Biochemical Pharmacology, Vol. 59, pp. 997–1005, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00406-2

Activation of Transcription Factors Activator Protein-1 and Nuclear Factor-κB by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

Alvaro Puga,† Sonya J. Barnes,* Ching-yi Chang,*‡ Huan Zhu,* Kenneth P. Nephew,§‖ Sohaib A. Khan§ and Howard G. Shertzer**

*Department of Environmental Health and §Department of Cell Biology, Neurobiology, and Anatomy, Center for Environmental Genetics, University of Cincinnati Medical Center, Cincinnati, OH, U.S.A.

ABSTRACT. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin), the prototype agonist of the aromatic hydrocarbon (Ah) receptor, is a potent tumor promoter as well as a complete liver carcinogen that produces an oxidative stress response in rodents and in cultured cell lines. It has been proposed that TCDD promotes neoplastic transformation through oxidative signal transduction pathways, which results in activation of immediate-early response transcription factors. To set the stage for a test of this hypothesis, we evaluated the effect of TCDD treatment on the activation of several transcription factors, including those in the nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) families, which are activated by changes in the redox state of cells. In an extension of prior results, we found that TCDD treatment produced a sustained overexpression of AP-1 for at least 72 hr in wild-type mouse hepatoma Hepa-1 cells, but not in the Ah receptor-deficient derivative *c*35 or in cytochrome P450-1A1 (CYP1A1)-negative *c*37 cells. In addition, TCDD treatment caused a significant increase in the DNA binding activity of NF-κB, but not in the activities of the other transcription factors tested. AP-1 and NF-κB activation were blocked by the thiol antioxidant *N*-acetylcysteine and by nordihydroguaiaretic acid, an antioxidant and lipooxygenase inhibitor and an inhibitor of the epoxygenase activity of CYP1A1, and did not take place in *c*35, *c*37, or in Ah nuclear translator-deficient *c*4 cells. Hence, sustained activation of these two transcription factors by TCDD is likely to result from a CYP1A1-dependent and Ah receptor complex-dependent oxidative signal. Electrophoretic mobility supershift analyses with specific antibodies showed that most of the increase in NF-κB binding activity could be accounted for by increases in p50/p50 complexes. Since these complexes are known to repress NF-κB-dependent gene transcription, our results delineate a second molecular mechanism, in addition to the recently found block of tumor necrosis factor-α-mediated p50/p65 activation, that may be responsible for the immunosuppresive effects of TCDD. BIOCHEM PHARMACOL **59**;8:997–1005, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. oxidative stress; Ah receptor; AP-1; NF-κB; dioxin; transcription

Polycyclic aromatic hydrocarbons including the polyhalogenated dibenzodioxins, dibenzofurans and biphenyls, form a group of environmental contaminants that are known or suspected carcinogens in animals and in humans [1–3]. Because of their slow rate of metabolic detoxification, these compounds have very long biological half-lives, and hence can produce biological effects long after the initial exposure [4]. TCDD,¶ a potent tumor promoter as well as a complete liver carcinogen [5–7], is the prototypic congener in this group. TCDD and related compounds bind to and activate the cytosolic Ah receptor, which subsequently migrates to the nucleus and forms heterodimers with the aromatic hydrocarbon nuclear translocator protein, ARNT. The AHR/ARNT complex functions as a transcription factor by binding to aromatic hydrocarbon response elements

† Corresponding author: Dr. A. Puga, Department of Environmental Health, Center for Environmental Genetics, University of Cincinnati Medical Center, Cincinnati, OH 45267-0056, U.S.A. Tel. (513) 558-0916; FAX (513) 558-0925; E-mail: Alvaro.Puga@UC.EDU

‡ Present Address: Department of Pharmacology, Duke University Medical Center, Durham, NC.

‖ Present Address: Department of Physiology and Biophysics, Indiana University, Bloomington, IN.

Received 17 June 1999; accepted 29 September 1999.

¶ *Abbreviations:* AHR, Ah receptor, aromatic hydrocarbon receptor; AP-1, activator protein-1; AP-2, activator protein-2; ARNT, aromatic hydrocarbon receptor nuclear translocator; CREB: cyclic AMP response element binding factor; CYP1A1, cytochrome P450-1A1; NAC, *N*-acetylcysteine; NDGA, nordihydroguaiaretic acid; NFI, nuclear factor I; Oct-1, octamer binding protein; NF-κB, nuclear factor-κB; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

(AhREs, also termed DREs and XREs) and inducing the expression of target genes [3]. Genes activated in this fashion include those encoding the cytochrome P450 monooxygenases CYP1A1, CYP1A2, and CYP1B1, and several phase II detoxification antioxidant enzymes [8, 9].

Changes in the cellular pro-oxidant status resulting from induction of cytochrome P450 monooxygenases may alter the activities of transcription factors involved in the oxidative stress response, such as NF-κB and AP-1, two factors modulated by alterations in the cellular redox state [10, 11]. NF-κB is rapidly activated by H_2O_2 treatment or by glutathione depletion, and activation is inhibited by antioxidants [12–15]. Paradoxically, NF-κB may also be activated by antioxidants such as *tert*-butylhydroquinone, butylated hydroxyanisole, and pyrrolidine dithiocarbamate. Activation can be blocked by antioxidant thiols, suggesting that these compounds in fact may also cause a pro-oxidative state in the cells that is responsible for the activation [10]. As a JUN–JUN homodimer or a JUN–FOS heterodimer, AP-1 binds to the regulatory regions of many genes responsive to environmental stimuli [16]. Induction of the *fos* and *jun* proto-oncogenes and activation of AP-1 are the result of many different types of stimuli that generate a change in cellular redox status, including phorbol esters, redox-cycling antioxidants, heat shock, and ionizing and non-ionizing radiation [10, 11, 17]. Clearly, NF-κB and AP-1 factors mediate to a large extent the cellular responses to environmental stimuli.

In female rats, TCDD produces an atypically large oxidative stress response characterized by enhanced hepatic lipid peroxidation and decreased hepatic membrane fluidity [1]. TCDD has been shown to cause CYP1A1-associated oxidative DNA damage in cultured mouse hepatoma cells [18] and in C57BL/6J mice [19]. In established tissue culture hepatoma cell lines, we have shown that TCDD exposure leads to an elevation of the pro-oxidant state of the treated cells [20], possibly as a result of induction of CYP1A1 and hydroxylation and epoxidation of arachidonic acid metabolites [21–25]. In fact, TCDD has been shown to induce the expression of cyclooxygenase-2, the inducible form of the rate-limiting enzyme in arachidonic acid peroxidation [26–28]. Hence, it is likely that TCDD-induced oxidative stress is responsible for the increased expression of c-*fos* and c-*jun* proto-oncogenes and for the concomitant increase in AP-1 DNA binding activity observed in TCDD-treated mouse hepatoma cells in culture [29,30].

