Oxidative stress interferes with several cellular functions, in particular transcriptional regulation. We show here that the human cytochrome P450 1A1 (CYP1A1) is down-regulated at the transcriptional level by oxidative stress. Basal as well as 2,3,7,8-tetrachloro-p-dioxin-induced promoter activities are strongly impaired by H$_2$O$_2$ treatment or glutathione depletion with L-buthionine-(S,R)-sulfoximine. Tumor necrosis factor $\alpha$ inhibits CYP1A1 expression, and this inhibition is prevented by the antioxidant pyrrolidine dithiocarbamate. We show that these regulations depend on the integrity of the nuclear factor 1 (NFI) site located in the proximal promoter. We therefore examined the redox regulation of this transcription factor. Treatment of human HepG2 or rat H4 hepatoma cells with H$_2$O$_2$ or L-buthionine-(S,R)-sulfoximine inactivates the binding of the NFI transcription factor to its DNA consensus sequence. Furthermore, H$_2$O$_2$ treatment leads to a dose-dependent decrease of reporter gene expressions driven by promoters containing NFI binding sites. Glutathione depletion and catalase inhibition also repress a NFI-driven promoter. Under the same conditions, the CP-1 transcription factor activity is not affected by oxidative stress. Thus, NFI seems particularly sensitive to oxidative stress. This accounts, at least partially, for the regulation of cyp1A1 gene expression.

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CYP1A1 expression. NFI plays an important role in viral (23) and cellular gene regulation. It was originally described as a factor required for the replication of adenovirus DNA (24). It was then shown to be part of a family of ubiquitous transcription factors encoded by four different genes (NFI A, NFI B, NFI C/CTF, and NFI X) that bind to the TGGC(N)xGCCA DNA consensus sequence after dimerization (25). The highly conserved 220 N-terminal residues containing the DNA binding domain are not homologous to any well characterized class of DNA binding domains (26). NFI is widely involved in the control of constitutive or inducible gene expression. In the liver, it has been shown to be critical for the expression of collagen I (27), albumin (28), and aspartate aminotransferase (29) and for the specific expression of α-fetoprotein (30) and vitellogenin (31). Members of the NFI family have been reported as possible targets for phosphorylation or glycosylation. Yet, this does not seem to affect their abilities to bind DNA in vitro (26). The NFI family members possess four conserved cysteines (32). Three of them were critical for DNA binding in vitro, which was abolished by their alkylation or oxidation (26). These observations suggest that NFI could be sensitive to modifications of the redox status within the cell. In this study, we have used hepatoma cell cultures to assess the functional impact of oxidative stress on the binding of NFI to DNA and its transcriptional activity and the consequences for the cyp1A1 gene promoter activity.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**H$_2$O$_2$ was used from a 30% stock (Merck). Other chemicals were obtained from Sigma (unless otherwise stated), and oligonucleotides were from Eurogentec (Belgium) and Genset (Paris, France). Human recombinant TNF-α was obtained from Tebu (Le Perray, France).

**Cell Culture—**The hepatoma cell line H4 II EC3 (33) and the human hepatoma cell line HepG2 (34) were maintained as described by Garlatti et al. (29). Cell viability was assessed using the trypan blue exclusion test. Cells from nonconfluent dishes were trypsinized and resuspended in 3 ml of phosphate-buffered saline. 1 ml of colorant (trypan blue 6.2 mM, NaCl 0.8 μM) was then added and gently mixed. After 2 min, cells were counted. Viability was expressed as (number of non-blue cells/total number of cells) × 100.

**Nuclear Extracts—**Confluent 10-cm dishes were trypsinized. All subsequent steps were performed at 4 °C. Cells were centrifuged and then resuspended in a hypotonic buffer (10 mM Heps, pH 8, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride) and allowed to swell for 15 min. Cells were lysed by adding 1.5% Nonidet P-40 and vortexing for 10 s. After centrifugation (7000 g, 10 min), the supernatant could be saved for cytosolic extract analysis and pellets were resuspended in a hypertonic buffer (20 mM Heps, pH 8, 1 mM EDTA, 1 mM EGTA, 0.4 mM NaCl, 1 mM phenylmethylsulfonyl fluoride). After a 15-min incubation, the mixture was centrifuged (13,000 × g, 10 min), and the supernatant was aliquoted and stored at −80 °C.

