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Activation of Nuclear Factor-κB by the Peroxisome Proliferator Ciprofibrate in H4IIEC3 Rat Hepatoma Cells and Its Inhibition by the Antioxidants *N*-Acetylcysteine and Vitamin E

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ABSTRACT. Peroxisome proliferators are a class of hepatic carcinogens in rodents and are proposed to act in part by increasing reactive oxygen species such as hydrogen peroxide. We previously showed that treatment of rats with ciprofibrate, a peroxisome proliferator, results in increased hepatic nuclear factor-κB (NF-κB) DNA binding activity. In this study, we have examined the link between peroxisome proliferators and NF-κB activation in hepatoma cell lines to test whether increased nuclear NF-κB levels activate NF-κB-regulated genes and to determine the mechanism of NF-κB activation. Electrophoretic mobility shift assays demonstrated NF-κB induction by ciprofibrate in peroxisome proliferator-responsive H4IIEC3 rat hepatoma cells but not in peroxisome proliferator-insensitive HepG2 human hepatoma cell lines. In addition, we found that stably transfected NF-κB-regulated reporter genes were activated by ciprofibrate in H4IIEC3 cells. This reporter gene activation was blocked by the antioxidants *N*-acetylcysteine and vitamin E. These studies suggest that hepatocytes are at least partially responsible for peroxisome proliferator-mediated hepatic NF-κB activation, and support the possibility that this activation is dependent upon reactive oxygen species. BIOCHEM PHARMACOL **59**;4:427–434, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. transcription; hepatoma cells; liver; NF-κB; peroxisome proliferators; hepatocarcinogenesis

The transcription factor NF-κB[‖] is activated in many different cell types in response to numerous stimuli [1, 2]. NF-κB is found normally in the cytosol as an inactive complex consisting of two subunits (p50 and p65, although other members of the Rel transcription factor family also can contribute to NF-κB complexes), which are bound to an inhibitory subunit (IκB; [3]). Upon activation, NF-κB releases from IκB and translocates to the nucleus, where it binds its cognate DNA recognition sequences and increases the transcription of specific genes. Reactive oxygen intermediates, including H_2O_2, are potent activators of NF-κB [4, 5].

Previous studies have shown that NF-κB is an important transcriptional regulator in the liver. NF-κB is activated in the liver rapidly after partial hepatectomy [6–8] or treatment with carbon tetrachloride [9], and during the "acute-phase" response [10, 11]. A more gradual induction of NF-κB is seen in the livers of rats and mice treated with phenobarbital [12] or the peroxisome proliferator ciprofibrate [13, 14].

Peroxisome proliferators are a group of chemical carcinogens that have been proposed to act by increasing reactive oxygen species [15]. These chemicals transiently increase hepatocyte proliferation and induce hepatocellular carcinomas in rodents but are themselves not genotoxic. In addition, these agents greatly increase the number and volume of peroxisomes in the cell as well as several enzymes of lipid metabolism [15]. Among other changes, enzymes of the peroxisomal β-oxidation pathway are highly induced, including FAO, which produces H_2O_2 as a by-product [16]. Catalase, the peroxisomal enzyme that detoxifies H_2O_2, is induced only slightly [16]. This has led to the suggestion that carcinogenesis by peroxisome proliferators is due, at least in part, to overproduction of H_2O_2, which then could cause lipid peroxidation or oxidative DNA damage [15]. Whether peroxisome proliferators induce either of these endpoints, however, remains controversial.

Peroxisome proliferators can induce the transcription of genes through their ability to activate PPARs, members of the nuclear hormone receptor family of transcriptional regulators [17]. These factors modulate transcription through

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‖ *Abbreviations*: NF-κB, nuclear factor κB; FAO, fatty acyl CoA oxidase; PPAR, peroxisome proliferator activated receptor; PPRE, peroxisome proliferator response element; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; and NAC, *N*-acetylcysteine.

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direct interactions with PPREs that are found in the 5′ regulatory regions of target genes [18–20]. PPAR activation occurs rapidly after treatment, leading to the rapid induction of PPAR-regulated genes.

