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Effects of the Peroxisome Proliferator Clofibric Acid on Superoxide Dismutase Expression in the Human HepG2 Hepatoma Cell Line

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ABSTRACT. We examined the effects of clofibric acid, a peroxisome proliferator, on the production of superoxide radicals, on the levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), and on the expression of superoxide dismutases (SODs) in the human HepG2 hepatoma cell line. To this end, HepG2 cells were treated for 1 or 5 days with 0.25, 0.50, or 0.75 mM clofibric acid. The production of superoxide radicals was only enhanced in HepG2 cells exposed for 5 days to the different clofibric acid concentrations. However, this overproduction of superoxide radicals was not accompanied by increased rates of lipid peroxidation, as the MDA and 4-HNE levels did not change significantly. Manganese (Mn) SOD activity was increased when HepG2 cells were treated for 1 day with 0.50 or 0.75 mM clofibric acid. For this duration of treatment, no change was observed in total SOD and copper/zinc (Cu/Zn) SOD activities. For a 5-day treatment, total SOD and MnSOD activities as well as the enzyme apoprotein and MnSOD mRNA levels increased whatever the clofibric acid concentration used. This transcriptional induction of the MnSOD gene was correlated with an activation of the activator protein-1 transcription factor for 1 and 5 days of treatment, but was independent of nuclear factor-kappa B and of peroxisome proliferator-activated receptor. On the other hand, the PP exerted very little effect if any on Cu,ZnSOD expression. In contrast to rodent data, PP treatment of human hepatoma cells induces MnSOD expression. BIOCHEM PHARMACOL **58**;6:1025–1033, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. superoxide dismutase; peroxisome proliferators; clofibric acid; human hepatoma cells; oxidative stress; transcription factors

Clofibric acid is a well-known PP† that increases the number of hepatic peroxisomes and the activities of peroxisomal enzymes involved in the production of superoxide radicals [1] and hydrogen peroxide [2, 3], in several rodent species. The control of gene expression by PPs is regulated via PPARs, which interact with specific response elements (PPREs) located upstream of responsive genes [4]. Chronic administration of PP in rats and mice results in the development of hepatocellular carcinomas [5–9]. PP-induced carcinogenicity has been postulated to be the consequence of an overproduction of $O_2^{\cdot}-$ and H_2O_2, leading to an oxidative stress [10]. This hypothesis has been recently supported by the fact that treatment with PP of monkey kidney CV1 cells transfected with a cDNA encoding fatty acyl-CoA oxidase (a peroxisomal H_2O_2-producing enzyme) induces their neoplastic transformation [11]. It has been reported that in rat hepatocytes PP-induced oxidative stress causes the formation of 8-hydroxydeoxyguanosine and the accumulation of lipofuscin, and end-product of the lipid peroxidation process [12–17]. However, the neoplastic transformation of PP-treated cells has also been attributed in several studies to a decrease in the activities of antioxidant defense enzymes which scavenge intracellular reactive oxygen species [18, 19]. In contrast with rodent data, the effect of PPs on human hepatocytes remains a matter of controversy [20]. Despite the lack of peroxisome proliferation, peroxisomal fatty acyl-CoA oxidase activity has been shown to be stimulated by fibrates in human hepatoma cells, whereas catalase activity remains unchanged [21, 22]. Whether or not the induction of peroxisomal and non-peroxisomal enzymes, which generate $O_2^{\cdot}-$ radicals and H_2O_2, leads to oxidative stress in human liver cells has not been established, as the expression of antioxidant enzymes (except catalase) has not yet been studied.

SODs (E.C.1.15.1.1) are metalloenzymes which catalyse the dismutation of superoxide anions ($O_2^{\cdot}-$) to H_2O_2 and O_2 [23, 24]. SODs are important as initial components in

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† *Abbreviations:* AP-1, activator protein-1; CA, clofibric acid; CoA, coenzyme A; Cu,ZnSOD, copper–zinc-dependent superoxide dismutase; H_2O_2, hydrogen peroxide; 4-HNE, 4-hydroxynonenal; MnSOD, manganese-dependent superoxide dismutase; MDA, malondialdehyde; NF-κB, nuclear factor-kappa B; PP, peroxisome proliferator; PPAR, peroxisome proliferator-activated receptor; and PPRE, peroxisome proliferator-response element.

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cellular defence against $O_2^{\cdot}-$ [24]. In mammals, three forms of SOD encoded by separate genes and differing in their amino acid sequences and localization have been identified: mitochondrial MnSOD, cytosolic and peroxisomal Cu,Zn-SOD, and extracellular Cu,ZnSOD [24–26]. Surprisingly, no information is available concerning the expression of superoxide dismutases in PP-treated human cells. The transcriptional induction of the human MnSOD gene has been found to be associated with an increased DNA binding of the AP-1 or NF-κB [27–29]. The AP-1 transcription factor consists of homo- or heterodimers of proteins belonging to the c-Fos and c-Jun families [30]. NF-κB is a heterodimer bound in its inactive cytoplasmic form to the inhibitory subunit IκB that can rapidly activate the expression of genes involved in inflammatory and acute phase responses [31].