**Glutathione Assay—**Glutathione using the GSH-400 kit from Oxys (Portland, OR). Proteins and allowed to swell for 15 min (7 min), and the supernatant was assayed for glutathione using the method of Bradford (35). Glutathione activity was measured with the ECL kit (Amersham Pharmacia Biotech). The rabbit polyclonal nonspecific NFI antibody targeting the N-terminal part was obtained from Tebu (Le Perray, France). Rabbit antibodies raised against NFI/CTF1 and NFI X3 were generous gifts of Drs. N. Tanese (New York University Medical Center) and B. Cortés (EPFL, Lausanne, Switzerland).

**Northern Blots—**RNA preparation and Northern blots were performed as already described (36). Probes were synthesized from cDNAs with the Megaprime DNA labeling kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Quantifications were performed with a phosphorimage and ImageQuant software (Molecular Dynamics).

**Plasmids—**The Firefly and Renilla luciferase expression plasmids (pGL3 basic and pRL-SV40) were purchased from Promega. The pRSV-FL plasmid was a gift from Dr M. Aggerbeck (INSERM, Paris). The pTk-pCAT plasmid, a gift from Dr. C. Forest (CNRS, Meudon, France), contains the thymidine kinase gene promoter (−105, +51) of the herpes simplex 1 virus upstream of the chloramphenicol acetyltransferase (CAT) reporter gene. Recombinant plasmids were constructed as follows (see Fig. 1A).

The −1566, +73 5’ region of the human cyp1A1 gene (a gift of Dr. Maurel, INSERM, Montpellier, France), was subcloned into the KpnI–HindIII double-digested pGL3 basic vector, upstream of the firefly luciferase reporter gene to yield p1A1-FL. The pmut1A1-FL is identical to the p1A1-FL except for a double mutation at positions −49 and −50 in the NFI site (GCGA → GCGG).

It was obtained by site-directed mutagenesis on p1A1-FL, using a mutated oligonucleotide and the GeneEdit kit (Promega) according to the manufacturer’s instructions.

A double-stranded oligomer derived from the 3’-end of the human α-globin proximal promoter (−72, +1) was synthesized with point mutations at the bases shown in italics, in order to change Sp1-like binding sites and BglII 5’ and HindIII 3’ ends: 5’-GATCTCGCAGGCGGCATGGCACGACGACGAC-3’. These were used as a control in transfection experiments. A NF1 site, present in the human α-globin gene promoter upstream of position −79, was not included in this sequence.

A double-stranded oligomer containing the rat cyp1A1 gene proximal promoter (−60, +1) was synthesized with Xhol 5’ and HindIII 3’ ends: 5’-GAGAAAGGGCGCCTGCCACAAGCAGATCTATATAGGTTGGTGGTGGCTCTCAGCCACAC-3’. It was subcloned into the Xhol–HindIII double-digested pGL3 basic vector, upstream of the firefly luciferase reporter gene to yield pNFI-FL.

For pmutNFI-FL, pTAT-FL, a similar strategy as for pNFI-FL was used: 5’-GAGAATGATCCGGCGGCGAGGCGGAGGCGGAGGCGGAG-3’.

The TriNF1 double-stranded oligomer (−5’AGGTTGGATTGGAAGGCGAAGATGGAGTGAAGGCGGAGGAAG-3’), containing three adjacent NFI consensus sites and HindIII-compatible ends was subcloned into the HindIII site located in the thymidine kinase promoter of pTk-CAT to give the pTRI-FL/NFI-cat.

All constructs were checked by DNA sequencing analysis.

**Transfection Experiments—** Transfection experiments were performed in HepG2 cells as described previously (29, 37). All cell cultures were taken from the human α-globin gene proximal promoter (−88, −58) containing both NF1 and CP-1 binding sites and mutations at the bases shown in italics, in order to change Sp1-like binding sites and BglII 5’ and HindIII 3’ ends) was subcloned into the XhoI–HindIII double-digested pGL3 basic vector, upstream of the firefly luciferase reporter gene to yield pNFI-FL.

