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Lipid Peroxidation Is Involved in the Activation of NF-κB by Tumor Necrosis Factor but Not Interleukin-1 in the Human Endothelial Cell Line ECV304

LACK OF INVOLVEMENT OF H_2O_2 IN NF-κB ACTIVATION BY EITHER CYTOKINE IN BOTH PRIMARY AND TRANSFORMED ENDOTHELIAL CELLS*

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It has been proposed that reactive oxygen species, and in particular H_2O_2, may be involved in the activation of NF-κB by diverse stimuli in different cell types. Here we have investigated the effect of a range of putative antioxidants on NF-κB activation by interleukin-1 and tumor necrosis factor as well as the ability of H_2O_2 to activate NF-κB in primary human umbilical vein endothelial cells and the transformed human endothelial cell line ECV304. Activation of NF-κB and stimulation of IκBα degradation by H_2O_2 was only evident in the transformed cells and required much longer contact times than that observed with interleukin-1 or tumor necrosis factor. Furthermore, only H_2O_2 was sensitive to *N*-acetyl-L-cysteine, and no increase in H_2O_2 was detected in response to either cytokine. Pyrrolidine dithiocarbamate has been purported to be a specific antioxidant inhibitor of NF-κB that acts independently of activating agent or cell type. However, we found that tumor necrosis factor- but not interleukin-1-driven NF-κB activation and IκBα degradation were sensitive to pyrrolidine dithiocarbamate in transformed cells, while neither pathway was inhibited in primary cells. Phorbol ester-mediated activation was sensitive in both transformed and primary cells. Other antioxidants failed to inhibit either cytokine, while the iron chelators desferrioxamine and 2,2,6,6-tetramethylpiperidine-1-oxyl mimicked the pattern of inhibition seen for the dithiocarbamate. This suggested that pyrrolidine dithiocarbamate was inhibiting NF-κB activation in endothelial cells primarily through its iron-chelating properties. Tumor necrosis factor, but not interleukin-1, was found to induce lipid peroxidation in ECV304 cells. This was inhibited by pyrrolidine dithiocarbamate and desferrioxamine. *t*-Butyl hydroperoxide, which induces lipid peroxidation, activated NF-κB. Finally, butylated hydroxyanisole, which inhibits lipid peroxidation but has no iron-chelating properties, inhibited NF-κB activation by tumor necrosis factor but not interleukin-1.

Taken together, the results argue against a role for H_2O_2 in NF-κB activation by cytokines in endothelial cells. Furthermore, tumor necrosis factor and interleukin-1 activate NF-κB through different mechanisms in ECV304 cells, with the tumor necrosis factor pathway involving iron-catalyzed lipid peroxidation.

The inducible, higher eukaryotic transcription factor NF-κB has an important role in the regulation of a number of genes involved in immune and inflammatory responses. It is activated in many cell types by a wide range of stimuli including the proinflammatory cytokines interleukin-1 (IL-1)[1] and tumor necrosis factor (TNF) and the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) (reviewed in Ref. 1).

In endothelial cells (ECs), activation of NF-κB is central to the regulation of many genes by IL-1 and TNF such as the cell adhesion molecules vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin (2) and tissue factor (3). Recently, NF-κB has been identified in an activated form in the ECs of atherosclerotic plaques (4), and it has been suggested that NF-κB may play a central role in the initiation of atherosclerosis (5).

NF-κB exists in a latent form in the cytoplasm of unstimulated cells comprising a transcriptionally active dimer bound to an inhibitor protein, IκB. The currently known subunit members of the NF-κB family in mammals are p50, RelA (p65), c-Rel, p52, and RelB, while multiple forms of IκB also exist, namely IκBα, β, γ, and Bcl-3 (reviewed in Ref. 6). The predominant form of NF-κB activated in cells is a p50/RelA heterodimer, which is associated with IκBα in resting cells. Upon stimulation with agents such as IL-1 and TNF, IκBα is rapidly phosphorylated on two serine residues (Ser^{32} and Ser^{36}), which targets the inhibitor protein for ubiquitination and subsequent degradation by the 26 S proteasome (reviewed in Ref. 7). This allows NF-κB to translocate to the nucleus and activate target genes by binding with high affinity to κB elements in their promoters. The phosphorylation and degradation of IκBα are tightly coupled events (7). Thus, it is likely that agents that activate NF-κB do so through the activation of a specific IκBα kinase or, alternatively, by inactivating a particular phosphatase. A high molecular mass kinase complex that phosphorylates IκBα on Ser^{32} and Ser^{36} has been identified (8).

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[1] The abbreviations used are: IL-1, interleukin-1; BHA, butylated hydroxyanisole; *t*-BHP, *tert*-butyl hydroperoxide; DDTC, diethyldithiocarbamate; DFO, desferrioxamine; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; MDA, malondialdehyde; NAC, *N*-acetyl-L-cysteine; PDTC, pyrrolidine dithiocarbamate; PGA, pyroglutamic acid; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substances; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TNF, tumor necrosis factor; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

The upstream events that lead to phosphorylation of IκBα are unclear. A model has been proposed whereby diverse agents all activate NF-κB by causing oxidative stress (an increase in intracellular reactive oxygen intermediates (ROS)) (9). In particular, H_2O_2 has been implicated as a common second messenger in the various pathways leading to NF-κB activation (10). This hypothesis is based on several of lines of evidence. First, in some cell types H_2O_2 has been shown to be released in response to agents that also activate NF-κB (9–11). Second, direct addition of H_2O_2 to culture medium has been shown to activate NF-κB in some cell lines (12, 13). Third, overexpression of the H_2O_2-metabolizing enzyme catalase in a mouse epidermal cell line was shown to attenuate the activation of NF-κB by TNF and okadaic acid, while overexpression of the H_2O_2-producing enzyme superoxide dismutase potentiated the activation, suggesting a role for H_2O_2 in these pathways to NF-κB (10). Other work has suggested that some of these observations are cell-specific. H_2O_2 had no stimulatory effect on NF-κB in a number of other cell types (14–17), while Suzuki *et al.* (18) showed that in COS-1 cells, overexpression of catalase did not block activation of NF-κB by either TNF or PMA. It has also been suggested that oxidative stress facilitates but does not mediate NF-κB activation (14).

Another line of evidence implicating oxidative stress and H_2O_2 as central to NF-κB activation has been the effect of antioxidants in inhibiting NF-κB activation in response to diverse stimuli. Two compounds in particular have been extensively used, the glutathione precursor and radical scavenger *N*-acetyl-L-cysteine (NAC) and the putative antioxidant pyrrolidine dithiocarbamate (PDTC). However, the effect of NAC on NF-κB is also somewhat cell-specific, in that although it has proved inhibitory in some cells (12, 13), we and others have reported NAC-insensitive pathways to NF-κB (16, 17, 19). PDTC seems to be a better general inhibitor of NF-κB and, in fact, has been proposed as a specific universal inhibitor of NF-κB that acts independently of the activating agent and cell type used (9). However, in addition to its radical scavenging and metal-chelating properties (9, 20), PDTC can also exert a pro-oxidant effect in some cells by increasing oxidized glutathione levels (21, 22), which also leads to an inhibition of NF-κB (22, 23).

Information on the role of oxidative stress and H_2O_2 in cytokine stimulation of NF-κB in ECs remains limited, mainly coming from studies using PDTC or NAC to perturb particular genes downstream of NF-κB activation (24–26). Given the importance of NF-κB in ECs together with the often cell-specific nature of the effect of H_2O_2 and antioxidants on NF-κB activation, we decided to investigate the role of H_2O_2 and oxidative stress in NF-κB activation in ECs using both primary and transformed ECs. Our results show that although H_2O_2 activates NF-κB in transformed ECs, it is unlikely to have a role in the cytokine-mediated pathways to NF-κB in transformed or primary ECs. In transformed cells, TNF but not IL-1 was sensitive to PDTC, while in primary cells neither stimulus was inhibited. Further, we show that the ability of PDTC to inhibit NF-κB activation by TNF in transformed ECs involves inhibition of iron-catalyzed lipid peroxidation that is not important for activation of NF-κB by IL-1.