In the present study, we first examined the effects of clofibric acid, a peroxisome proliferator, on the production of superoxide radicals and on the occurrence of lipid peroxidation, before analysing the expression of superoxide dismutase enzymes in human HepG2 hepatoma cells. The production of superoxide radicals was increased only when the cells were treated for 5 days, whatever the concentration of clofibric acid used. In contrast to rodent data, no lipid peroxidation was observed. We found that MnSOD, but not Cu,ZnSOD, was induced at the transcriptional and translational levels, independently of PPAR. Clofibric acid also induced an increase in the DNA-binding activity of AP-1 (but not NF-κB) after a treatment for at least 1 day. These results show that in contrast to rodent data, PP treatment of human hepatoma cells induces MnSOD expression.

MATERIALS AND METHODS

Reagents and Chemicals

Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Eurobio. DMSO was purchased from Merck. Clofibric acid, poly(dI-dC), tumor necrosis factor alpha, reagents used for superoxide radical and SOD assays, and the rabbit anti-sheep immunoglobulin G polyclonal antibody conjugated with peroxidase were provided by Sigma Chemical Co. Sheep polyclonal antibodies against human MnSOD and Cu,ZnSOD were from Janssen Pharmaceutica and Biodesign, respectively. The chemiluminescent substrate used in ECL Western analysis and DNA size markers were purchased from Boehringer Mannheim. Molecular mass markers for proteins and the Sephadex G50 for column chromatography were obtained from Pharmacia. Polyvinylidene difluoride membranes for Western blot analysis and nylon membranes for Northern blot analysis were purchased from NEN and Amersham, respectively. Primers for reverse transcriptase-polymerase chain reaction were obtained from Eurogentec and probes containing AP-1 or NF-κB motifs and used in gel retardation assay were purchased from GIBCO BRL. Guanidium isothiocyanate and water-saturated phenol for RNA extractions were purchased from Appligene. The Gene Amp kit was from Cetus, the protein assay and Prep A gene DNA purification matrix kits from BioRad. [α-^{32}P]dCTP and the β-actin cDNA were provided by Isotopchim and Clontech, respectively. Trimax XM films were from Imagerie Scientifique Européenne.

Cell Lines and Culture Conditions

Experiments were carried out using human HepG2 hepatoma cells [32] which were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, at 37°, in a water-saturated atmosphere with 5% CO_2/95% air. The culture medium was renewed each day. Cells were detached weekly for transfer with 0.1% trypsin and 10 μM EDTA in PBS.

Clofibric Acid Treatments

Human hepatoma cells were seeded at 2.10^4 cells/cm^2. Two days later, the medium was replaced by one containing clofibric acid at a final concentration of 0.25, 0.50, or 0.75 mM in 0.1% (v/v) DMSO. The latter did not induce any modifications in cell growth or SOD expression. Cells were exposed to the fibrate drug for 1 or 5 days. Shorter treatments (1, 3, 6, and 12 hr) were applied in some experiments. As a negative control, cells were cultured in a medium supplemented with only 0.1% DMSO. For each culture, the viability of clofibric acid-treated cells was examined using the trypan blue exclusion test.

Measurement of Superoxide Radical Production

The production of superoxide radicals by HepG2 cells treated with different concentrations of clofibric acid for 1 and 5 days was spectrophotometrically assayed by following the reduction of acetyl-cytochrome *c* at 550 nm [33]. The cells were rinsed twice with PBS, harvested, and then disrupted using a Dounce homogeniser. After centrifugation at 600 *g* for 5 min at 4°, the supernatant containing whole cellular particles was used for superoxide radical assays. The latter were carried out in 1 mL of PBS containing 0.4 mM NADPH, 3 mM succinate, 60 μM acetyl-cytochrome *c*, and the homogenate. The superoxide radical production was inhibited by the addition of bovine erythrocyte SOD (500 units) to the reaction mixture. The rate of superoxide radical formation was expressed as nanomoles of acetyl-cytochrome *c* reduced per minute and per mg of protein.

Measurement of Lipid Peroxidation

MDA and 4-HNE levels were determined in cells treated with different concentrations of clofibric acid for 1 or 5 days. At the end of treatments, the cells were rinsed twice with PBS, scraped, collected in glass test tubes containing H_2O ($2 \cdot 10^6$ cells/500 μL H_2O), and placed in ice. Assays were performed using LPO-586, a colourimetric substrate manufactured by Bioxytech. The reagents were mixed with 200 μL of cell sample and the reaction mixture was incubated at 45° for 40 min, then centrifuged at 15,000 *g*

for 10 min. The supernatant was monitored at 586 nm. The concentrations of MDA and 4-HNE were estimated from standard curves. Results were expressed as nanomoles per mg of protein.

