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H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor

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We show that AP-1 is an antioxidant-responsive transcription factor. DNA binding and transactivation by AP-1 were induced in HeLa cells upon treatment with the antioxidants pyrrolidine dithiocarbamate (PDTC) and *N*-acetyl-L-cysteine (NAC), and upon transient expression of the antioxidative enzyme thioredoxin. While PDTC and NAC enhanced DNA binding and transactivation of AP-1 in response to phorbol ester, the oxidant H_2O_2 suppressed phorbol ester activation of the factor. H_2O_2 on its own was only a weak inducer of AP-1. Activation of AP-1 by PDTC was dependent on protein synthesis and involved transcriptional induction of c-*jun* and c-*fos* genes. Transcriptional activation of c-*fos* by PDTC was conferred by the serum response element, suggesting that serum response factor and associated proteins function as primary antioxidant-responsive transcription factors. In the same cell line, the oxidative stress-responsive transcription factor NF-κB behaved in a manner strikingly opposite to AP-1. DNA binding and transactivation by NF-κB were strongly activated by H_2O_2, while the antioxidants alone were ineffective. H_2O_2 potentiated the activation of NF-κB by phorbol ester, while PDTC and NAC suppressed PMA activation of the factor. PDTC did not influence protein kinase C (PKC) activity and PKC activation by PMA, indicating that the antioxidant acted downstream of and independently from PKC.

Key words: AP-1/H_2O_2/NF-κB/SRE/thioredoxin

Introduction

Eukaryotic cells continuously produce reactive oxygen intermediates (ROIs) as side products of electron transfer reactions (reviewed in Halliwell and Gutteridge, 1989). Major ROI species are H_2O_2, superoxide (O_2^-), and hydroxyl radicals ($OH\cdot$). Intracellular levels of ROIs become unphysiologically low in the absence of oxygen, for instance during ischemia. This condition is called hypoxia or anoxia. Above-normal levels of ROIs are referred to as oxidative stress (reviewed in Sies, 1991). This condition occurs frequently in cells exposed to UV light, γ rays or low concentrations of H_2O_2, but also upon stimulation of cells with cytokines and other natural ligands for cell surface receptors (reviewed in Schreck *et al.*, 1992a). Only high levels of ROIs, as produced by stimulated neutrophils, are strictly cytocidal as they cause irreversible damage to DNA, proteins and lipids (reviewed in Baggiolini and Wyman, 1990). Such concentrations of ROIs serve primarily to kill parasites in the organism.

The intracellular concentrations of ROIs seem to be finely tuned. Evidence for regulation of ROI homeostasis comes from the observation that reperfusion of hypoxic tissue leads to severe oxidative damage (reviewed in Korthuis and Granger, 1986; Zweier *et al.*, 1988). Hypoxia apparently results in downregulation of enzymes and antioxidative metabolites involved in controlling ROI levels. A hypoxic state can also be induced by exposure of cells to antioxidants. Diverse antioxidants were shown to induce expression of glutathione S-transferase (GST) Ya subunit and NAD(P)H:quinone reductase (NQO_1) genes (Rushmore *et al.*, 1991; Li and Jaiswal, 1992a), depending on *cis*-acting antioxidant response elements (AREs).

Oxidative stress triggers reactions counteracting ROIs and ROI-induced damage. These involve induction of enzymes with radical scavenging and repair activities. The oxidative stress response is well studied in bacteria. Two ROI-responsive transcription factor systems called oxyR (reviewed in Storz *et al.*, 1990) and soxRS (reviewed in Demple, 1991) have been investigated in detail. They control the expression of multiple antioxidative enzymes in response to H_2O_2 and O_2^-, respectively. The mechanisms and factors regulating oxidant and antioxidant responses in eukaryotic cells are poorly understood.

One eukaryotic inducible transcription factor activated by H_2O_2 treatment of cultured cells is nuclear factor κB (NF-κB) (Schreck *et al.*, 1991). NF-κB binds DNA as a heterodimer composed of structurally related DNA-binding subunits (for reviews see Baeuerle, 1991; Blank *et al.*, 1992; Nolan and Baltimore, 1992). Activation of NF-κB in response to extracellular signals involves release of the inhibitory subunit IκB from a cytoplasmic complex with the heterodimer (Baeuerle and Baltimore, 1988). An extreme variety of other agents can activate NF-κB, including phorbol esters, inflammatory cytokines, UV light, γ rays, antibodies to cell surface receptors, viral and bacterial proteins, lipopolysaccharide, double-stranded RNA and reduced protein synthesis (reviewed in Baeuerle, 1991). Most if not all inducers of NF-κB seem to rely on the production of ROIs, as is evident from the inhibitory effect of antioxidants on induction of NF-κB by all inducers tested so far. Butyl peroxide, like H_2O_2, activates NF-κB, but agents leading to the production of O_2^- are not effective (reviewed in Schreck *et al.*, 1992a). With respect to ROI specificity, NF-κB is thus more closely related to the bacterial oxyR than to the soxRS system. Cysteine and derivatives (Staal *et al.*, 1990; Mihm *et al.*, 1991; Schreck *et al.*, 1991), metal

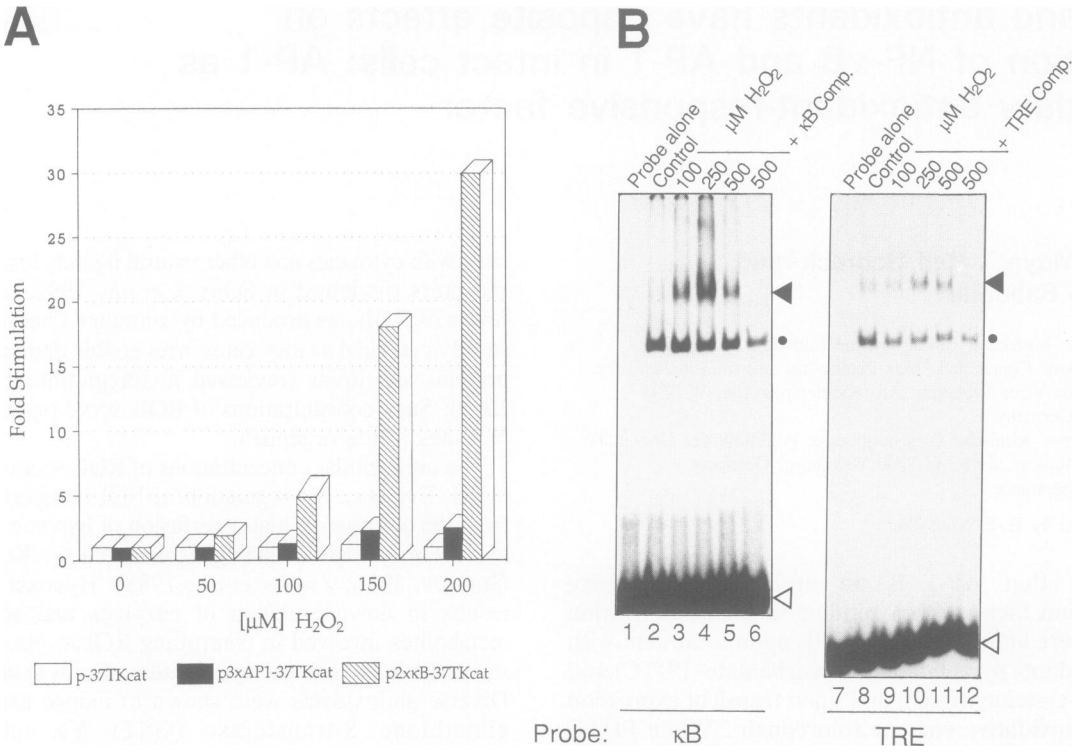

Fig. 1. The effects of H_2O_2 on the activities of NF-$\varkappa$B and AP-1 in HeLa cells. (**A**) The effect of H_2O_2 on $\varkappa$B- and TRE-dependent gene expression. HeLa cells transfected with a basal (p-37TKcat; open columns), a $\varkappa$B-dependent (p2x$\varkappa$B-37TKcat; hatched columns) or a TRE-dependent reporter CAT construct (p3xAP1-37TKcat; black columns) were treated with the indicated concentrations of H_2O_2 for 8–12 h prior to harvesting. The amounts of CAT protein were determined by ELISA. (**B**) The effect of H_2O_2 on the DNA-binding activities of AP-1 and NF-$\varkappa$B. Nuclear extracts were prepared from HeLa cells treated for 90 min with the indicated amounts of H_2O_2 and incubated with ^{32}P-labeled oligonucleotides encompassing NF-$\varkappa$B (lanes 1–6) or AP-1 consensus motifs (lanes 7–12) followed by analysis with EMSA. In lanes 6 and 12, a 100-fold molar excess of unlabeled specific oligonucleotide was added to the binding reactions. Fluorographs of native gels are shown. Filled arrowheads indicate the positions of specific complexes and black dots the positions of non-specific complexes. The open arrowheads mark the positions of uncomplexed DNA probes.