The ability of PPARs to modulate transcriptional changes does not eliminate additional mechanisms of transcriptional control in response to peroxisome proliferator treatment. For example, we demonstrated previously that peroxisome proliferators can activate NF-κB in the livers of rats and mice [13, 14]. This observation raised several questions. First, what hepatic cell population is responsible for the change in nuclear NF-κB levels? The liver is composed of multiple cell types, including Kupffer, parenchymal, stellate, bile duct, and oval cells. Of these, previous studies have shown that hepatocytes and Kupffer cells contain NF-κB [21]. A second question is whether the increased nuclear NF-κB levels can activate NF-κB-regulated genes. A third question is whether reactive oxygen species are involved in peroxisome proliferator-mediated NF-κB activation. To address these issues, we analyzed NF-κB induction in cultured cell lines. These studies demonstrated NF-κB induction in a peroxisome proliferator-responsive rat hepatoma cell line but not in a peroxisome proliferator-insensitive human hepatoma cell line, suggesting that hepatocytes are at least partially responsible for NF-κB activation in the liver. Second, we showed that a NF-κB-regulated reporter gene is activated by ciprofibrate in rat hepatoma cells. This activation can be blocked by the antioxidants vitamin E or NAC, consistent with a role for reactive oxygen intermediates in this process. These data further support our hypothesis that NF-κB may contribute to the cellular changes that occur in response to peroxisome proliferators.

MATERIALS AND METHODS

Plasmids and DNA Fragments

The pGL2 luciferase reporter construct, obtained from the Promega Corp., contains the SV40 promoter 5′ of the luciferase gene and will be referred to as SV40-Luc. An oligonucleotide containing two tandem copies of an NF-κB binding site flanked by GATC overhangs, GATCCGTC GACAGATCCAAGGGGACTTTCCATGGATCCAA GGGGACTTTCCATGGATCTCGAGGATC, was inserted into the *Bgl*II site of the modified pUC9 vector, pUC9.BglII [22]. The NF-κB 2-mer was excised as a *Sma*I–*Xho*I fragment and inserted into *Sma*I–*Xho*I linearized pGL2 to generate $(NF\text{-}\kappa B)_2$-SV40-Luc.

Cell Treatments and Transfections

The rat hepatoma H4IIEC3 cell line was obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% FBS, 5% horse serum, 4 mM glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (pen/strep). HepG2 cells (from the American Type Culture Collection) were maintained in DMEM/F12 (1:1) supplemented with 10% FBS, 4 mM glutamine, pen/strep, and 10 μg/mL of insulin. Unless otherwise indicated, all tissue culture reagents were obtained from GIBCO-BRL Life Technologies. Ciprofibrate, a gift from Sanofi-Winthrop, Inc., was dissolved in 100% ethanol as a 1 M stock solution; the stock solution was mixed with medium to obtain the desired ciprofibrate concentrations. In all ciprofibrate treatments, including untreated cells, the final concentration of ethanol in the medium was adjusted to 0.1%. Vitamin E (α-tocopherol acetate) and NAC were obtained from the Sigma Chemical Co.

Stable transfections of H4IIEC3 cells were performed using the calcium phosphate procedure [23] as described [24]. Three micrograms of pSV_2Neo and 15 μg of $(NF\text{-}\kappa B)_2$-SV40-Luc or SV40-Luc reporter plasmid were added to H4IIEC3 cells at 70% confluence in 10-cm dishes. Six hours after the addition of DNA, cells were washed once in PBS, and fresh medium was added. Twenty-four hours after the addition of DNA, the selection agent G418 was added to a final concentration of 400 μg/mL. Medium containing G418 was changed every 3–4 days; single colonies were visible after 3 weeks and were clonally expanded. Stable clones were maintained in the presence of 100 μg/mL of G418 to avoid the loss of the transfected DNA.

Enzyme Analysis

FAO activity in hepatoma cell homogenates was determined using lauroyl CoA as a substrate as described [25]. Luciferase activity was monitored using 100 μg of cellular extract in 96-well microtiter plates with the substrate luciferin, using a Dynatech luminometer as described [26].

EMSAs

Nuclear extracts were prepared as described [27] using subconfluent monolayers of H4IIEC3 or HepG2 cells that were harvested by scraping plates with a rubber policeman in 1 mL of PBS and then were transferred to 1.5-mL microcentrifuge tubes. EMSAs were performed to monitor the presence of NF-κB DNA binding activity as described [13]. The NF-κB probe contained two NF-κB binding sites. A size-matched fragment from the pUC9 polylinker region was used as a nonspecific competitor fragment. A DNA fragment containing the HNF-3 binding site of the transthyretin promoter (−111 to −85; [28]) was used as an HNF-3 probe. Binding reactions were performed using 10 μg of protein. Antisera for supershift experiments were purchased from Santa Cruz Biotech., Inc.