Superoxide Dismutase Analysis

The human hepatoma cells treated or not with clofibric acid were harvested and disrupted in 10 mM Tris–HCl, pH 7.4, containing 0.1% (w/v) Triton X100 by three cycles of freezing/thawing. After centrifugation at 17,000 *g* for 20 min at 4°, the supernatants were used for protein determination, SOD assays, and Western blot analyses. The SOD activities in HepG2 cells were assayed as previously described [34], using the autoxidation of pyrogallol by superoxide radicals. Human erythrocyte superoxide dismutase was used as a standard. One unit of SOD is defined as the amount of enzyme that inhibits the rate of autoxidation of pyrogallol by 50%. For the determination of MnSOD activity, Cu,ZnSOD activity was inhibited by preincubation of the hepatoma cell homogenates for 5 min with 2 mM potassium cyanide (KCN). The residual activity corresponded to that of MnSOD. Protein was measured according to the method of Lowry *et al.* [35], using BSA as a standard. The apoprotein levels of MnSOD and Cu,ZnSOD were quantified using the ECL-Western blot procedure. After electrophoresis on SDS-PAGE [36], proteins were transferred to polyvinylidene difluoride membrane as described [37]. MnSOD and Cu,ZnSOD proteins were detected using specific polyclonal antibodies diluted 1:5,000 and 1:80,000, respectively, and visualized by using an immunoglobulin G polyclonal antibody conjugated to peroxidase exposed to a chemiluminescent substrate. The intensities of the bands were quantified by densitometry with a computerised image processing system (Biocom 200, France). Results were expressed as percentages of control values.

Production and Characterisation of MnSOD and Cu,ZnSOD cDNA Probes

Probes were produced by reverse transcription coupled to polymerase chain reaction (RT-PCR) according to Jones [38]. The primers used were selected from published nucleotide sequences in the open reading frames encoding MnSOD and Cu,ZnSOD [39, 40]. Sense primers for MnSOD and Cu,ZnSOD were 5′-CTTCAGCCTGCACTGAAGTTCAAT-3′ (nt 316–339) and 5′-GCGACGAAGGCCGTGTGCGTGC-3′ (nt 84–105), respectively. Antisense primers for MnSOD and Cu,ZnSOD were 5′-CTGAAGGTAGTAAGCGTGCTCCC-3′ (nt 620–642) and 5′-ACTTTCTTCATTTCCACCTTTGCC-3′ (nt 1449–1469), respectively. The following PCR program was used: 94° for 1 min, 56° for 1 min, and 72° for 1 min. A total of 35 cycles was performed using the Gene Amp kit and the automated thermal cycler (Perkin Elmer). DNA fragments for MnSOD (327 bp) and Cu,ZnSOD (451 bp) were purified with the Prep A gene DNA purification matrix kit and sequenced according to the dideoxy chain termination method [41]. The probes were labelled with [α-^{32}P] dCTP by random priming and further purified on Sephadex G50 column chromatography.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted by the guanidium isothiocyanate–phenol–chloroform procedure [42] and used for Northern blot analysis. Gel electrophoresis was performed with glyoxal–DMSO-treated RNA and samples were blotted to nylon membranes. The membranes were hybridised with the probes and autoradiographed at −80° using Trimax XM films. Membranes were reprobed with [α-^{32}P] dCTP-labelled β-actin cDNA used as an internal control for load and integrity of the RNA. Autoradiograms were analysed by densitometry using the signal from β-actin as an internal control. Northern blot data correspond to the ratio of the SOD signal to that of β-actin and results are expressed as percentages of control values.

Gel Retardation Assay

HepG2 cells were incubated with 0.50 mM clofibric acid for 1, 3, 6, 12 or 24 hr, or 5 days. Nuclear extracts were then prepared as described previously [43], with the following modifications. The nuclei were resuspended in 20 mM HEPES, pH 7.9, containing 450 mM NaCl, 0.2 mM EDTA, 0.15 mM $MgCl_2$, 25% (v/v) glycerol, 0.5 mM dithiothreitol and 0.5 mM PMSF, and then homogenised with Dounce and incubated for 30 min at 4° after addition of 0.25% (v/v) Nonidet P-40. Nuclear extracts were cleared by centrifugation for 20 min at 4° at 15,000 *g*, and the resulting supernatant was immediately frozen in liquid nitrogen and stored at −70° until used for gel retardation assays. Distinct oligonucleotides containing complementary sequences at their 3′ termini and binding motifs for AP-1, NF-κB, and PPAR, respectively, were annealed and the protruding single-stranded regions filled in with [α-^{32}P] dCTP using Klenow fragment of DNA polymerase I. The sequences of the oligonucleotides used were:

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AP-1     5′-CGCTTGATGAGTCA-3′
                3′-TACTCAGTCGGCCTT-5′
NF-κB         5′-AGTTGAGGGGACTTTCC-3′
                      3′-CCCTGAAAGGGTCCG-5′
PPAR          5′-GCAGCCTCCTTTCTCCCGT-3′
                     3′-AGGAAAGAGGGCACGGGTAAC-5′
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(a putative PPRE found in the 5′ non-coding region of the MnSOD gene)

The binding reaction mixture (10 μL) for gel retardation assays contained 1 ng of DNA probe, 2 μg poly(dI-dC), and 10 μg of the different nuclear extracts in 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 0.5 mM dithiothreitol, and 1 mM $MgCl_2$. The mixture was incubated for 30 min at 0°. The DNA–protein complexes were resolved in native 5% polyacrylamide gels in 0.5 X

TABLE 1. Effects of clofibric acid on the rate of superoxide radical formation in the HepG2 cell line

Treatment	Superoxide radical formation (nmol/min/mg protein)	
	1 day	5 days
Control	2.5 ± 0.1	2.7 ± 0.1
0.25 mM CA	2.6 ± 0.1	3.1 ± 0.2*
0.50 mM CA	2.5 ± 0.1	3.2 ± 0.1*
0.75 mM CA	2.6 ± 0.1	3.3 ± 0.3*

The cells were treated for 1 or 5 days with different concentrations of clofibric acid (CA). The rate of formation of intracellular superoxide radicals was spectrophotometrically assessed by following the reduction of acetyl-cytochrome *c* at 550 nm. Results are means ± standard deviations from 3 independent experiments, each performed in triplicate. They are expressed as nanomoles of reduced acetyl-cytochrome *c* per min and per mg protein.

Statistically significant difference from the control was indicated as $*P < 0.05$.

Tris-borate EDTA at 100 V for 45 min. The dried gels were exposed to Trimax XM film overnight at −80°. In order to test the specificity of the binding, competition studies were performed in the presence of a 100-fold molar excess of the specific unlabelled probe prior to the addition of 1 ng of the radiolabelled probe.

Statistical Analysis

Data concerning superoxide radical formation, lipid peroxidation, and SOD activity were expressed as means ± standard deviations of three independent experiments, each performed in triplicate. Densitometric analyses of Northern and Western blots were carried out from three independent experiments. Evaluation of statistical significances was assessed using analyses of variance (ANOVA) and the Fisher protected least significant difference test (multiple comparisons) [44]. Statistical significance was indicated as $*P < 0.05$.

TABLE 2. Effects of clofibric acid on lipid peroxidation in the HepG2 cell line

Treatment	MDA + 4-HNE (nmol/mg protein)	
	1 day	5 days
Control	1.5 ± 0.3	1.4 ± 0.3
0.25 mM CA	1.5 ± 0.1	1.4 ± 0.3
0.50 mM CA	1.8 ± 0.5	1.5 ± 0.3
0.75 mM CA	1.8 ± 0.2	1.4 ± 0.3
0.5 mM H_2O_2	3.8 ± 0.3*	

HepG2 cells were incubated for 1 or 5 days with different concentrations of clofibric acid (CA). Susceptibility to lipid peroxidation was spectrophotometrically detected at 586 nm by malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) formation. As a positive control, the human hepatoma cells were exposed to 0.5 mM H_2O_2 for 5 hr. Results are means ± standard deviations from 3 independent experiments, each performed in triplicate. Data are expressed as nanomoles of MDA and 4-HNE per mg of protein.

Statistically significant difference from the control was indicated as $*P < 0.05$.

TABLE 3. Effects of clofibric acid on SOD activities in the HepG2 cell line

Treatment	Total SOD activity (U/mg protein)		Mitochondrial SOD activity (U/mg protein)	
	1 day	5 days	1 day	5 days
Control	17.0 ± 1.2	18.3 ± 1.1	3.3 ± 0.6	2.8 ± 0.7
0.25 mM CA	18.6 ± 2.0	22.8 ± 2.4*	3.8 ± 0.7	5.3 ± 1.1*
0.50 mM CA	18.8 ± 1.5	22.3 ± 2.5*	5.3 ± 0.5*	5.5 ± 1.0*
0.75 mM CA	18.2 ± 1.6	22.3 ± 2.2*	6.2 ± 1.8*	4.8 ± 1.0*

HepG2 cells were incubated for 1 or 5 days with different concentrations of clofibric acid (CA). The results concerning total SOD and MnSOD activities are the means ± standard deviations from 3 independent experiments, each performed in triplicate.

Statistically significant difference from the control was indicated as $*P < 0.05$.