chelators and dithiocarbamates (Schreck *et al.*, 1992b), vitamin E and quinone derivatives (Israel *et al.*, 1992) and α-lipoic acid (Suzuki *et al.*, 1993) were shown to suppress activation of NF-$\varkappa$B in response to diverse stimuli. We have therefore proposed that ROIs serve as common messengers in the activation of NF-$\varkappa$B (Schreck *et al.*, 1991; Schreck and Baeuerle, 1991), and that NF-$\varkappa$B is primarily an oxidative stress-responsive transcription factor (Schreck *et al.*, 1992a).

In cell cultures, micromolar amounts of pyrrolidine dithiocarbamate (PDTC) and related compounds are potent inhibitors of NF-$\varkappa$B activation (Schreck *et al.*, 1992a,b). The antioxidative effect of dithiocarbamates might on one hand rely on their metal-chelating properties (Bartoli *et al.*, 1983, and references therein), as chelation of free iron and copper is considered to be an important protective mechanism against oxidants (reviewed in Halliwell and Gutteridge, 1986). On the other hand, dithiocarbamates can act directly as free radical scavengers (Zanocco *et al.*, 1989, and references therein). Another inhibitor of NF-$\varkappa$B activation is the cysteine derivative and glutathione (GSH) precursor *N*-acetyl-L-cysteine (NAC; Staal *et al.*, 1990; Schreck *et al.*, 1991). However, in cell culture experiments, high concentrations of NAC in the millimolar range are required to observe inhibitory effects on NF-$\varkappa$B activation.

The DNA binding of the transcription factor AP-1 is weakly responsive to H_2O_2 treatment of cells (Devary *et al.*, 1991). Induction of AP-1, a heterodimer of the Jun and Fos proteins, relies primarily on novel synthesis of the two DNA-binding subunits, which is controlled by pre-existing (called here 'primary') transcription factors, including serum response factor (SRF), CREB and c-Jun homodimers (for a recent review, see Karin, 1991). The weak induction of AP-1 DNA binding by H_2O_2 is in apparent contrast to the strong induction of the c-*jun* and c-*fos* mRNAs by H_2O_2 (Crawford *et al.*, 1988; Shibanuma *et al.*, 1988; Devary *et al.*, 1991; Nose *et al.*, 1991; Amstad *et al.*, 1992). In the case of c-*fos*, mRNA induction by H_2O_2 and UV light relies on the serum response element (SRE) (Stein *et al.*, 1989; Nose *et al.*, 1991; Amstad *et al.*, 1992). In the case of c-*jun*, Devary *et al.* (1991) suggested an involvement of the AP-1 binding site (TRE) in H_2O_2 induction. Also in lower eukrayotes, AP-1-like proteins are involved in responses to ROIs. There is genetic evidence that the PAR1 gene product, a c-Jun homologue, is involved in oxygen metabolism in yeast (Schnell *et al.*, 1992).

Both NF-$\varkappa$B and AP-1 are activated upon treatment of cells with the PKC activator phorbol 12-myristate 13-acetate (PMA, also called TPA). The activation of NF-$\varkappa$B by PMA has been shown to be suppressed by antioxidants, suggesting that ROIs produced in response to PKC activation are necessary (Schreck *et al.*, 1991, 1992b). In the case of AP-1, PMA is thought to induce a nuclear protein phosphatase which dephosphorylates and thereby activates pre-existing c-Jun homodimers (Boyle *et al.*, 1991; Papavassiliou *et al.*, 1992).

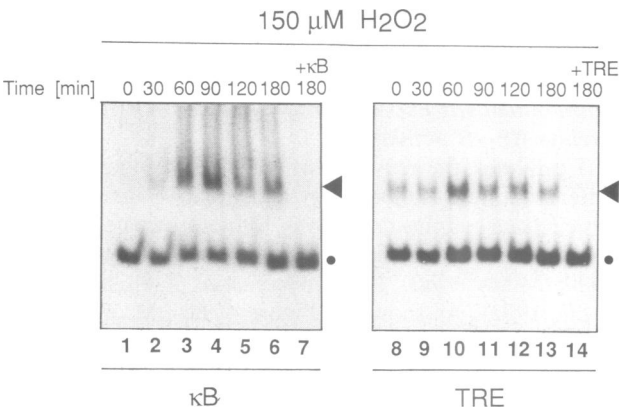

Fig. 2. Kinetics of induction of NF-$\varkappa$B and AP-1 upon H_2O_2 treatment. Nuclear extracts were prepared from HeLa cells treated with 150 μM H_2O_2 after the indicated time points and incubated with ^{32}P-labeled oligonucleotides encompassing NF-$\varkappa$B (lanes 1–7) or AP-1 consensus motifs (lanes 8–14), and analyzed by EMSA. In lanes 7 and 14, 100-fold molar excesses of unlabeled specific oligonucleotides were added to the binding reactions. Sections of fluorographs are shown. Filled arrowheads indicate the positions of specific complexes and black dots the positions of non-specific complexes.

The similarities between NF-$\varkappa$B and AP-1 activation prompted us to test the responsiveness of the two factors to H_2O_2 and antioxidants, and to test the effect of H_2O_2 and antioxidants on the activation of NF-$\varkappa$B and AP-1 by PMA. NF-$\varkappa$B was strongly activated in H_2O_2-treated HeLa cells and activation of NF-$\varkappa$B by PMA was potently suppressed by the antioxidants PDTC and NAC. H_2O_2 potentiated the effect of PMA, supporting the idea that PMA and H_2O_2 signals converge into a common ROI-dependent pathway. In contrast to NF-$\varkappa$B, both DNA binding and transactivation by AP-1 showed a comparatively weak response to H_2O_2 treatment of HeLa cells. The oxidant even suppressed the induction of AP-1 by PMA, while antioxidants enhanced the PMA effect. Surprisingly, treatment with the antioxidant PDTC and transient expression of the antioxidative enzyme thioredoxin alone efficiently activated AP-1. The effect of PDTC was protein synthesis dependent and involved transcriptional induction of c-*fos* and c-*jun* genes with slightly different kinetics. c-*fos* gene induction by PDTC involved the SRE. This shows that AP-1 is involved in the mammalian antioxidant response as a 'secondary' antioxidant-responsive transcription factor requiring *de novo* synthesis. We discuss these findings in the light of recently published reports on *in vitro* redox regulation of AP-1 (Abate *et al.*, 1990), and induction of c-*fos* and c-*jun* genes by the oxidant H_2O_2 and by UV light (Stein *et al.*, 1989; Devary *et al.*, 1991; Nose *et al.*, 1991; Amstad *et al.*, 1992).