Statistical Analysis

For the analysis of NAC and vitamin E effects on luciferase activity in stably transfected H4IIEC3 cells, one-way ANOVA and then Tukey's test were used. For the analysis of FAO activity in H4IIEC3 cells and luciferase activity in stably transfected H4IIEC3 cells, one-way ANOVA and

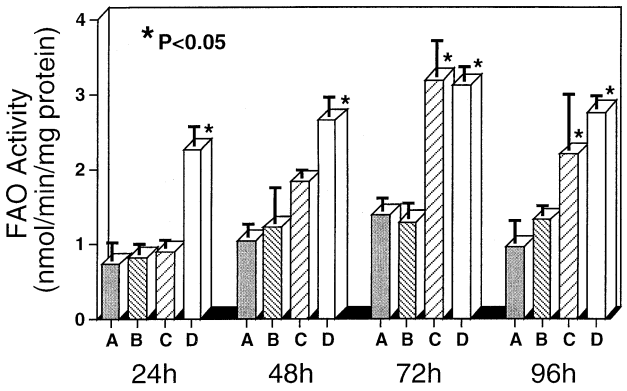

FIG. 1. FAO activity in cultured rat hepatoma H4IIEC3 cells following exposure to ciprofibrate. Cells were maintained in DMEM supplemented with 10% FBS, glutamine, and pen/strep and were treated with the following concentrations of ciprofibrate: (A) 0 μM (control), (B) 30 μM, (C) 120 μM, and (D) 480 μM. Cells were harvested at the designated times, and FAO activity in cell homogenates was determined using lauroyl CoA as the substrate. Key: (*) indicates values significantly different from untreated controls at $P < 0.05$.

then Dunnett's test were used. The level of significance was $\alpha = 0.05$ [29].

RESULTS

To further investigate the basis for peroxisome proliferator-mediated hepatic NF-κB induction, we tested whether NF-κB would be induced in established liver cell lines. We used rat hepatoma H4IIEC3 cells, since previous studies have shown that these cells respond to peroxisome proliferators [20, 30–32]. To ensure that H4IIEC3 cells were responsive to ciprofibrate under the culture conditions used in our laboratory, FAO levels were measured after incubation with this peroxisome proliferator. Cells were untreated or treated with 30, 120, or 480 μM ciprofibrate, and were harvested after 24, 48, 72, or 96 hr. FAO activity was increased in a concentration–dependent manner; the increase was significant at all time points in cells treated with 480 μM ciprofibrate and at 72 and 96 hr in cells treated with 120 μM ciprofibrate (Fig. 1). No significant increase was seen at any time point in cells treated with 30 μM ciprofibrate. This result confirmed that H4IIEC3 cells were responsive to ciprofibrate treatment. Since the highest increase in FAO levels was observed in cells treated for 72 hr (3- to 4-fold), this time point was chosen for subsequent experiments.

To test whether H4IIEC3 cells showed an increase in NF-κB activity in response to peroxisome proliferators, nuclear extracts were prepared from cells that were untreated or treated with 30, 120, or 480 μM ciprofibrate for 72 hr. NF-κB levels in these extracts were measured using EMSAs with a radiolabeled probe containing two copies of an NF-κB binding site. Low but detectable levels of NF-κB were present in extracts from untreated cells and remained unchanged in cells treated with 30 μM ciprofibrate (Fig. 2A). A dramatic increase in NF-κB was seen in extracts from cells treated with 120 or 480 μM ciprofibrate. Interestingly, this result is similar to what was seen with FAO induction (Fig. 1) in that increased NF-κB levels were seen only with the higher concentrations of ciprofibrate but not with 30 μM ciprofibrate. Additional faster migrating bands also were seen in Fig. 2A. These appear to bind nonspecifically to the NF-κB probe, as they could not be competed out with unlabeled self and non-self DNA fragments (see Fig. 3, below).

