.01 mM dithiothreitol/5 mM $MgCl_2$/2.5 mM ATP/0.01% Triton X-100/1 mM KCN/15 μM palmitate (substrate), and aliquots (0.1–1.0 μg) of a gradient-purified liver peroxisome fraction from control or Wy-14643-treated male rats. These mixtures were incubated for various times at 37°C; 25 μl of buffer A (10 mM Tris·HCl, pH 7.2/5 mM NaCl/1 mM EDTA) containing 40% glycerol and trace bromcresol green was then added to the chilled reactions, and the mixtures were applied to individual wells of a 0.8% agarose Tris borate gel (50 mM Tris, pH 8.3/55 mM borate/1

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Abbreviation: SV40, simian virus 40.

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Table 1. H_2O_2 and associated DNA strand breaks generated by peroxisomes purified from livers of control or hypolipidemic drug-treated rats

Group	H_2O_2 production, nmol/min per mg of peroxisomal protein	Peroxisome density, mg of peroxisomal protein per g of liver	Liver H_2O_2 production, nmol/min per g of liver	DNA damage, ng of SV40 DNA nicked × 10^{-3} per g of liver
Control	65	0.079	5.1	2
Hypolipidemic drug-treated	412	0.381	157.0	101
Purified fatty acyl-CoA oxidase	112	—	—	21.5*

Incubations contained purified peroxisomes (15), palmitate, cofactors, and ^{3}H-labeled SV40 DNA. To measure H_2O_2 production, incubation aliquots were removed and mixed with 5% trichloroacetic acid; after centrifugation, the H_2O_2 concentration in the supernate was measured by the ferrithiocyanate method (17). Peroxisomes were gradient-purified from 20 g of liver from control or Wy-14643-treated rats; mean weights of livers derived from 200 g control or Wy-14643 rats were 7 g and 19 g, respectively. Fatty acyl-CoA oxidase was purified to apparent gel homogeneity as described (18). Under conditions of linear formation of DNA nicks versus concentration of control or Wy-14643 peroxisomes, we determined the concentration of peroxisomal protein required to give 50% conversion (i.e., 250 ng) of supercoiled to nicked DNA; these peroxisome concentrations were then multiplied by mg of peroxisomal protein recovered per g of starting liver weight to indicate (last column) total *in vitro* DNA damaging capacity for control of Wy-14643-treated liver peroxisomes.
*Value indicated corresponds to mg of purified enzyme protein.

mM EDTA) containing 0.1 μg of ethidium bromide per ml. Samples were electrophoresed in the dark at 1.6 V/cm for 18 hr. The mobility of the SV40 species was supercoiled > linear > nicked. Gels were photographed, and specific gel bands were cut out, dissolved in scintillation vials (1 ml; 1 M HCl; 60°C; 1 hr), and counted to quantitatively determine the conversion of supercoiled to nicked DNA.

RESULTS

Induced Peroxisomal H_2O_2 Production. Treatment of animals with the hypolipidemic drug induced a large increase in the level of liver peroxisomal fatty acid oxidation and an associated increase in the generation of H_2O_2 (Table 1). This drug-associated increase in H_2O_2 generation was the combined result of an increase in the specific activity of peroxisomal H_2O_2-generating enzymes (6.3-fold, Table 1) as well as an increase in the number of peroxisomes per unit of liver volume (4.8-fold by peroxisome recovery, Table 1; or 11-fold by morphometric analysis of liver sections, see ref. 19). As a result, the overall peroxisome production of H_2O_2 was increased 30- to 70-fold in the hypolipidemic drug-treated rat livers, depending on the method used to assess the numerical increase in peroxisome density (Table 1). The level of peroxisomal catalase is also increased after Wy-14643 treatment (3, 20), but the increase in H_2O_2 levels observed here is in excess of any induced catalase degradation.

H_2O_2-Induced DNA Damage. Pure H_2O_2 (in Tris EDTA alone) was able to induce single strand nicks in a dose-dependent manner (Fig. 1*A*), with 9.3 mM H_2O_2 giving 50% conversion to nicked DNA during the 4-hr incubation. Prior addition of pure catalase to these H_2O_2 incubations diminished the H_2O_2-induced damage in a dose-dependent manner with 400 units of catalase per ml totally preventing the effects of 24 mM H_2O_2 (Fig. 1*B*).