Results

Distinct responsiveness of NF-$\varkappa$B and AP-1 to H_2O_2 in HeLa cells

HeLa cells were transiently transfected with chloramphenicol acetyl transferase (CAT) reporter constructs driven by a thymidine kinase (TK) promoter under the control of either two NF-$\varkappa$B binding sites (p2x$\varkappa$B-37TKcat) or three AP-1 binding sites (p3xAP1-37TKcat). Transfected cells were treated with increasing concentrations of H_2O_2 and transactivation determined by CAT ELISA. As reported for Jurkat cells (Schreck *et al.*, 1991), micromolar amounts of H_2O_2 led to a potent transactivation of the $\varkappa$B-dependent reporter construct (Figure 1A). Addition of 200 μM H_2O_2 to the culture medium resulted in a 30-fold induction of CAT protein. Activation of NF-$\varkappa$B in HeLa cells was slightly less responsive toward H_2O_2 than in Jurkat cells. The latter cell line already showed strong transactivation of an HIV-1 LTR-driven CAT reporter construct at 30 μM H_2O_2 (Schreck *et al.*, 1991).

The reporter gene controlled by three AP-1 sites gave only a weak transactivation of the CAT gene under identical conditions. At 150–200 μM H_2O_2, between 2.5- (Figure 1A) and 5-fold (see Figure 3) increased levels of CAT protein were determined by ELISA. The CAT reporter construct controlled solely by the TK promoter showed virtually no responsiveness to treatment of HeLa cells with the oxidant.

We tried to relate the results from the transactivation experiments to the effects of H_2O_2 on the DNA-binding activities of NF-$\varkappa$B and AP-1. The same nuclear extracts were examined by electrophoretic mobility shift assays (EMSAs) for $\varkappa$B- and TRE(AP-1)-binding activities. Consistent with the results from the transactivation assays, the $\varkappa$B-binding activity showed a dramatic increase upon treatment of cells with 100 or 250 μM H_2O_2 (Figure 1B, lanes 3 and 4), whereas the TRE-binding activity showed only a subtle increase upon treatment of cells with 250 and 500 μM H_2O_2 (Figure 1B, lanes 10 and 11). As shown by competition with an excess of unlabeled homologous oligonucleotide, the slow-migrating activities binding to $\varkappa$B and TRE probes, respectively, were specific, whereas the faster migrating complexes were not (Figure 1B, lanes 6 and 12). The level of AP-1 induction by H_2O_2 was in the range reported previously for HeLa cells (Devary *et al.*, 1991). The AP-1 activity seemed considerably less susceptible to inhibition at 500 μM H_2O_2 than the NF-$\varkappa$B activity (Figure 1B, compare lanes 5 and 11). The non-specific activities showed no striking variation in response to H_2O_2.

In order to exclude that AP-1 activity was determined at an unfavorable time point following H_2O_2 stimulation, a kinetic analysis was performed. The strongest activation of AP-1 was seen after a 60 min treatment with 150 μM H_2O_2 (Figure 2, lane 10). In contrast, NF-$\varkappa$B activity increased up to 60 min and remained more or less constant thereafter (Figure 2, lanes 1–6). The non-specific faster migrating activities were not responsive. In conclusion, NF-$\varkappa$B and AP-1 showed a markedly distinct responsiveness to treatment of HeLa cells with the oxidant H_2O_2. There were differences with respect to the strength of the response, kinetics as well as susceptibility to high levels of the oxidant.

H_2O_2 potentiates activation of NF-$\varkappa$B but suppresses activation of AP-1 in response to PMA

We tested by transactivation and DNA-binding assays whether H_2O_2 influences the activation of NF-$\varkappa$B and AP-1 by PMA. In this experiment, treatment of HeLa cells with 150 μM H_2O_2 led to a 15-fold increase in $\varkappa$B-dependent transactivation of the CAT reporter gene, while treatment with 120 nM PMA gave an almost 60-fold induction (Figure 3, left panel). A combination of H_2O_2 and PMA gave a 130-fold induction of CAT protein which was significantly higher than the induction observed with either stimulus alone. This synergistic effect of the two stimuli was also reflected at the level of DNA binding. More $\varkappa$B-binding

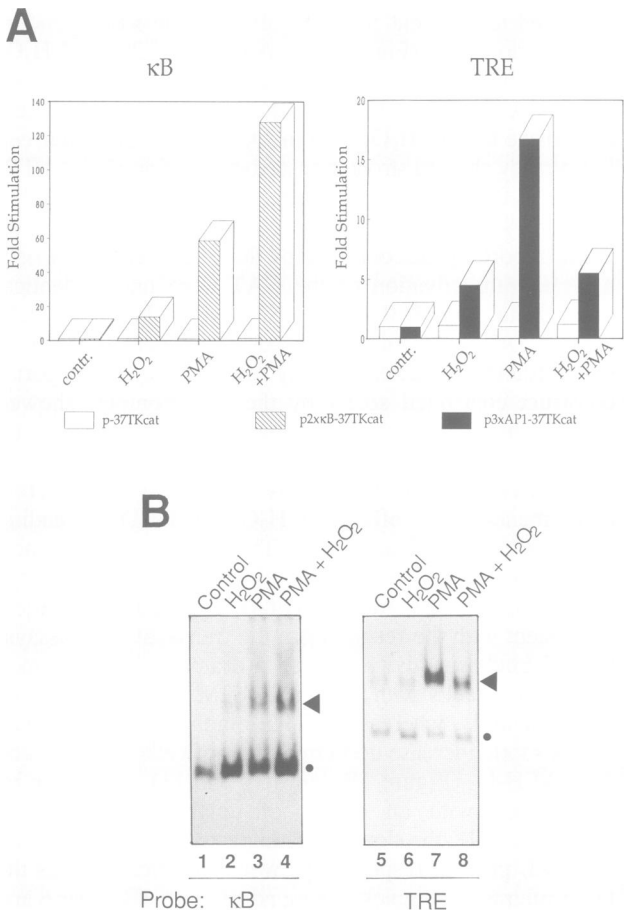

Fig. 3. The effect of H_2O_2 on the activation of NF-$\varkappa$B and AP-1 by PMA. (**A**) The effect of H_2O_2 on PMA-induced gene induction by NF-$\varkappa$B and AP-1. HeLa cells transfected with a basal (p-37TKcat; open columns), a $\varkappa$B-dependent (p2x$\varkappa$B-37TKcat; hatched columns) or a TRE-dependent reporter CAT construct (p3xAP1-37TKcat; black columns) were treated with 150 μM H_2O_2 or 125 nM PMA or a combination of both for 8–12 h prior to harvesting. The amounts of CAT protein were determined by ELISA. (**B**) The effect of H_2O_2 on the PMA-induced DNA-binding activities of AP-1 and NF-$\varkappa$B. Nuclear extracts from HeLa cells treated for 90 min with 150 μM H_2O_2 (lanes 2 and 6), 125 nM PMA (lanes 3 and 6), or a combination thereof (lane 4 and 8) were incubated with ^{32}P-labeled oligonucleotides encompassing NF-$\varkappa$B (lanes 1–4) or TRE consensus motifs (lanes 5–8), and analyzed by EMSA. Sections of fluorographs from native gels are shown. Filled arrowheads indicate the positions of specific complexes and the black points the positions of non-specific complexes.

activity was detected in nuclear extracts from HeLa cells subjected to a combined H_2O_2/PMA treatment (Figure 3B, lane 4) than to either stimulus alone (lanes 2 and 3). Similar results were obtained with two sublones of Jurkat T cells (Schreck *et al.*, 1992a).

In sharp contrast to NF-$\varkappa$B, H_2O_2 did not support the inducing effect of PMA on AP-1 (Figure 3A, right panel). Upon H_2O_2/PMA treatment, transactivation by AP-1 was not higher than that seen with H_2O_2 alone. Also, DNA binding by AP-1 was reduced when cells were stimulated with PMA in the presence of H_2O_2 (Figure 3B, right panel). These findings support the notion that the activation of NF-$\varkappa$B by PMA relies on the production of ROI (Schreck *et al.*, 1991), whereas the activation of AP-1 by PMA in HeLa cells might involve a distinct pathway, such as the dephosphorylation of pre-existing c-Jun (Boyle *et al.*, 1991; Papavassiliou *et al.*, 1992).