If NF-κB activation was indeed due to the action of ciprofibrate, then a peroxisome proliferator-insensitive cell line should not respond to treatment. To test this, nuclear extracts were prepared from human hepatoma HepG2 cells and used in EMSAs. HepG2 cells are non-responsive to peroxisome proliferators [33], but do exhibit changes in NF-κB activity in response to other stimuli [34]. No difference in nuclear NF-κB levels was observed in HepG2 cells that were untreated or treated with 30, 120, or 480 μM ciprofibrate for 72 hr (Fig. 2B). No change in FAO activity was observed in these treated cells when compared with untreated cells (data not shown), consistent with the idea that ciprofibrate responsiveness is required for NF-κB activation.

It was possible that ciprofibrate treatment resulted in a widespread increase in transcription factor binding activity in H4IIEC3 cells. HNF-3 is an abundant liver-enriched factor that comprises several members [35]. HNF-3 activity remained unchanged in H4IIEC3 cells treated with increasing concentrations of ciprofibrate (data not shown). This suggests that the ciprofibrate-induced increase in NF-κB levels was not due to a general change in transcription factors.

To confirm that the ciprofibrate-induced complex in H4IIEC3 extracts contained NF-κB, several controls were performed. EMSAs were performed in the presence of an excess of unlabeled self and non-self fragments to determine if the complex was bound to the consensus NF-κB site. Twenty- and forty-fold molar excesses of the homologous fragment could compete for binding effectively, whereas the heterologous fragment did not diminish binding to the radiolabeled probe at either concentration (Fig. 3A). The faster migrating complex seen in all five lanes could not be competed out by an excess of either the self or non-self fragments, suggesting that this complex contained factors that were bound nonspecifically to DNA. In addition, EMSAs were carried out with antiserum against the NF-κB p65 subunit (Fig. 3B). The presence of pre-immune rabbit serum did not alter the NF-κB complex, whereas the addition of the anti-p65 antiserum reduced the intensity of the NF-κB complex. The antibody likely has disrupted the NF-κB–DNA complex, indicating that p65 is a component of the ciprofibrate-induced complex. These results confirmed that ciprofibrate can increase nuclear NF-κB levels in H4IIEC3 cells.

Whereas these *in vitro* data demonstrated increased nuclear NF-κB DNA binding activity in treated cells, it was also important to demonstrate an increase in NF-κB tran-

