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 H₂O₂ 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 H₂O₂, 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 H₂O₂ 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 IkB degradation by H₂O₂ 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 H₂O₂ was sensitive to inhibitors 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 IkB 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. Pyrrolidine dithiocarbamate has been purported to be a specific antioxidant inhibitor of NF-κB that acts independently of activating agent or cell type. Therefore, we found that tumor necrosis factor- but not interleukin-1-driven NF-κB activation and IkB 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 H₂O₂ 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) 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 IκBa, 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 IκBa in resting cells. Upon stimulation with agents such as IL-1 and TNF, IκBa is rapidly phosphorylated on two serine residues (Ser32 and Ser36), which targets the inhibitor protein for ubiquitination and subsequent degradation by the 26S 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 IκBa are tightly coupled events (7). Thus, it is likely that agents that activate NF-κB do so through the activation of a specific IκBa kinase or, alternatively, by inactivating a particular phosphatase. A high molecular mass kinase complex that phosphorylates IκBa on Ser32 and Ser36 has been identified (8).

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; PGα, pyrogulatic 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, H2O2 has been implicated as a common second messenger in the various pathways leading to NF-κB activation (10). This hypothesis is based on several lines of evidence. First, in some cell types H2O2 has been shown to be released in response to agents that also activate NF-κB (9–11). Second, direct addition of H2O2 to culture medium has been shown to activate NF-κB in some cell lines (12, 13). Third, overexpression of the H2O2-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 H2O2-producing enzyme superoxide dismutase potentiated the activation, suggesting a role for H2O2 in these pathways to NF-κB (10). Other work has suggested that some of these observations are cell-specific. H2O2 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 H2O2 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 pyrroldine 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 H2O2 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 H2O2 and antioxidants on NF-κB activation, we decided to investigate the role of H2O2 and oxidative stress in NF-κB activation in ECs using both primary and transformed ECs. Our results show that although H2O2 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 Dr. Steve Foster (Zeneca Pharmaceuticals, Macclesfield, UK). The 22-base pair oligonucleotide, 5′-AGT GA GGC GAC TTC CCC AGG C-3′, containing the NF-κB consensus sequence (underlined), T4 polynucleotide kinase, and the Cy3 fluorochrome were radioactivity-cytotoxicity assay from Promega Corporation (Madison, WI). The 22-base pair oligonucleotide, 5′-AGT GA GGC GAC TTC 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 p65 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-32P]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, pyrogallol acid, NAC, desferoxamine, allopurinol, the spin traps 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), and 5,5-dimethyl-1-pyrroline N-oxide (DMPO), FeCl3, H2O2, PMTA, butylated hydroxyanisole (BHA), tert-butyldihydroperoxide (t-BHP), thiobarbituric acid, anti-mouse IgG peroxidase conjugate, scopolenin, horseradish peroxidase (type II), and catalase were from Sigma (Poole, UK).

Cell Culture and Treatment—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% CO2. Cells were pretreated with the test compounds or left untreated before the addition of IL-1, TNF, PMA, or H2O2 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; pyrogallol acid (PGA), desferoxamine (DFO), and FeCl3, were dissolved in H2O; NAC was dissolved in 25 mM Tris-HCl, pH 7.5; BHA was dissolved in ethanol; and PMTA, allopurinol, TEMPO, and DMPO were dissolved in MeSO. 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 chelators were used. The effect of the compounds on cell viability was assessed using the CellToxTM 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 540 nm in the presence of 75 mM Tris-HCl, pH 7.2, containing 50 mM NaH2PO4 and 4 mM MgCl2.

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 Determination of Nuclear Extracts—Nuclear extracts were prepared using a modified version of the method of O’Doherty et al. (25). 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 mM NaCl, 0.027 mM 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 MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). Stimulation of Jurkat T cells (5 × 106 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-32P]ATP (10 μCi/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 antisera 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-IgBo 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 radiolabeled probe 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 Na3VO4. Following further disruption of cells by passage through a 21-gauge needle (5 strokes), an additional 0.1 μg/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 IgBo immunoblot analysis was performed as described previously (30).