Until recently, activation of AP-1 and NF-κB has been considered to be a sensitive biomarker of the oxidative stress response in higher eukaryotes [31]; however, it has become apparent that NF-κB can also be activated under non-oxidant conditions [32, 33]. To test the hypothesis that TCDD produces a sustained activation of AP-1 and NF-κB via a redox signal, we evaluated the DNA binding activities of these two transcription factors in mouse hepatoma cells in culture. Our results indicate that TCDD activates a CYP1A1- and AHR-dependent oxidative stress signal that results in the prolonged activation of NF-κB and AP-1.

MATERIALS AND METHODS

Cell Lines and Treatments

The mouse Hepa-1c1c7 cell line, hereafter referred to as Hepa-1, is a subclone of the Hepa-1 hepatoma cell line [34]. Three Hepa-1 mutant derivatives, a gift from O. Hankinson (UCLA), were used in these studies; *c35*, lacking Ah receptor protein expression; *c37*, containing a mutant *Cyp1a1* gene that codes for an inactive CYP1A1 enzyme; and *c4*, lacking ARNT expression [35]. Exponentially growing cells were cultured at 37° in alpha-minimal essential medium supplemented with 5% fetal bovine serum, 0.1% gentamicin, and 26 mM $NaHCO_3$. When indicated, cultures were treated with TCDD to a final concentration of 5 nM in DMSO, and control cultures, hereinafter referred to as *untreated*, were treated with an equivalent volume of DMSO vehicle (never to exceed 0.05% v/v). In some experiments, cells were also treated for 1 hr with 10 mM NAC brought to pH 7.1 immediately before use, or with 30 μM NDGA.

Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared according to procedures described previously [36, 37], with minor modifications. Nuclear extracts were obtained in a final volume of 100 μL buffer containing 2 mM EDTA, 2 mM dithiothreitol, 0.4 M KCl, 10% glycerol, and 25 mM HEPES, pH 7.9, at a protein concentration of 10–20 μg/μL. DNA binding reactions were performed in a 20-μL reaction volume with 10,000 dpm (approximately 0.1 ng) of double-stranded probe and 5–15 μg nuclear protein, in a buffer containing 1 mM EDTA, 1 mM dithiothreitol, 240 mM KCl, 10% glycerol, 1 μg poly(dI-dC)-poly(dI-dC) carrier, and 20 mM HEPES, pH 7.8. The transcription factors and their cognate nucleotide sequences that were used as probes are shown in Table 1. One strand of each complementary pair of oligonucleotides was end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and annealed to an excess of the unlabeled complementary oligonucleotide. Binding reactions were allowed to proceed for 20 min at room temperature and samples were loaded onto non-denaturing 4% polyacrylamide gels. Following electrophoresis at 200 V for 2–3 hr in 0.5X Tris–borate buffer, the gels were dehydrated and exposed to x-ray film. For antibody supershift assays, 2 μL of 1 μg/μL NF-κB/p65/RelA or 2 μL of NF-κB/p50 subunit antibodies (Santa Cruz Technologies) was added to the DNA binding reaction mixture and incubated at room temperature for an additional 20 min prior to electrophoresis. Quantitation was by phosphorimaging in a Storm 860 phosphorimager (Molecular Dynamics).

TABLE 1. Oligonucleotides used for electrophoretic mobility shift assays

Factor	Sequence	Reference
AP-1	5′-CTAGTGATGAGTCAGCCGGAT-3′	[77]
AP-2	5′-GATCGAACTGACCGCCCGCGGCCCGT-3′	[45]
AHR/ARNT	5′-TCCGGCTCTTCTCACGCAACTCCGAGCTCA-3′	[78]
CREB	5′-GATTGGCTGACGTCAGAGAGCT-3′	[79]
NFI	5′-ATTTTGGCTTGAAGCCAATATG-3′	[80]
NF-κB	5′-GATCGAGGGGACTTTCCCTAGC-3′	[31]
Oct-1	5′-GATCGAATGCAAATCACTAGCT-3′	[81]

RNA Isolation and Northern Blot Analysis

Cells were lysed directly in each culture dish with 3 mL TRI Reagent (Molecular Research Center), and the cell lysate was homogenized by passing through a pipette several times. To the homogenates 0.6 mL chloroform was added, and the mixture was shaken vigorously for 15 sec and centrifuged at 4500 *g* for 15 min. RNA remained in the aqueous phase, whereas DNA and protein partitioned to the interphase and organic phase. RNA was precipitated from the aqueous phase by mixing with 1.5 mL isopropanol, collected by centrifugation, and washed with 75% ethanol. Concentration and quality of the RNA preparations were confirmed by examination of the 28S and 18S ribosomal RNA species resolved by electrophoresis in agarose–formaldehyde gels.

For the Northern blot shown in Fig. 2, 40-μg aliquots of each RNA sample were separated by electrophoresis in 1% formaldehyde–agarose gels and transferred to nitrocellulose filters by capillary blotting. A probe of the 2.2-kb *PstI* fragment of mouse c-*jun* cDNA [29] (sp. act. ca. 2×10^9 cpm/μg) was prepared by random primer labeling (New England Nuclear) using [α-^{32}P]dCTP as the labeled precursor. Prehybridization, hybridization with denatured probe at a concentration of 1.5×10^6 cpm/mL, and washes were as previously described [37]. For autoradiographic detection, filters were exposed to x-ray film at −70° with intensifying screens. Quantitation was by phosphorimaging in a Storm 860.

RESULTS

Sustained Induction of Ap-1 Binding Activity by TCDD

Exposure of Hepa-1 cells to TCDD has previously been shown to induce transcription of c-*fos* and c-*jun* and to activate the DNA binding activity of AP-1 [29]. In those experiments, AP-1 activation was only measured within 2–4 hr after treatment, and it was never determined whether the effect of TCDD treatment would be prolonged beyond that time interval. It could be argued that if the effect of TCDD on AP-1 was short-lived, it would be less critical to the overall physiologic outcome of TCDD exposure than if the effect were sustained over a longer period of time. To determine whether TCDD treatment would induce elevated AP-1 levels over long periods of time, we used electrophoretic mobility shift assays from nuclear extracts of Hepa-1 cells treated with 5 nM TCDD for increasing lengths of time. Within 1 hr of treatment, the AP-1 DNA binding activity increased by more than 3-fold over untreated control cells, and within 6 hr after treatment the increase was almost 5-fold. Subsequently, AP-1 DNA binding activity remained at 3-fold higher levels than control for at least 72 hr (Fig. 1, A and B). Sustained AP-1 induction by TCDD was dependent on the presence of the Ah receptor and on the enzymatic activity of the CYP1A1 enzyme, since it was almost completely blocked in CYP1A1-negative *c*37 cells and did not occur in Ah receptor-negative *c*35 cells (Fig. 1C). Concomitant Northern blot hybridization experiments in Hepa-1 cells showed a significant dose-dependent increase in accumulation of c-jun mRNA 24 hr after TCDD treatment, reaching a 5-fold induction level over the untreated control (Fig. 2). Within this time frame, similar effects on the accumulation of c-fos mRNA were also observed (data not shown). These results indicate that TCDD can maintain high levels of AP-1 DNA binding activity and of expression of AP-1 components for extended periods of time and that the Ah receptor and CYP1A1 are required for this effect of TCDD.

