Cooperativity between Oxidants and Tumor Necrosis Factor in the Activation of Nuclear Factor (NF)-κB

Requirement of Ras/Mitogen-Activated Protein Kinases in the Activation of NF-κB by Oxidants

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The transcription factor nuclear factor (NF)-κB is activated by oxidative stress or cytokines and is critical to the activation of inflammatory genes. Here, we report that hydrogen peroxide or 3-morpholinosydnonimine, which simultaneously releases nitric oxide and superoxide, synergize with the cytokine tumor necrosis factor (TNF)-α to activate NF-κB in rat lung epithelial cells, suggesting that signaling pathways elicited by reactive oxygen species (ROS)/reactive nitrogen species (RNS) are different from TNF-induced signaling. These findings were substantiated by observations that levels of IκB-α did not change after exposure to ROS/RNS, whereas a rapid depletion of IκB-α was observed in cells exposed to TNF. In addition, the proteosome inhibitor MG132 did not affect activation of NF-κB by ROS/RNS, whereas it abolished the TNF response. Transfection of a dominant negative Ras construct prevented the activation of NF-κB by ROS/RNS, demonstrating the requirement for Ras in the activation of NF-κB by oxidants. In contrast, TNF activated NF-κB in a Ras-independent fashion. Evaluation of members of the mitogen-activated protein kinase (MAPK) family as downstream effectors of Ras revealed the requirement of MAPK/extracellular-regulated kinase (ERK) kinase kinase (MEKK)1 and c-Jun N-terminal kinases in the induction of NF-κB by both oxidants and TNF, whereas the MEK–ERK pathway negatively regulates NF-κB. Our findings demonstrate that cytokines and oxidants cooperate in the activation of transcription factors through distinct pathways, and suggest that anti-inflammatory and antioxidant therapies may be required in concert to prevent the activation of NF-κB–regulated genes important in the development of inflammatory diseases. **Janssen-Heininger, Y. M. W., I. Macara, and B. T. Mossman. 1999. Cooperativity between oxidants and tumor necrosis factor in the activation of nuclear factor (NF)-κB: requirement of Ras/mitogen-activated protein kinases in the activation of NF-κB by oxidants. Am. J. Respir. Cell Mol. Biol. 20:942–952.**

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Abbreviations: β-galactosidase, β-gal; dimethyl sulfoxide, DMSO; dithiothreitol, DTT; extracellular regulated kinase, ERK; glutathione-S-transferase, GST; hydrogen peroxide, H_2O_2; hemagglutinin, HA; interleukin, IL; c-Jun N-terminal kinase, JNK; JNK 1 and 2 expression constructs, JNK1,2+/+; mitogen-activated protein kinase, MAPK; MAPK kinase kinase, MAPKKK; MAPK/ERK kinase, MEK; dominant negative MEK kinase 1, MEKK K-M; newborn bovine serum, NBS; nuclear factor, NF; NF-κB–inducing kinase, NIK; nitric oxide, $NO^{\cdot}$; ([Z]-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio) butyl]-amino}-diazen-1-ium-1,2-diolate), NONOate; phosphate-buffered saline, PBS; rat lung epithelial, RLE; reactive nitrogen species, RNS; reactive oxygen species, ROS; 3-morpholinosydnonimine, SIN-1; tumor necrosis factor, TNF; TNF receptor–associated factor, TRAF.

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Inflammatory diseases are accompanied by the chronic release of cytokines and reactive oxygen and nitrogen species (ROS and RNS, respectively), which are important in the causation and aggravation of clinical symptoms (1). These agents are known to activate the transcription factor nuclear factor (NF)-κB, which controls the expression of genes with multiple functions in inflammatory processes (2). Thus, the role of NF-κB in lung is complex and may involve the transcriptional control of inflammatory as well as survival genes (3–5). We previously reported that the oxidant-generating mineral dust, asbestos, or hydrogen peroxide (H_2O_2), activates NF-κB in lung epithelial cells and pleural mesothelial cells (6, 7). Moreover, increases in levels of p65, the major transactivating member of the NF-κB family, occur in rat lungs during the development of inflammation and pulmonary fibrosis (6). The cytokine tumor necrosis factor (TNF), a major activator of NF-κB (8), is an important mediator of inflammation or fibrosis asso-

ciated with inhalation of mineral dusts (9, 10). Exposure to mineral dusts enhances TNF release *in vitro* and in lung following inhalation (11, 12). Furthermore, studies employing an antibody against TNF or a soluble TNF receptor demonstrated an amelioration of inflammation and development of fibrosis observed after inhalation of inflammatory agents (10, 13). Therefore, the activation of NF-κB by TNF or oxidants that are generated during inflammation (9, 14) may regulate the clinical outcome.