Peroxisome-Induced DNA Damage. Purified supercoiled (form I) SV40 DNA was used to characterize and quantitate the DNA damage induced by reaction products generated during peroxisomal fatty acid β-oxidation. Peroxisomes isolated from untreated rat liver (Fig. 2), when incubated with palmitate and cofactors, did not induce strand breaks and thus did not alter the electrophoretic mobility of the SV40 DNA (Fig. 3). When peroxisomes isolated from the same amount of liver from a rat treated with Wy-14643 were incubated under the same reaction conditions, the majority of the supercoiled SV40 DNA was damaged (Fig. 3). The mobility of this peroxisome-damaged DNA was compared to the SV40 DNA products generated in *Eco*RI or xanthine–xan-

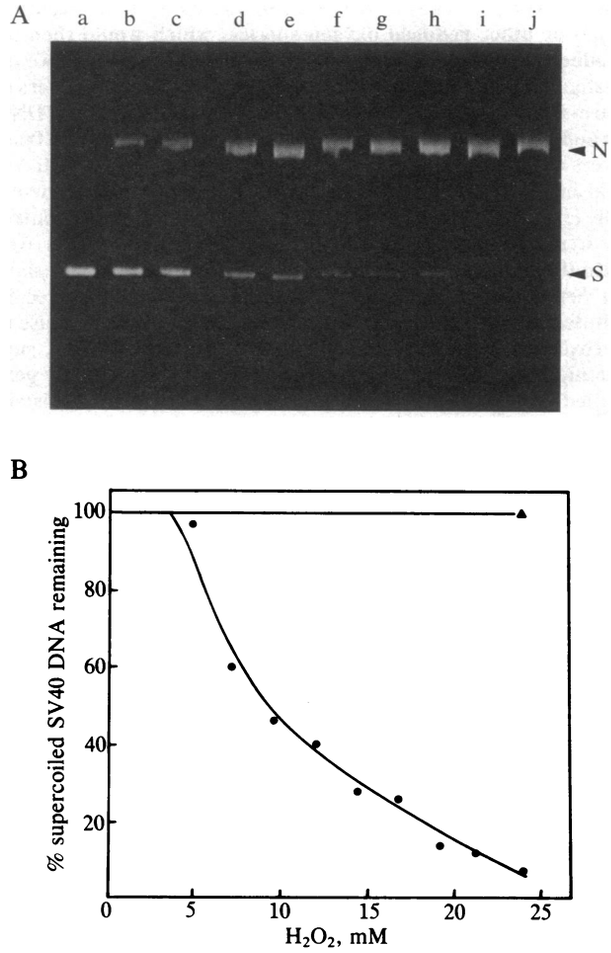

FIG. 1. Strand scission of supercoiled SV40 DNA in H_2O_2 incubations. Incubations containing 500 ng (15,000 dpm) of supercoiled ^{3}H-labeled SV40 DNA in buffer A containing 0 (lane a), 4.8 (lane b), 7.2 (lane c), 9.6 (lane d), 12.0 (lane e), 14.4 (lane f), 16.8 (lane g), 19.2 (lane h), 21.6 (lane i), or 24.0 (lane j) mM H_2O_2 were incubated at 37°C for 4 hr. Samples were electrophoresed (*A*) in 0.8% agarose, Tris borate gels. Gel bands that contained the remaining supercoiled SV40 DNA were then cut out, dissolved, and counted to quantitate the H_2O_2-dependent conversion of supercoiled to nicked SV40 DNA (*B*). In one reaction tube, 400 units of catalase per ml was added prior to the addition of 24 mM H_2O_2 (▲). S, supercoiled; N, nicked.

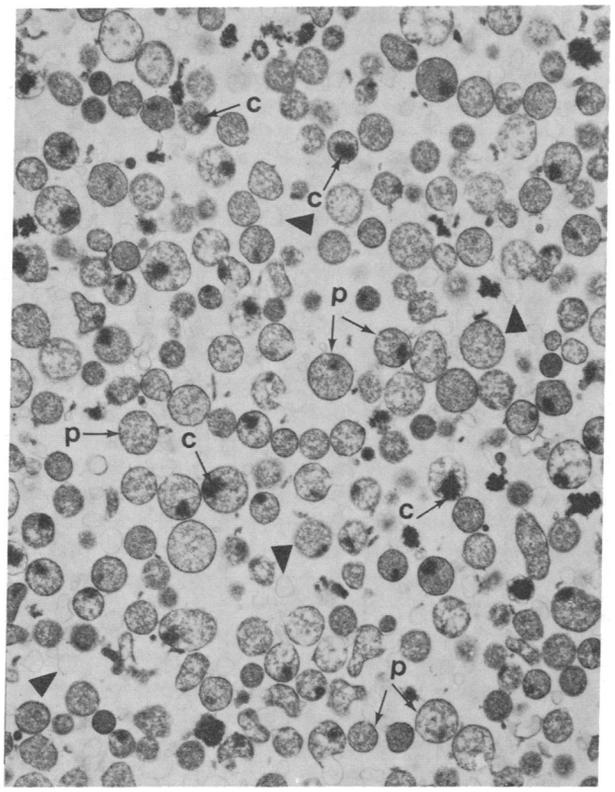

FIG. 2. Electron micrograph showing purified (>99%) peroxisomes (p) with a unilamellar membrane, diffuse contents, and characteristic electron dense core (c); in many of the peroxisomes, this electron dense core does not appear in the same plane of this thin section. Arrowheads point to vesiculation or protrusion of peroxisomal membranes, some in cross-section, without any apparent contact with peroxisomes. (×13,500.)