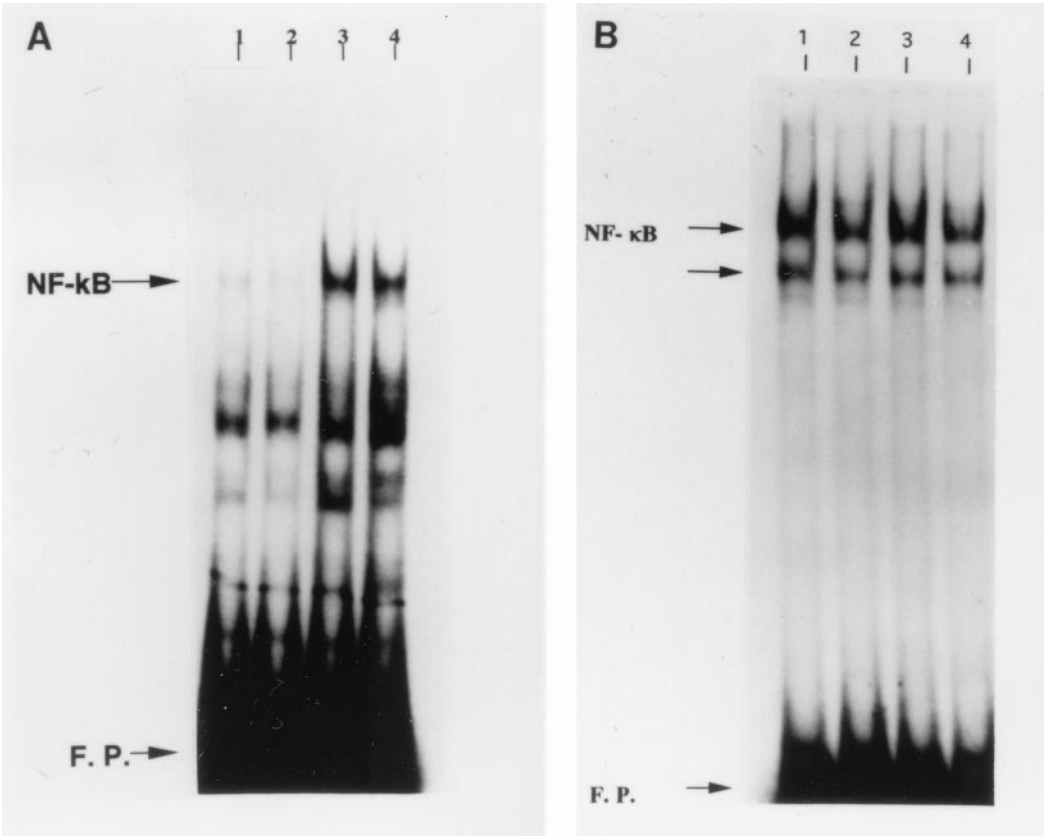

FIG. 2. NF-κB levels in H4IIEC3 cells and HepG2 cells treated with ciprofibrate. EMSAs were performed using 10 μg of nuclear extracts from peroxisome proliferator-responsive H4IIEC3 (A) or peroxisome proliferator-nonresponsive HepG2 (B) cells that were untreated (lane 1) or treated with ciprofibrate at 30 μM (lane 2), 120 μM (lane 3), or 480 μM (lane 4). After 72 hr, cells were harvested and nuclear extracts were prepared. The higher mobility bands found in H4IIEC3 extracts are nonspecific complexes that cannot be competed out with an excess of unlabeled specific or nonspecific competitor fragments (see Fig. 3A). The NF-κB arrows designate the single NF-κB complex in H4IIEC4 cells and two NF-κB complexes in HepG2 cells. F.P., free probe.

scriptional activity in these cells. To test this, $(NF\text{-}\kappa B)_2$-SV40-Luc and the parental SV40-Luc reporter constructs were stably transfected into H4IIEC3 cells with the selectable pSV_2Neo gene by the calcium phosphate method. $G418^r$ colonies were clonally expanded and assayed for luciferase activity after treatment with ciprofibrate (Fig. 4). As expected, control cells that were transfected solely with pSV_2Neo (Neo1) showed no luciferase activity, regardless of treatment. Two independent $G418^r$ clones that contained SV40-Luc, lines P9 and P14, had measurable luciferase levels in the absence of inducer; this is due presumably to the action of the moderately active SV40 promoter. However, no change in luciferase activity was observed when these two lines were treated with ciprofibrate. Two $G418^r$, $(NF\text{-}\kappa B)_2$-SV40-Luc clones, lines N33 and N38, exhibited higher luciferase activity in untreated cells than did the untreated SV40-Luc lines. This was not unexpected, since untreated H4IIEC3 cells contain moderate NF-κB levels. With 30 μM ciprofibrate, no increase in luciferase activity was seen in either N33 or N38 cells. However, clone N38 exhibited a significant increase in luciferase activity when treated with 120 μM ciprofibrate, while both N33 and N38 exhibited increased luciferase levels with 480 μM ciprofibrate. Thus, despite the higher basal level of luciferase in these lines, both N33 and N38 showed a concentration-dependent increase in NF-κB regulated luciferase activity in response to ciprofibrate. This demonstrated that increased nuclear NF-κB could transactivate a NF-κB regulated reporter gene in cells.

Previous studies have shown that reactive oxygen species are potent activators of NF-κB [4]. That peroxisome proliferators act, in part, by inducing enzymes of the β-oxidation pathway is consistent with the idea that NF-κB is being activated by H_2O_2. If this notion is correct, antioxidants should inhibit ciprofibrate-mediated changes in nuclear NF-κB levels. The P9 and N38 lines were treated with ciprofibrate in the presence of the antioxidants vitamin E or NAC to test this possibility. When the SV40-Luc line P9 was treated with ciprofibrate, either in the presence or absence of vitamin E, no change in luciferase activity was observed (Fig. 5, top panel). Since this reporter gene is not regulated by NF-κB, we would not expect to see any change in these cells. Luciferase activity was induced by ciprofibrate in the $(NF\text{-}\kappa B)_2$-SV40-Luc clone N38, consistent with the results shown earlier. This induction was blocked completely by the presence of the antioxidant, as luciferase