RESULTS

Effects of Clofibric Acid on Superoxide Radical Production and Lipid Peroxidation

The production of superoxide radicals and the occurrence of lipid peroxidation were first studied to estimate the oxidant effects of clofibric acid in human HepG2 hepatoma cells. The superoxide radicals were quantified spectrophotometrically using their capacity to reduce acetyl-cytochrome *c*. Whatever the concentration of clofibric acid used, the formation of superoxide radicals did not change significantly when HepG2 cells were treated for 1 day (Table 1). A slight but significant increase was observed for the rate of superoxide radical production when the cells were exposed for 5 days to clofibric acid. This increase was not drug-dose-dependent (Table 1). Susceptibility to lipid peroxidation was detected by MDA and 4-HNE formation. The production of MDA and 4-HNE did not change significantly in HepG2 cells exposed to clofibric acid whatever the concentration of the fibrate drug used and the duration of treatment (Table 2). On the other hand, when the human hepatoma cells were incubated with 0.5 mM H_2O_2 for 5 hr, lipid peroxidation occurred as attested by the increased formation of MDA and 4-HNE.

Effects of Clofibric Acid on SOD Activities

The results concerning the time- and dose-dependent effects of clofibric acid in HepG2 cells are shown in Table 3. When the human hepatoma cells were treated for 1 day with clofibric acid, the total SOD activity did not change significantly whatever the concentration used. However, when the MnSOD activity was assayed after addition of KCN to the reaction mixture, increases of 1.6- and 1.9-fold were found for cells treated with 0.50 and 0.75 mM clofibric acid, respectively. For a 5-day treatment, the total SOD and MnSOD activities were significantly increased for all clofibric acid concentrations used. A 2-fold maximal increase was obtained for the mitochondrial SOD activity (Table 3). Similar results were obtained for a 3-day treatment (data not shown).

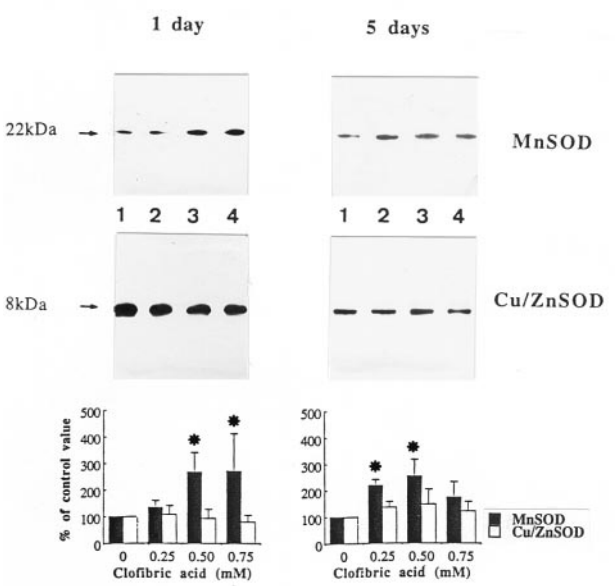

FIG. 1. Effects of clofibric acid on MnSOD and Cu,ZnSOD apoprotein levels. HepG2 cells were treated for 1 or 5 days with different concentrations of clofibric acid (CA). Total protein was subjected to Western blotting using polyclonal anti-human MnSOD or anti-human Cu,ZnSOD antibodies. Each lane was loaded with 25 μg of protein. The blots for MnSOD and Cu,ZnSOD apoproteins result from the same experiments. Lane 1, control cells; lane 2, 0.25 mM CA; lane 3, 0.50 mM CA; lane 4, 0.75 mM CA. Arrows, position of 22 kDa and 18 kDa HepG2 cell MnSOD and Cu,ZnSOD, respectively. The protein band intensities for MnSOD and Cu,ZnSOD were quantified by densitometry from the ECL immunoblots and represented by histograms. Values were expressed as percentages of control values. Results are means ± standard deviations from 3 independent experiments. Statistically significant difference from the control was indicated as *$P < 0.05$.

Effects of Clofibric Acid on SOD Apoprotein Levels

Proteins extracted from HepG2 cells treated or not with clofibric acid were submitted to Western blotting using specific antibodies for MnSOD and Cu,ZnSOD (Fig. 1). For the two enzymes, a unique band was detected with M_r of 22,000 for MnSOD and 18,000 for Cu,ZnSOD. The protein band intensities were quantified by scanning densitometry (Fig. 1). When the HepG2 cells were treated for 1 day with clofibric acid at a 0.25 mM concentration, a 1.3-fold increase in the MnSOD apoprotein level was observed. The latter was increased by 2.7-fold in treatments with 0.50 or 0.75 mM clofibric acid. The induction of the MnSOD apoprotein level was time-dependent only for a 0.25 mM concentration of clofibric acid. For a 5-day treatment with 0.25 mM clofibric acid, the protein level of the enzyme was significantly increased (2.2-fold). With 0.50 mM clofibric acid, the SOD apoprotein level was not different from that found when the human hepatoma cells were treated for 1 day. The longest treatment with 0.75 mM clofibric acid gave a lower increase in enzyme content than that observed for the shortest one. The Cu,ZnSOD protein content did not change significantly when the HepG2 cells were exposed for 1 and 5 days to the different concentrations of clofibric acid (Fig. 1).