Sustained Induction of NF-κB DNA Binding Activity in Hepa-1 Cells by TCDD

In addition to its role in tumor promotion and cellular differentiation, TCDD causes a plethora of other biological effects, including immunosuppression [38–41] and craniofacial developmental abnormalities [42–44], suggesting that TCDD may extensively interfere with the molecular mechanisms that regulate more than one single pathway of gene expression. To test this hypothesis and to determine whether TCDD treatment could alter the expression of transcription factors other than AP-1, we examined TCDD-treated Hepa-1 cells for effects on AP-2, CREB, NFI, NF-κB, and Oct-1, chosen for their established roles in immune system regulation and in craniofacial morphogenesis [45–49]. As a positive control, we analyzed the same Hepa-1 nuclear extracts for the activation of the AHR/ARNT transcription factor complex. When compared with nuclear extracts from vehicle-treated cells, only the DNA binding activities of AHR/ARNT and NF-κB showed statistically significant increases after treatment with TCDD, albeit with very different kinetics. In agree-

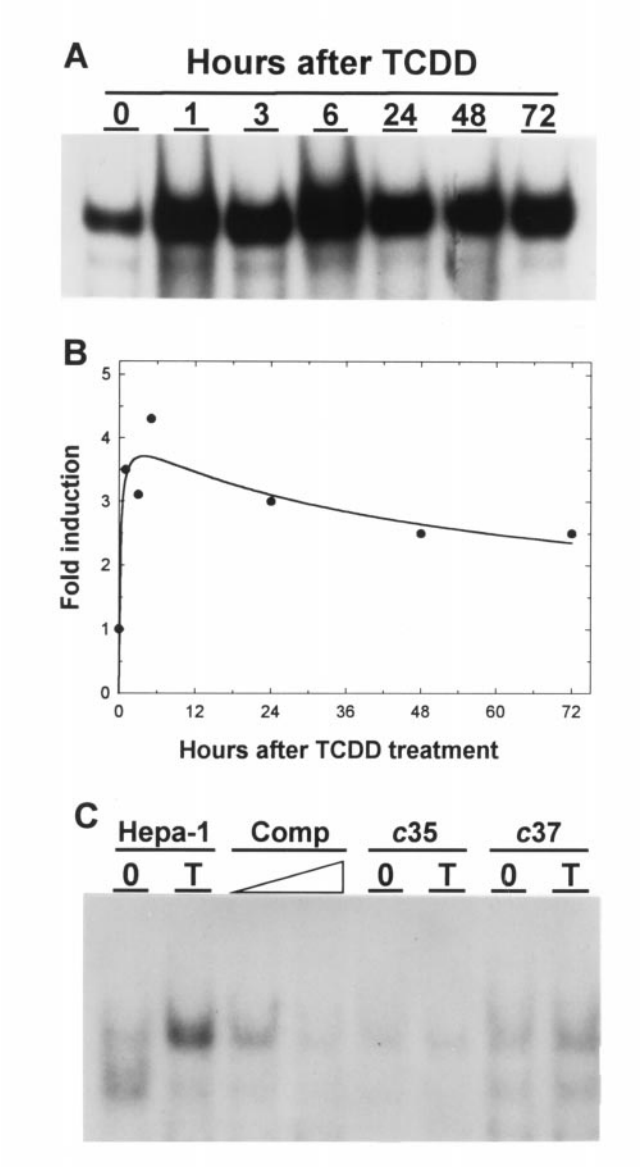

FIG. 1. Induction of AP-1 DNA binding activity by TCDD. (A) At the indicated times, Hepa-1 cells were treated with DMSO (0) or with 5 nM TCDD for various periods of time, and nuclear extracts from these cells were analyzed by electrophoretic mobility shift using a ^{32}P-labeled TPA response element oligonucleotide containing the AP-1 binding domain (Table 1). Time–course studies indicated that vehicle (DMSO) treatment had no effect on AP-1 binding activity. This experiment was independently repeated three times. (B) The data in (A) were quantitated and plotted relative to the time after TCDD addition. (C) Hepa-1, *c*35, and *c*37 cells were treated with 5 nM TCDD (T) or with DMSO vehicle (0) for 5 hr and analyzed as in (A). To determine probe specificity, binding to Hepa-1 extracts was competed with increasing amounts of unlabeled double-stranded TRE oligonucleotide (lanes labeled *Comp*).

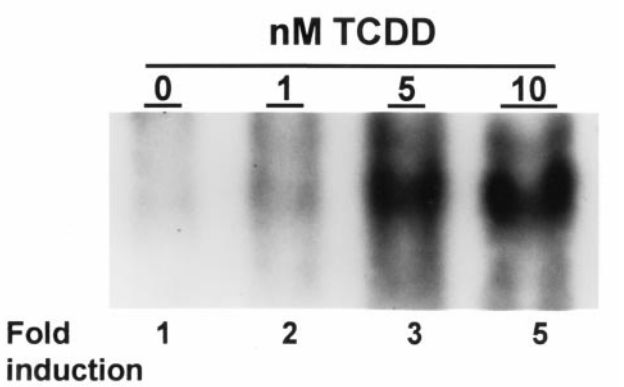

FIG. 2. Induction of c-Jun mRNA by TCDD. Hepa-1 cells were treated for 24 hr with TCDD in 0.05% DMSO at the indicated concentrations. Total RNA was extracted from these cells, fractionated in agarose–formaldehyde gels, and transferred and hybridized to a mouse c-jun probe as described in the Methods Section. Fold induction, determined by densitometry, is indicated below each lane.

ment with prior observations by other authors and by ourselves [50–52], AHR/ARNT activation increased by 40- to 50-fold within 2 hr after exposure and then decreased to a plateau level 3 times lower, which was maintained for at least 72 hr (Fig. 3). NF-κB activity levels, on the other hand, increased for a period of 10 hr to a level approximately 2-fold higher than in vehicle-treated cells, and this level was maintained or slightly decreased for the next 60–70 hr (Fig. 3). None of the other factors tested showed statistically significant changes due to TCDD treatment (Fig. 3).

The NF-κB complexes shown in Fig. 3 consisted of a single electrophoretic mobility species, in contrast with the more commonly found banding pattern of a doublet of complexes formed by the binding of p50/p50 and p50/p65(relA) dimers to the cognate binding site [53]. To determine whether TCDD activated the formation of p50/p50 or p50/p65 dimers, we used specific anti-p50 and anti-p65/relA antibodies in gel supershift assays. Positive control cells, treated with TPA, showed the formation of p50/p50 and p50/p65 dimers, both of which were supershifted by anti-p50 and anti-p65 antibodies, respectively (Fig. 4). In contrast, anti-p50, but not anti-p65/relA antibodies, supershifted practically all the NF-κB activity in vehicle-treated cells as well as in cells treated with TCDD for 2 or 5 hr. Residual complexes at the p50/p50 position seen after supershift with anti-p50 antibodies are likely to be p50/p50 homodimers, since they could be completely supershifted by larger amounts of antibody. We cannot rule out, however, the possibility that they are homodimers of cross-reacting p52 species. At the later time points, a trace of p50/p65 heterodimers could also be detected by the anti-p65 antibodies (Fig. 4). These results indicate that TCDD activates p50/p50 homodimers to a considerably larger extent than p50/p65 heterodimers.