EXPERIMENTAL PROCEDURES

Materials—The immortalized human endothelial cell line ECV304 (27) and human Jurkat T cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, United Kingdom). Pooled human umbilical vein endothelial cells (HUVECs) were obtained at first passage from Clonetics Corporation (San Diego, CA). RPMI 1640 medium, heat-inactivated fetal bovine serum (FBS), trypsin-EDTA, and penicillin-streptomycin-glutamine were from Life Technologies, Inc. (Paisley, Scotland). Human recombinant IL-1α was a gift from NCI, National Institutes of Health (Frederick, MD), while human recombinant TNFα was a gift from Dr. Steve Foster (Zeneca Pharmaceuticals, Macclesfield, UK). The 22-base pair oligonucleotide, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, containing the NF-κB consensus sequence (underlined), T4 polynucleotide kinase, and the Cytotox 96™ nonradioactive cytotoxicity assay were from Promega Corp. (Madison, WI). The 22-base pair oligonucleotide, 5′-AGT TGA GGC GAC TTT CCC AGG C-3′, containing the mutated NF-κB consensus sequence (underlined), the rabbit polyclonal antibody to human IκBα, and the antisera to the NF-κB subunits p50 and c-Rel were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antiserum to the NF-κB subunit RelA (p65) was a gift from Dr. Jean Imbert (INSERM, Marseille, France). [γ-^{32}P]ATP (3000 Ci/mmol) and enhanced chemiluminescence (ECL) reagent were from Amersham International (Aylesbury, UK). Poly(dI·dC) was from Pharmacia Biosystems (Milton Keynes, UK). All other reagents, including heparin (sodium salt), human recombinant acidic fibroblast growth factor, medium 199 (HEPES modification), PDTC (ammonium salt), diethyldithiocarbamate (DDTC), pyrrolidine, pyroglutamic acid, NAC, desferrioxamine, allopurinol, the spin traps 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), $FeCl_3$, H_2O_2, PMA, butylated hydroxyanisole (BHA), *tert*-butylhydroperoxide (*t*-BHP), thiobarbituric acid, anti-mouse IgG peroxidase conjugate, scopoletin, horseradish peroxidase (type II), and catalase were from Sigma (Poole, UK).

Cell Culture and Treatments—ECV304 cells were grown in medium 199 (HEPES modification) containing 10% (v/v) FBS, and passaged when confluent using 0.05% (w/v) trypsin, 0.02% (w/v) EDTA. HUVECs were grown in medium 199 containing 20% FBS, 10 ng/ml acidic fibroblast growth factor, and 90 μg/ml heparin. The medium was changed every 48 h, and cells were passaged when 80–90% confluent using trypsin-EDTA. Jurkat T cells were grown in RPMI 1640 medium containing 10% FBS. All media were supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine, and cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were pretreated with the test compounds or left untreated before the addition of IL-1, TNF, PMA, or H_2O_2 as described in the figure legends. All experiments were carried out in complete medium at 37 °C. PDTC, DDTC, and pyrrolidine were dissolved in PBS; pyroglutamic acid (PGA), desferrioxamine (DFO), and $FeCl_3$ were dissolved in H_2O; NAC was dissolved in 25 mM Tris-HCl, pH 7.5; BHA was dissolved in ethanol; and PMA, allopurinol, TEMPO, and DMPO were dissolved in Me_2SO. NAC and PGA were adjusted to pH 7.4 with 1 N NaOH. None of the vehicles had any effect on NF-κB alone at the concentrations used. No metal spatulas were used. The effect of the compounds on cell viability was assessed using the Cytotox 96™ nonradioactive cytotoxicity assay, as described by the manufacturers. This system uses lactate dehydrogenase release as an index of cell toxicity. At the concentrations used here, NAC interfered with this assay, and thus the magnitude of release of lactate dehydrogenase activity from NAC-treated cells was determined directly by monitoring spectrophotometrically the decrease in absorbance at 340 nm in the presence of 75 mM Tris-HCl, pH 7.2, containing 150 mM KCl, 0.2 mM NADH, and 4.8 mM sodium pyruvate. Measurement of LDH release from intact cells and examination of monolayer morphology revealed that none of the compounds used were toxic to the cells at the exposure times and concentrations used here.

Cell Fractionation and Preparation of Nuclear Extracts—Nuclear extracts were prepared using a modified version of the method of Osborn *et al.* (28). Confluent ECV304 cells or HUVECs in six-well plates (3-ml volume) were treated as described in the figure legends. Stimulation was terminated by removal of medium followed by washing twice with 3 ml of ice-cold PBS (0.145 M NaCl, 0.027 M phosphate buffer, pH 7.6). Washed cells were then scraped into 1 ml of hypotonic buffer (10 mM Hepes buffer, pH 7.9, containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). Stimulation of Jurkat T cells (5×10^6 in 1 ml of medium) was terminated by the removal of cells into 5 ml of ice-cold PBS, followed by centrifugation (170 g, 10 min). Cell pellets were resuspended in 1 ml of hypotonic buffer. Nuclear extracts were subsequently prepared as described previously (17). Protein concentrations were determined using the method of Bradford (29). Extracts were then stored at −20 °C and assayed for NF-κB activity the next day.

Electrophoretic Mobility Shift Assay—Nuclear extracts (2 μg of protein) were incubated with 10,000 cpm of a 22-base pair oligonucleotide containing the NF-κB consensus sequence that had previously been labeled with [γ-^{32}P]ATP (10 mCi/mmol) by T4 polynucleotide kinase. Incubations were performed for 30 min at room temperature, in the presence of 2 μg of poly(dI·dC) as nonspecific competitor and 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 5 mM dithio-

threitol, 4% glycerol, and 100 μg/ml nuclease-free bovine serum albumin. For competition studies, unlabeled wild type or mutant NF-κB oligonucleotides were added to the binding reaction 30 min before the addition of the radiolabeled probe. In experiments involving antisera to NF-κB subunits, 0.5 μl of a specific antiserum to p50, RelA, or c-Rel was incubated with nuclear extracts for 20 min on ice prior to the binding reaction. All incubation mixtures were subjected to electrophoresis on native 5% (w/v) polyacrylamide gels, which were subsequently dried and autoradiographed.

Anti-IκBα Immunoblot Analysis—Confluent ECV304 cells in six-well plates (3-ml volume) were treated as described in the figure legends. Treatment was terminated by washing monolayers twice with ice-cold PBS. Cells were then scraped into 1 ml of ice-cold radioimmune precipitation buffer (1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS in PBS) containing 10 μg/ml phenylmethylsulfonyl fluoride, 7 μg/ml aprotinin, and 1 mM Na_3VO_4. Following further disruption of cells by passage through a 21-gauge needle (5 strokes), an additional 0.1 mg/ml phenylmethylsulfonyl fluoride was added to samples, which were then incubated on ice for 45 min. Samples were then centrifuged at 14,000 × *g* for 20 min at 4 °C, and the supernatant was removed as cell lysate. Supernatants were assayed for protein (29). Equal amounts of protein (2–4 μg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose, and IκBα immunoblot analysis was performed as described previously (30).

Determination of H_2O_2—H_2O_2 released from ECV304 cells was measured by the horseradish peroxidase-mediated oxidation of the fluorescent probe scopoletin to its nonfluorescent oxidized form (31). Since hydrogen peroxide freely diffuses across the cell membrane, measuring extracellular release is an indication of intracellular levels. Confluent monolayers of ECV304 cells in six-well plates (3-ml volume) were washed twice with Hanks' balanced salt solution to remove phenol red and serum. Monolayers were incubated with 3 ml of assay solution containing stimulant (IL-1 (10 ng/ml), TNF (10 ng/ml), or PMA (100 ng/ml)), sodium azide (1 mM), scopoletin (1 μM), and horseradish peroxidase (0.2 units/ml) in Hanks' balanced salt solution, for 30 min at 37 °C. The supernatant was then transferred to a test tube and cooled to room temperature, and the fluorescence was measured on a Perkin-Elmer LS 50B fluorimeter using an excitation wavelength of 390 nm and an emission wavelength of 460 nm. Controls included cell-free plates with full assay mix and cells incubated in the absence of peroxidase. The specific decrease in fluorescence due to H_2O_2 was assessed by measuring scopoletin oxidation for each sample in the presence and absence of catalase (50 units/ml). H_2O_2 concentration was then determined against a standard curve, using concentrations of 0.05–0.5 μM H_2O_2, as determined spectrophotometrically (ϵ_{240} = 43.6 M^{-1} cm^{-1}). Protein concentrations of samples were determined (29) following alkali digestion of monolayers. H_2O_2 release was then expressed as pmol/min/mg of protein.