**Electrophoretic Mobility Shift Assay (EMSA)—** The NF1 and CP-1 probes contained, respectively, a NF1 site from adenovirus 2 (5’TATTTTTTGATGAGGCCAGGATATGATA-3’) and the CP-1 site from the mouse α-globin gene proximal promoter (−104, −75, 5’-GCCAACAACAGAGGCGCCCAATAGAAGGG-3’). The Duo probe was taken from the human α-globin gene proximal promoter (−88, −58) containing both NF1 and CP-1 binding sites (5’TGTCGCCCGTGCGCGCCGACGCACTGAGCGGCCAGGAGGAAGGAAGAAGGAGGAGGAGG-3’, containing three adjacent NF1 consensus sites and HindIII-compatible ends) was subcloned into the HindIII site located in the thymidine kinase promoter of pTk-CAT to give the pTRI-FL/NFI-cat.

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All constructs were checked by DNA sequencing analysis.
Oxidative Stress Down-regulates CYP1A1 mRNAs. HepG2 (left panels) and H4 (right panels) cells were either untreated or treated for 6 h with 10 nM TCDD after pretreatment without or with 50 μM BSO for 20 h or 1.5 mM H2O2 for 30 min. Northern blots were hybridized with a CYP1A1 probe then actin probe. Diagrams show CYP1A1 mRNA level normalized to actin mRNA levels (100% corresponds to the mRNA ratio in cells induced with TCDD alone).

Table I

| Glutathione assay following H2O2 or BSO treatment |
|-----------------------------------------------|
| Control | H2O2 | BSO |
| GSH (%) | 100 | 55 ± 9.2 | 25 ± 7.6 |

Redox Regulation of the cyp1A1 Promoter—To determine whether the CYP1A1 mRNAs down-regulation by oxidative stress was accounted for by a modification of the gene promoter activity, we transfected the p1A1-FL plasmid containing the firefly luciferase reporter gene controlled by the human cyp1A1 gene promoter (~1566, +73 fragment) in HepG2 cells. The treatment of cells by increasing concentrations of H2O2 caused a dose-dependent inhibition of the reporter gene expression (Fig. 3A, curve a). A BSO-induced GSH depletion also impaired the expression driven by this promoter (Fig. 3B, open bars). Dioxin (TCDD) induced cyp1A1 promoter activity 20-fold (not shown). As shown in Fig. 3C (open bars), the induced activity was also strongly inhibited by H2O2, suggesting that the decrease in steady-state mRNAs levels are mainly due to a decrease in promoter activity. The effect of H2O2 was more potent on the TCDD-induced activity than on the basal activity. In some cellular systems, the cytokine TNFα is known to increase cellular H2O2 levels (40, 41). In HepG2 cells, TNFα decreased cyp1A1 promoter activity by 40% (Fig. 3D, open bars). This effect is prevented in the presence of the antioxidant PDTC, suggesting that it is mediated by a redox mechanism. In the same experiments, TNFα did not affect the expression driven by a CP-1/TATA promoter (not shown).

Critical Contribution of the Proximal NFI Site to the Redox Regulation—Since both basal and induced activities were altered by oxidative stress, we hypothesized that the proximal NFI site may be implicated in the H2O2 effect. In order to test this hypothesis, we mutated the proximal NFI site in the cyp1A1 promoter (3A). While the mutated promoter displayed decreased basal activity (8-fold decrease, Fig. 3A), it also displayed decreased TCDD inducibility, 11- versus 20-fold induction for pmu1A1-FL and p1A1-FL, respectively (not shown).

The role of the NFI site in the basal and induced activities of the 1.6-kilobase pair-long human promoter is in agreement with previous studies using the mouse promoter (3).

Interestingly, the effect of H2O2 and BSO on the basal activity was abolished by the mutation of the NFI site (Fig. 3A, curve a).
Oxidative Stress Down-regulates cyp1A1 through NFI