Activation of NF-κB by TCDD did not take place in AHR-negative *c*35 cells, and was greatly ~diminished, particularly at the early time points, in *c*4 and *c*37 cells lacking functional ARNT and CYP1A1 proteins, respectively (Fig. 5). In contrast, TPA treatment showed a robust induction of high levels of NF-κB DNA binding activity in all three cell lines (Fig. 5), suggesting that the signaling

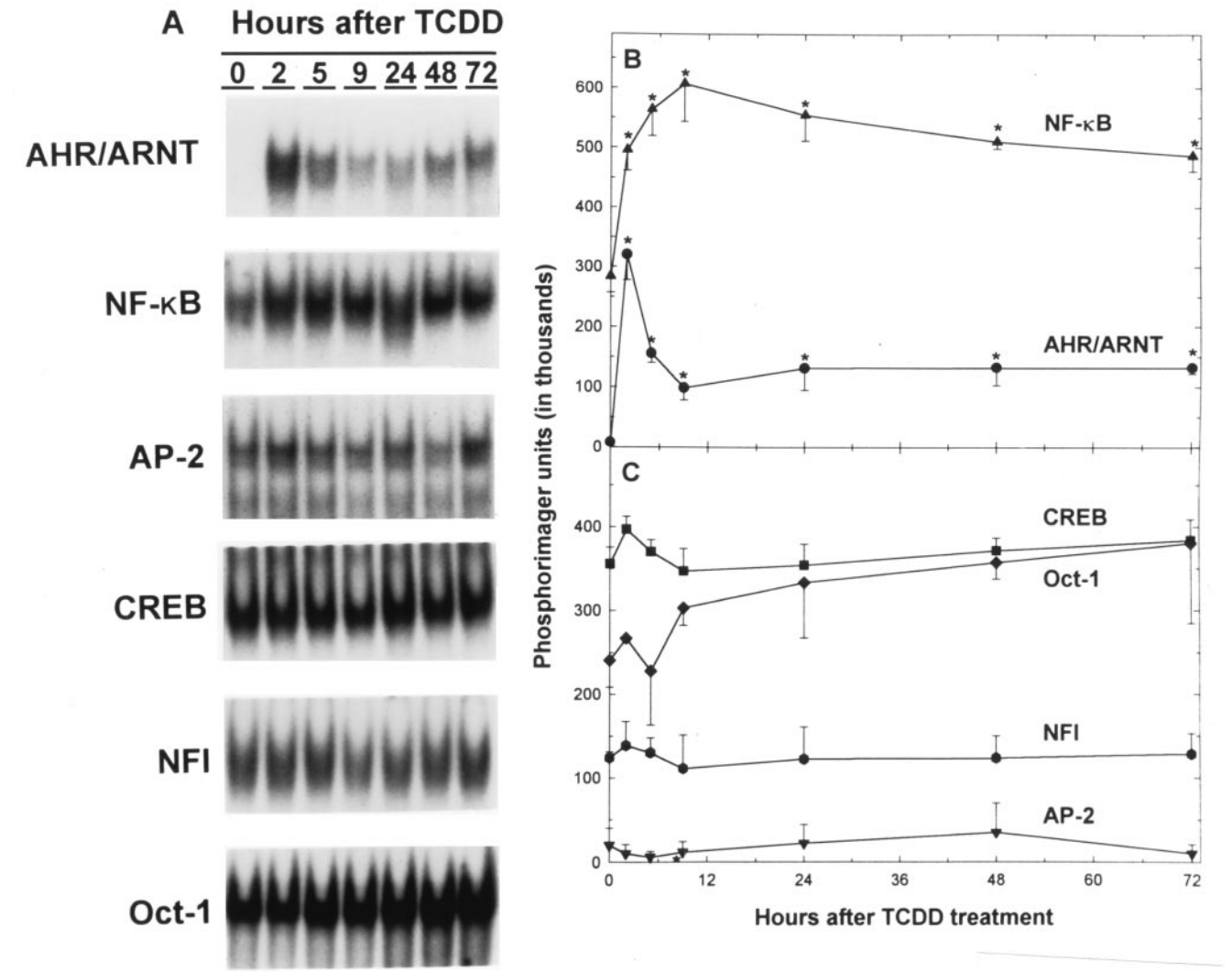

FIG. 3. Electrophoretic mobility shift analyses of several transcription factors in Hepa-1 cells treated with TCDD. (A) Nuclear extracts from Hepa-1 cells treated with 5 nM TCDD for the indicated lengths of time or with vehicle were assayed for DNA binding with probes for the transcription factors indicated to the left. The sequences of the oligonucleotide probes used are shown in Table 1. (B) The mobility shift analyses were repeated 3–4 times and quantitated by phosphorimaging. Phosphorimager units, representative of the number of radioactive counts bound to each transcription factor, are plotted as a function of the time after TCDD addition. *The asterisk denotes values significantly different ($P < 0.05$) from vehicle-treated controls.

mechanisms followed by the two inducers are fundamentally different.

TCDD-Induced AP-1 and NF-κB by Subtle Changes in Cellular Pro-oxidant Status

As described earlier, both AP-1 and NF-κB are transcription factors activated by oxidative stress. To determine whether TCDD caused a direct biochemical effect in Hepa-1 cells, we evaluated several parameters indicative of a sustained oxidative stress response. At doses of 1 and 10 nM, well into the range of full biological effects, TCDD was not cytotoxic and did not alter the thiol status of the cells, as evidenced by the absence of changes in intracellular GSH, cysteine, or protein thiol levels in TCDD-treated

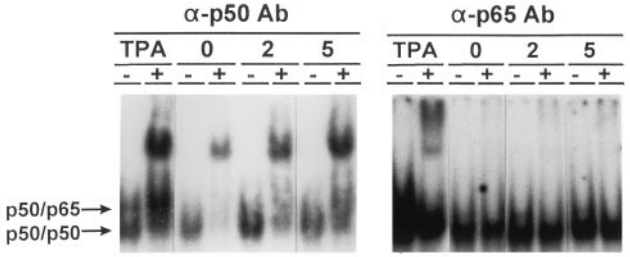

FIG. 4. Electrophoretic mobility supershift assays with specific anti-NF-κB antibodies. Hepa-1 cells were treated with 50 nM TPA for 4 hr or with 5 nM TCDD for the indicated lengths of time. Electrophoretic mobility shift assays with nuclear extracts from these cells used the NF-κB probe in Table 1 and were done in the presence (+) or absence (−) of 2 μg of anti-p50 or anti-p65 polyclonal antibodies. The *arrows* indicate the position of the p50/p50 and p50/p65 dimers.