The antioxidant PDTC strongly induces AP-1 but inhibits NF-$\varkappa$B activation

In T cell lines, the activation of NF-$\varkappa$B in response to many different stimuli is blocked by antioxidative agents such as cysteine and cysteine derivatives, dithiocarbamates, iron and copper chelators or butylated hydroxyanisole (Staal *et al.*, 1990; Mihm *et al.*, 1991; Israel *et al.*, 1992b; Schreck *et al.*, 1992). As shown in Figure 4, 60 μM of the metal chelator and radical scavenger PDTC strongly interfered with the activation of NF-$\varkappa$B by PMA in HeLa cells. This was evident from a complete block of $\varkappa$B-dependent transactivation in CAT ELISAs (Figure 4A, left panel) and lack of NF-$\varkappa$B activity in nuclear extracts, as detected by EMSAs (Figure 4B, compare lanes 2 and 4). Treatment with 30 mM NAC also suppressed $\varkappa$B-dependent expression of the CAT protein (Figure 4A, left panel) and nuclear appearance of NF-$\varkappa$B following PMA stimulation of HeLa cells (Figure 4C, compare lanes 4 and 5). NAC was, however, less efficient than PDTC. Treatment with PDTC or NAC alone had no stimulating effect on NF-$\varkappa$B, but reduced the baseline levels of NF-$\varkappa$B activities seen in CAT ELISAs as well as EMSAs.

In contrast to NF-$\varkappa$B, treatment of HeLa cells with 60 μM PDTC alone strongly induced TRE-dependent transactivation (Figure 4A, right panel) as well as the DNA-binding activity of AP-1 (Figure 4B, compare lanes 6 and 8). In CAT ELISAs, the induction of the reporter gene by PDTC and PMA was almost equal, whereas in EMSAs, PDTC-treated cells showed a significantly stronger increase in DNA binding of AP-1 than PMA-treated cells (compare lanes 7 and 8). When HeLa cells were treated with both PMA and PDTC, the nuclear DNA binding of AP-1 was further augmented (Figure 4B, lane 9). A 50% higher level of the reporter gene product CAT was detected in cells treated with a combination of PMA and PDTC compared with cells treated with either agent alone (Figure 4A, right panel). Although NAC-treated cells showed increased AP-1 binding activity (Figure 4C, lane 7), only a very small increase in CAT reporter protein was detectable (Figure 4A, right panel). A PMA treatment in the presence of NAC resulted in further increased DNA binding of AP-1 (Figure 4C, lane 10) and TRE-dependent transactivation (Figure 4A). The quantitative differences between the data from EMSAs and CAT ELISA might stem from kinetic differences inherent to the two experimental approaches (see legend to Figure 4). In conclusion, antioxidants were found to be activators of AP-1 while, under identical conditions, they inhibit activation of NF-$\varkappa$B. Similar results were obtained in Jurkat T cells (data not shown).

Transcriptional induction of c-fos and c-jun genes by PDTC

A possible mechanism of AP-1 induction by PDTC in HeLa cells may involve a post-translational reaction, such as dephosphorylation of pre-existing c-Jun. In this case, one would expect that induction of AP-1 by PDTC is independent of new protein synthesis. As shown in Figure 5, this is clearly not the case. Treatment of cells with the protein synthesis inhibitor cycloheximide completely prevented the appearance of the TRE-binding activity in nuclear extracts

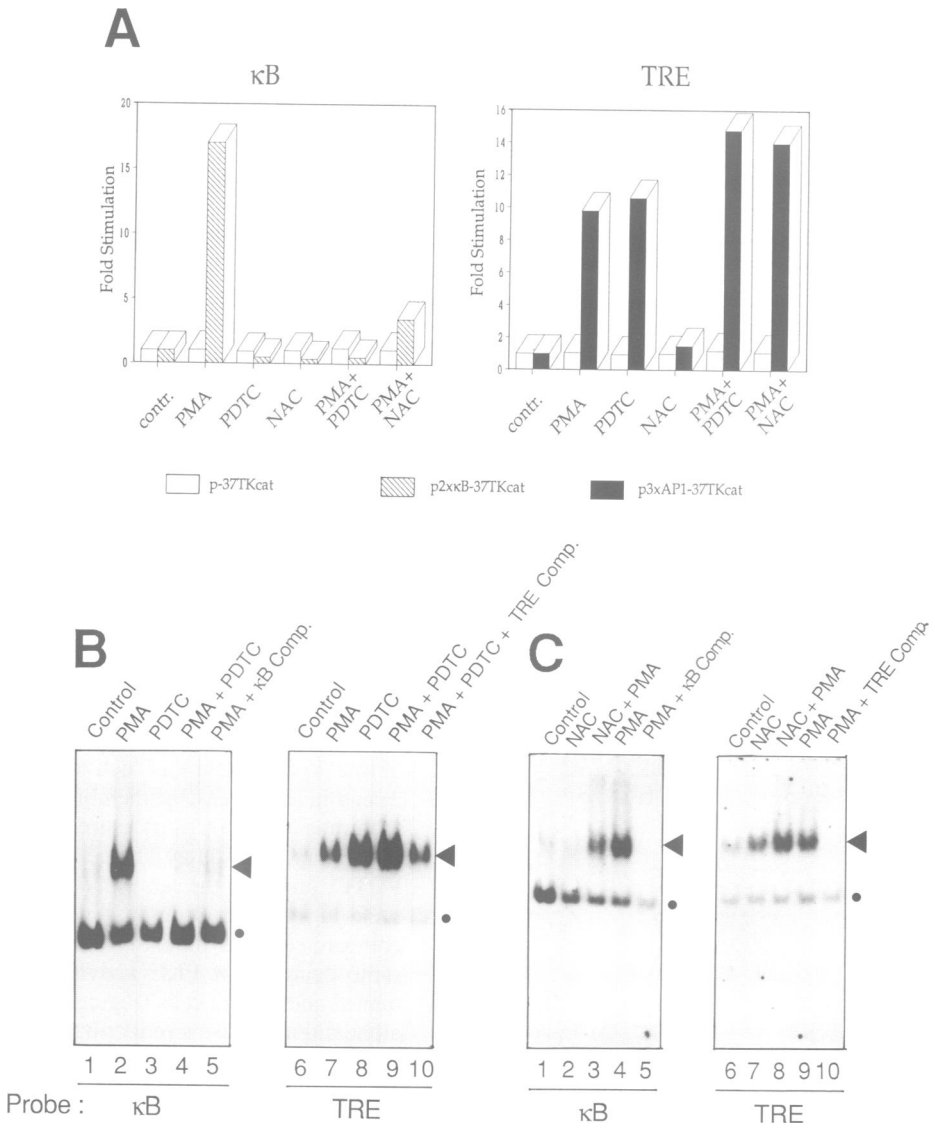

Fig. 4. The effects of PMA and the antioxidants PDTC and NAC on NF-$\varkappa$B and AP-1. (**A**) The effects of PMA, PDTC and NAC on $\varkappa$B- and TRE-dependent gene expression. HeLa cells transfected with a basal (p-37TKcat; open columns), a $\varkappa$B-dependent (p2x$\varkappa$B-37TKcat; hatched columns) or a TRE-dependent reporter CAT construct (p3xAP1-37TKcat; black columns) were treated with 125 nM PMA, 60 μM PDTC and 30 mM NAC or the indicated combinations thereof for 8−12 h prior to harvesting. The amounts of CAT protein were determined by ELISA. (**B**) The effect of PMA and PDTC on the DNA-binding activities of NF-$\varkappa$B and AP-1. Nuclear extracts from HeLa cells treated for 90 min with 125 nM PMA (lanes 2,5 and 7,10), 60 μM PDTC (lanes 3 and 8), or a combination thereof (lanes 4 and 9) were incubated with ^{32}P-labeled oligonucleotides encompassing NF-$\varkappa$B (lanes 1−5) or AP-1 consensus motifs (lanes 6−10), and analyzed by EMSA. In lanes 5 and 10, 100-fold molar excesses of unlabeled specific oligonucleotides were added to the binding reactions. Sections of fluorographs from native gels are shown. Filled arrowheads indicate the positions of specific complexes, the black dots the positions of non-specific complexes. (**C**) The effect of PMA and NAC on the DNA binding activity of NF-$\varkappa$B and AP-1. Nuclear extracts from HeLa cells treated for 90 min with 30 mM NAC (lanes 2 and 7), 125 nM PMA (lanes 4,5 and 9,10), or a combination thereof (lanes 3 and 8) were analyzed by EMSA. For details, see the legend to Figure 4B.