Determination of Thiobarbituric Acid-reactive Substances (TBARS)—Lipid peroxidation was assessed by the TBARS assay, which detects mainly malondialdehyde (MDA), an end product of the peroxidation of polyunsaturated fatty acids and related esters. TBARS were measured by a modification of the method of Ohkawa *et al.* (32). Confluent monolayers of ECV304 cells in 100-mm dishes were treated as described in the figure legends and washed in PBS before undergoing three cycles of freeze-thawing in 200 μl of water. A 20-μl aliquot was subsequently removed for Bradford protein determination (29), and 800 μl of assay mix (0.4% (w/v) thiobarbituric acid, 0.5% (w/v) SDS, 9.4% (v/v) acetic acid, pH 3.5) was added to the remaining sample. Samples were incubated for 60 min at 95 °C, cooled to room temperature, and centrifuged at 14,000 × *g* for 10 min, and the absorbance of the supernatants was read at 532 nm against a standard curve prepared using the MDA standard (10 mM 1,1,3,3-tetramethoxypropane in 20 mM Tris-HCl, pH 7.4). Results were calculated as nmol of MDA equivalents/mg of protein and expressed as a percentage of matched control values.

Statistical Analysis—Significance was evaluated by Student's *t* test.

RESULTS

Hydrogen Peroxide Activates NF-κB in ECV304 Cells but Not HUVECs and with Different Kinetics Compared with IL-1 and TNF—Fig. 1*A* shows that nuclear extracts from untreated ECV304 cells contained trace amounts of NF-κB, the levels of which varied slightly between experiments (*lanes 1*, *9*, *17*, and *25*). Following treatment with 10 ng/ml IL-1 (*lanes 2–8*) or 10 ng/ml TNF (*lanes 10–16*), NF-κB was activated, as evidenced by the increased retardation of the DNA probe containing the κB motif. This activation was evident from 5 min (*lanes 2* and *10*) and peaked at 30 min (*lanes 4* and *12*). NF-κB activity was strong for at least 4 h (*lanes 6* and *14*) and still detectable at 24 h (*lanes 8* and *16*). Treatment of cells with 100 ng/ml PMA (*lanes 18–24*) gave a somewhat different time course of activation in that active NF-κB only became detectable at 30 min (*lane 20*) and was greatly reduced by 24 h (*lane 24*). Prolonged treatment of ECV304 cells with H_2O_2 led to activation of NF-κB. When cells were treated with 0.2 mM H_2O_2 for 4 h, a strong activation was observed (compare *lanes 28* and *25*). In contrast to the rapid response seen for IL-1 and TNF, the H_2O_2-stimulated activation was not detectable after 1 h (compare *lanes 26* and *25*) and did not peak until 4 h (*lane 29*). H_2O_2-mediated activation was also more transient than that seen for IL-1 and TNF, with activity greatly decreased by 8 h (compare *lanes 29* and *28*) and identical to control levels at 24 h (compare *lanes 30* and *25*). A concentration of 0.2 mM H_2O_2 was optimal in this effect. Fig. 1*B* confirms that the protein-DNA complexes activated by IL-1, TNF, PMA, and H_2O_2 were all specific for NF-κB, since 18 or 180 fmol of unlabeled NF-κB wild type consensus sequence effectively competed with each binding activity, while the same concentrations of a mutant NF-κB oligonucleotide containing a single base pair change in the consensus sequence failed to compete with binding.

Since this is the first study reporting NF-κB activation in ECV304 cells, we also characterized the NF-κB subunits present in the complexes activated by the four stimuli. Fig. 1*C* demonstrates that IL-1, TNF, PMA, and H_2O_2 activated similar NF-κB complexes. Using specific antisera to p50, RelA, and c-Rel, the same pattern of supershifting was seen for IL-1-, TNF-, PMA-, and H_2O_2-activated NF-κB (*lanes 1–4*, *5–8*, *9–12*, and *13–16*, respectively). This revealed the presence of two main NF-κB complexes. Antiserum to p50 affected both the lower and upper complex (*lanes 2*, *6*, *10*, and *14*), while RelA antiserum only reacted with the upper complex (*lanes 3*, *7*, *11*, and *15*). There was no detectable reaction with c-Rel antiserum (*lanes 4*, *8*, *12*, and *16*). Hence, it was likely that IL-1, TNF, PMA, and H_2O_2 were activating two NF-κB complexes, tentatively identified as p50/p50 homodimers and p50/RelA heterodimers.

Fig. 1*D* shows that IL-1, TNF, and PMA also activated NF-κB in HUVECs as has been well characterized by others (2). IL-1 (10 ng/ml) gave a similar time course of activation of NF-κB to that seen in ECV304 cells, with activity detectable after 5 min (compare *lanes 2* and *1*), maximal at 1 h (*lane 5*) and still apparent at 24 h (*lane 8*). Activation of NF-κB by TNF (10 ng/ml) and PMA (100 ng/ml) at a single time point (1 h) is also shown (compare *lanes 10* and *11*, respectively, with *lane 9*). In contrast to ECV304 cells, H_2O_2 failed to activate NF-κB in HUVECs. No activation was apparent upon a 2- or 4-h incubation of HUVECs with either 0.2 or 0.4 mM H_2O_2 (compare *lanes 12–15* with *lane 9*). Higher doses of H_2O_2 also failed to activate NF-κB (not shown). These results highlight an important difference between the transformed (ECV304) and primary (HUVEC) ECs in terms of responsiveness of NF-κB to H_2O_2.

Cytokines and Hydrogen Peroxide Stimulate IκBα Degradation in ECV304 Cells, but with Different Kinetics—Since degradation of the inhibitory subunit IκBα is a common key event in the activation of NF-κB by diverse stimuli, the effect of IL-1, TNF, and H_2O_2 on IκBα levels in ECV304 cells was examined. Fig. 2 shows a marked difference in the kinetics of IL-1-, TNF-, and H_2O_2-stimulated IκBα degradation. Although there was a large decrease in IκBα levels in response to IL-1 by 20 min (compare *lanes 2* and *1*) and to TNF by 1 h (compare *lanes 6* and *1*), H_2O_2-stimulated degradation was only detectable at 2 h (*lane 10*), and obvious by 3 h (*lane 13*). By 4 h, IκBα levels were

