Effect of Dexamethasone on Ciprofibrate-Induced Cell Proliferation and Peroxisome Proliferation

M. Sambasiva Rao and V. Subbarao

Department of Pathology, Veterans Affairs Lakeside Medical Center and Northwestern University Medical School, Chicago, Illinois 60611

Received May 1, 1996; accepted October 1, 1996


Peroxisome proliferators cause liver cell proliferation in addition to other pleiotropic effects such as peroxisome proliferation and induction of certain peroxisomal and cytosolic enzymes in liver. Since dexamethasone has been shown to inhibit mitogen-induced liver cell hyperplasia, we examined whether dexamethasone inhibits only cell proliferation without affecting peroxisome proliferation induced by peroxisome proliferators such as ciprofibrate. Livers of rats fed a diet containing ciprofibrate (0.025%) with or without added dexamethasone (0.5 mg or 1 mg/kg diet) for 1 week were evaluated for hepatocyte proliferation and peroxisome proliferation. Dexamethasone administration resulted in abrogation of ciprofibrate-induced cell proliferation as shown by bromodeoxyuridine (BrdU) labeling and mitoses counts. The hepatocyte proliferative index measured after administration of a single dose of BrdU was 18.3 ± 1.1 and 2.3 ± 0.7% (p < 0.01) in ciprofibrate and ciprofibrate + dexamethasone treated rats, respectively. With multiple injections of BrdU (daily injections for 7 days) the proliferative index was 225 ± 10 and 183 ± 2% (p < 0.02), respectively, in these two groups. Interestingly, whereas the levels of peroxisome proliferator-induced M, 80,000 polypeptide and catalase and peroxisomal bifunctional enzyme, and the corresponding mRNAs and peroxisome volume density were unaffected. These results show that dexamethasone selectively inhibits only cell proliferation without inhibiting the peroxisome proliferation caused by ciprofibrate. This model should be useful for examining the role of cell proliferation versus oxidative stress in peroxisome proliferator-induced hepatocarcinogenesis. © 1997 Society of Toxicology.

Peroxisome proliferators are a diverse group of synthetic and naturally occurring compounds that induce predictable pleiotropic responses in the liver of rats and mice (Reddy and Lalwani, 1983; Moody 1994). These responses include hepatomegaly, hepatocyte proliferation, and peroxisome proliferation associated with induction of certain peroxisomal enzymes and transcriptional activation of genes involved in peroxisomal fatty acid β-oxidation (Reddy and Lalwani, 1983; Reddy et al., 1986). The peroxisome proliferative activity of these compounds is shown to be a receptor-mediated process (Green, 1992). In addition, long-term administration of peroxisome proliferators causes hepatocellular carcinomas (Reddy et al., 1980; Rao and Reddy, 1987). Since peroxisome proliferators are nongenotoxic agents, the mechanism by which they cause liver tumors remains controversial. The suggested mechanisms include oxidative stress resulting from sustained induction of H2O2 generating peroxisomal fatty acyl-CoA (Reddy and Rao, 1989) and increased cell proliferation (Butterworth et al., 1992). To resolve the issue of the primacy of oxidative stress versus cell proliferation in peroxisome proliferator-induced hepatocarcinogenesis, an experimental model is needed in which the cell proliferative and peroxisome proliferative properties can be dissociated.

Hepatocyte proliferation is induced either after cell loss (partial hepatectomy or necrosis caused by hepatotoxic agents), referred to as compensatory hyperplasia, or after mitogen stimulation (lead nitrate, ethylene bromide, etc.), referred to as direct hyperplasia (Fausto and Webber, 1994; Ledda-Columbano et al., 1992). It has been shown that during compensatory hyperplasia there is activation of immediate early genes and production of cytokines and growth factors (Fausto and Webber, 1994). The mitogen-induced liver cell hyperplasia appears to be dependent mostly on tumor necrosis factor-α (TNFα) and not other factors (Coni et al., 1993; Shinozuka et al., 1994). The glucocorticoid dexamethasone has been shown to inhibit mitogen-induced liver cell proliferation through abrogation of TNFα production (Ledda-Columbano et al., 1994). Since peroxisome proliferators also induce liver cell proliferation through a mitogenic effect without causing liver necrosis, we hypothesized that dexamethasone inhibits only cell proliferation, and not the peroxisome proliferation induced by ciprofibrate, a potent peroxisome proliferator. The results demonstrate that dexamethasone prevents cell proliferation in liver but not the peroxisome proliferation induced by ciprofibrate in the rat.