thine oxidase incubations (21), which are known to convert supercoiled SV40 DNA to linear or nicked DNA, respectively. As can be seen (Fig. 3), nicked SV40 DNA species were generated in the hypolipidemic drug-induced peroxisome incubations. Control incubations containing buffer, cofactors, and peroxisomes without palmitate showed only negligible background conversion (<5%), which was subtracted from peroxisome and palmitate incubations.

In incubations containing peroxisomes from hypolipidemic drug-treated rats, a time- and enzyme-dependent conversion to nicked SV40 DNA was observed (Fig. 4) that paralleled the level of H_2O_2 production in these peroxisome incubations. Incubations containing peroxisomes purified from an equivalent amount of untreated rat liver showed no measurable damage to the SV40 DNA. At much higher concentrations of control peroxisomal protein, we could then observe measurable SV40 DNA damage; this allowed quantitative comparison of control and drug-treated peroxisomes, showing that the factor increase in DNA nicking capacity was roughly paralleled by the capacity of the respective peroxisomes to oxidize palmitate and produce H_2O_2 (Table 1). This same relationship between H_2O_2 production and DNA nicking also held true when H_2O_2 production was mediated by purified fatty acyl-CoA oxidase (Table 1), now independent of the other peroxisome components. In the Wy-14643-induced peroxisome incubations in which 50% nicking occurred, significantly less H_2O_2 was measurable than what was required to induce 50% nicking in the pure H_2O_2 incubations (9.3 mM, Fig. 1*B*). Related to this, catalase at 400 units/ml had only a marginal quenching effect on the peroxisome-initiated DNA damage (Fig. 4).

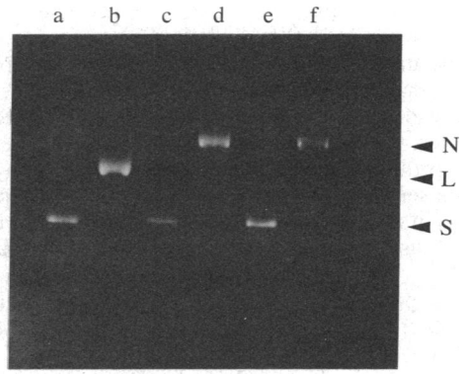

FIG. 3. Agarose gel electrophoresis separation of supercoiled (S), nicked (N), or linear (L) SV40 DNA molecules. Lanes: a, 500 ng of supercoiled SV40 DNA incubated in buffer A (10 mM Tris, pH 7.2/5 mM NaCl/1 mM EDTA); b, 500 ng of supercoiled SV40 DNA digested with 5 units of *Eco*RI at 37°C for 4 hr; c, 500 ng of supercoiled SV40 DNA incubated for 4 hr with 0.5 nM xanthine oxidase/50 μM xanthine/0.1 mM EDTA/50 mM potassium phosphate, pH 7.8; d, 500 ng of supercoiled SV40 DNA incubated with 29 mM H_2O_2 in buffer A for 4 hr; e, 500 ng of supercoiled SV40 DNA incubated in H_2O_2-generating reaction mixture containing purified liver peroxisomes from untreated rats; f, 500 ng of supercoiled SV40 DNA incubated in H_2O_2-generating reaction mixture containing purified liver peroxisomes from Wy-14643-treated rats.

DISCUSSION

Damage to DNA resulting from covalent modification by a carcinogenic chemical or its metabolites is generally regarded as the initial biochemical alteration leading to neoplastic transformation in the case of the majority of chemical carcinogens (22–26). Carcinogenic peroxisome-proliferating compounds do not induce mutations (11, 12) in the standard *Salmonella* microsome assay (22) or in the newly identified tester strains TA102 and TA104, which detect oxidative