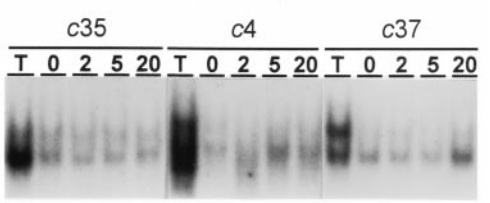

FIG. 5. Activation of NF-κB by TCDD in different Hepa-1 variant cell lines. *c*35 (AHR$^-$), *c*4 (ARNT$^-$), and *c*37 (CYP1A1$^-$) cells were treated for varying lengths of time with 0.05% DMSO (0) or 5 nM TCDD in 0.05% DMSO. As a control, cells were also treated with 50 nM TPA for 4 hr. Nuclear extracts from these cells were probed for NF-κB DNA binding activity with the same probe used in Figs. 3 and 4.

cells relative to untreated controls. In addition, no substantial increases of thiobarbituric acid-reactive species, indicative of lipid peroxidation, or of plasma membrane protein carbonyls were detectable in TCDD-treated cells (negative data not shown). These results led us to conclude that TCDD did not produce any gross alterations in the redox status of Hepa-1 cells. However, NAC, a thiol antioxidant and cysteine precursor, and NDGA, also an antioxidant and a strong inhibitor of arachidonic acid metabolism by lipooxygenases and epoxygenases, effectively prevented the activation of AP-1 and NF-κB by TCDD (Fig. 6), suggesting that TCDD causes a subtle oxidative stress response that can be blocked by thiol antioxidants and that is likely to be dependent on arachidonate metabolism.

DISCUSSION

In this report, we show that TCDD activates the DNA binding activities of NF-κB and AP-1, two immediate-early transcription factors whose expression is modulated by alteration in cellular redox state and strongly implicated in cellular responses to oxidative stress. In clear distinction to the effect of phorbol esters, activation of these two transcription factors by TCDD was sustained for as long as 72 hr, possibly due to the slow rate of TCDD detoxification, which results in a long biological half-life for this compound. Sustained TCDD-dependent activation of AP-1 and NF-κB requires functional AHR, ARNT, and CYP1A1 proteins, suggesting that a product of transcriptional upregulation by the Ah receptor complex, possibly CYP1A1 itself, is partly responsible for activation of these factors. The ultimate effector molecules are likely to be reactive oxygen species generated by the CYP1A1 monooxygenase activity and possibly also by cyclooxygenases (see below).

TCDD is known to induce enhanced hepatic lipid peroxidation in female rats [1] and oxidative DNA damage in cultured cells [18] and in mice [19]. In medaka fish, the DNA-damaging effects of TCDD are CYP1A1-dependent

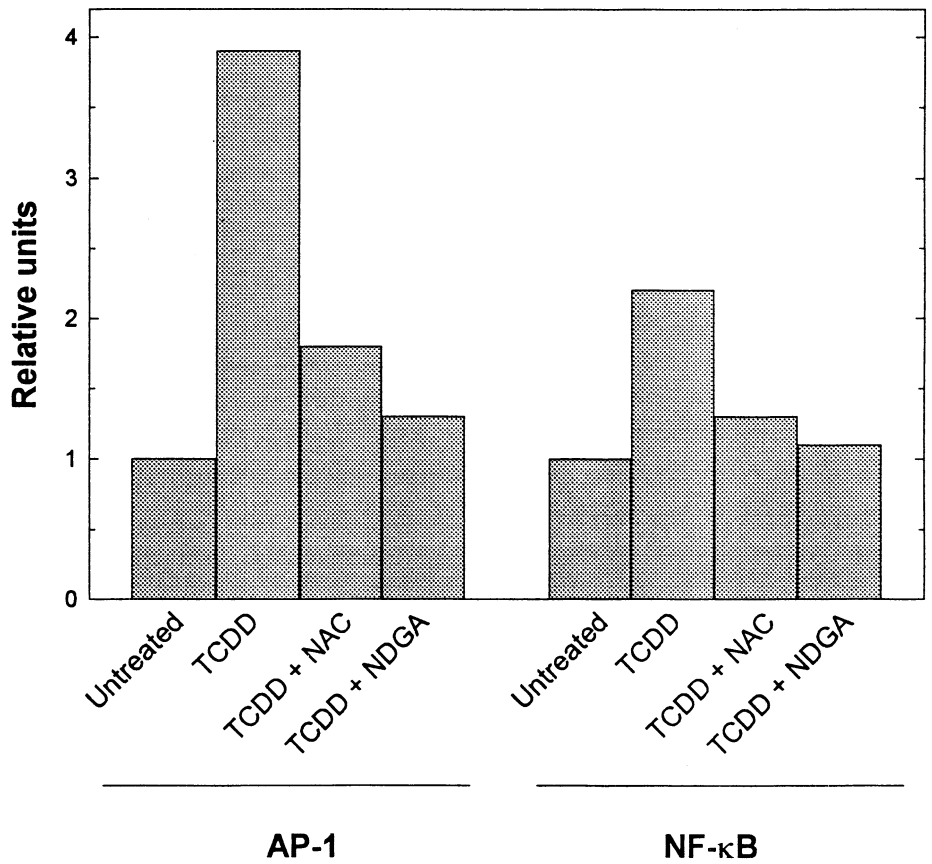

FIG. 6. The effect of NAC and NDGA on the activation of AP-1 and NF-κB by TCDD. Hepa-1 cells were pretreated with 10 mM NAC, with 30 μM NDGA, or with DMSO vehicle for 1 hr and then with 5 nM TCDD for 24 hr. Control cells were treated with vehicle throughout. Nuclear extracts from these cells were probed with AP-1 and NF-κB probes and the results were quantitated by densitometry. The histogram shows the fold-induction over the DMSO-treated control in arbitrary units.

and can be significantly blocked by treatment with the thiol antioxidant NAC [54, 55]. In our experiments, NAC strongly inhibited activation of AP-1 and NF-κB by TCDD, pointing to the possibility that the activation mechanism involves redox cycling at disulfide bonds. Although we did not observe a change in steady-state levels of reduced glutathione or total protein–cysteine mixed disulfides in TCDD-treated cells, we cannot exclude the possibility that specific proteins may be S-thiolated with cysteine or glutathione and may mediate an oxidative stress response responsible for transcription factor activation. Reactive cysteinyl residues, for example, are critical in bZIP and DNA binding of dimeric transcription factors, including FOS and JUN [56].