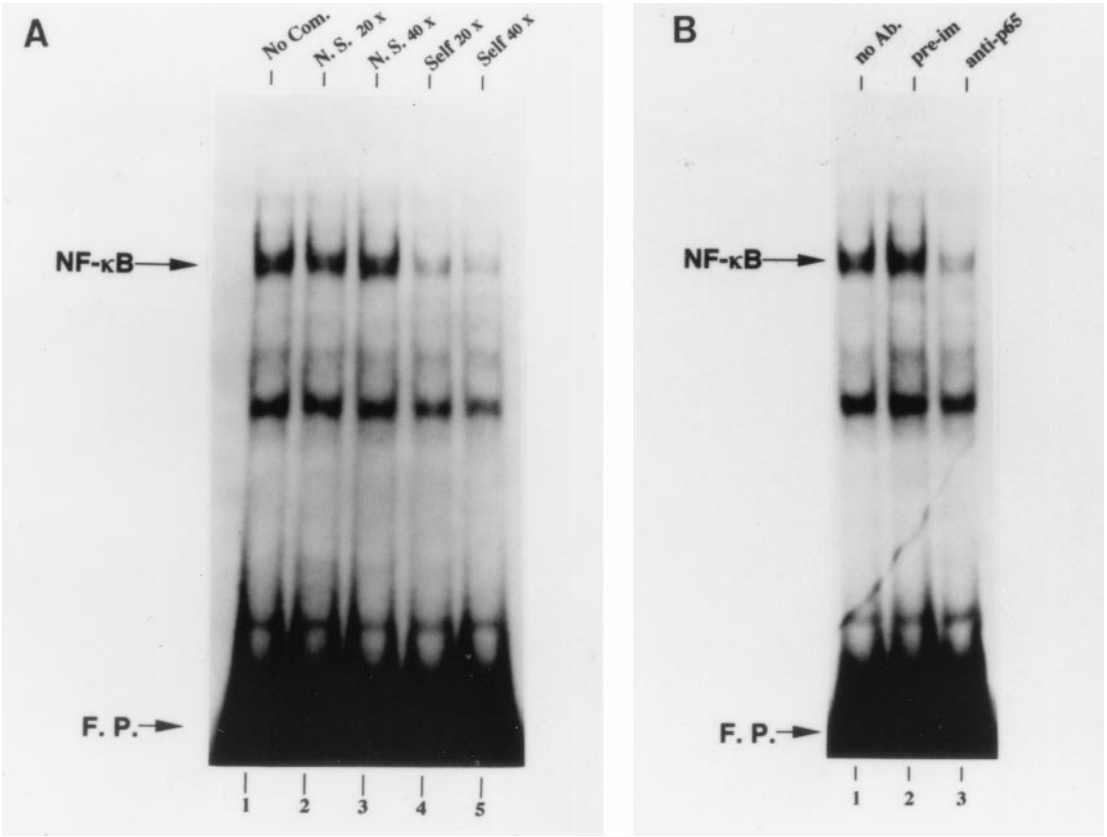

FIG. 3. Unlabeled competition and antibody supershift assays for NF-κB with extracts from ciprofibrate-treated H4IIEC3 cells. EMSAs were performed using 10 μg of nuclear extracts from H4IIEC3 cells that had been treated with 120 μM ciprofibrate for 72 hr. (A) Unlabeled competition assays. Nuclear extracts were incubated in the absence (lane 1) or presence of 20-fold (lanes 2 and 4) or 40-fold (lanes 3 and 5) molar excess of nonspecific (N.S., lanes 2 and 3) or specific (Self, lanes 4 and 5) unlabeled fragments prior to the addition of 1 ng of the radiolabeled fragment containing two copies of the NF-κB binding site. (B) Antibody supershift assays. Nuclear extracts were incubated with no antibody (lane 1), nonspecific rabbit antiserum (lane 2), or rabbit antiserum against the NF-κB p65 subunit (lane 3). The higher mobility bands in both A and B are nonspecific complexes that cannot be competed out with an excess of unlabeled specific or nonspecific competitor fragments. The NF-κB arrows designate the complex that is competed out by the specific probe in A and is diminished by the anti-p65 antibodies in B. F.P., free probe.

levels in cells treated with ciprofibrate and vitamin E were the same as in untreated control cells. When the same experiment was performed in P9 and N38 cells treated with NAC, similar results were obtained; luciferase activity remained unchanged in P9 cells regardless of the presence of ciprofibrate and/or NAC, whereas the ciprofibrate-induced luciferase increase in N38 cells was blocked by NAC (Fig. 5, bottom panel). The inhibitory effect of these two antioxidants indicated that reactive oxygen species were involved in ciprofibrate-mediated NF-κB activation.