(Figure 5A, compare lanes 3 and 4). In support of a transcriptional induction of the c-*fos* and c-*jun* genes in response to PDTC, we observed a rapid increase of c-*fos* and c-*jun* mRNA following treatment of HeLa cells with PDTC in Northern blots (Figure 5B). While the c-*fos* mRNA already became detectable after 30 min, induction of c-*jun* mRNA was not observed before 60 min. Similar kinetics have been observed for induction of c-*fos* and c-*jun* genes by H_2O_2 in MC3T3 cells (Nose *et al.*, 1991) and HeLa cells (data not shown). This suggests that antioxidants and oxidants activate AP-1 by overlapping pathways.

The SRE as a primary antioxidant response element

Induction of AP-1 by H_2O_2 and UV-A light, which might also involve production of ROIs (Tyrrell, 1991), has been shown to rely on the SRE in the upstream promoter region of the c-*fos* gene (Stein *et al.*, 1989; Nose *et al.*, 1991; Amstad *et al.*, 1992). Here we tested whether the induction of c-*fos* transcription by PDTC also relies on the SRE. As shown in Figure 6A, the CAT reporter gene controlled by upstream sequences of the c-*fos* gene from position +41 to −771 (pFC-771) was induced 15-fold upon treatment of HeLa cells with 60 μM PDTC. Also, a CAT reporter

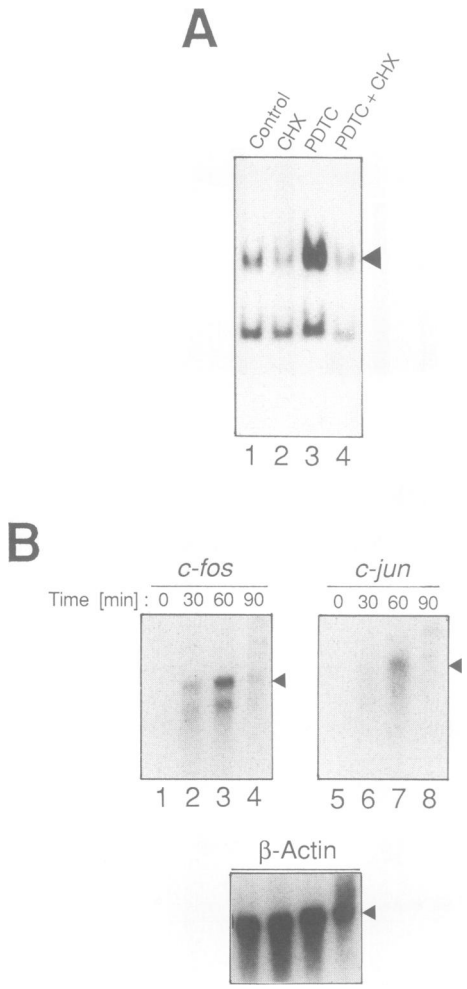

Fig. 5. Transcriptional induction of AP-1 by PDTC. (**A**) The effect of cycloheximide on PDTC induction of AP-1 DNA binding. HeLa cells (lane 1) were treated for 15 min with 10 μg/ml cycloheximide (CHX; lanes 2 and 4) followed by a 90 min incubation with 60 μM PDTC (lanes 3 and 4). Nuclear extracts were prepared, incubated with ^{32}P-labeled oligonucleotides encompassing an AP-1 consensus motif and analyzed by EMSA. A section of a fluorograph from a native gel is shown. The filled arrowhead indicates the position of the specific complex, the black dot the position of the non-specific complex. (**B**) The effect of PDTC on c-*fos* and c-*jun* mRNA levels. HeLa cells were treated with 60 μM PDTC for the indicated time points. Isolated mRNA was detected by Northern blot analysis. Poly(A)$^+$ mRNAs were hybridized with ^{32}P-labeled DNA probes encompassing 1.4 kb of the mouse c-*fos* gene (left panel), 1.1 kb of the mouse c-*jun* gene (right panel) and 1.15 kb of the mouse β-actin gene (lower panels), applying high-stringency conditions. Sections of fluorograms are shown. The arrowheads indicate positions of c-*fos*-, c-*jun*- and actin-specific signals, respectively.

construct with a TK promoter controlled by two copies of a SRE (positions −338 to −298 of c-*fos*) lacking adjacent AP-1 sites was induced 7-fold by 60 μM PDTC (Figure 6B). These values from CAT ELISAs are divided by values obtained with a respective vector lacking the SRE sites. A titration showed that already 10 μM PDTC led to transactivation of the SRE. However, a CAT gene under control of a truncated c-*fos* promoter missing the SRE element, but which retains the cAMP-response element (pFC-149), lost its inducibility to the antioxidant treatment (Figure 6A). This suggests that the SRE acted as primary antioxidant-responsive *cis*-acting element for the c-*fos* gene.

DNA binding of AP-1 and NF-κB are not influenced by PDTC in vitro

c-Jun, c-Fos and the NF-κB subunits p50 and c-Rel have been reported to be susceptible to oxidizing agents in cell-free systems (Abate *et al.*, 1990; Bannister *et al.*, 1991; Frame *et al.*, 1991; Toledano and Leonard, 1991; Kumar *et al.*, 1992; Matthews *et al.*, 1992). Treatment of nuclear extracts with high concentrations of dithiothreitol was shown to increase the DNA-binding activity of these factors. This was attributed to reduction of redox-sensitive cysteine residues within the DNA-binding domains of the proteins. Here, we tested whether PDTC can increase the DNA-binding activities of AP-1 and NF-κB when added to nuclear extracts. Virtually no change of DNA-binding activity of NF-κB (Figure 7, left panel) or AP-1 (right panel) in nuclear extracts from control, PMA- or PDTC-treated HeLa cells was observed following an *in vitro* incubation with 60 μM PDTC. This shows that PDTC could activate AP-1 or inhibit NF-κB activation only in intact cells.

PDTC does not influence PKC activity or PKC activation by PMA

PDTC could act either as an inducer of PKC in the case of AP-1, or as inhibitor of PKC in the case of NF-κB. As shown in Figure 8, neither possibility could be demonstrated. Treatment of Jurkat T cells with PMA resulted in a decrease of total cytoplasmic PKC activity (Figure 8, columns 1 and 2) and in an increase of membrane-associated PKC activity (columns 5 and 6), as measured by a PKC-specific peptide phosphorylation assay. In cells pre-treated with 100 μM PDTC, precisely the same amount of total PKC activity was seen in the cytoplasm as in untreated cells (Figure 8, compare columns 1 and 3). Following PMA stimulation, the same decrease of PKC activity was observed in PDTC-treated and control cells (Figure 8, columns 3 and 4). PDTC alone did not cause a redistribution of PKC activity as seen with PMA. We detected about twice as much PKC activity in membrane fractions from PDTC/PMA-treated cells compared with PMA-treated cells (compare columns 6 and 8). This was reproducible and seems to be due to an improved recovery or stabilization of PKC activity in membranes from PDTC/PMA-treated cells. The findings that PDTC on its own cannot activate PKC and does not significantly influence PKC redistribution in response to PMA suggest that the antioxidant acts independently of PKC.