FIG. 3. Effect of oxidative stress generated by various compounds on the activity of the cyp1A1 gene promoter in HepG2 cells. In all experiments, cells were cotransfected with pglob-RL as internal control. Results are expressed as firefly luciferase activity/ Renilla luciferase activity. A, transfection with p1A1-FL (open bars) or pmut1A1-FL (shaded bars). Cells were treated or not treated with 50 μM BSO for 20 h. Results are expressed as mean ± S.E. (n = 13). C, effect of oxidative stress on TCDD-induced activity. Cells were transfected with either p1A1-FL (open bars) or pmut1A1-FL (shaded bars) and were induced by 3 μM TCDD after pretreatment with increasing H2O2 concentrations for 30 min or 50 μM BSO for 8 h. Cells were harvested 16 h later. Results are expressed as mean ± S.E. (n = 8). 100% corresponds to the activity in cells transfected with p1A1-FL and treated with TCDD only. D, effect of TNFα on the activity of the cyp1A1 gene promoter. Cells were transfected with p1A1-FL (open bars) and were treated with TNFα (5 ng/ml) with or without prior treatment with the antioxidant PDTC (50 μM) for 1 h. In the same experiments, cells were transfected with pmut1A1-FL (shaded bars) and also treated with TNFα. They were harvested 19 h after TNFα treatment, and luciferase activities were assayed. Results are expressed as mean ± S.E. (n = 9).

b and Fig. 3B, shaded bars). Similarly, the activity of the dioxin-induced mutated promoter was not altered by oxidative stress (Fig. 3C, shaded bars). We conclude that NFI site integrity is required for the inhibitory effect caused by oxidative stress. Furthermore, this site is also required for the inhibition of promoter activity by TNFα, since this cytokine had no effect on the activity of the mutated promoter (Fig. 3D, shaded bars).

NFI Activity Is Down-regulated by Oxidative Stress—In order to further characterize the contribution of NFI to the oxidative regulation of gene expression, the activity and regulation of short promoter fragments containing critical NFI sites were tested. We first tested a promoter containing a NFI site upstream of a TATA box (pNFI/TATA-FL plasmid containing the 60-base pair-long proximal promoter of the cyp1A1 gene). As shown in Fig. 4A, increasing concentrations of H2O2 down-regulated this promoter activity in a dose-dependent manner. When the NFI site of this promoter fragment was mutated, the inhibitory effect of H2O2 was prevented (the basal activity of the mutated promoter was decreased but remained at least 3 times higher than the one obtained with the pGL3 basic vector). The effect of other compounds modifying the redox status within the cell was also tested. BSO inhibited this promoter activity (not shown). Aminotriazole, an inhibitor of the H2O2 scavenging enzyme catalase, also decreased this promoter activity, which was further repressed by H2O2. BSO down-regulated the activity of the mutated NFI site (Fig. 4B, filled diamonds, curve b). Cells were harvested 16 h after H2O2 treatment. Results are expressed as mean ± S.E. (n = 6). 100% corresponds to the firefly luciferase/Renilla luciferase ratio in control cells. C, effect of TNFα on the activity of the NFI/TATA promoter. Cells were transfected with the pNFI/TATA-FL plasmid. They were treated with TNFα (5 ng/ml) with or without prior treatment with the antioxidant PDTC (50 μM) for 1 h. They were then harvested, and luciferase activities were assayed. Results are expressed as mean ± S.E. (n = 9). In the same experiments, cells were cotransfected with pNFI/TATA-FL (proximal promoter with a mutated NFI site; filled diamonds, curve b). Cells were harvested 16 h after H2O2 treatment. Results are expressed as mean ± S.E. (n = 6). 100% corresponds to the NFI-driven promoters in HepG2 cells. Cells were cotransfected with pglob-RL as internal control, and results were expressed as firefly luciferase activity/Renilla luciferase activity. A, effect of H2O2 on cells transfected with pNFI/TATA-FL (open squares, curve a) or pmutNFI/TATA-FL (proximal promoter with a mutated NFI site; filled diamonds, curve b). Cells were harvested 16 h after H2O2 treatment. Results are expressed as mean ± S.E. (n = 6). 100% corresponds to the firefly luciferase/Renilla luciferase ratio in control cells transfected with pNFI/TATA-FL. B, effect of aminotriazole on cells transfected with pNFI/TATA-FL. Cells were treated with aminotriazole (ATA) for 20 h with or without subsequent H2O2 addition. Cells were harvested 16 h after H2O2 treatment. Results are expressed as mean ± S.E. (n = 9). 100% corresponds to the firefly luciferase/ Renilla luciferase ratio in control cells. C, effect of TNFα on the activity of the NFI/TATA promoter. Cells were transfected with the pNFI/TATA-FL plasmid. They were treated with TNFα (5 ng/ml) with or without prior treatment with the antioxidant PDTC (50 μM) for 1 h. They were then harvested, and luciferase activities were assayed. Results are expressed as mean ± S.E. (n = 9). 100% corresponds to the firefly luciferase/Renilla luciferase ratio in control cells. D, HepG2 were transiently transfected with pTrNFI-CAT (open squares, curve a) or pTk-CAT (filled diamonds, curve b). In this experiment, cells were cotransfected with pRSV-FL as internal control and were harvested 16 h after H2O2 treatment. Results are expressed as CAT activity/firefly luciferase activity (mean ± S.E., n = 5). 100% corresponds to the activity in control cells transfected with pTrNFI-CAT.