METHODS

Male F-344 rats were obtained from Charles River Laboratories (Wilmington, MA) and housed in plastic cages on San-i-cel bedding in an air-conditioned room with a 12-hr dark and light cycle. After a week of acclimatization, rats were divided into six groups (4 to 8 rats/group) and fed a...
Dexamethasone and Liver Cell Proliferation

RESULTS

Body and liver weights, proliferative index of hepatocytes, and peroxisome volume density in rats given ciprofibrate diet with or without dexamethasone are presented in Table 1. Body weight gain in control rats and rats fed diet containing ciprofibrate and ciprofibrate + dexamethasone (0.5 mg/kg diet) increased approximately by 52, 34, and 13%, respectively, in 7 days, whereas no weight gain was observed in rats that were fed diet containing higher dose of dexamethasone. As expected, ciprofibrate treatment caused a significant increase in relative liver weight when compared to that of controls (9.0 g vs 4.7 g/100 g body weight, p < 0.001). Similarly, rats that received both dexamethasone and ciprofibrate also showed a significant increase in absolute and relative liver weights over those of controls. In rats that were fed diet containing only dexamethasone there was no increase in absolute liver weight, although the relative weight increased by 36 to 60% due to a relative reduction in body weight gain.

BrdU, a pyrimidine analogue, is incorporated into DNA synthesizing nuclei and can be easily identified immunohistochemically using anti-BrdU antibodies. Immunohistochemical studies performed after administration of single or multiple doses of BrdU demonstrated a significant increase in the number of labeled hepatocytes in rats given ciprofibrate, when compared to controls and rats given ciprofibrate + dexamethasone diet (Figs. 1a and 1b). The labeling index of hepatocytes after pulse labeling for 1 hr was 18.3 and 8.5/2000 in ciprofibrate treated and control rats, respectively (p < 0.01). Interestingly, in rats given dexamethasone and ciprofibrate or dexamethasone alone there was a marked decrease in the number of labeled cells. Dexamethasone at 0.5 mg and 1 mg/kg diet dose levels completely prevented ciprofibrate-induced cell proliferation. In the dexamethasone-treated groups the labeling indices ranged from 2 to 5/2000 cells. It is not clear why the 0.5 mg dexamethasone dose level caused more inhibition of cell proliferation than the 1 mg dose level. In rats that were given multiple doses of BrdU the labeling index was 164,225, 183, and 41/2000 cells in the control, ciprofibrate, and ciprofibrate + dexamethasone groups, respectively. Similarly, the mitotic index was also 5- to 12-fold higher in the ciprofibrate group when compared to the dexamethasone + ciprofibrate group (Table 1).

Ultrastructural analysis of hepatocytes from rats fed diet containing ciprofibrate alone or ciprofibrate and dexamethasone (1 mg/kg diet) showed a marked increase in the number of peroxisomes (Figs. 2a and 2b). Morphometric analysis in both the groups revealed a 10-fold increase in the volume density of peroxisomes over that of control animals (p < 0.001), which received normal chow or chow containing dexamethasone (1 mg/kg diet).

SDS–PAGE analysis of postnuclear fractions prepared

Morphometric analysis of peroxisome.