Transient expression of human thioredoxin activates AP-1 but not NF-κB

The gene encoding the enzyme ADF/thioredoxin is activated upon infection of T cells with HTLV-I (Tagaya *et al.*, 1989). Thioredoxins can repair proteins that underwent disulfide linkage as a consequence of oxidative damage (reviewed in Holmgren, 1985), and protect cells from the cytotoxic effects of H_2O_2, TNF and monoclonal antibodies directed against the apoptosis receptor Fas (reviewed in Yodoi and Uchiyama, 1992). Here, we tested whether transient expression of human thioredoxin (ADF) under the control of a CMV promoter/enhancer leads to activation of a TRE-dependent CAT reporter construct. As shown in Figure 9, transient expression of thioredoxin caused a strong maximally 23-fold activation of AP-1-dependent transactivation. The response was biphasic; high-level expression of the antioxidative enzyme showed a reduced

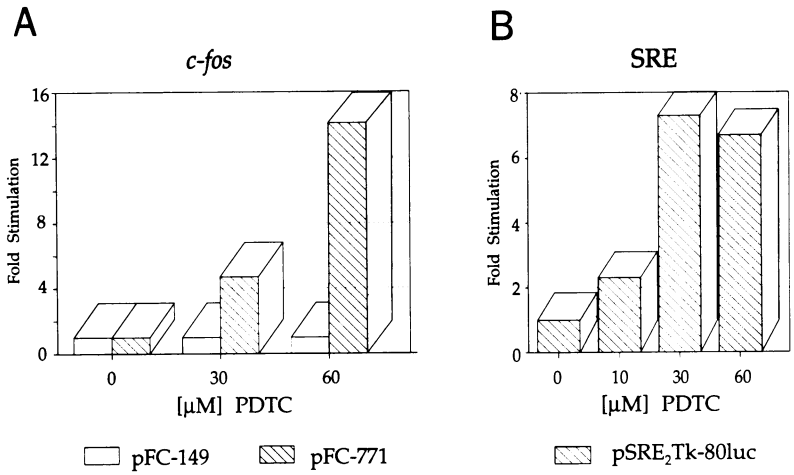

Fig. 6. The effect of PDTC on c-*fos* promoter elements. (**A**) The effect of PDTC on the human c-*fos* promoter. HeLa cells were transfected with 1.5 μg of pFC-149 (open columns) or pFC-771 (hatched columns) and treated with the indicated amounts of PDTC 10 h prior to harvesting. The amount of CAT protein was determined by ELISA. (**B**) The effect of PDTC on the SRE. Cells were transfected with 1.5 μg of a plasmid containing two copies of the human c-*fos* SRE ($pSRE_2Tk$-80luc) and treated with the indicated amounts of PDTC 10 h prior to harvesting of cells. Luciferase activity was determined in a luminometer. The CAT ELISA values shown were divided by values obtained in control experiments with pKS+/Ltk80-luc lacking the SRE sites.

effect. The expression of a κB-controlled CAT reporter construct was not activated by thioredoxin. Rather, the basal levels of κB-dependent CAT expression were reduced in a dose-dependent fashion. These results suggest that AP-1 is activated in the course of a physiological antioxidant response and not only by antioxidative chemicals.

Discussion

AP-1 as antioxidant-responsive factor

This study shows that treatment of cells with the antioxidant PDTC or transient expression of thioredoxin strongly activates the transcription factor AP-1 in HeLa cells. Under the same experimental conditions in the same cells, the oxidative-stress responsive factor NF-κB was not activated. On the contrary, antioxidants impaired activation of NF-κB by PMA, while supporting activation of AP-1 by PMA. These observations rule out that the antioxidants in fact induced oxidative stress, as was observed as a consequence of ischemic hypoxia. Furthermore, it is unlikely that oxidative stress can build up within a short time after addition of antioxidants. However, it is possible that long-term incubation with PDTC (and other antioxidants) causes—in a counter-reaction—oxidative stress once the antioxidant is depleted by oxidation.

Our results indicate that AP-1 binding sites (TREs) represent antioxidant response elements (AREs). This idea is supported by recent studies analyzing the transcriptional control of NQO_1 and GST Ya genes in response to the antioxidants 3-(2-)-*tert*-butyl-4-hydroxyanisole (BHA) and β-naphthoflavone (Rushmore *et al.*, 1991; Li and Jaiswal, 1992a). Both genes contain AREs in their 5′-flanking regions. The ARE controlling the human NQO_1 gene contains a perfect consensus AP-1 site (5′-TGACTCA-3′). EMSAs and CAT assays supported an involvement of AP-1 in the antioxidant response by the human NQO_1 ARE (Li and Jaiswal, 1992a,b). Surprisingly, the AP-1 motif is not conserved in the ARE from the rat GST Ya gene. A point mutation analysis suggested an ARE consensus motif in

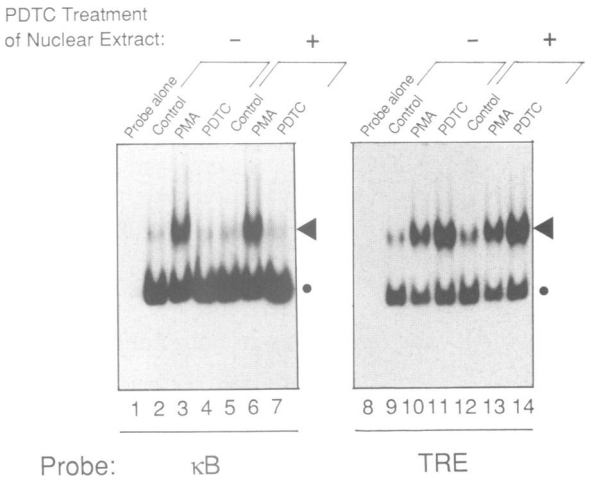

Fig. 7. The *in vitro* effect of PDTC on DNA-binding activities of NF-κB and AP-1 in nuclear extracts. Nuclear extracts from HeLa cells treated for 90 min with 125 nM PMA (lanes 3, 6 and 10,13) or 60 μM PDTC (lanes 4,7 and 11,14) were incubated for 30 min with 60 μM PDTC in PBS (+) or with PBS alone (−), followed by incubation with ^{32}P-labeled oligonucleotides encompassing NF-κB (lanes 1−7) or AP-1 binding motifs (lanes 8−14) and analysis by EMSA. Sections of fluorographs from native gels are shown. Filled arrowheads indicate the positions of specific complexes, the black dots the positions of non-specific complexes.

which only one half-site of the AP-1 motif is required: 5′-puGTGACNNNGC-3′ (Rushmore *et al.*, 1991). Like TREs, the ARE from the rat GST Ya gene was also weakly responsive to treatment of cells with micromolar concentrations of H_2O_2. It will be interesting to find out whether PDTC and thioredoxin can also induce NQO_1 and GST Ya expression and, on the other hand, whether BHA and β-naphthoflavone induce a classical TRE-controlled CAT reporter gene. Schmalbach *et al.* (1992) have observed that IL-1 and TNF genes are induced 3 h after incubation with the diethyl derivative of dithiocarbamate. It is possible that the AP-1 sites in the 5′-flanking regions of these genes were involved in this antioxidant response.

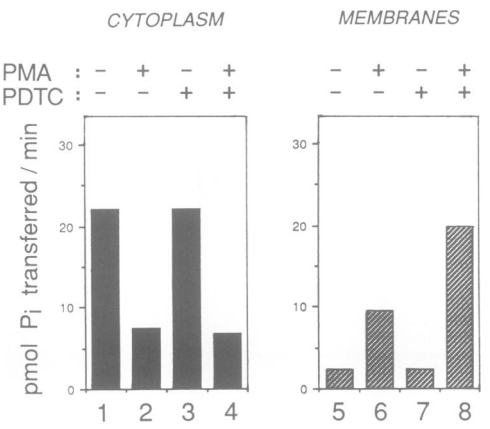

Fig. 8. The effect of PMA and PDTC on the total activity and PMA activation of protein kinase C. Cytoplasmic (black columns 1–4) and membrane fractions (hatched columns 5–8) from untreated Jurkat T cells (columns 1 and 5), cells treated with 75 ng/ml PMA (columns 2 and 6), 100 μM PDTC (columns 3 and 7) or a combination of PMA and PDTC (columns 4 and 8) were analyzed for PKC-specific kinase activity by a peptide phosphorylation assay.