To study the influence of H2O2 on NFI activity in a heterologous context, we transiently transfected HepG2 cells with a recombinant Tk-CAT plasmid. The pTrNFI-CAT construct (three NFI sites inserted upstream of the thymidine kinase promoter) displayed a basal CAT expression 4 times higher than the pTk-CAT. The reporter gene expression decreased dramatically when cells were treated with increasing concentrations of H2O2. The dose-dependent inhibition of NFI transcriptional activity was maximal at a 1 μM concentration (75% decrease) as shown in Fig. 4D. Under the same conditions, the CAT gene expression driven by the HSV-Tk promoter (pTk-CAT) was also decreased but to a lesser extent (see Fig. 4D, inset). It has been reported that the HSV-Tk promoter contains a CCAAT box (42), which could bind NFI, and two Sp1 binding sites, which could account for this observation.

H2O2 Doses and Cytotoxicity—In the experiments reported here, the concentration of H2O2 used was within the 100 μM to
2 mM range. However, we observed, by a spectrophotometric method (43), a rapid degradation of H$_2$O$_2$ in the culture medium, in agreement with previous studies in liver cells (6, 44). Thus, only a minor fraction of the initial H$_2$O$_2$ introduced in the medium could reach the intracellular location. In our experiments, cells did not suffer significant irreversible cytotoxicity after exposure to H$_2$O$_2$ (Fig. 5) or to BSO and aminotriazole (data not shown). These data are consistent with published studies on hepatoma cells undergoing H$_2$O$_2$ treatment (44–46). It should be noted that sensitivity to H$_2$O$_2$ depends on the cell type and that liver cells appear to be particularly resistant to such a treatment.

Characterization of NFI Complexes and Effect of H$_2$O$_2$—The binding of NFI to DNA was evaluated by EMSA using nuclear extracts of hepatoma cells treated or not with H$_2$O$_2$. In H4 cell nuclear extracts, four NFI complexes were observed in gel retardation assays (labeled as a, b, c, and d in Fig. 6). These complexes are specific as demonstrated by competition experiments (Fig. 6, A and B). In order to further characterize these complexes, we performed supershift experiments. Using a NFI C/CTF antibody, we observed that the intensity of all four complexes was decreased (Fig. 6C). However, the effect of the antibody on the different complexes was variable, and only three supershifted complexes were clearly detected (labeled as A, B, and C in Fig. 6C). The NFI complex b was entirely supershifted. Interestingly, this complex was almost completely down-regulated in nuclear extracts of cells treated with H$_2$O$_2$ (Fig. 6C). Using an anti-NFI X antibody, only a faint supershift was observed, and the intensity of the four NFI complexes was not modified (data not shown). Thus, several NFI/DNA complexes, particularly complexes a and b, contain the NFI C/CTF isoform. In addition, these complexes are the most sensitive to H$_2$O$_2$ treatment. We show below that NFI C/CTF is the most abundant isoform.

In HepG2 cells, NFI complexes could not be as clearly resolved as in H4 cells. Nonetheless, we also observed the formation of four complexes that were displaced in a competition experiment (Fig. 7A). The NFI C/CTF antibody also decreased NFI/DNA interaction (not shown). Treatment of HepG2 cells with a 1 mM H$_2$O$_2$ concentration led to a 50% decrease in NFI/DNA binding (Fig. 7A).