The antioxidant and arachidonate oxygenation inhibitor NDGA also strongly inhibited the TCDD-induced expression of NF-κB and AP-1, suggesting that the induction mechanism may also involve the TCDD-dependent elevation of the pro-oxidant status of the cell, possibly by the generation of arachidonic acid metabolites. Endogenous arachidonic acid, released from membrane phospholipids by TCDD-inducible phospholipase C [57], provides substrates for TCDD-inducible enzymes with peroxidase and epoxygenase activities, such as CYP1A1, or with dioxygenase activity, such as cyclooxygenase-2 [28, 58]. Monooxygenase and dioxygenase activities would ultimately tend to produce more reactive oxygen in cells and, in addition, TCDD-mediated induction of CYP1A1 epoxygenase may facilitate the hydroxylation and epoxidation of arachidonic acid metabolites into reactive epoxyeicosatrienoic acids, hydroxyeicosatetraenoic acids, and ω-hydroxy arachidonate and ω-1-hydroxy arachidonate products [21–24]. Metabolites produced by all these enzymatic routes induced by TCDD may produce secondary cellular responses, including ω-hydroxy fatty-generated peroxisomal proliferation, marked by the induction of acyl-CoA oxidase involved in peroxisomal β-oxidation [59].

Other investigators have observed the transient induction of NF-κB in the immature rat thymus by TCDD [60]. In our experiments in cultured Hepa-1 hepatocytes, induction was sustained for at least 3 days and principally involved the formation of nuclear p50/p50 homodimers, which are believed to be repressors of p50/p65-dependent transcription [61, 62]. In this context, Tian *et al.* have recently provided an elegant mechanistic explanation for the immunosuppressive effects of TCDD exposure. These authors have found that the activated Ah receptor forms protein complexes with the p65 subunit of NF-κB, but not with p50, and blocks tumor necrosis factor (TNF)-alpha-mediated activation of p50/p65 complexes and transcriptional activation of a NF-κB reporter gene [63]. Our results are not in conflict with these findings, but rather uncover a parallel mechanism to block NF-κB function. As a consequence of AHR activation by TCDD and of NF-κB by TNF-α, AHR and p50/p65 dissociate from HSP90 and NF-κB inhibitory factor, respectively, and allow AHR to sequester p65 in the cytoplasm [63], leaving an excess of p50 subunits free to interact with each other. Subsequent generation of reactive oxygen metabolites by AHR/ARNT-dependent gene activation would lead to higher levels of repressive p50/p50 homodimers, reinforcing the inhibition of NF-κB-dependent gene expression. On the other hand, p50/p50 homodimers do not always function as repressors, but in some tissues also function as inducers. In particular, p50/p50 complexes have been shown to induce transcription of the amyloid precursor protein in cerebellar neurons [64, 65]. Hence, the toxic effects of TCDD exposure might also include positive regulatory responses in some NF-κB-regulated genes.

NF-κB and AP-1 regulate the expression of genes which are critical for proliferative responses [66–70], immune responses [71, 72], and cell adhesion [73, 74], which in turn are of paramount importance in tumor cell growth and tumor metastasis [75, 76]. It is appealing to speculate that the oxidative stress response elicited by TCDD treatment, subtle yet sustained for a long period of time, leads to the persistent expression of these immediate-early response genes and hence to altered patterns of expression of the genes regulated by these factors. Such an effect might also be expected for other chemically and biologically recalcitrant environmental AHR agonists, such as the polyhalogenated dibenzodioxins, dibenzofurans, and biphenyls. A chronic change in expression of genes involved in cell cycle regulation may eventually increase cancer risk by altering the control of cell proliferation in the course of clonal expansion and metastasis.

We thank Oliver Hankinson (UCLA) for the gift of the Hepa-1 hepatoma variant cell lines. We also wish to acknowledge the excellent help of Cameron Q. Sheeler. This work was supported by NIH grants ES06273, P30-ES06096, and CA72039.

References

1. Stohs SJ, Oxidative stress induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *Free Radic Biol Med* **9:** 79–90, 1990.
2. Hébert CD, Harris MW, Elwell MR and Birnbaum LS, Relative toxicity and tumor-promoting ability of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PCDF), and 1,2,3,4,7,8-hexachlorodibenzofuran (HCDF) in hairless mice. *Toxicol Appl Pharmacol* **102:** 362–377, 1990.
3. Whitlock JP Jr, Mechanistic aspects of dioxin action. *Chem Res Toxicol* **6:** 754–763, 1993.
4. Pirkle JL, Wolfe WH, Patterson DG, Needham LL, Michalek JE, Miner JC, Peterson MR and Phillips DL, Estimates of the half-life of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in Vietnam veterans of Operation Ranch Hand. *J Toxicol Environ Health* **27:** 165–171, 1989.
5. Pitot HC, Goldsworthy T, Campbell HA and Poland A, Quantitative evaluation of the promotion by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin of hepatocarcinogenesis from diethylnitrosamine. *Cancer Res* **40:** 3616–3620, 1980.
6. Toth K, Somfai-Relle S, Sugar J and Bence J, Carcinogenicity testing of herbicide 2,4,5-trichlorophenoxyethanol containing dioxin and of pure dioxin in Swiss mice. *Nature* **27:** 548–549, 1979.