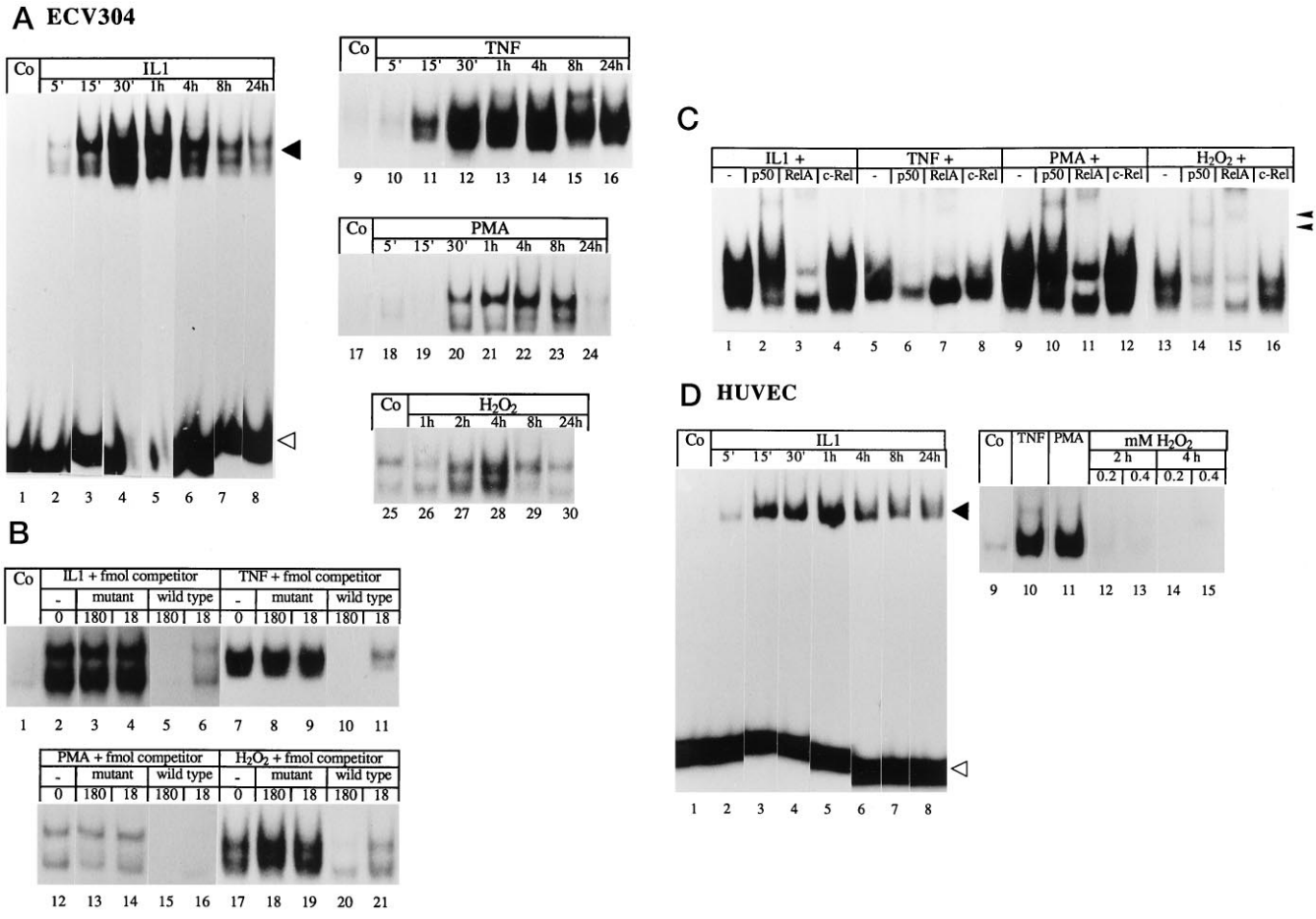

FIG. 1. **Kinetics of the effect of IL-1, TNF, PMA, and H_2O_2 on NF-κB activation in ECV304 cells and HUVECs.** Confluent monolayers of ECV304 cells (*A*, *B*, and *C*) or HUVECs (*D*) were treated as follows before preparation of nuclear extracts. *A*, ECV304 cells were treated with vehicle (*Co*), 10 ng/ml IL-1, 10 ng/ml TNF, 100 ng/ml PMA, or 0.2 mM H_2O_2 for the times indicated *above* the *lanes*. *B*, nuclear extracts from ECV304 cells stimulated for 1 h with IL-1, TNF, or PMA or for 4 h with H_2O_2 were incubated with 18 or 180 fmol of unlabeled wild type or mutant NF-κB probe as indicated for competition analysis (described under "Experimental Procedures"). *C*, nuclear extracts from ECV304 cells stimulated for 1 h with IL-1, TNF, or PMA or for 4 h with H_2O_2 were incubated with antisera to p50, RelA, or c-Rel as indicated for supershift analysis (described under "Experimental Procedures"). The position of supershifted complexes is indicated with an *upper* (RelA) and *lower* (p50) *arrowhead*. *D*, HUVECs were treated with vehicle (*Co*), 10 ng/ml IL-1 for the stated times, or 10 ng/ml TNF or 100 ng/ml PMA for 1 h. In *lanes 12–15*, cells were treated with 0.2 or 0.4 mM H_2O_2 for 2 or 4 h as indicated. Control (*Co*) levels of NF-κB remained unchanged at different time points in all experiments. In all cases, nuclear extracts were assessed for the presence of NF-κB as described under "Experimental Procedures." In *A* and *D*, *lanes 1–8*, retarded protein-DNA complexes are shown by a *filled arrowhead*, and unbound DNA probe is shown by an *open arrowhead*. Subsequently, only retarded NF-κB·DNA complexes are shown. Results shown are representative of 2–4 experiments.

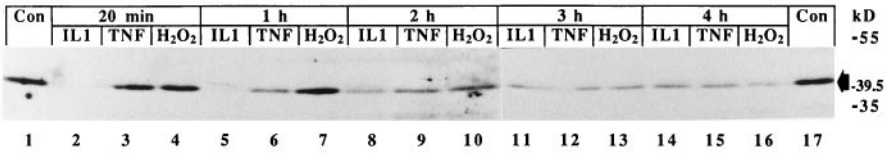

FIG. 2. **IL-1, TNF, and H_2O_2 stimulate IκBα degradation in ECV304 cells but with different kinetics.** Confluent ECV304 monolayers were stimulated with 10 ng/ml IL-1, 10 ng/ml TNF, or 0.2 mM H_2O_2 for the indicated times. Control cells were left untreated (*Con*) and remained unchanged over the time course of the experiment. Cell lysates were prepared and assessed for the presence of IκBα by Western blotting as described under "Experimental Procedures." The band marked with an *arrow* was the only one detected and corresponds to IκBα. The positions of the relevant molecular weight markers are shown. Results are representative of two experiments.

increasing again in the case of IL-1 and TNF (*lanes 14* and *15*), whereas levels were still decreasing in response to H_2O_2 (*lane 16*). This slower degradation of IκBα induced by H_2O_2 in comparison with IL-1 and TNF was consistent with the different time course of NF-κB activation for the cytokines and H_2O_2 described above.

Hydrogen Peroxide Levels Do Not Increase in Response to IL-1, TNF, or PMA in ECV304 Cells—We next determined the effect of IL-1, TNF, and PMA on H_2O_2 production by ECV304 cells. The rate of H_2O_2 production over 30 min was measured. Table I shows that there was no significant increase above basal H_2O_2 release from ECV304 cells upon exposure to IL-1, TNF, or PMA over five separate experiments. This suggested that an increase in intracellular H_2O_2 levels is not necessary for IL-1, TNF, or PMA activation of NF-κB in ECV304 cells.

PDTC Differentially Inhibits NF-κB Activated by IL-1, TNF, and PMA in ECV304 Cells and HUVECs—PDTC has been shown to inhibit NF-κB activated by a variety of agents in different cell types. We therefore tested the effect of this inhibitor on NF-κB activation in both ECV304 cells and HUVECs. Surprisingly, PDTC failed to inhibit NF-κB binding activity stimulated by IL-1 in ECV304 cells. Fig. 3*A* shows that the

IL-1-stimulated activity was completely insensitive to a range of PDTC concentrations (*lanes 2–6*). Interestingly, TNF and PMA were inhibited. Pretreatment of cells for 1 h with 0.01–10 mM PDTC caused a dose-dependent inhibition of the NF-κB binding activity stimulated by TNF or PMA (compare *lanes 8–11* with lane *7* and lanes *13–16* with lane *12*). This inhibition was partially relieved at 10 mM PDTC in the case of PMA (*lane 16*). Activation of NF-κB by H_2O_2 in ECV304 cells was also inhibited by PDTC (compare *lanes 18* and *17*).

A different result was obtained in HUVECs, however. Both IL-1- and TNF-activated NF-κB were completely insensitive to PDTC. 0.01–10 mM PDTC had no effect on IL-1 activation (Fig. 3*B*, compare *lanes 3–6* with *lane 2*) as was the case for ECV304 cells, while in contrast to ECV304s, 0.1–10 mM PDTC also failed to inhibit TNF (compare *lanes 8–12* with *lane 7*). Activation of NF-κB by PMA in HUVECs was potently inhibited by PDTC, however (compare *lanes 15–18* with *lane 14*), demonstrating that the compound was active in these cells and that at least one pathway to NF-κB in HUVECs was still sensitive to PDTC.

Hence, in endothelial cells, only some pathways to NF-κB were sensitive to PDTC, depending on the particular stimulants used and on whether the cells were primary (HUVECs) or transformed (ECV304s).

Inhibition of NF-κB by PDTC in ECV304 Cells Is Due to the Compound's Dithiocarboxy Group—To elucidate the mechanism of inhibition of PDTC, studies were carried out involving structural and functional analogues. Fig. 4*A* shows that another dithiocarbamate, diethyldithiocarbamate (DDTC) mimicked the pattern of inhibition seen for PDTC in ECV304 cells in that, over the concentration range 0.01–10 mM, IL-1 activation of NF-κB proved insensitive to DDTC (compare *lanes 3–6* with *lane 2*), whereas both TNF- and PMA-activated NF-κB were inhibited (compare *lanes 8–11* with *lane 7* and *lanes 13–16* with *lane 12*). Similar to PDTC, inhibition was partially relieved at 10 mM DDTC for PMA (*lane 16*). Two other compounds with similar structures to PDTC, but lacking the dithiocarboxy group, PGA, and pyrrolidine, failed to inhibit either TNF or PMA activation of NF-κB at 1 mM (Fig. 4, *B* and *C*), a concentration at which PDTC and DDTC were inhibitory. Fig. 4*D* shows the structures of PDTC, DDTC, PGA, and pyrrolidine for comparison. These results indicated that the inhibitory activity of PDTC was due to its dithiocarboxy group.

TABLE I

IL-1, TNF, and PMA do not increase extracellular H_2O_2 release in ECV304 cells

Confluent monolayers of ECV304 cells were left untreated (control) or stimulated with the indicated concentration of IL-1, TNF, or PMA for 30 min. H_2O_2 release from the cells was measured as described under "Experimental Procedures." The data are expressed as the mean ±S.E. for five experiments, each performed in triplicate. There was no significant difference between values for stimulated and control cells.