Effect of Antioxidants on the H$_2$O$_2$-altered NFI/DNA Complexes—Antioxidant molecules prevented in vivo the action of oxidative stress on NFI/DNA binding. N-Acetylcysteine is a precursor of GSH and contributes to raising its level in hepatocytes (6). Pretreatment of H4 cell cultures with 5 mM N-acetylcysteine or 10 mM dithiothreitol prevented the effect of H$_2$O$_2$ (data not shown).

Effect of Intracellular GSH Content on NFI/DNA Binding—To focus on the role of thiols during oxidative stress, we induced GSH depletion with BSO (cf. Table I) in both H4 and HepG2 cells. An overnight treatment of H4 cell cultures with 5 mM BSO led to a dose-dependent alteration of NFI/DNA binding. The percentage decreases were 20, 33, and 64% at the doses 10, 20, and 50 μM, respectively (Fig. 7B, lanes 5–7). H$_2$O$_2$ alone had a similar effect (lane 3). We observed that a pretreatment of cells for 20 h by BSO strengthened the inhibition of DNA binding caused by H$_2$O$_2$. The BSO pretreatment effect occurred in a dose-dependent manner (Fig. 7B, lanes 1–3). With 50 μM BSO and 2 mM H$_2$O$_2$ (30 min), the formation of the protein-DNA complexes was almost totally blunted (under this particular condition, cells suffered a limited loss of viability of less than 20%). Thus, BSO-pretreated cells are more sensitive to H$_2$O$_2$, reflecting the predominance of a glutathione-based antioxidant mechanism. It is noticeable that among the four NFI complexes, the slowest migrating ones were the most sensitive to H$_2$O$_2$, since they disappeared first when the intensity of the oxidative treatment increased. This is consistent with experiments described above (Fig. 6C).

Differential Regulation of NFI and CP-1—To further investigate the specificity of the effects of oxidative stress on the DNA-binding abilities of NFI, we used the Duo probe (see “Experimental Procedures”) in EMSA experiments. This se-

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**FIG. 5. Effect of H$_2$O$_2$ on cell viability.** The irreversible cytotoxic effect of a 1-h H$_2$O$_2$ treatment on either H4 or HepG2 cells was assessed as described under “Experimental Procedures.” The proportion of viable cells after H$_2$O$_2$ treatment is expressed as a percentage of the value obtained with control cells. Results are expressed as mean ± S.E. (n = 5). No statistical difference with control is observed up to the 3 mM dose (p = 0.05). Results obtained after a 16-h treatment are similar (not shown).

**FIG. 6. Characterization of NFI-DNA complexes.** EMSAs were performed with H4 cell nuclear extracts and the NFI probe. A, competition experiment with a 40- or 80-fold excess of unlabeled NFI oligonucleotide. B, competition experiment with a 300-fold excess of unlabeled NFI or CP-1 oligonucleotide. C, supershift experiment with the anti-NFI C/CTF antibody. Before the addition of the NFI probe, nuclear extracts of control cells (lanes 1–3) were incubated or not incubated (lane 1) for 2 h at 4 °C with 1 μg of preimmune IgG (lane 2) or 1 μg of IgG from the anti-NFI C/CTF serum (lane 3). Lane 4 shows nuclear extracts of cells treated with 1.5 mM H$_2$O$_2$ for 1 h. NS, nonspecific complex; a, b, c, and d, specific NFI complexes; A, B, and C, supershifted complexes.
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Fig. 7. Effect of oxidative stress on NFI DNA binding. A, EMSA with HepG2 cell nuclear extracts and the NFI probe. Lane 1, control cell; lane 2, competition with a 100-fold excess of unlabeled NFI oligonucleotide; lane 3, control cell. B, EMSA with H4 cell nuclear extracts and the NFI probe. Lanes 1–3, cells treated with H2O2 (2 mM, 30 min) after pretreatment (20 h) by BSO concentrations of 50, 20, and 0 μM, respectively; lane 4, control (untreated cells); lanes 5–7, cells treated for 20 h by increasing BSO concentrations of 10, 20, and 50 μM, respectively.

Fig. 8. Differential regulation of NFI and CP-1 transcription factors by oxidative stress. EMSA performed with H4 cells nuclear extracts and the Duo probe containing adjacent NFI and CP-1 binding sites (see “Experimental Procedures”). Lanes 1–3, competition with unlabeled Duo (lane 1), CP-1 (lane 2), and NFI (lane 3) oligonucleotides (100-fold excess); lane 4, control; lane 5, cells treated with BSO (50 μM, 22 h); lane 6, cells pretreated with BSO (5 μM, 22 h) and treated with H2O2 (2 mM, 45 min). Dashes and arrows show NFI and CP-1 complexes, respectively.