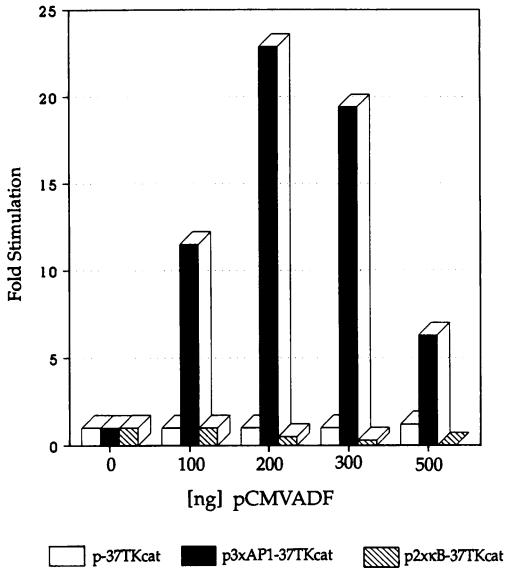

Fig. 9. The effect of transient expression of thioredoxin (ADF) on TRE- and κB-dependent gene expression. HeLa cells were co-transfected with a basal (p-37TKcat; open columns), a TRE(AP-1)-dependent (p3xAP1-37TKcat; black columns) or a κB-dependent (p2xκB-37TKcat; hatched columns) CAT reporter constructs and the indicated amounts of the thioredoxin expression vector pCMVADF. CAT protein was quantitated by ELISA.

Why have cells evolved an antioxidant response? Most likely antioxidants induce in cells a hypoxic state similar to the one occurring upon decreased perfusion of tissue under injury and ischemia. A major danger to hypoxic tissue is oxidative stress occurring upon reperfusion (reviewed in Korthuis and Granger, 1986). It is thus very advantageous if genes encoding antioxidative enzymes and other defense proteins are induced under hypoxia such that their products can alleviate oxidative damage occurring during a subsequent reperfusion. This study suggests AP-1 plays a role as transcriptional regulator in this adaptative process.

How can AP-1 be responsive to both antioxidant and oxidant?

An intriguing fact is that AP-1 genes are also induced by conditions causing a pro-oxidant state of cells, such as treatment with H_2O_2 (Devary *et al.*, 1991; Nose *et al.*, 1991; Amstad *et al.*, 1992), UV light (Stein *et al.*, 1989; Devary *et al.*, 1991), leukotriene B4 (Stankova and Rola-Pleszczynski, 1992, and references therein), lipopolysaccharide (Kaminska *et al.*, 1992), ionizing radiation (Datta *et al.*, 1992) and IL-1 (Munoz *et al.*, 1992a). All these agents also activate NF-κB in the appropriate cell lines (reviewed in Baeuerle, 1991; Schreck *et al.*, 1992a). Even more intriguing is that the induction of c-*fos* and c-*jun* mRNAs by H_2O_2 and by the antioxidant PDTC occurred with very similar kinetics and acted on the same *cis*-acting element, the SRE. This suggests that signals emerging from effector proteins sensing either pro-oxidant or antioxidant conditions in the cell funnel into the same pathway. This might occur at the level of the SRE, or earlier, at the level of protein kinases.

Devary *et al.* (1992) provided evidence that UV induction of c-*jun* required activation of tyrosine kinases and Raf-like serine/threonine kinases. Because UV-A light is known to induce a pro-oxidant state (reviewed in Tyrrell, 1991) and UV effects are blocked with NAC (Devary *et al.*, 1992), UV-induced signals might converge into the same ROI-dependent pathway as H_2O_2 and other inducers of AP-1. Consistent with this notion is that tyrosine kinases are also activated upon treatment of cells with H_2O_2, diamide and IL-1 (Heffetz *et al.*, 1990; Bauskin *et al.*, 1991; Munoz *et al.*, 1992b). Future experiments will investigate whether activation of AP-1 by antioxidants also depends on protein kinases. Because activation of the SRE in the c-*fos* gene involves phosphorylation of Ets-like accessory proteins of SRF (Gille *et al.*, 1992), this possibility is very likely.

Why is a transcription factor activated under such contrary conditions as oxidative stress and hypoxia? Most studies on the induction of AP-1 by various stimuli focused on investigating c-*fos* and c-*jun* mRNA levels, which were apparently assumed to reflect the factor's DNA-binding and transactivating capability. In this study, we became aware of a discrepancy between the strong mRNA induction of c-*jun* and c-*fos* by H_2O_2 (Nose *et al.*, 1991; Devary *et al.*, 1991; data not shown) and the weak DNA-binding and transactivating capacity of AP-1 in response to H_2O_2. Although the mRNA induction by PDTC was comparable to that seen with H_2O_2, the activation of AP-1 DNA binding and transactivation by the antioxidant was much more dramatic than with the oxidant, and even stronger than with PMA. It thus appears that both under antioxidant and pro-oxidant conditions AP-1 genes are induced, but that that more active AP-1 protein is produced under the antioxidant condition. Under the pro-oxidant condition, AP-1 protein might exist in a latent form which is only fully active when cells regain a 'normoxic' or hypoxic state. This would allow production of AP-1 under very diverse conditions, but restrict its biological activity to a defined state of the cell.

How is AP-1 activated by antioxidants?

c-Jun and c-Fos contain conserved redox-sensitive cysteine residues in their DNA-binding domains (Abate *et al.*, 1990; Bannister, 1991; Frame *et al.*, 1991). In nuclear extracts from cells, these cysteine residues are found partially oxidized which causes a loss in DNA-binding activity. Treatment of nuclear extracts or purified protein with

dithiothreitol or the repair enzyme Ref-1 (Xanthoudakis *et al.*, 1992) restores DNA-binding activity of Jun and Fos proteins. A physiological significance of these *in vitro* observations has not yet been demonstrated. It is possible that the millimolar concentrations of GSH in cytoplasm and nuclei do not allow for a redox regulation of AP-1 in intact cells. Consistent with this idea is the observation that treatment of cells with 500 μM H_2O_2 did not abolish AP-1 DNA binding, but interfered substantially with DNA binding and/or activation of NF-$\varkappa$B (see Figure 1B). On the assumption that pre-existing c-Jun occurs in HeLa cells in an oxidized form, an antioxidant treatment of cells could directly or indirectly result in reduction of cysteine residues leading to increased DNA binding and transactivation by c-Jun. We could not obtain evidence that this post-translational mechanism is the primary cause for activation of AP-1 in response to antioxidants. The induction of AP-1 by antioxidants was dependent on new protein synthesis and involved rapid up-regulation of c-*fos* and c-*jun* mRNA levels. As observed for the oxidant H_2O_2 (Nose *et al.*, 1991), PDTC induced the c-*fos* gene slightly faster than the c-*jun* gene. The delay could come from a dependence of c-*jun* expression on newly synthesized c-Fos proteins.

A transcriptional mechanism of AP-1 activation requires a pre-existing transcription factor inducing the c-*fos* gene in response to antioxidants. This primary factor appears to recruit the SRE in the 5′-flanking region of the c-*fos* gene. The SRE can thus be considered a primary antioxidant response element. SRF and ternary complex-forming proteins belonging to the *ets* proto-oncogene family are in fact pre-existing factors (Herrera *et al.*, 1989; Shaw *et al.*, 1989; Hipskind *et al.*, 1991; Dalton and Treisman, 1992) and the latter were recently shown to confer stimulatory effects on the SRE upon phosphorylation by MAP kinase (Gille *et al.*, 1992). Future studies have to explore whether Ets-like proteins associated with SRF undergo covalent modification in response to antioxidant as well as oxidant treatment of cells.

PMA activates AP-1 and NF-$\varkappa$B by distinct pathways

Activation of AP-1 in response to H_2O_2 and UV light has been shown to be independent of PKC (Büscher *et al.*, 1988; Nose *et al.*, 1991; Devary *et al.*, 1992), suggesting that the ROI-dependent pathway does not require PKC. Rather, activation of PKC is one of several mechanisms inducing oxidative stress in cells (reviewed in Cerutti, 1985). Likewise, the antioxidant response was apparently independent of PKC since PDTC did not influence PKC activity and PKC redistribution by PMA in intact cells.