Treatment	H_2O_2 release
	pmol/min/mg protein
Control	6.7 ± 1.6
10 ng/ml IL-1	6.6 ± 1.4
10 ng/ml TNF	7.6 ± 1.7
100 ng/ml PMA	8.2 ± 2.3

Effect of Other Antioxidants and Iron Chelators on NF-κB Activation by IL-1, TNF, and PMA in Endothelial Cells—We next examined structurally unrelated, functional analogues of PDTC. Dithiocarbamates are known to have antioxidant (9) and iron-chelating properties (20). We therefore tested the effect of other antioxidants on NF-κB activation by IL-1, TNF, and PMA. The radical scavenger and glutathione precursor NAC has been shown to inhibit NF-κB in some cell types (12, 13) but not others (16, 17). Fig. 5, *A* and *B*, show that NAC failed to inhibit the IL-1-, TNF-, or PMA-stimulated pathways to NF-κB in either ECV304 cells or HUVECs. Pretreatment of either endothelial cell type for 1 h with 10–40 mM NAC had no effect on NF-κB activated by IL-1, TNF, or PMA. However, NAC was found to inhibit activation of NF-κB by H_2O_2 in ECV304 cells (Fig. 5*A*, compare *lanes 14* and *13*), highlighting a further difference between this pathway to NF-κB activation and the cytokine-mediated pathways. NAC also inhibited activation of NF-κB by TNF in Jurkat T cells within the same concentration range (Fig. 5*C*, compare *lanes 3–5* with *lane 2*), as has been previously reported (12), pointing to a difference between TNF responses in Jurkat cells and ECs.

Another antioxidant, the xanthine oxidase inhibitor allopurinol (tested up to 1 mM), also failed to inhibit NF-κB activated by IL-1, TNF, and PMA in ECV304 cells (not shown).

We next tested iron chelators. ECV304 cells were pretreated with the specific ferric iron chelator DFO for 18 h (the longer preincubation time was necessary, since DFO is taken up slowly by mammalian cells (33)) before a 1-h stimulation with IL-1, TNF, or PMA. Fig. 6 shows that activation of NF-κB by both TNF and PMA was dose-dependently inhibited between 0.1 and 1 mM DFO (compare *lanes 6–9* with *lane 5*, and compare *lanes 12–15* with *lane 11*), while concentrations of up to 1

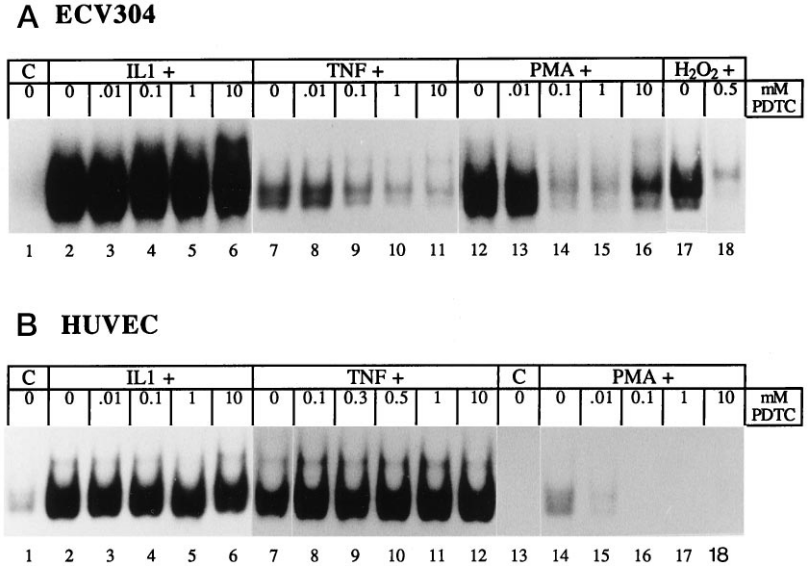

FIG. 3. **Effect of PDTC on NF-κB activation by IL-1, TNF, PMA, and H_2O_2 in ECV304 cells and HUVECs.** Confluent monolayers of ECV304 cells (*panel A*) or HUVECs (*panel B*) were pretreated for 1 h with 0, 0.01, 0.1, 1, or 10 mM PDTC, as indicated. Cells were subsequently stimulated as follows before preparation of nuclear extracts. *Panel A*, ECV304 cells were treated with vehicle (*C*), 10 ng/ml IL-1, 10 ng/ml TNF, or 100 ng/ml PMA for 1 h or with 0.2 mM H_2O_2 for 4 h. *Panel B*, HUVECs were treated with vehicle (*C*), 10 ng/ml IL-1, 10 ng/ml TNF, or 100 ng/ml PMA for 1 h. In all cases, nuclear extracts were assessed for the presence of NF-κB as described under "Experimental Procedures." Retarded protein-DNA complexes are shown. Results are representative of two or three experiments.

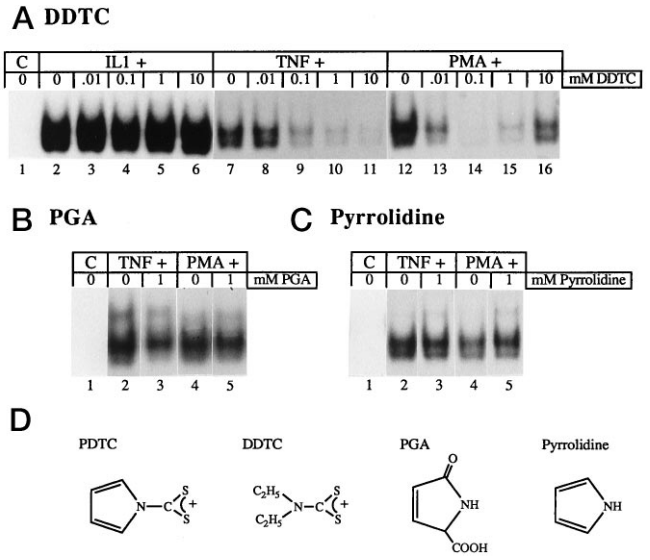

FIG. 4. **PDTC inhibition of NF-κB activation is due to its dithiocarbamate moiety.** Confluent ECV304 monolayers were pretreated with the indicated concentrations of DDTC (*panel A*), PGA (*panel B*), or pyrrolidine (*panel C*) before stimulation with 10 ng/ml IL-1, 10 ng/ml TNF, or 100 ng/ml PMA for 1 h as indicated. Control cells were left unstimulated, indicated by *C above* the *lanes*. Nuclear extracts were prepared and assessed for the presence of NF-κB as described under "Experimental Procedures." Retarded protein-DNA complexes are shown. Results are representative of two experiments. In *panel D*, the structure of the compounds is shown for comparative purposes.

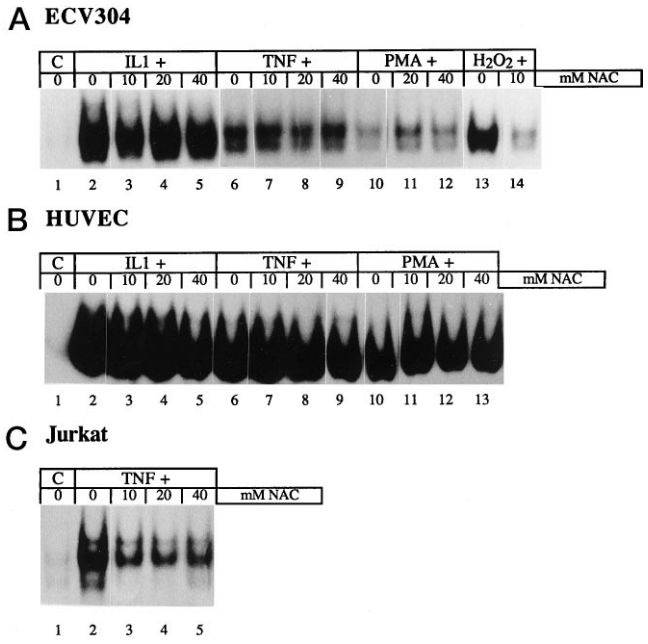

FIG. 5. **Effect of *N*-acetyl-L-cysteine on NF-κB activation in ECV304 cells, HUVECs, and Jurkat T cells.** Cells were pretreated with 0, 10, 20, or 40 mM NAC for 1 h as indicated and were subsequently stimulated as follows. *Panel A*, ECV304 cells were treated with 10 ng/ml IL-1, 10 ng/ml TNF, or 100 ng/ml PMA for 1 h or with 0.2 mM H_2O_2 for 4 h. *Panel B*, HUVECs were treated with 10 ng/ml IL-1, 10 ng/ml TNF, or 100 ng/ml PMA for 1 h. *Panel C*, Jurkat T cells were treated with 10 ng/ml TNF for 1 h. Control cells were left unstimulated (*C*, *first lane* of each *panel*). Nuclear extracts were prepared and assessed for the presence of NF-κB as described under "Experimental Procedures." Retarded protein-DNA complexes are shown. Results are representative of two or three experiments.