Fragmented sections of liver from four rats in each group were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 4 hr and processed for transmission electron microscopy. Thin sections were cut on an LKB ultratome, stained with uranyl acetate and lead citrate, and examined in a JOEL JEM-100CS 11 electron microscope. Morphometric analysis of peroxisomes was carried out as described before (Rao et al., 1982), according to the procedure of Weibel et al. (1966). Briefly, micrographs were taken at ×5000 and magnified 2.5 times at printing. Points of intersection overlying cytoplasm and peroxisomes were counted using a 5-mm-spaced lattice grid. The volume density of peroxisomes was determined in relation to cytoplasmic volume. Ten photographs were counted from each animal.

Analysis of proliferative indices.

For BrdU immunohistochemistry, portions of liver from all animals were fixed in 70% alcohol overnight at 4°C and processed for light microscopy. Five-micrometer-thick paraffin sections were stained immunohistochemically by the avidin–biotin–peroxidase complex method using Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) with monoclonal antibodies specific for BrdU (Becton–Dickinson) at a dilution of 1 in 300 (Ward et al., 1988). The reaction product was visualized with the addition of diaminobenzidine tetrahydrochloride and the slides were counterstained with hematoxylin. The labeling index was obtained by counting 2000 hepatocytes in each liver.

To obtain a mitotic index 2000 hepatocytes were counted from each liver on hematoxylin and eosin stained sections.

SDS–polyacrylamide gel electrophoresis.

Ten percent (W/V) liver homogenates were prepared in ice-cold 0.25 M sucrose containing 10 mM EDTA (pH 7.5) using a Potter–Elvehjem homogenizer. Postnuclear fractions were obtained by centrifuging the homogenate at 700g for 10 min in a Beckman J-21 centrifuge at 4°C. The protein concentration in the homogenate was determined by the method of Lowry et al. (1951). Electrophoresis was performed on 10% SDS–polyacrylamide slab gels according to the method of Laemmli (1970), using 30 μg protein (Nemali et al., 1988). After completion of the electrophoretic run (20 mA/Slab), the proteins were fixed by immersing the gels in 10% trichloroacetic acid for 30 min and then stained with 0.1% Coomassie blue in 50% methanol/10% acetic acid.

Northern blot analysis.

Total RNA was extracted from livers using the method of guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi, 1987). Electrophoresis of glyoxal-denatured total RNA (30 μg) in 1% agarose gel and transfer to nitrocellulose filters were performed (Heilman et al., 1982). Filters were hybridized with nick-translated 32P-labeled catalase and peroxisomal bifunctional enzyme (PBE) cDNA at 42°C for 18 hr (Nemali et al., 1988). The filters were washed in 2x SSC/0.1% SDS at room temperature for 2 hr, dried, and exposed to film at −70°C. The relative mass of specific RNAs was measured by densitometric scanning of the autoradiographs.

diet containing ciprofibrate (0.025%; Sterling–Winthrop Research Institute, Rensselaer, NY) and/or dexamethasone (Sigma) for 7 days as follows: group 1, control diet; group 2, ciprofibrate; group 3, ciprofibrate + dexamethasone (1 mg/kg diet); group 4, ciprofibrate + dexamethasone (0.5 mg/kg diet); group 5, dexamethasone (1 mg/kg diet); and group 6, dexamethasone (0.5 mg/kg diet). At the beginning of experiments rats weighed between 80 and 90 g. At the end of 7 days all animals were given bromodeoxyuridine (BrdU) intraperitoneally (100 mg/kg body weight) 1 hr prior to euthanization. In addition, 16 rats of the same age and weight were divided into four equal groups and fed a diet containing ciprofibrate, ciprofibrate + dexamethasone (0.5 mg/kg diet), dexamethasone (0.5 mg/kg diet), and control diet for 7 days. These animals were given daily i.p. injections of BrdU (100 mg/kg body weight); a total of seven doses, and euthanized 1 hr after the last injection. At the time of euthanization body weights and liver weights were obtained and portions of liver were used for light microscopy, electron microscopy, preparation of postnuclear fraction, and total RNA. Quantitative data were analyzed by Student’s t test.

Morphometric analysis of peroxisome.