DISCUSSION

We previously reported that NF-κB is activated in the livers of rats and mice treated with the peroxisome proliferator ciprofibrate [13, 14]. We have demonstrated here that NF-κB also can be activated in a peroxisome proliferator-responsive cultured hepatocyte cell line in response to ciprofibrate. This activation occurs at the level of increased nuclear NF-κB DNA binding activity, as measured by EMSAs. In addition, we have shown that a NF-κB regulated reporter gene is activated by ciprofibrate in stably transfected cells. This increase in NF-κB can be blocked by the antioxidants NAC or vitamin E. Taken together, these results extend our previous findings and demonstrate a mechanism by which ciprofibrate can activate NF-κB.

Our previous study in rats could not ascertain which cells within the liver were responsible for the increased hepatic NF-κB activity. Although hepatocytes make up the majority of cells in the liver, numerous other cell types are present. NF-κB has been demonstrated to be present in both Kupffer cells and hepatocytes after isolation from rat liver [21]. Cressman *et al.* [7], however, found NF-κB to be localized mainly in hepatocytes following partial hepatectomy in rodents. NF-κB was shown recently to be activated in Kupffer cells by the hepatotoxin acetaminophen [36]. Our results in H4IIEC3 hepatoma cells suggest that NF-κB is being activated by ciprofibrate in hepatocytes. To ascertain whether this peroxisome proliferator also activates

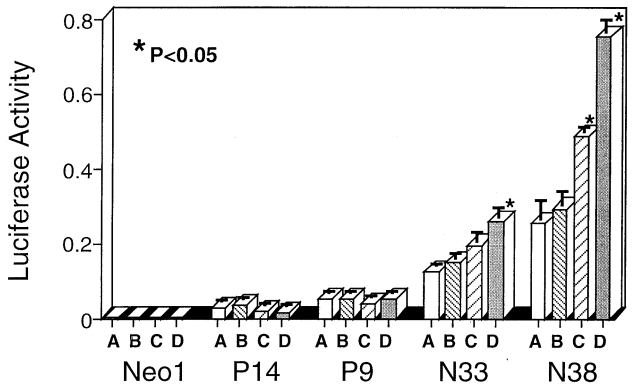

FIG. 4. Luciferase activity in H4IIEC3 lines after treatment with ciprofibrate. Stable G418^r transfectants were generated with pSV$_2$Neo alone (Neo1 clone), pSV$_2$Neo and SV40-Luc (P9 and P14 clones), or pSV$_2$Neo and (NF-κB)$_2$-SV40-Luc (clones N33 and N38). Cloned lines were maintained in the presence of 400 μg/mL of G418 to ensure the stable maintenance of transfected genes. Cells were treated with the following concentrations of ciprofibrate: (A) 0 μM (control), (B) 30 μM, (C) 120 μM, and (D) 480 μM. After 72 hr, cells were harvested, and luciferase activity in cell homogenates was determined using a luminometer. Key: (*) indicates values significantly different from untreated controls within each cell line at $P < 0.05$.