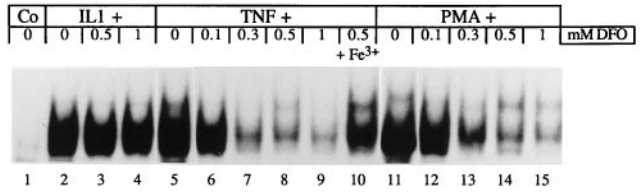

FIG. 6. **Desferrioxamine mimics the pattern of NF-κB inhibition seen for PDTC in ECV304 cells.** Confluent monolayers of ECV304 cells were pretreated for 18 h with the indicated concentrations of DFO (0, 0.1, 0.3, 0.5, and 1 mM) before stimulation for 1 h with 10 ng/ml IL-1, 10 ng/ml TNF, or 100 ng/ml PMA. In *lane 10*, 0.5 mM $FeCl_3$ was added to cells at the same time as DFO. Control cells were left unstimulated (*Co*). Nuclear extracts were prepared and assessed for the presence of NF-κB as described under "Experimental Procedures." Retarded protein-DNA complexes are shown. Results are representative of four experiments.

mM DFO had no effect on IL-1 activation (compare *lanes 3* and *4* with *lane 2*). The inhibitory effect of DFO was confirmed to be due to its iron-chelating properties, since co-incubation of DFO with an equimolar concentration of ferric ions prevented its inhibitory effect on NF-κB activation by TNF (compare *lanes 10* and *8*).

Another compound with activity toward metals mimicked the pattern of PDTC and DFO inhibition in ECV304 cells, namely TEMPO, a nitroxide spin trap that can maintain iron as Fe^{3+} and thus prevent redox cycling of the transition metal (34). Fig. 7*A* shows that, similar to PDTC and DFO, IL-1-activated NF-κB was insensitive to 1–2 mM TEMPO (compare *lanes 3* and *4* with *lane 2*), while both TNF- and PMA-activated NF-κB proved sensitive to the compound over this concentration range (compare *lanes 6* and *7* with *lane 5*, and compare *lanes 9* and *10* with *lane 8*, respectively). In addition, DMPO, another commonly used spin trap that does not have such activity against iron but can trap hydroxyl and superoxide radicals (35), had no effect on IL-1-, TNF-, or PMA-stimulated NF-κB activation up to 40 mM (Fig. 7*B*).

These results suggested that the basis of the PDTC inhibition is iron chelation rather than a general antioxidant effect and that the TNF pathway in ECV304 cells has a requirement for iron that is able to redox cycle.

PDTC and DFO Inhibit NF-κB Activation by Preventing IκBα Degradation—The effect of the inhibitors on IL-1-, TNF-, and H_2O_2-stimulated IκBα degradation was determined. ECV304 cells were pretreated with 0.5 mM PDTC for 1 h or 0.5 mM DFO for 18 h before stimulation with IL-1 or TNF for 1 h or H_2O_2 for 4 h, as was the case for measurement of NF-κB activation in the inhibitor studies above. Fig. 8 shows that PDTC or DFO had no effect on IL-1-mediated IκBα degradation (compare *lanes 3* and *4* with *lane 2*), as would be expected from the lack of effect of these compounds on IL-1-mediated NF-κB activation. In contrast, both PDTC and DFO strongly inhibited TNF-stimulated IκBα degradation (compare *lanes 6* and *7* with *lane 5*), consistent with their effect on TNF-mediated NF-κB activation. These results confirm the different sensitivities of the IL-1 and TNF pathways to PDTC and DFO in ECV304 cells and also suggest that PDTC was inhibiting NF-κB activation in ECs by preventing IκBα degradation. PDTC also strongly inhibited degradation of IκBα induced by a 4-h treatment with H_2O_2 (compare *lanes 9* and *8*).

TNF, but Not IL-1 or H_2O_2, Increases Lipid Peroxidation in ECV304 Cells—DFO, DDTC, and TEMPO have well documented inhibitory effects on lipid peroxidation (33, 36, 37). Since lipid peroxidation has been implicated in NF-κB activation in some systems (14, 38–40), we therefore determined whether this was important for the difference between IL-1 and TNF here. Lipid peroxidation was assessed by the TBARS assay, which detects mainly MDA, an end product of the peroxidation of polyunsaturated fatty acids and related esters. This method has been shown to be a sensitive index of lipid peroxidation (41–43).

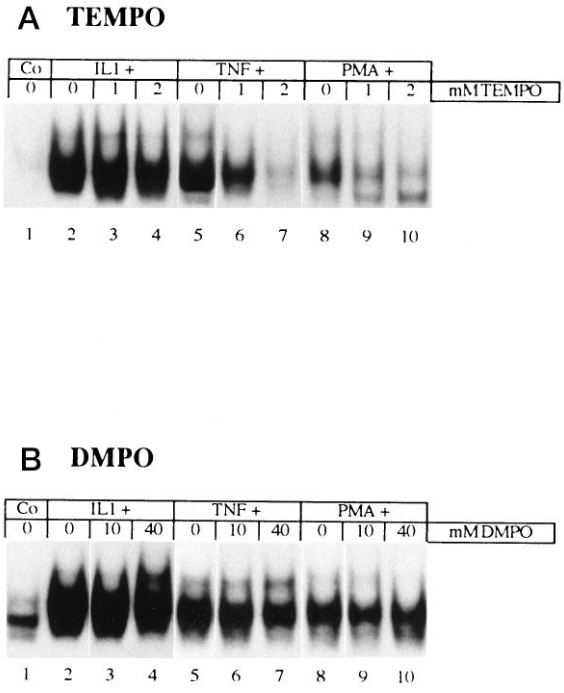

FIG. 7. **TEMPO mimics the pattern of NF-κB inhibition seen for PDTC in ECV304 cells, while DMPO has no effect.** Confluent monolayers of ECV304 cells were pretreated for 2 h with the indicated concentrations of TEMPO (*A*) (0, 1, and 2 mM) or DMPO (*B*) (0, 10 and 40 mM) before stimulation for 1 h with 10 ng/ml IL-1, 10 ng/ml TNF, or 100 ng/ml PMA. Control cells were left unstimulated (*Co*). Nuclear extracts were prepared and assessed for the presence of NF-κB as described under "Experimental Procedures." Retarded protein-DNA complexes are shown. Results in *A* are representative of three experiments, while those in *B* are representative of two experiments.

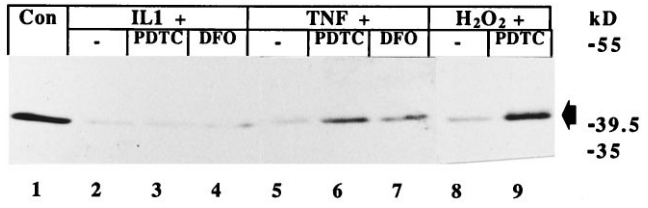

FIG. 8. **Effect of PDTC and desferrioxamine on IL-1-, TNF-, and H_2O_2-induced IκBα degradation in ECV304 cells.** Confluent ECV304 monolayers were pretreated with 0.5 mM PDTC for 1 h or 0.5 mM DFO for 18 h as indicated before stimulation with vehicle control (*Con*), 10 ng/ml IL-1, or 10 ng/ml TNF for 1 h or with 0.2 mM H_2O_2 for 4 h. Cell lysates were prepared and assessed for the presence of IκBα by Western blotting as described under "Experimental Procedures." The band marked with an *arrow* was the only one detected and corresponds to IκBα. The position of the relevant molecular weight markers is shown. Results are representative of three experiments.

TABLE II
Effect of NF-κB activators and inhibitors on TBARS formation in ECV304 cells

Top, confluent monolayers of ECV304 cells were stimulated with 40 ng/ml IL-1 or TNF for 30 min or 0.4 mM H_2O_2 for 4 h. Middle, cells were pretreated for 18 h with 0.5 mM DFO before stimulation with or without TNF. Bottom, cells were pretreated for 1 h with 0.5 mM PDTC before stimulation with or without TNF. In all cases, TBARS levels were measured in triplicate as described under "Experimental Procedures," and results were calculated as nmol of MDA equivalents/mg of protein. Results shown are mean ± S.E. TBARS levels are expressed as a percentage of matched controls (untreated cells). The number of experiments is indicated in parentheses. The mean ± S.E. control value over 26 experiments was 0.25 ± 0.02 nmol of MDA equivalents/mg of protein.