Several proteins are revealed by the anti-NFI general antibody (Fig. 9, right panels). A 54-kDa protein appears to be the major isoform. An isoform displaying the same molecular weight is revealed by the anti-CTF antibody (Fig. 9, left panels). Two minor bands (50 and 67 kDa) correspond to the isoforms recognized by the anti-NFI X antibody (compare middle and right panels). Oxidative stress, generated by treatment with either 2 mM H2O2 for 30 min or 50 μM BSO for 20 h, did not affect the nuclear levels of any NFI isoforms (cf. the Anti NFI general panels). In particular, the major isoforms NFI C/CTF and NFI X showed stable levels (see left and middle panels). Furthermore, Western blots performed with total or cytosolic extracts showed no significant variations of the contents of NFI C/CTF and NFI X (data not shown).

Discussion

Most studies of gene regulation by oxidative stress have focused on induction of immediate early genes (8, 47) or phase II enzymes (9, 11, 46). Few studies have addressed the down-regulation of genes. CYP1A1 mRNAs have been shown to be under negative control by H2O2 in rat hepatocytes (6). Furthermore, these mRNAs are decreased in human hepatocytes by inflammatory cytokines (5, 48) known to generate reactive oxygen species within the cell (40, 41). We thus hypothesized that the cyp1A1 gene promoter could be negatively regulated by oxidative stress and that this may explain, at least partially, the biological effect of these cytokines. We show here that this is indeed the case and that the transcription factor NFI mediates this negative effect. The role of other transacting elements, such as the Ah pathway, in this redox regulation, although not excluded, seems unlikely. Indeed, the mutation that inhibits NFI binding to its cognate DNA sequence located in the proximal promoter totally abolishes the redox regulation on basal as well as TCDD-induced activity. In the mutated promoter, the fold induction by TCDD is decreased by half but remains potent. This inducibility is unimpaired by oxidative stress. This study establishes the role of NFI in gene regulation by reactive oxygen species, which could be relevant for the cyp1A1 gene promoter down-regulation by cytokines. We report...
that the cytokine TNFα down-regulates CYP1A1 expression through a redox mechanism involving NFI. This cytokine is known, at least in some cellular models, to elicit reactive oxygen species production within the cell (41). Furthermore, in hepatocytes, it has been reported to activate nitric-oxide synthase expression (49), which may cause endogenous oxidative stress. It can also lead to glutathione depletion (50).

In order to generate an oxidative stress, we used either H$_2$O$_2$, which diffuses through membranes, or BSO, which inhibits glutathione synthesis. The alteration of cellular mechanisms regulating the redox homeostasis, particularly through GSH, elicits an endogenous oxidative stress because the basal metabolism generates oxidant species (51). These endogenous species are essentially trapped by specific enzymes such as catalase, glutathione peroxidase, or superoxide dismutase. Depleting GSH thus leads to a modification of the intracellular redox status. We show here that a severe depletion of GSH following BSO treatment tends to mimic exogenous oxidative stress and down-regulate cyp1A1 gene promoter activity through NFI. Aminothiazole, which inhibits catalase, also down-regulates the NFI/TATA promoter. These experiments show that xenobiotics that are not oxidants themselves can induce oxidative stress and have the same consequence on NFI activity. This down-regulation elicited by different species such as H$_2$O$_2$, aminothiazole, BSO, or TNFα converge on a common target via a redox mechanism.