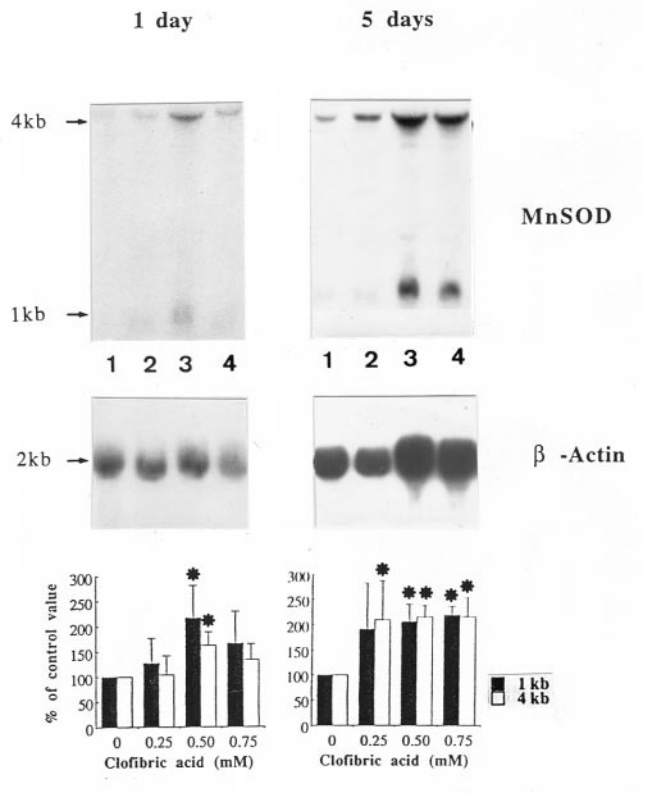

FIG. 2. Northern blot analysis of MnSOD mRNA expression. Total RNA was extracted from control and HepG2 cells exposed to clofibric acid (CA) for 1 or 5 days. RNA (15 μg) was loaded, separated, transferred to a nylon membrane, and hybridised with ^{32}P-labelled DNA probes for human MnSOD and β-actin. The autoradiograph shown is representative of the results obtained from 3 Northern blot analyses. RNA sizes are given on the left. Exposures were for 2 days (MnSOD) and 12 hr (β-actin). Lane 1, control cells; lane 2, 0.25 mM CA; lane 3, 0.50 mM CA; lane 4, 0.75 mM CA. The intensities of the hybridising bands obtained by Northern blotting were densitometrically quantified and compared to those of the β-actin signal. Data represented in the histogram expressed as percentages of control values are means ± standard deviations from 3 independent experiments. Statistically significant difference from the control was indicated as *$P < 0.05$.

Effects of Clofibric Acid on SOD mRNA Levels

As a first step in the expression analyses of MnSOD and Cu,ZnSOD mRNAs, the specificity of the DNA probes were verified by sequencing. They were found suitable for Northern blot analyses on human hepatoma cell RNA, since their sequences were identical to those of the previously published sequences [39, 40]. The MnSOD and Cu,ZnSOD mRNA levels were investigated during clofibric acid treatment of HepG2 cells using Northern blotting. Densitometric analyses were performed from three independent experiments, and data were internally controlled relative to the β-actin mRNA signal and then plotted as percentages of untreated cell SOD mRNAs (Fig. 2). As