Treatment	TBARS (percentage of control)
Effect of IL-1, TNF, and H_2O_2 on TBARS	
	%
IL-1	102 ± 4 (6)
TNF	123 ± 2 (19)[a]
H_2O_2	97 ± 7 (4)
Effect of DFO on basal and TNF-stimulated TBARS	
	%
DFO	59 ± 5 (3)[a]
TNF	117 ± 2 (3)[a]
TNF + DFO	50 ± 3 (3)[a,b]
Effect of PDTC on basal and TNF-stimulated TBARS	
	%
PDTC	82 ± 17 (2)
TNF	115 ± 2 (3)[a]
PDTC + TNF	84 ± 3 (3)[a,b]

[a] $p < 0.05$ *versus* control.
[b] $p < 0.05$ *versus* TNF.

Table II (top) shows that treatment of ECV304 cells with TNF for 30 min (which strongly activates NF-κB) did indeed increase lipid peroxidation. Levels of TBARS showed a small but highly significant increase of 123 ± 2% ($p < 0.01$) above control levels. The magnitude of the effect is consistent with a noncytotoxic increase in lipid peroxidation. Importantly, stimulation of cells with IL-1 for 30 min failed to increase TBARS above control values. Interestingly, treatment of cells with H_2O_2 for 4 h also did not increase TBARS, suggesting that ECV304 cells have very effective antioxidant defense mechanisms and that lipid peroxidation is unlikely to have a role in H_2O_2-mediated NF-κB activation in these cells. In total, 19 separate experiments were performed using TNF, some of which involved PDTC, DFO, or BHA as inhibitors (see below). TNF was also used as a positive control in separate experiments involving IL-1 and H_2O_2.

PDTC and DFO both inhibited lipid peroxidation in the cells. Table II (middle and bottom) shows experiments for DFO and PDTC, respectively. DFO decreased basal levels of TBARS to 59 ± 5% of control values. PDTC was less potent, decreasing levels to 82 ± 17%. Both compounds were able to effectively block the TNF-mediated increase in TBARS, since when cells were preincubated with DFO and PDTC before stimulation with TNF, TBARS values recorded were similar to those seen for the compounds alone.

These results pointed to iron-catalyzed lipid peroxidation as the basis for the differential sensitivity of TNF and IL-1 to PDTC and DFO.

Effects of t-BHP and BHA on NF-κB and TBARS—We next examined whether a compound used to increase lipid peroxidation within cells, *t*-BHP, can itself activate NF-κB. Fig. 9*A* shows that treatment of ECV304 cells for 4 h with 0.2–1.0 mM *t*-BHP led to a dose-dependent activation of NF-κB. We confirmed that *t*-BHP was increasing lipid peroxidation within the cells, since, although it only gave a slight reaction itself with TBA, in the presence of cells TBARS levels increased to 149 ± 6% of control levels ($n = 6$). Thus, activation of NF-κB is possible by simply increasing lipid peroxidation.

Finally, we explored the potential role of lipid peroxidation in the TNF but not the IL-1 pathway by testing BHA, a chain-breaking antioxidant that inhibits lipid peroxidation but has no iron-chelating properties. Fig. 9*B* shows that pretreatment of cells with 0.2 mM BHA for 2 h strongly inhibited TNF-stimulated NF-κB activation (compare *lanes 3* and *2*). In contrast, IL-1-stimulated activation was totally insensitive to this pretreatment (compare *lanes 5* and *4*). We also confirmed that BHA, like PDTC and DFO, could indeed inhibit lipid peroxidation and block the TNF-mediated increase in TBARS (Table

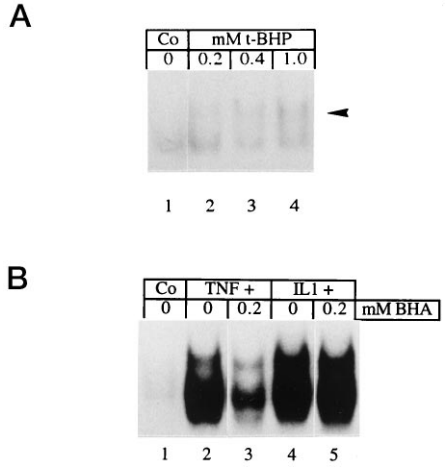

FIG. 9. **Effect of *t*-BHP and BHA on NF-κB in ECV304 cells.** *A*, confluent ECV304 monolayers were treated with 0.2, 0.4, or 1.0 mM *t*-BHP for 4 h. Control cells were left untreated (*Co*). Nuclear extracts were prepared and assessed for the presence of NF-κB as described under "Experimental Procedures." Retarded protein-DNA complexes are shown. The band marked with an *arrow* corresponds to a p50/Rel A heterodimer (see Fig. 1*C*). Results are representative of four experiments. *B*, confluent monolayers of ECV304 cells were pretreated for 2 h with 0.2 mM BHA (*lanes 3* and *5*) before stimulation for 1 h with or without 10 ng/ml TNF or IL-1. Control cells were left unstimulated (*Co*). Nuclear extracts were prepared and assessed for the presence of NF-κB as described under "Experimental Procedures." Retarded protein-DNA complexes are shown. Results are representative of three experiments.

III). These results provide strong evidence that lipid peroxidation is a necessary part of the signal activated by TNF that leads to NF-κB activation.

Taken together, the data indicate that activation of NF-κB by TNF but not IL-1 involves increases in iron-catalyzed lipid peroxidation.

DISCUSSION

In this study, we have investigated the role of H_2O_2 as well as the effect of a range of antioxidants on IL-1- and TNF-stimulated NF-κB activation in both primary and transformed human ECs. We first investigated the particular role played by H_2O_2 as a signal for NF-κB activation. We found that H_2O_2 release from ECV304 was not increased upon stimulation with IL-1, TNF, or PMA, as measured at concentrations and times of exposure to stimulants that gave strong activation of NF-κB in these cells. This is consistent with a study by Royall *et al.* (44) in bovine aortic endothelial cells, where they showed that TNF had no effect on intra- or extracellular H_2O_2 levels over a period of 12 h. Adding H_2O_2 to ECV304, however, activated NF-κB and induced IκBα degradation. Both responses were significantly slower than those seen for the cytokines. In addition, the peroxide-mediated activation proved sensitive to the antioxidant NAC, unlike that mediated by cytokines. These differences called into question the relevance of H_2O_2 in cytokine activation of NF-κB in ECs. Similar slow kinetics for NF-κB activation by H_2O_2 and other signals that induce oxidative stress have been reported by other groups in lymphoid cells (45–47). This may be characteristic of a response of NF-κB to agents that induce oxidative stress.

The failure of H_2O_2 to activate NF-κB in HUVECs was in agreement with the findings of Bradley *et al.* (15). It is difficult to discern why H_2O_2 activated NF-κB in the transformed and not the primary ECs. It has been known for some time that transformed cells have lower levels of antioxidant enzymes and subsequently a higher cellular oxidative potential than primary cells (48), implying that transformed cells would be more susceptible to the effects of H_2O_2. Chiao *et al.* (49) recently

TABLE III
Effect of BHA on basal and TNF-stimulated TBARS

Confluent monolayers of ECV304 cells were pretreated with 0.2 mM BHA for 2 h before stimulation with or without 40 ng/ml TNF for 30 min. Control cells were left untreated. TBARS levels were measured in triplicate as described under "Experimental Procedures," and results were calculated as nmol of MDA equivalents/mg of protein. Results shown are mean ± S.E. TBARS levels are expressed as a percentage of control. The number of experiments is indicated in parentheses.

Treatment	TBARS (percentage of control)
	%
BHA	74 ± 4 (3)[a]
TNF	121 ± 6 (4)[a]
TNF + BHA	84 ± 2 (4)[a,b]

[a] $p < 0.02$ *versus* control.
[b] $p < 0.02$ *versus* TNF.

demonstrated that primary rat embryo fibroblasts were resistant to H_2O_2-induced apoptosis, while their adenovirus-transformed counterparts were sensitive. Sen *et al.* (50) have shown that differences in the kinetics of an increase in intracellular $[Ca^{2+}]$ in response to H_2O_2 may be the basis of the difference in sensitivity of NF-κB to H_2O_2 in sensitive and insensitive Jurkat subclones, which could conceivably be important in the primary-transformed difference observed in our study. Interestingly, they also treated cells for 4 h, with 0.25 mM H_2O_2, similar to the conditions used here.