7. Kociba RJ, Keyes DG, Beyer JE, Carreon RM, Wade CE, Dittenber DA, Kalnins RP, Frauson LE, Park CN, Barnard SD, Hummel RA and Humiston CG, Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats. *Toxicol Appl Pharmacol* **46:** 279–303, 1978.
8. Liu RM, Vasiliou V, Zhu H, Duh JL, Tabor MW, Puga A, Nebert DW, Sainsbury M and Shertzer HG, Regulation of [*Ah*] gene battery enzymes and glutathione levels by 5,10-dihydroindeno[1,2,-*b*]indole in mouse hepatoma cell lines. *Carcinogenesis* **15:** 2347–2352, 1994.
9. Nebert DW, The *Ah* locus: Genetic differences in toxicity, cancer, mutation, and birth defects. *Crit Rev Toxicol* **20:** 153–174, 1989.
10. Pinkus R, Weiner LM and Daniel V, Role of oxidants and antioxidants in the induction of AP-1, NF-kappaB, and glutathione *S*-transferase gene expression. *J Biol Chem* **271:** 13422–13429, 1996.
11. Tissieres A, Georgopoulos C, Kullik I and Storz G, Transcriptional regulators of the oxidative stress response in prokaryotes and eukaryotes. *Redox Report* **1:** 23–29, 1994.
12. Staal FJ, Roederer M and Herzenberg LA, Intracellular thiols regulate activation of nuclear factor κB and transcription of human immunodeficiency virus. *Proc Natl Acad Sci USA* **87:** 9943–9947, 1990.
13. Scheck R, Rieber P and Baeuerle PA, Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO J* **10:** 2247–2258, 1991.
14. Toledano MB and Leonard WJ, Modulation of transcription factor NF-κB binding activity by oxidation-reduction *in vitro*. *Proc Natl Acad Sci USA* **88:** 4328–4332, 1991.
15. Schreck R, Meier B, Mannel DN, Droge W and Baeuerle PA, Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. *J Exp Med* **175:** 1181–1194, 1992.
16. Karin M, The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* **270:** 16483–16486, 1995.
17. Choi HS and Moore DD, Induction of c-*fos* and c-*jun* gene expression by phenolic antioxidants. *Mol Endocrinol* **7:** 1596–1602, 1993.
18. Park JY, Shigenaga MK and Ames BN, Induction of cytochrome P4501A1 by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or indolo(3,2-*b*)carbazole is associated with oxidative DNA damage. *Proc Natl Acad Sci USA* **19:** 2322–2327, 1996.
19. Shertzer HG, Nebert DW, Puga A, Ary M, Sonntag D, Dixon K, Robinson LJ, Cianciolo E and Dalton TP, TCDD causes a sustained oxidative stress response in C57BL/6J mice. *Biochem Biophys Res Commun* **253:** 44–48, 1998.
20. Yao Y, Hoffer A, Chang CY and Puga A, Dioxin activates HIV-1 gene expression by an oxidative stress pathway requiring a functional cytochrome P450 CYP1A1 enzyme. *Environ Health Perspect* **103:** 366–371, 1995.
21. Capdevila J, Gil L, Orellana M, Marnett LJ, Mason JI, Yadagiri P and Falck JR, Inhibitors of cytochrome P-450-dependent arachidonic acid metabolism. *Arch Biochem Biophys* **261:** 257–263, 1988.
22. Capdevila J, Falck JR and Estabrook RW, Cytochrome P450 and the arachidonate cascade. *FASEB J* **6:** 731–736, 1992.
23. Rifkind AB, Gannon M and Gross SS, Arachidonic acid metabolism by dioxin-induced cytochrome P-450: A new hypothesis on the role of P-450 in dioxin toxicity. *Biochem Biophys Res Commun* **172:** 1180–1188, 1990.
24. Nakai K, Ward AM, Gannon M and Rifkind AB, β-Naphthoflavone induction of a cytochrome P-450 arachidonic acid epoxygenase in chick embryo liver distinct from the aryl hydrocarbon hydroxylase and from phenobarbital-induced arachidonate epoxygenase. *J Biol Chem* **267:** 19503–19512, 1992.
25. Lee CA, Lawrence BP, Kerkvliet NI and Rifkind AB, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin induction of cytochrome P450-dependent arachidonic acid metabolism in mouse liver microsomes: Evidence for species-specific differences in responses. *Toxicol Appl Pharmacol* **153:** 1–11, 1998.
26. Puga A, Bohm J, Hoffer A, Leikauf GD, Shertzer HG and Zhou S, Dioxin alters calcium homeostasis and the regulation of arachidonate metabolism in mouse hepatoma cells. *Proc 15th Int Symp Chlor Dioxins* **25:** 381–386, 1995.
27. Puga A, Hoffer A, Zhou S, Bohm JM, Leikauf GD and Shertzer HG, Sustained increase in intracellular free calcium and activation of cyclooxygenase-2 expression in mouse hepatoma cells treated with dioxin. *Biochem Pharmacol* **54:** 1287–1296, 1997.
28. Kraemer SA, Arthur KA, Denison MS, Smith WL and DeWitt DL, Regulation of prostaglandin endoperoxide H synthase-2 expression by 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin. *Arch Biochem Biophys* **330:** 319–328, 1996.
29. Puga A, Nebert DW and Carrier F, Dioxin induces expression of c-*fos* and c-*jun* proto-oncogenes and a large increase in transcription factor AP-1. *DNA Cell Biol* **11:** 269–281, 1992.
30. Hoffer A, Chang CY and Puga A, Dioxin induces *fos* and *jun* gene expression by Ah receptor dependent- and independent-pathways. *Toxicol Appl Pharmacol* **141:** 238–247, 1996.
31. Schreck R, Albermann K and Baeuerle PA, Nuclear factor κB: An oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radic Res Commun* **17:** 221–237, 1992.
32. Krejsa CM, Nadler SG, Esselstyn JM, Kavanagh TJ, Ledbetter JA and Schieven GL, Role of oxidative stress in the action of vanadium phosphotyrosine phosphatase inhibitors. Redox independent activation of NF-kappaB. *J Biol Chem* **272:** 11541–11549, 1997.
33. Piette J, Piret B, Bonizzi G, Schoonbroodt S, Merville MP, Legrand-Poels S and Bours V, Multiple redox regulation in NF-κB transcription factor activation. *Biol Chem* **378:** 1237–1245, 1997.
34. Bernard HP, Darlington GJ and Ruddle FH, Expression of liver phenotypes in cultured mouse hepatoma cells: Synthesis and secretion of serum albumin. *Dev Biol* **35:** 83–96, 1973.
35. Hankinson O, The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* **35:** 307–340, 1995.
36. Dignam JD, Lebovitz RM and Roeder RG, Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11:** 1475–1489, 1983.
37. Carrier F, Owens RA, Nebert DW and Puga A, Dioxin-dependent activation of murine *Cyp1a-1* transcription requires protein kinase C-dependent phosphorylation. *Mol Cell Biol* **12:** 1856–1863, 1992.
38. Kerkvliet NI, Immunological effects of chlorinated dibenzo-*p*-dioxins. *Environ Health Perspect* **103**(Suppl 9): 47–53, 1995.
39. Yamaguchi K, Matulka RA, Shneider AM, Toselli P, Trombino AF, Yang S, Hafer LJ, Mann KK, Tao XJ, Tilly JL, Near RI and Sherr DH, Induction of PreB cell apoptosis by 7,12-dimethylbenz[*a*]anthracene in long-term primary murine bone marrow cultures. *Toxicol Appl Pharmacol* **147:** 190–203, 1997.
40. Morris DL, Jeong HG, Jordan SD, Kaminski NE and Holsapple MP, Characterization of the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin B6C3F1 and DBA/2 mice following single and repeated exposures. *Arch Toxicol* **72:** 157–168, 1998.
41. Sulentic CE, Holsapple MP and Kaminski NE, Aryl hydrocarbon receptor-dependent suppression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin of IgM secretion in activated B cells. *Mol Pharmacol* **53:** 623–629, 1998.