This and earlier studies (see Introduction) suggest that NF-$\varkappa$B relies on the ROI-inducing effect of the pleiotropically acting PKC. In contrast, AP-1 appears not to rely on this effect of PKC, as is evident from the enhancing effect of diverse antioxidants on PMA induction of the factor. Further, consistent with the notion that PKC does not activate AP-1 via ROIs, H_2O_2 inhibits PMA stimulation. This shows that under pro-oxidant conditions activation of AP-1 by a second independent stimulus is impaired. PMA is thought to activate pre-existing c-Jun by dephosphorylation (Boyle *et al.*, 1991; Papavassiliou *et al.*, 1992). H_2O_2 might interfere with this process upstream from c-Jun or act on the newly dephosphorylated c-Jun.

In conclusion, the transcription factors AP-1 and NF-$\varkappa$B behave strikingly differently in response to oxidant and antioxidant treatment of HeLa cells. NF-$\varkappa$B behaved under all conditions as primary oxidative stress-responsive factor. AP-1 showed a much more complex response pattern due to its regulation at the transcriptional level and the involvement and cross-talk of at least three different signalling pathways: the PMA-induced pathway, the ROI-dependent pathway and a novel antioxidant-responsive pathway.

Materials and methods

Cell culture

HeLa cells (ATCC CCl2) were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 8% fetal calf serum and 100 μg/ml kanamycin. In experiments using H_2O_2, DMEM was replaced by iron-free minimum essential medium (MEM, Gibco-BRL).

Constructs

A CAT reporter gene under control of the herpes simplex thymidine kinase (TK) promoter, called pTK-37cat, was obtained by exchanging a *Bam*HI−*Bgl*II fragment of pBLcat2 (Luckow and Schütz, 1987) harboring the entire TK promoter fragment (from nts −109 to +51) with a *Bam*HI−*Bgl*II fragment, containing a minimal promoter region from −37 to +51. The constructs p2x$\varkappa$B-37Tkcat and p3xAP1-37TKcat were obtained by insertion of double-stranded oligonucleotides representing two spaced NF-$\varkappa$B binding sites with the sequence 5′-GGGACTTTCC-3′, or three spaced AP-1/TRE motifs with the sequence 5′-TGACTCA-3′, respectively, into the *Hin*dIII−*Xba*I-opened vector pTK-37cat. Plasmid pFC-771 was constructed by insertion of a *Hin*dIII-fragment harboring the promoter/enhancer sequences of the human c-*fos* gene from −771 to +41 that was derived from plasmid pFC3 (Deschamps *et al.*, 1985) into the *Hin*dIII-opened plasmid pGCAT-C (Frebourg and Breson, 1988). pFC-149 was obtained by ligation of an *Alu*I−*Hin*dIII fragment (nts −149 to +41) derived from pFC-771 into *Sma*I−*Hin*dIII-cleaved vector pGCAT-C. Plasmids pKS+/Ltk80-luc and pSRE$_2$Tk-80luc were kindly given by Prof. Nordheim.

For the construction of pCMVADF, a DNA fragment encompassing the entire coding region of ADF was amplified by PCR from a bacterial expression vector encoding ADF (kindly given by Markus Gütlich, GSF). The 5′-primer contained a complete *Not*I site and 18 bases annealing to the sequence surrounding the start codon of ADF. The 3′-primer introduced a complete *Xba*I site, three stop codons in three different frames and contained 10 bases annealing to the 3′-end of the ADF coding sequence. The eukaryotic expression vector pRc/CMV (Invitrogen, San Diego) was cleaved with *Not*I and *Xba*I, and subsequently ligated with the *Not*I−*Xba*I-digested PCR product.

Transfections and CAT ELISA

HeLa cells were seeded out the day prior to transfection at a density of 3.5×10^5 cells/60 mm dish. CAT reporter plasmid (1−3 μg) was transfected using a modification of the calcium phosphate method described by Graham and van der Eb (1973). Cells were treated with H_2O_2, PMA, NAC, PDTC, or combinations thereof for 8−12 h prior to harvesting; i.e. 26−30 h after transfection (for concentrations see the figure legends). NAC and PDTC were dissolved in PBS, the solutions freed from gas and sterile filter. The NAC solution was adjusted to pH 7.4 by the addition of 1 N NaOH. For quantitation of expressed CAT protein, cells were detached 36−38 h after transfection, with PBS, 10 mM EDTA, collected by centrifugation, resuspended in 200 μl of 250 mM Tris−HCl (pH 7.8), 5 mM EDTA, and lysed by four cycles of freeze/thawing. A total of 50 μg of total cellular protein was assayed by a CAT ELISA (Boehringer Mannheim), according to the manufacturer's instructions. In one experiment (Figure 6), luciferase was used as reporter. Luciferase activity was determined in a type 2010 luminometer (ALL, San Diego), using a commercial assay system (Promega, Heidelberg). All transfections were performed in duplicates and assayed at least three times with $<10\%$ deviation from the mean.

Electrophoretic mobility shift assay

HeLa cells (1.2×10^6/100 mm dish) were incubated with H_2O_2, PMA, NAC or PDTC as indicated in the figure legends. Nuclear extracts were isolated as described by Dignam *et al.* (1983), with the modification that buffer D was supplemented with 0.1% NP-40. Binding reactions were

performed for 25 min on ice with 3–5 μg total protein in 20 μl of 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 10% glycerol, 1–1.5 μg acetylated bovine serum albumin (Gibco BRL), 2 μg poly(dI-dC) (Boehringer Mannheim), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 30 000 c.p.m. of ^{32}P-labeled oligonucleotides (Promega Heidelberg) labeled with T4 kinase (Boehringer Mannheim) and [γ-^{32}P]ATP (3000 Ci/mmol; Amersham). In addition, the binding reactions detecting AP-1 activity contained 5 mM $MgCl_2$. DNA–protein complexes were separated from unbound DNA probe on native 4.5% polyacrylamide gels at 20 mA in 34 mM Tris–HCl (pH 7.5), 17 mM sodium acetate and 0.5 mM EDTA (pH 8.0). Gels were vacuum dried and exposed to Amersham MP films at −80°C for 16–48 h. The sequences of the oligonucleotides were as follows (factor binding sites are underlined):

κB: 5′-AGTTGAGGGGACTTTCCCAGGC-3′
3′-TCAACTCCCCTGAAAGGGTCCG-5′
AP-1: 5′-TTCCGGCTGACTCATCAAGCG-3′
3′-AAGGCCGACTGAGTAGTTCGC-5′

Northern blot analysis

HeLa cells (1.2×10^6/100 mm dish) were incubated with 60 μM PDTC and harvested after various time points. Total cellular RNA (~100 μg) was isolated from guanidinium isothiocyanate-lysed cells and purified using an acid phenol extraction method (Chomcynski and Sacchi, 1987). Polyadenylated RNA was purified using oligo(dT)$_{25}$-coated super paramagnetic polystyrene beads (Dynal, Hamburg) and a magnetic particle concentrator. The RNA was electrophoresed in 1% agarose gels with 20 mM 3-[*N*-morpholino]propanesulfonic acid, 1 mM EDTA, 660 mM formaldehyde, and blotted onto Genescreen-Plus nylon membranes (NEN-Dupont). Hybridization was performed in 50% (v/v) formamide, 5×SSPE, 10% (w/v) dextran sulfate, 1% (v/v) sarcosyl, 0.1% (w/v) SDS and 1×10^6 c.p.m./ml of an [α-^{32}P]dCTP-labeled DNA probe generated by random priming (Feinberg and Vogelstein, 1983). Blots were washed in 2×SSC, 0.5% (w/v) SDS at room temperature and 65°C, and exposed to Amersham X-ray films at −80°C for 12–48 h. For re-probing, bound radioactive probe was removed from the blots by boiling in water for 20 min.

Protein kinase C assay

Jurkat T cells (1×10^7) were treated as described in the legend to Figure 7, and subsequently fractionated into cytoplasmic and membrane fractions as described by Thomas *et al.* (1987). Protein kinase C activity in cell equivalents of cytoplasm and detergent-soluble membrane components was determined with a commercial system (RPN 77, Amersham) following the instructions of the manufacturer. Three independent assays were performed. The deviations from the mean values were <5%.

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