The ability of NFI to mediate a negative regulation by oxidative stress was not specific to the cyp1A1 gene promoter. It could also be observed in the context of the heterologous HSV-Tk promoter containing consensus NFI binding sites. Furthermore, EMSA experiments allowed us to show that oxidative stress induced in vitro by H$_2$O$_2$ or glutathione depletion leads to an alteration of NFI binding to its cognate DNA sequence. We could identify the 54-kDa NFI C/CTF as an isoform that is particularly sensitive to oxidative stress. It seems unlikely that this effect involves a regulation of the amounts of the NFI protein themselves. Indeed, Western blots showed that neither H$_2$O$_2$ nor BSO treatment of the cells was followed by a loss of the NFI proteins in the nuclear (and cytosolic) location, suggesting that oxidative stress alters the efficiency of NFI/DNA interaction. In addition, the pretreatment of cells with antioxidants prevents the effect of H$_2$O$_2$. Those data are in agreement with in vitro studies that correlated the efficiency of NFI binding to DNA with a reversible oxidation of strategic cysteines (26, 52). However, our data suggest that the alteration of the DNA binding only partially accounts for the repression of NFI transactivation function by oxidative stress. Indeed, the transactivating function of NFI is more sensitive to oxidative stress than its DNA binding activity. Submillimolar H$_2$O$_2$ concentrations (this work) as well as TNFα treatment (53) do not affect the binding, yet we show that they clearly inhibit the transcription driven by NFI. In the case of other ubiquitous transcription factors such as AP-1 or NF-kB, the effects of reactive oxygen species on their transactivating functions (which are increased) and on their DNA binding abilities (which are impaired by the oxidation of a strategic cysteine) are opposite (10, 54). As for NFI, both DNA binding and transactivating function are altered by oxidative stress, which makes this transcription factor particularly sensitive to changes of the redox status within the cell.

Reactive oxygen species play an important role in signal transduction (7, 55). Cysteine thiol groups (the main redox-sensitive moiety) may react differently to a wave of oxidative stress according to the protein conformation and to their own redox potentials. Thus, a change of the intracellular redox status may alter some transcription factors, while others are unaffected. In this study, we report that both the transactivating function and DNA binding of CP-1, a ubiquitous transcription factor, remained unaffected by oxidative conditions that altered NFI function. The CP-1 DNA-binding domain contains two cysteines, but their mutation to serines does not affect DNA binding (15), contrary to the mutations in NFI (26). When CP-1 and NFI compete for two close DNA binding sites, as in the human a-globin gene proximal promoter, we observed an interesting NFI/CP-1 redox switch. NFI and CP-1 are known to compete for the binding to at least another promoter sequence in the gene of the α1b adrenergic receptor (56). Furthermore, NFI and AP-2 (which is activated by oxidative stress (57)) compete for the binding to the promoter region of the human growth hormone gene (58). Thus, further investigations have to be undertaken with other promoter sequences containing both binding sites to assess the potential biological relevance of redox switches involving NFI.

The differential response of various transcription factors toward the imbalance of the redox state within the cell may be a new regulatory pathway for gene regulation. We report here that NFI mediates the human cyp1A1 gene inhibition by oxidative stress. Furthermore, the CP-1 transcription factor has been reported to have a negative regulatory function on this expression (4). Since we show here that it is insensitive to oxidative stress, this could further explain the strong inhibition of CYP1A1 by oxidative stress. NFI plays an important role in the regulation of many genes (see the Introduction). The down-regulation of its activity by redox mechanisms could have diverse physiological consequences. Interestingly, NFI has been shown to be a repressor of the expression of two enzymes (glutathione S-transferase and metallothionein) that are induced by oxidative stress (59).

As previously mentioned, cyp1A1 belongs to the Ah-inducible gene battery, which comprises other phase I and phase II drug-metabolizing enzymes. In all these genes, including cyp1A1, induction by TCDD is mediated by Ah receptor binding sites (xenobiotic-responsive elements) (2). Interestingly, four of the phase II genes of this battery (among them UDP glucuronosyltransferase, menadione oxidoreductase, and aldehyde dehydrogenase) have been reported to be up-regulated by oxidative stress through an electrophile response element/antioxidant-responsive element (60). In this respect, the regulation of cyp1A1 is opposite, and it is mediated by the NFI site present in its proximal promoter (besides, the cyp1A1 promoter does...
not contain any electrophile response element site). Such a negative regulation could be metabolically relevant, since several cytochromes P450 generate H₂O₂ as an effect of the reactions they catalyze (61). In the case of CYP1A1, it has been associated with oxidative DNA damage (62). It is thus conceivable that H₂O₂ generation could result in a retrocontrol loop limiting the expression or induction of this gene. An alternative mechanism could be suggested by a study showing that TCDD increases TNFα mRNA levels (63), which could also lead to CYP1A1 down-regulation.

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