Our experiments with PDTC revealed for the first time a receptor-mediated, PDTC-insensitive pathway to NF-κB (activated by IL-1) in the same cell type (ECV304) as a receptor-mediated pathway that is sensitive (activated by TNF). It has been reported recently that the activation of NF-κB by IL-1 in epithelial cell lines is also insensitive to PDTC (47). We also found a difference in sensitivity between a particular pathway (TNF) in a transformed and primary cell line. This implies that PDTC inhibition of NF-κB is neither stimulus- nor cell type-independent as has been previously postulated (9). Different TNF responses between primary and transformed cells have been reported by others. For example, TNF is cytotoxic to many tumor cells but not primary cells (51).

The pathway activated by PMA was sensitive to PDTC in both cell types. This was an important control, demonstrating that PDTC was active in the primary cells. It also indicated that the PMA response consistently activated NF-κB in a PDTC-sensitive manner, irrespective of whether the cells were primary or transformed, presumably through a protein kinase C-dependent pathway.

PDTC could have a number of effects within cells, including scavenging of ROS (9), chelation of divalent metal ions (20), alteration of intracellular thiol levels (52), and modification of proteins by decomposition products (53). The comparison with structural and functional analogues of PDTC demonstrated that its inhibitory effect on TNF was probably due to metal chelation rather than a general antioxidant effect. The failure of the widely used antioxidant NAC to inhibit the cytokines while inhibiting H_2O_2 was further evidence against a role for oxidative stress in NF-κB activation by the cytokines. We and others have shown insensitivity to NAC in response to IL-1 and/or TNF in other cell types (16, 17, 47). We were unable to find a single report of NAC inhibiting NF-κB in ECs. Like PDTC, an effect of NAC on gene expression has often been used to implicate NF-κB in that pathway, but it has been shown that both compounds can inhibit protein expression independent of an effect on NF-κB (17, 54, 55).

Because of the lack of effect of NAC, it was possible that the metal-chelating properties of PDTC were responsible for its inhibitory effect, as distinct from its antioxidant properties. We therefore examined two metal chelators, DFO and TEMPO.

Both of these mimicked the pattern of inhibition of NF-κB by PDTC in ECV304 cells. DFO is a specific ferric iron chelator (33). The addition of ferric ions prevented the inhibition of the TNF-mediated activation, indicating that DFO was inhibiting via its iron-chelating properties. This is the first report of TEMPO inhibiting NF-κB activation. In addition to scavenging superoxide anion and hydroxyl radical, this nitroxide spin trap compound can maintain iron in its ferric form and thus prevent it from redox cycling (34, 56). Since the other ROS scavengers NAC and DMPO did not inhibit activation of NF-κB by TNF, it is likely that TEMPO was inhibiting due to its ability to trap iron in its ferric form. This ability of TEMPO to chelate iron is a key difference between it and DMPO. Taken together, these results suggest that the property of PDTC (and DDTC) that is important in its inhibitory effect on NF-κB in ECs is its ability to chelate iron. Both PDTC and DDTC have high affinities for iron, with log β_3 values for ferrous ions of 12.7 and 11.3, respectively (57).

PDTC and DFO were found to block TNF-stimulated IκBα degradation. Since degradation of IκBα is tightly coupled to its phosphorylation and subsequent release from the latent complex, PDTC was likely to be inhibiting the TNF (and PMA) pathway(s) at a point upstream of IκBα phosphorylation. Thus, inhibition by PDTC in ECV304 cells defines a requirement of the TNF, but not the IL-1 pathway to NF-κB for iron, at a point upstream of IκBα phosphorylation.

We suspected that the role of iron in TNF-mediated NF-κB activation in ECV304 cells was to promote lipid peroxidation and that the failure of PDTC and DFO to inhibit NF-κB activation by IL-1 was due to the lack of involvement of lipid peroxidation in the IL-1 pathway. Inhibition by DFO and TEMPO suggested that the iron required was available to these compounds and that redox cycling of iron was necessary for NF-κB activation by TNF. This could suggest an involvement of the Fenton reaction, but this was felt to be unlikely since we found no role for H_2O_2 in any of the pathways to NF-κB in these cells. The lack of effect of NAC and DMPO on the pathways would also argue against this. However, iron-dependent lipid peroxidation could be important, since this process requires redox cycling of iron and does not necessarily require H_2O_2 or ROS (58). Indeed, iron has a key role in both the initiation and propagation of lipid peroxidation, leading to the generation of peroxyl and alkoxyl radicals as well as lipid peroxides (58). A role for iron-catalyzed lipid peroxidation was further suggested by the fact that DDTC, DFO, and TEMPO all have well documented inhibitory effects on iron-catalyzed membrane lipid peroxidation (33, 36, 37). In addition, there are a number of reports in the literature implicating lipid peroxidation in pathways to NF-κB. TNF-stimulated NF-κB binding activity was inhibited by lipid peroxide inhibitors such as BHA and anetholdithiolthione in T cell lines (14, 38). The addition of linoleic acid to porcine pulmonary endothelial cells led to an increase in lipid peroxide levels and activation of NF-κB, both responses being inhibited by pretreatment of cells with the lipid peroxide scavenger vitamin E (39). There is also strong evidence that NF-κB mediates the induction of inflammatory genes known to be activated by lipid-peroxide species, such as those generated *in vivo* by oxidized low density lipoprotein (5, 40, 59).

In this study, several lines of evidence implicated iron-catalyzed lipid peroxidation in TNF- but not IL-1-mediated NF-κB activation in transformed ECs. First, stimulation of ECV304 cells with TNF led to an increase in TBARS, with no effect observed with IL-1. Second, PDTC and DFO limited lipid peroxidation in the cells, as indicated by a decrease in TBARS, and further blocked the TNF-mediated increase in TBARS. Third, *t*-BHP, an agent well known to increase lipid peroxidation, activated NF-κB. Finally and most importantly, similar to DFO and PDTC, a nonchelating inhibitor of lipid peroxidation, BHA, also inhibited lipid peroxidation and TNF- but not IL-1-stimulated NF-κB activation. Particular lipid peroxides or radicals, generated via an iron-dependent mechanism, are therefore likely to have a key role in mediating NF-κB activation by TNF in ECV304 cells. The fact that *t*-BHP gave a larger increase of TBARS than TNF while being a weaker activator of NF-κB may suggest that lipid peroxidation is necessary but not sufficient for the TNF pathway. *t*-BHP may give rise to a different subset of lipid peroxides that may include only low levels of the putative lipid peroxides required by TNF. This would be consistent with the low levels of NF-κB in control cells despite detectable levels of TBARS. The precise mechanism whereby TNF induces lipid peroxidation in an iron-dependent manner remains to be determined. TNF could stimulate the release of iron from a heme protein or from the mitochondrial redox chain. Alternatively, 5-lipoxygenase may be involved, since this enzyme requires DFO-chelatable, redox-active iron and leads to the generation of lipid peroxides (60). Interestingly, this enzyme has been implicated in TNF-mediated cytotoxicity (61) and also in NF-κB activation by CD28 in primary T cells (11).

As neither IL-1 nor H_2O_2 increased lipid peroxidation and yet activated NF-κB, other processes can clearly activate NF-κB in ECs. The inhibitory effect of PDTC on H_2O_2 was most likely due to the antioxidant properties of PDTC.

Other differences are beginning to emerge between TNF and IL-1 signaling pathways, particularly with regard to early components involved in NF-κB activation. TRAF-2 has been shown to be critical for NF-κB activation by TNF but not IL-1 (62), while TRAF-6 has been implicated in NF-κB activation by IL-1 alone (63). A common kinase for both cytokine pathways has been identified, however, termed NF-κB-inducing kinase, which lies downstream of TRAF-2 and possibly of TRAF-6 (64). We speculate that lipid peroxidation occurs on the TRAF-2 pathway upstream of NF-κB-inducing kinase.

A role for lipid peroxidation in the activation of NF-κB in ECs may have wider implications. Several groups have suggested and provided evidence that activated NF-κB has a role in the pathogenesis of atherosclerosis and that activation may be mediated by lipid peroxides released from oxidized low density lipoprotein, since this molecule is atherogenic and can activate NF-κB (5, 24, 40, 59). Coupled with the fact that activated NF-κB has recently been identified in endothelial cells in the atherosclerotic lesion (4), it will be important to fully investigate the role of lipid peroxides in NF-κB activation in ECs.

In conclusion, this study argues against a role for H_2O_2 or ROS in NF-κB activation by IL-1 or TNF in ECs. However, the mechanisms by which TNF and IL-1 activate NF-κB differ, with the TNF pathway in ECV304 cells involving iron-catalyzed lipid peroxidation.

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