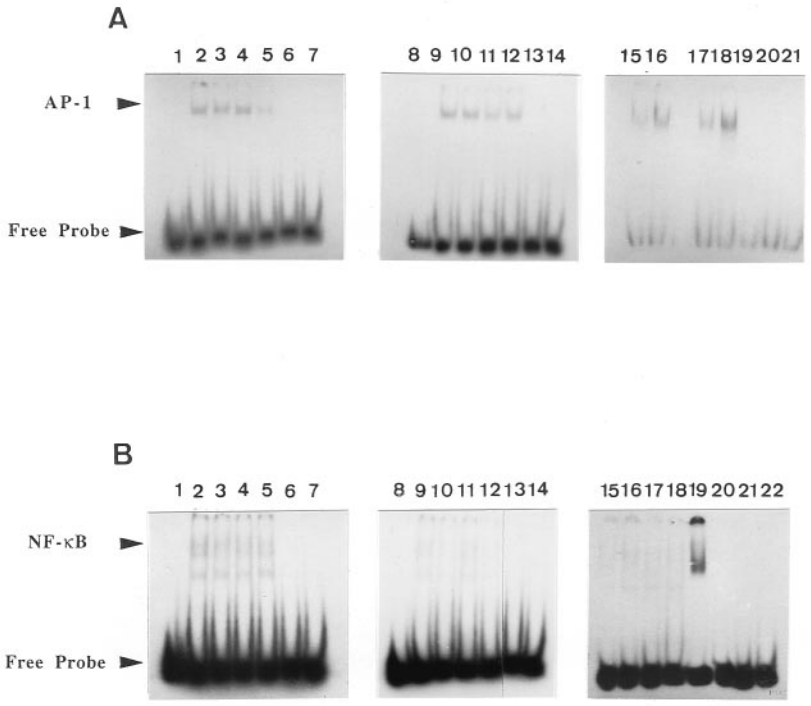

FIG. 3. DNA-binding activity of AP-1 and NF-κB transcription factors during clofibric acid (CA) treatment of HepG2 cells. Nuclear extracts were prepared from cells treated with 0.50 mM CA for 1, 3, 6, 12, or 24 hr, or 5 days. Binding reactions were performed with 10 μg proteins of nuclear extracts and 1 ng of radiolabelled fragment containing the AP-1- or NF-κB-binding site. The DNA protein complexes for AP-1 (A) and NF-κB (B) were resolved in native polyacrylamide gels. Lane 1, probe alone; lane 2, control cells 1 hr; lane 3, CA-treated cells for 1 hr; lane 4, control cells 3 hr; lane 5, CA-treated cells for 3 hr; lanes 6 and 7, CA-treated cells for 1 and 3 hr, respectively with a 100-fold molar excess of non-radioactive AP-1- or NF-κB-binding site; lane 8, probe alone; lane 9, control cells 6 hr; lane 10, CA-treated cells for 6 hr; lane 11, control cells 12 hr; lane 12, CA-treated cells for 12 hr; lanes 13 and 14, CA-treated cells for 1 and 3 hr, respectively with a 100-fold molar excess of non-radioactive AP-1- or NF-κB-binding site; lane 15, control cells 24 hr; lane 16, CA-treated cells for 24 hr; lane 17, control cells 5 days; lane 18, CA-treated for 5 days. (A) lanes 19 and 20, CA-treated cells for 24 hr and 5 days, respectively with a 100-fold molar excess of non-radioactive AP-1-binding site; lane 21, probe alone. (B) lane 19, positive control corresponding to cells treated with 100 U/mL with TNFα for 6 hr; lanes 20 and 21, TNFα-treated cells for 6 hr and CA-treated cells for 5 days, respectively with a 100-fold molar excess of non-radioactive NF-κB-binding site; lane 22, probe alone. In the case of A, lanes 15 to 21, the free probe is not visualised on the figure.

shown in Fig. 2, two major transcripts were found for MnSOD mRNAs with sizes of 1.0 and 4.0 kb, respectively. There was no significant change in the MnSOD mRNA levels when the human hepatoma cells were cultured for 1 day in the presence of 0.25 and 0.75 mM clofibric acid. On the other hand, for this duration of treatment, the 1 kb and 4 kb mRNA levels were increased 2.2- and 1.6-fold, respectively, when HepG2 cells were cultured with 0.50 mM clofibric acid. When cells were exposed for 5 days, the fibrate drug at a concentration of 0.25 mM only significantly induced the level of the 4 kb MnSOD mRNA 2.1-fold. The contents of the two transcripts were significantly increased 2.2-fold when HepG2 cells were treated with 0.50 or 0.75 mM clofibric acid for 5 days (Fig. 2). Densitometric analyses showed that treatment of the human hepatoma cells with clofibric acid did not significantly induce the level of the Cu,ZnSOD mRNAs (data not shown).

Effect of Clofibric Acid on the DNA-Binding Activity of PPAR, AP-1, and NF-κB

We also investigated the activation of AP-1, NF-κB, and PPAR, transcription factors which could be involved in the induction of the MnSOD gene. *In vitro* DNA-binding assays were carried out with nuclear extracts from cells treated with 0.50 mM clofibric acid for 1, 3, 6, or 12 hr, or 1 or 5 days. Figure 3 shows that the DNA-binding activity of AP-1 was increased after a 1-day treatment with clofibric acid. The increase was more obvious after 5 days of treatment. Whatever the duration of treatment, the PP did not induce the DNA-binding activity of NF-κB (Fig. 3). Using a polyclonal antibody which recognises both the different PPAR subtypes [45] and a putative PPRE sequence found in the regulatory region (from −221 bp to −194 bp) of the human MnSOD gene and used as a probe, no binding activity was observed for PPARs (data not shown).

DISCUSSION

In the present study, we have shown an increased level of superoxide radicals in human cells treated with clofibric acid. In rodent species, PPs induce the microsomal cytochrome P450 system, leading to a high rate of superoxide radical formation [46]. Xanthine oxidase and cytochrome

P450 reductase, two peroxisomal enzymes, generate more superoxide radicals under the action of PPs [1, 46, 47]. The activities of rat mitochondrial enzymes cause an oxidative stress in hepatocytes following clofibrate treatment [48]. These effects are considered to be specific to rodent species and therefore of no consequence to humans. However, activities of peroxisomal H_2O_2-producing enzymes have been found to be increased in human hepatoma cells exposed to PPs [21, 22]. Until now, no data were available on the effects of PPs on the activity of superoxide radical-producing enzymes in human hepatocytes. Thus, the present study demonstrates for the first time that the production of reactive oxygen species is enhanced in human HepG2 cells exposed to clofibric acid for 5 days.