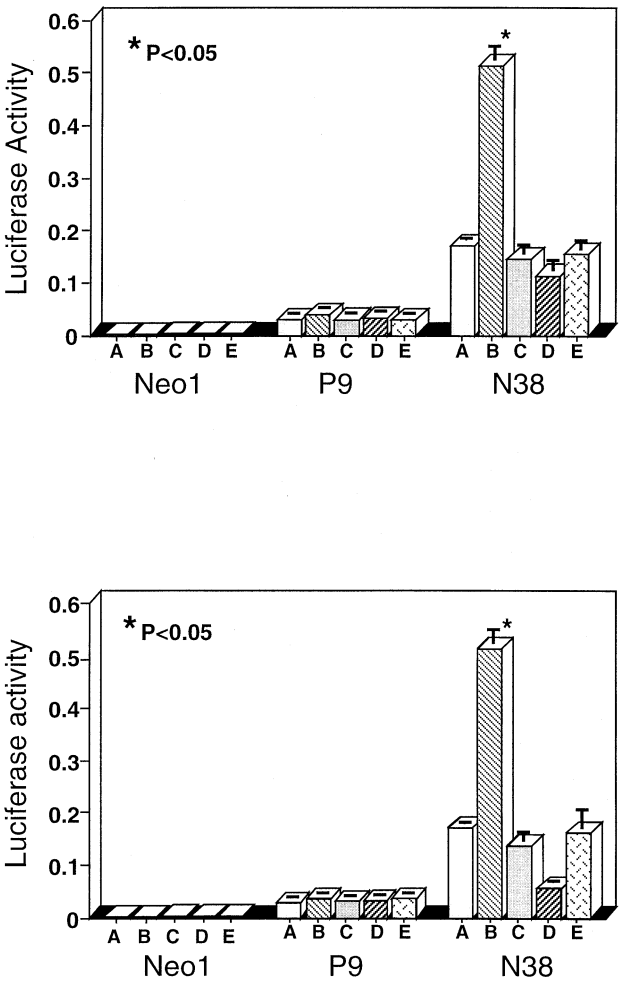

FIG. 5. Inhibition of ciprofibrate-induced activation of an NF-κB-regulated reporter gene in H4IIEC3 cells by the antioxidants vitamin E and NAC. Top panel: G418^r cloned lines [Neo 1, pSV$_2$Neo alone; P9, pSV$_2$Neo and SV40-Luc; N38, pSV$_2$Neo and (NF-κB)$_2$-SV40-Luc] were untreated (A), or treated with 480 μM ciprofibrate alone (B) or in combination with vitamin E at 100 μM (C) or 1 mM (D), or with 1 mM vitamin E alone (E). After 72 hr, cells were harvested, and luciferase activity in cell homogenates was determined. Key: (*) indicates values significantly different from untreated controls within each cell line at $P < 0.05$. Bottom panel: G418^r cloned lines [Neo 1, pSV$_2$Neo alone; P9, pSV$_2$Neo and SV40-Luc; N38, pSV$_2$Neo and (NF-κB)$_2$-SV40-Luc] were untreated (A), or treated with 480 μM ciprofibrate alone (B) or in combination with NAC at 1 μM (C) or 10 μM (D), or with 10 μM NAC alone (E). After 72 hr, cells were harvested, and luciferase activity in cell homogenates was determined. Key: (*) indicates values significantly different from untreated controls within each cell line at $P < 0.05$.

NF-κB in other hepatic cell populations will require additional studies.

It is likely that reactive oxygen is involved in ciprofibrate-mediated NF-κB activation, since either NAC or vitamin E can block this activation effectively. Vitamin E is a lipid-soluble free radical scavenger located in lipid membranes throughout the cell. NAC is a precursor to reduced GSH, which is a substrate for the enzymes glutathione peroxidase and glutathione-*S*-transferase. NAC administration increases GSH concentrations *in vitro* [37]. Antioxidants also have been found to inhibit NF-κB activation in other cellular systems [4, 38]. Since FAO is highly induced by peroxisome proliferators and can generate H_2O_2, we suggest that increased levels of peroxisomal enzymes are responsible for increased H_2O_2, which subsequently leads to NF-κB activation. In support of this possibility, we have shown that FAO overexpression in Cos1 cells activates NF-κB [39]. Other enzymes also are activated by peroxisome proliferators, including those of the cytochrome P450 4A family. P450 enzymes can generate active oxygen species, including H_2O_2 [40], and may contribute to NF-κB activation.

The results presented here raise the question of what role NF-κB might have in the biological processes that occur in rodents in response to peroxisome proliferator treatment, including the role NF-κB might have in the carcinogenic and tumor promoting effects of this class of agents. NF-κB is known to regulate genes involved in growth control; identifying changes in the levels of these or other genes that are known to be regulated by NF-κB would be of great interest. NF-κB also may be involved in the regulation of apoptosis in the liver; knocking out the p65 gene results in massive apoptosis in the liver during development [41]. Finally, the possibility that FAO is a link between peroxisome proliferators and NF-κB activation raises the possibility that NF-κB is involved in the tumor promoting activities of these agents. This idea is consistent with the data of Chu *et al.* [42, 43], who found that overexpressing the hydrogen peroxide-generating enzymes FAO or urate oxidase in CV1

cells led to anchorage-independent cell growth and allowed these cells to develop into tumors in nude mice.

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