Four rats in each group were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 4 hr and processed for transmission electron microscopy. Thin sections were cut on an LKB ultratome, stained with uranyl acetate and lead citrate, and examined in a JOEL JEM-100CS 11 electron microscope. Morphometric analysis of peroxisomes was carried out as described before (Rao et al., 1982), according to the procedure of Weibel et al. (1966). Briefly, micrographs were taken at ×5000 and magnified 2.5 times at printing. Points of intersection overlying cytoplasm and peroxisomes were counted using a 5-mm-spaced lattice grid. The volume density of peroxisomes was determined in relation to cytoplasmic volume. Ten photographs were counted from each animal.

Analysis of proliferative indices.

For BrdU immunohistochemistry, portions of liver from all animals were fixed in 70% alcohol overnight at 4°C and processed for light microscopy. Five-micrometer-thick paraffin sections were stained immunohistochemically by the avidin–biotin–peroxidase complex method using Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) with monoclonal antibodies specific for BrdU (Becton–Dickinson) at a dilution of 1 in 300 (Ward et al., 1988). The reaction product was visualized with the addition of diaminobenzidine tetrahydrochloride and the slides were counterstained with hematoxylin. The labeling index was obtained by counting 2000 hepatocytes in each liver.

To obtain a mitotic index 2000 hepatocytes were counted from each liver on hematoxylin and eosin stained sections.

SDS–polyacrylamide gel electrophoresis.

Ten percent (W/V) liver homogenates were prepared in ice-cold 0.25 M sucrose containing 10 mM EDTA (pH 7.5) using a Potter–Elvehjem homogenizer. Postnuclear fractions were obtained by centrifuging the homogenate at 700g for 10 min in a Beckman J-21 centrifuge at 4°C. The protein concentration in the homogenate was determined by the method of Lowry et al. (1951). Electrophoresis was performed on 10% SDS–polyacrylamide slab gels according to the method of Laemmli (1970), using 30 μg protein (Nemali et al., 1988). After completion of the electrophoretic run (20 mA/Slab), the proteins were fixed by immersing the gels in 10% trichloroacetic acid for 30 min and then stained with 0.1% Coomassie blue in 50% methanol/10% acetic acid.

Northern blot analysis.

Total RNA was extracted from livers using the method of guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi, 1987). Electrophoresis of glyoxal-denatured total RNA (30 μg) in 1% agarose gel and transfer to nitrocellulose filters were performed (Heilman et al., 1982). Filters were hybridized with nick-translated 32P-labeled catalase and peroxisomal bifunctional enzyme (PBE) cDNA at 42°C for 18 hr (Nemali et al., 1988). The filters were washed in 2x SSC/0.1% SDS at room temperature for 2 hr, dried, and exposed to film at −70°C. The relative mass of specific RNAs was measured by densitometric scanning of the autoradiographs.
TABLE 1
Effect of Dexamethasone on Ciprofibrate-Induced Liver Weight, Peroxisome Volume Density, and Hepatocyte Proliferation*

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight</th>
<th>Liver weight (g)/100 g body weight</th>
<th>Peroxisome volume density</th>
<th>BrdU, single dose</th>
<th>BrdU, 7 doses</th>
<th>Mitotic index/2000 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>137 ± 3*</td>
<td>0.7 ± 0.1</td>
<td>8.5 ± 1.5*</td>
<td>164 ± 15*</td>
<td>1.8 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Ciprofibrate</td>
<td>116 ± 2</td>
<td>0.7 ± 0.1</td>
<td>8.5 ± 1.5*</td>
<td>164 ± 15*</td>
<td>1.8 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Ciprofibrate + dexamethasone (1 mg/kg diet)</td>
<td>84 ± 1</td>
<td>9.6 ± 0.2</td>
<td>6.8 ± 1.1</td>
<td>4.7 ± 0.9</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone (1 mg/kg diet)</td>
<td>84 ± 1</td>
<td>9.6 ± 0.2</td>
<td>6.8 ± 1.1</td>
<td>4.7 ± 0.9</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Ciprofibrate + dexamethasone (0.5 mg/kg diet)</td>
<td>84 ± 2</td>
<td>9.4 ± 0.1</td>
<td>—</td>
<td>2.3 ± 0.7</td>
<td>183 ± 2</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone (0.5 mg/kg diet)</td>
<td>84 ± 1</td>
<td>7.9 ± 0.3</td>
<td>0.7 ± 0.1</td>
<td>3.5 ± 0.7</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

* Ciprofibrate (0.025%) diet with or without dexamethasone was fed for 7 days.
* Mean ± SEM of 8 animals.
* Mean ± SEM of 4 animals.