42. Abbott BD, Perdew GH and Birnbaum LS, Ah receptor in embryonic mouse palate and effects of TCDD on receptor expression. *Toxicol Appl Pharmacol* **126:** 16–25, 1994.
43. Birnbaum LS, The mechanism of dioxin toxicity: Relationship to risk assessment. *Environ Health Perspect* **102**(Suppl 9): 157–67, 1994.
44. Birnbaum LS, Developmental effects of dioxins. *Environ Health Perspect* **103**(Suppl 7): 89–94, 1995.
45. Imagawa M, Chiu R and Karin M, Transcription factor AP-2 mediates induction by two different signal-transduction pathways: Protein kinase C and cAMP. *Cell* **51:** 251–260, 1987.
46. Kemler I, Schreiber E, Müller MM, Matthias P and Schaffner W, Octamer transcription factors bind to two different sequence motifs of the immunoglobulin heavy chain promoter. *EMBO J* **8:** 2001–2008, 1989.
47. Kemler I and Schaffner W, Octamer transcription factors and the cell type-specificity of immunoglobulin gene expression. *FASEB J* **4:** 1444–1449, 1990.
48. Liou HC and Baltimore D, Regulation of the NF-κB/rel transcription factor and IκB inhibitor system. *Curr Opin Cell Biol* **5:** 477–487, 1993.
49. Wanner R, Zhang J, Henz BM and Rosenbach T, AP-2 gene expression and modulation by retinoic acid during keratinocyte differentiation. *Biochem Biophys Res Commun* **223:** 666–669, 1996.
50. Pollenz RS, The aryl-hydrocarbon receptor, but not the aryl-hydrocarbon receptor nuclear translocator protein, is rapidly depleted in hepatic and non-hepatic culture cells exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Mol Pharmacol* **49:** 391–398, 1996.
51. Roman BL, Pollenz RS and Peterson RE, Responsiveness of the adult male rat reproductive tract to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure: Ah receptor and ARNT expression, CYP1A1 induction, and Ah receptor down-regulation. *Toxicol Appl Pharmacol* **150:** 228–239, 1998.
52. Chang CY and Puga A, Constitutive activation of the aromatic hydrocarbon receptor. *Mol Cell Biol* **18:** 525–535, 1998.
53. Xie QW, Kashiwabara Y and Nathan C, Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem* **269:** 4705–4708, 1994.
54. Cantrell SM, Lutz LH, Tillitt DE and Hannink M, Embryotoxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD): The embryonic vasculature is a physiological target for TCDD-induced DNA damage and apoptotic cell death in Medaka (Orizias latipes). *Toxicol Appl Pharmacol* **141:** 23–34, 1996.
55. Cantrell SM, Joy-Schlezinger J, Stegeman JJ, Tillitt DE and Hannink M, Correlation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced apoptotic cell death in the embryonic vasculature with embryotoxicity. *Toxicol Appl Pharmacol* **148:** 24–34, 1998.
56. Xanthoudakis S, Miao GG and Curran T, The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains. *Proc Natl Acad Sci USA* **91:** 23–27, 1994.
57. Beebe L, Park SS and Anderson LM, Differential enzyme induction of mouse liver and lung following a single low or high dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *J Biochem Toxicol* **5:** 211–219, 1990.
58. Puga A, Hoffer A, Zhou S, Bohm JM, Leikauf GD and Shertzer HG, Sustained increase in intracellular free calcium and activation of cyclooxygenase-2 expression in mouse. *Biochem Pharmacol* **54:** 1287–1296, 1997.
59. Ortiz de Montellano PR, Chan WK, Tuck SF, Kaikaus RM, Bass NM and Peterson JA, Mechanism-based probes of the topology and function of fatty acid hydroxylases. *FASEB J* **6:** 695–699, 1992.
60. Olnes MJ, Verma M and Kurl RN, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-mediated gene expression in the immature rat thymus. *Exp Clin Immunogenet* **11:** 102–109, 1994.
61. Kang SM, Tran AC, Grilli M and Lenardo MJ, NF-kappa B subunit regulation in nontransformed CD4+ T lymphocytes. *Science* **256:** 1452–1456, 1992.
62. Franzoso G, Bours V, Park S, Tomita-Yamaguchi M, Kelly K and Siebenlist U, The candidate oncoprotein Bcl-3 is an antagonist of p50/NF-kappa B-mediated inhibition. *Nature* **359:** 339–342, 1992.
63. Tian Y, Ke S, Denison MS, Rabson AB and Gallo MA, Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity. *J Biol Chem* **274:** 510–515, 1999.
64. Grilli M, Goffi F, Memo M and Spano PF, Interleukin-1β and glutamate activate the NF-κB/Rel binding site from the regulatory region of the amyloid precursor protein gene in primary neuronal cultures. *J Biol Chem* **271:** 15002–15007, 1996.
65. Grilli M and Memo M, Nuclear factor-κB/Rel proteins: A point of convergence of signaling pathways relevant in neuronal function and dysfunction. *Biochem Pharmacol* **57:** 1–7, 1998.
66. Curran T and Franza BR Jr, Fos and Jun: the AP-1 connection. *Cell* **55:** 395–397, 1988.
67. Cohen DR and Curran T, The structure and function of the fos proto-oncogene. *Crit Rev Oncog* **1:** 65–88, 1989.
68. Bravo R, Growth factor-responsive genes in fibroblasts. *Cell Growth Differ* **1:** 305–309, 1990.
69. Morgan JI and Curran T, Proto-oncogene transcription factors and epilepsy. *Trends Pharmacol Sci* **12:** 343–349, 1991.
70. Holbrook NJ and Fornace AJ Jr, Response to adversity: Molecular control of gene activation following genotoxic stress. *New Biol* **3:** 825–833, 1991.
71. Schulze-Osthoff K, Los M and Baeuerle PA, Redox signalling by transcription factors NF-κB and AP-1 in lymphocytes. *Biochem Pharmacol* **50:** 735–741, 1995.
72. Stein B, Baldwin AS Jr, Ballard DW, Greene WC, Angel P and Herrlich P, Cross-coupling of the NF-kappa B p65 and Fos/Jun transcription factors produces potentiated biological function. *EMBO J* **12:** 3879–3891, 1993.
73. Bereta J, Cohen MC and Bereta M, Stimulatory effect of ouabain on VCAM-1 and iNOS expression in murine endothelial cells: Involvement of NF-kappa B. *FEBS Lett* **377:** 21–25, 1995.
74. Gille J, Swerlick RA, Lawley TJ and Caughman SW, Differential regulation of vascular cell adhesion molecule-1 gene transcription by tumor necrosis factor alpha and interleukin-1 alpha in dermal microvascular endothelial cells. *Blood* **87:** 211–217, 1996.
75. Albelda SM and Buck CA, Integrins and other cell adhesion molecules. *FASEB J* **4:** 2868–2880, 1990.
76. Hynes RO, Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* **69:** 11–25, 1992.
77. Lee W, Mitchell P and Tjian R, Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* **49:** 741–752, 1987.
78. Reyes H, Reiz-Porszasz S and Hankinson O, Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science* **256:** 1193–1195, 1992.
79. Sheng M, Thompson MA and Greenberg ME, CREB: A Ca^{2+}-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* **252:** 1427–1430, 1991.
80. Borgmeyer U, Nowock J and Sippel AE, The TGGCA-binding protein: A eukaryotic nuclear protein recognizing a symmetrical sequence on double-stranded linear DNA. *Nucleic Acids Res* **12:** 4295–4311, 1984.
81. Verrijzer CP, Kal AJ and van der Vliet PC, The oct-1 homeo domain contacts only part of the octamer sequence and full oct-1 DNA-binding activity requires the POU-specific domain. *Genes Dev* **4:** 1964–1974, 1990.