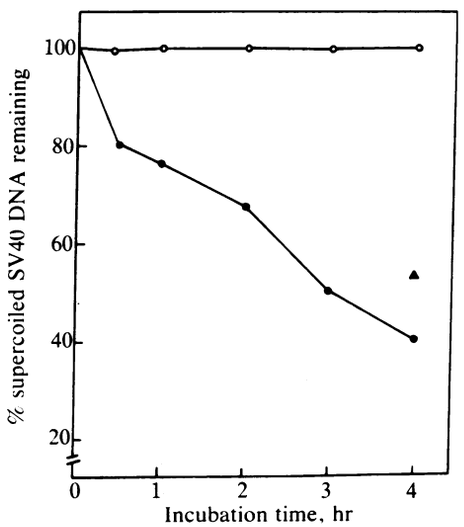

FIG. 4. SV40 DNA strand scission in incubations containing purified peroxisomes from livers of control (○) or hypolipidemic drug-treated (●) rats. Incubations containing 500 ng of ^{3}H-labeled SV40 DNA per 30 μl, purified liver peroxisomes, substrate (palmitate, 15 μM), and cofactors were incubated at 37°C for 4 hr. At the specified time points, aliquots were removed and chilled, mixed with buffer A/glycerol/bromcresol green, and applied to individual wells of a 0.8% agarose gel. Electrophoresis and quantitation of the remaining supercoiled ^{3}H-labeled SV40 DNA on the gel were done as described. In one reaction tube, 400 units of catalase per ml was added prior to initiating the reaction with hypolipidemic drug-treated peroxisomes (▲).

mutagens (27). Likewise, these compounds do not covalently modify rat liver DNA (13). In aggregate, these results suggested that, unlike the majority of chemical carcinogens, peroxisome proliferating agents were not carcinogenic because of any electrophilic metabolites that were formed from them. Rather, these observations led to the hypothesis that persistent proliferation of peroxisomes induced by these compounds served as an endogenous initiator of hepatocarcinogenesis in rodents by increasing the intracellular production of H_2O_2 as a by-product of the peroxisomal oxidases (5, 6, 10).

Hydrogen peroxide is generated as a by-product of the FAD-dependent oxidation of fatty acyl-CoA by fatty acyl-CoA oxidase, the first step in the chain reaction of peroxisomal fatty acid oxidation (2, 5, 6). Peroxisomes oxidize long-chain and medium-chain fatty acids, and the removal of two carbons results in the generation of one molecule of H_2O_2 (2). The results of our experiments clearly demonstrate that supercoiled SV40 DNA received extensive strand breaks when incubated *in vitro* with Wy-14643-induced peroxisomes and palmitate. The DNA nicking capacity was proportional to the ability of the peroxisomes to oxidize palmitate and generate H_2O_2. This result for biologically generated H_2O_2 (Table 1) is clearly consistent with previous reports showing the ability of pure H_2O_2 to cause DNA strand breakage as well as chemical alteration of the DNA bases (28). H_2O_2 has also been shown to be carcinogenic in rats and mice (29). Furthermore, H_2O_2 and related oxygen free radicals are known to be mutagenic in repair-deficient strains of *Escherichia coli* (30, 31) and *Salmonella typhimurium* (27, 32), underlining the importance of a primary, oxygen-mediated, DNA-damaging event in the biological response to these reduced oxygen species (33–35).

The observation that catalase exerted only a minor quenching effect on the peroxisome-initiated DNA damage under these *in vitro* conditions raises some interesting possibilities. Although this observation does not rule out the direct DNA-damaging role of H_2O_2 in these peroxisome-containing incubations, it does suggest that rapid reduction of H_2O_2 to OH^- and $OH^{\cdot}$ may be occurring, probably by metal ion mediated interactions (21, 32–36). Recently, Tolbert and Gee (37) identified a peroxisomal membrane oxidase that oxidizes NADH, xanthine, glycerol phosphate, or aldehydes. This peroxisomal membrane oxidase exhibits similar properties to milk xanthine oxidase and may thus produce superoxide ($O_2^{\cdot-}$) and H_2O_2. It is well known that $O_2^{\cdot-}$ and H_2O_2 react rapidly to yield the hydroxyl radical. It is pertinent to note that catalase is not highly effective in destroying H_2O_2 present at low concentrations (33, 38) and that some of the H_2O_2 generated in peroxisomes can escape degradation by catalase even in the normal liver (39). Since it is evident that H_2O_2 is formed in excess in the liver as a consequence of drug-induced peroxisome proliferation and that these livers exhibit increased lipid peroxidation (40), it will now be of interest to determine the extent to which endogenously generated H_2O_2 as well as $O_2^{\cdot-}$ produced by peroxisomal membrane oxidase contributes to $OH^{\cdot}$ generation and DNA damage *in vivo*. Further studies with specific reactive oxygen scavengers (21, 33, 41–44) will be necessary to define the molecular species responsible for peroxisome-initiated DNA damage. Preliminary studies in which diethylenetriamine pentaacetic acid, a metal ion chelator (21), was added to the peroxisome–DNA incubations have shown inhibition of DNA strand scission.

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