We have demonstrated both a sustained induction of MnSOD activity and significant increases in enzyme apoprotein and MnSOD mRNA levels after treatment of HepG2 cells with clofibric acid. The catalytic activity, amounts of apoprotein, and abundance of mRNA for MnSOD appear to be regulated in a congruent way. In good agreement with a previous work [49], two MnSOD transcripts were detected in Northern blot analyses. This result is at variance with Ciriolo *et al.* [18], who found that total superoxide dismutase activity is decreased in rat liver during long-term treatment with clofibrate. No information concerning MnSOD activity, protein and mRNA levels are provided in their work. The difference between MnSOD rodent and human data may be due to a species- or time-dependent response as is the case for peroxisomal oxidases [21, 22]. In addition, we found that Cu,ZnSOD, which has been shown to be a peroxisomal enzyme [26], was not induced by clofibric acid in HepG2 cells. The activity, apoprotein level, and mRNA content of Cu,ZnSOD did not change markedly when the human hepatoma cells were exposed to the PP for 1 or 5 days.

Concerning MnSOD gene induction, we studied the DNA-binding activities of three transcription factors, AP-1, NF-κB, and PPAR. Previous reports have revealed a correlation between the activation of the first two of these transcription factors and the induction of the MnSOD gene [27, 28]. The AP-1 transcription factor could be involved in the regulation of the human MnSOD gene via the presence of an AP-1 consensus binding site in the 5′ flanking region of this gene [50]. On the other hand, PPs control the transcriptional activity of their target genes via PPARs [4]. Thus, the DNA-binding activities of these transcription factors were examined according to the duration of clofibric acid treatment using HepG2 cell nuclear extracts. No activation of AP-1 was observed before 1 day of treatment. Thereafter, a marked increase in DNA-binding activity was found for AP-1. On the other hand, the induction of MnSOD gene transcription occurred independently of NF-κB. This result is at variance with a recent work reporting an increased activation of NF-κB in rats treated with ciprofibrate [51]. This discrepancy may be ascribed to differences between rodents and humans in the regulation of MnSOD gene expression by NF-κB. In contrast to rodent data, our results are in good agreement with a previous work demonstrating that induction of MnSOD gene transcription is not mediated through NF-κB activation in human cervical Hela and colonic HT-29 carcinoma cells [29]. Using the only putative PPRE found in the regulatory sequence of the MnSOD gene, we investigated the DNA-binding activity of PPAR with nuclear extracts from control and PP-treated cells. No complex was observed and supershifted with polyclonal antibodies directed against the different PPAR subtypes (data not shown).

The activity of the H_2O_2-producing enzyme fatty acyl-CoA oxidase is increased in human hepatoma cells treated with clofibrate [22] or ciprofibrate [21]. This finding, taken together with the present data concerning MnSOD induction by clofibric acid, raises the question as to whether enhanced hydrogen peroxide production may cause oxidative damage in human hepatoma cells treated with PPs. Increased membrane lipid peroxidation generally results from increased cellular levels of reactive oxygen species such as hydrogen peroxide and superoxide or hydroxyl radical. Levels of MDA and 4-HNE were used as a measure of lipid peroxidation and oxidative stress occurring during incubation of HepG2 cells with clofibric acid. We found no evidence of lipid peroxidation in HepG2 cells cultured with clofibric acid for 1 or 5 days, as the intracellular contents in MDA and 4-HNE did not change. Our results are consistent with the observations of Arnaiz *et al.* [52], showing that increased H_2O_2 steady-state concentrations are not correlated with increased rates of lipid peroxidation in livers of mice fed with fenofibrate for 1–3 weeks. However, *in vivo* [8, 12, 14] and *in vitro* [15] studies provide evidence for lipid peroxidation in rodent hepatocytes following PP treatment. The duration of treatment, potency of the inducer, and magnitude of the peroxisome proliferative response and peroxisomal oxidase induction appear to determine the levels of lipid peroxidation.

In summary, the present study extends previous investigations [21, 22] on the effects of PPs on human hepatoma cells. Our results indicate that clofibric acid treatment induces MnSOD and increases the production of superoxide radicals, but does not produce lipid peroxidation in human HepG2 cells. An increased DNA-binding activity was observed for AP-1 during clofibric acid treatment, whereas no change was observed in that of NF-κB. Furthermore, PPARs do not seem to be involved in the regulation of MnSOD gene expression. Taken together, these results illustrate the pleiotropic responses induced by PPs in human hepatoma cells.

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