**Determination of H2O2—**H2O2 released from ECV304 cells was measured by 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 Hank's 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), sodium fluoride, 7% DMSO, and 2 mg/ml sodium deoxycholate, 0.5% (w/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 Na3VO4). Following further disruption of cells by passage through a 21-gauge needle (5 strokes), an additional 0.1 μg/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 IgBo immunoblot analysis was performed as described previously (30).

**Determination of Thiobarbituric Acid-reactive Substances (TBARS)—**Lipid peroxidation was assessed by the TBARS assay, which detects the fluorescent product 1,1,3,3-tetramethoxypropane in 20 ml 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. 1A 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 H2O2 led to activation of NF-κB. When cells were treated with 0.2 mM H2O2 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 H2O2-stimulated activation was not detectable after 1 h (compare lanes 26 and 25) and did not peak until 4 h (lane 29). H2O2-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 H2O2 was optimal in this effect. Fig. 1B demonstrates that the protein-DNA complexes activated by IL-1, TNF, PMA, and H2O2 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. 1C demonstrates that IL-1, TNF, PMA, and H2O2 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 H2O2-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 antisemir only reacted with the upper complex (lanes 3, 7, 11, and 15). There was no detectable reaction with c-Rel antisemir (lanes 4, 8, 12, and 16). Hence, it was likely that IL-1, TNF, PMA, and H2O2 were activating two NF-κB complexes, tentatively identified as p50/p50 homodimers and p50/RelA heterodimers.

Fig. 1D 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, H2O2 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 H2O2 (compare lanes 12–15 with lane 9). Higher doses of H2O2 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 H2O2.

**Cytokines and Hydrogen Peroxide Stimulate IκBo Degradation in ECV304 Cells, but with Different Kinetics—**Since degradation of the inhibitory subunit IκBo is a common key event in the activation of NF-κB by diverse stimuli, the effect of IL-1, TNF, and H2O2 on IκBo levels in ECV304 cells was examined. Fig. 2 shows a marked difference in the kinetics of IL-1-, TNF-, and H2O2-stimulated IκBo degradation. Although there was a large decrease in IκBo 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), H2O2-stimulated degradation was only detectable at 2 h (lane 10), and obvious by 3 h (lane 13). By 4 h, IκBo levels were
increasing again in the case of IL-1 and TNF (lanes 14 and 15), whereas levels were still decreasing in response to H₂O₂ (lane 16). This slower degradation of IκBα induced by H₂O₂ in comparison with IL-1 and TNF was consistent with the different time course of NF-κB activation for the cytokines and H₂O₂ 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, or PMA on H₂O₂ production by ECV304 cells. The rate of H₂O₂ production over 30 min was measured. Table I shows that there was no significant increase above basal H₂O₂ release from ECV304 cells upon exposure to IL-1, TNF, or PMA over five separate experiments. This suggested that an increase in intracellular H₂O₂ 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. PDTC failed to inhibit NF-κB binding activity stimulated by IL-1 in ECV304 cells. Fig. 3A 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$_2$O$_2$ 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. 3B, 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. 4A 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 pyrroldine, 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. 4D shows the structures of PDTC, DDTC, PGA, and pyrroldine for comparison. These results indicated that the inhibitory activity of PDTC was due to its dithiocarboxy group.

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 was found 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 activation by IL-1, TNF, or PMA. However, NAC was found to inhibit activation of NF-κB by H$_2$O$_2$ in ECV304 cells (Fig. 5A, 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. 5C, 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 activation by IL-1, TNF, and PMA in ECV304 cells (not shown).

We next tested iron chelators. ECV304 cells were pretreated with the specific ferric ion 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

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**Table I**

| Treatment       | H$_2$O$_2$ 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                            |

**Fig. 3.** Effect of PDTC on NF-κB activation by IL-1, TNF, PMA, and H$_2$O$_2$ 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$_2$O$_2$ 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.
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**Fig. 4.** PDTC inhibition of NF-κB activation is due to its dithiocarbamate moiety. Confluent ECV304 monolayers were pre-treated with the indicated concentrations of PDTC (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 H2O2 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 FeCl3 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.