DISCUSSION

The present study, which was designed to examine the effect of the glucocorticoid dexamethasone on ciprofibrate-induced peroxisome proliferation and hepatocyte proliferation, clearly demonstrates that dexamethasone inhibits cell proliferation without affecting the peroxisome proliferation and PBE induction caused by ciprofibrate. Peroxisome volume density and the levels of catalase and PBE mRNAs were identical in the ciprofibrate group and the dexamethasone + ciprofibrate groups. These findings indicate that dexamethasone did not inhibit activation of the peroxisome proliferator-activated receptor isoforms implicated in the modulation of peroxisome proliferator-induced pleiotropic effects. Nonetheless, the effect of dexamethasone on ciprofibrate-induced

FIG. 1. Immunoperoxidase stain for BrdU in the liver of ciprofibrate-treated (a) and control (b) rat. Several labeled nuclei are seen in ciprofibrate treated rat. Original magnification, ×200.
cell proliferation was completely different. Dexamethasone at both dose levels (0.5 mg and 1 mg/kg diet), as revealed by immunohistochemistry after administration of a single dose and multiple doses of BrdU, completely abrogated the cell proliferation induced by ciprofibrate. In addition, dexamethasone also suppressed cell proliferation in control rats.

Liver cell proliferation is dependent on a multitude of regeneration signals derived from hepatic and extrahepatic sources. These signals include hormones, growth factors, and cytokines (Bucher, 1995; Fausto et al., 1995). It appears that cytokines and growth factors play a major role during compensatory hyperplasia, whereas cytokine TNFα mostly

FIG. 2. Electron micrograph of hepatocyte (a) from a rat treated with ciprofibrate and dexamethasone (1 mg/kg diet) showing a marked increase in the number of peroxisomes (arrows) and (b) from dexamethasone-alone treated rat showing an occasional peroxisome (arrow). ×14,000.
plays a crucial role during mitogen-induced direct hyperplasia (Fausto and Webber, 1994; Coni et al., 1993; Shinozuka et al., 1994). TNFα that is produced by Kupffer cells in the liver, through a paracrine effect, stimulates transcription factor (NF-κB), leading to priming of hepatocytes (Censue et al., 1991; Fausto and Webber, 1994). In addition, it has also been shown that TNFα induces DNA synthesis in cultured hepatocytes (Beyer and Theologides, 1993). Dexamethasone, a known inhibitor of TNFα, was shown to inhibit mitogen-induced liver cell proliferation (Leddà-Columbano et al., 1994). Remick et al. (1989) have shown that dexamethasone markedly reduced TNF induction caused by lipopolysaccharide in mice. It is possible that inhibition of ciprofibrate-induced cell proliferation by dexamethasone is also through inhibition of TNFα. Earlier studies have shown that peroxisome proliferators cause proliferation of both hepatocytes and Kupffer cells (Ward et al., 1988; Rao, personal observation). In this study we have noticed a marked decrease in the number of labeled Kupffer cells in the dexamethasone and ciprofibrate group when compared to that of the ciprofibrate treated group (data not shown).

We have presented an experimental model where peroxisome proliferator-induced cell proliferation was selectively inhibited without affecting peroxisome proliferation and induction of peroxisomal enzymes. This model should serve as an ideal system for investigating the role of oxidative stress in peroxisome proliferator-induced hepatocarcinogenesis.

ACKNOWLEDGMENT

This research was supported by a Veterans Affairs Merit Review Grant.

REFERENCES