**A** DDTC

**B** PGA

**C** Pyrrolidine

**D**

**Fig. 4.** PDTC inhibition of NF-κB activation is due to its dithiocarbamate moiety. Confluent ECV304 monolayers were pre-treated with the indicated concentrations of PDTC (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.

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 Fe2+ and thus prevent redox cycling of the transition metal (34). Fig. 7A 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. 7B).

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 H2O2-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 H2O2 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 H2O2 (compare lanes 9 and 8).

**TNF, but Not IL-1 or H2O2 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 assayed by the TBARS assay, which detects mainly MDA, an end product of the per-oxidation of polyunsaturated fatty acids and related esters. This method has been shown to be a sensitive index of lipid peroxidation (41–43).
**NF-κB Activation in Endothelial Cells**

**A TEMPO**

**B DMPO**

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**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 preincubated with 0.5 mM TEMPO (A) or 0.5 mM DMPO (B) for 1, 10, and 40 min 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.

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 nontoxic 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$_2$O$_2$ for 4 h also did not increase TBARS, suggesting that above control values. Interestingly, treatment of cells with IL-1 for 30 min failed to increase TBARS above control values. Importantly, stimulation of cells with IL-1 for 30 min failed to increase TBARS above control values. Interestingly, treatment of cells with IL-1 for 30 min failed to increase TBARS above control values. Importantly, stimulation of cells with IL-1 for 30 min failed to increase TBARS above control values. Importantly, stimulation of cells with IL-1 for 30 min failed to increase TBARS above control values. Importantly, stimulation of cells with IL-1 for 30 min failed to increase TBARS above control values. Importantly, stimulation of cells with IL-1 for 30 min failed to increase TBARS above control values. Importantly, stimulation of cells with IL-1 for 30 min failed to increase TBARS above control values. Importantly, stimulation of cells with IL-1 for 30 min failed to increase TBARS above control values. Importantly, stimulation of cells with IL-1 for 30 min failed to increase TBARS above control values. Importantly, stimulation of cells with IL-1 for 30 min failed to increase TBARS above control values.

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 82% of control values. PDTC was less potent, decreasing levels to 59% of control values. PDTC was less potent, decreasing levels to 59% of control values. PDTC was less potent, decreasing levels to 59% of control values. PDTC was less potent, decreasing levels to 59% of control values.

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. 9A 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. 9B 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 II).
NF-κB Activation in Endothelial Cells

**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. 1C). 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 H₂O₂ 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 H₂O₂ as a signal for NF-κB activation. We found that H₂O₂ 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 H₂O₂ levels over a period of 12 h. Adding H₂O₂ 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 H₂O₂ in cytokine activation of NF-κB in ECs. Similar slow kinetics for NF-κB activation by H₂O₂ 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 H₂O₂ to activate NF-κB in HUVECs was in agreement with the findings of Bradley et al. (15). It is difficult to discern why H₂O₂ 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 H₂O₂. Chiao et al. (49) recently demonstrated that primary rat embryo fibroblasts were resistant to H₂O₂-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 [Ca²⁺] in response to H₂O₂ may be the basis of the difference in sensitivity of NF-κB to H₂O₂ 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 H₂O₂, 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 is H₂O₂-dependent, similar to the conditions used here. 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 H₂O₂ 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 \( p_d \) values for ferrous ions of 12.7 and 11.3, respectively (57).

PDTC and DFO were found to block TNF-stimulated IκB 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\(_2\)O\(_2\) 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\(_2\)O\(_2\) 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 anetholethioliathione 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 \textit{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\(_2\)O\(_2\) increased lipid peroxidation and yet activated NF-κB, other processes can clearly activate NF-κB in ECs. The inhibitory effect of PDTC on H\(_2\)O\(_2\) 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\(_2\)O\(_2\) 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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