NF-κB is sequestered in the cytoplasm, where it is complexed to members of the IκB family of inhibitor proteins. Release of IκB unmasks the nuclear localization signal and causes translocation of NF-κB to the nucleus, allowing activation of transcription (15). Activation of NF-κB is observed after exposure to diverse agents, including cytokines or proinflammatory agents, oxidants, phorbol esters, and others. Numerous studies have demonstrated that activation of NF-κB can often be prevented by antioxidants, and have led to the prevailing theory that NF-κB is an oxidant-sensitive transcription factor (16).

Signaling pathways that precede the dissociation of IκB, which is intrinsic to NF-κB activity, are beginning to be defined. For instance, binding of TNF or interleukin (IL)-1 to their respective receptors causes activation of members of the TNF receptor–associated factor (TRAF) family of adaptor proteins (17) and their interaction with NF-κB–inducing kinase (NIK) (18), which precedes phosphorylation of IκB. Although activation of NIK, a protein with sequence similarities to the mitogen-activated protein kinase kinase kinase (MAPKKK) family, is required for the TNF- and IL-1–mediated activation of NF-κB, NIK does not phosphorylate IκB (18). Independent studies have demonstrated that MAPK/extracellular-regulated kinase (ERK) kinase kinase (MEKK)1, another MAPKKK that acts as an upstream activator of c-Jun N-terminal kinases (JNK), activates the IκB kinase activity of a multiprotein complex of 900 kD that is poorly defined (19). Recently, the serine threonine kinase, conserved helix loop helix ubiquitous kinase (CHUK), was shown to interact with NIK and was identified as an IκB kinase capable of phosphorylating IκB-α at serines 32 and 36 (20, 21), events required for polyubiquitination and degradation through the proteosome pathway. Therefore, CHUK has been renamed IκB kinase-α (IKK-α) (21) or IKK-1, and the closely related IKK-2 has also been identified (22).

It is unknown whether mechanisms of NF-κB activation by oxidants or cytokines are similar. Studies employing pervanadate as a model of oxidative stress have demonstrated that tyrosine phosphorylation of IκB also results in dissociation of the NF-κB–IκB complex, which appears independent of ubiquitination and proteosome-dependent degradation (23). These findings suggest that activation of NF-κB may be regulated through multiple pathways and that ROS and RNS may trigger unique cascades that lead to activation of NF-κB. Depending on the reactivity of the oxidant encountered, the species can traverse the membrane and elicit intracellular responses, or react primarily with cell surface structures. For example, H_2O_2 can traverse the membrane, whereas 3-morpholinosydnonimine (SIN-1), which simultaneously releases superoxide (O_2^-) and nitric oxide ($NO^{\cdot}$) (24), will act primarily as an extracellular oxidant. The site of formation of oxidants could be critical in the activation of transcription factors such as NF-κB and determine the signaling cascades that are involved.

Recent observations have demonstrated that Ras is an important sensor of redox stress (25, 26), and that nitrosylation of one critical cysteine moiety by $NO^{\cdot}$ is responsible for guanosine triphosphate loading and activation of downstream signaling (26). We therefore wanted to investigate the involvement of Ras in the activation of NF-κB in rat lung epithelial (RLE) cells exposed to ROS or RNS that are encountered during inflammation. Because mitogen-activated protein kinases (MAPKs) are activated downstream of Ras (27), we also determined whether this family of signaling proteins was involved in the activation of NF-κB by oxidants. We previously showed the activation of ERK by H_2O_2 in pleural cells (28), and others have reported activation of MAPK family members in response to $NO^{\cdot}$ (29). Numerous agents that induce the JNK member of the MAPK family also activate NF-κB. The MAPKKK MEKK1, which activates the IκB kinase, also induces JNK (19), observations suggesting that MAPKs may be involved in the activation of NF-κB by oxidants.

Lung epithelial cells are a major target of oxidative stress, and oxidant stresses both activate NF-κB and upregulate inflammatory genes in this cell type (30, 31). Because oxidants and cytokines are intrinsic to inflammation, we determined patterns of NF-κB activation following exposure to ROS/RNS and TNF. Our studies demonstrate that simultaneous exposure to oxidants and cytokines causes a synergistic activation of NF-κB in RLE cells, suggesting that signaling pathways may be different. In contrast to TNF, ROS and RNS activate NF-κB in a Ras-dependent manner that does not involve degradation of IκB-α through a proteosome-dependent pathway. Examination of MAPK pathways revealed that MEKK1 and JNK positively regulate NF-κB activation in RLE cells exposed to oxidants or TNF, whereas MEK-dependent pathways are negative regulators of NF-κB. Our data are unique in that they demonstrate that multiple pathways, including MEKK1 and JNK, cooperate in the activation of NF-κB by oxidants and cytokines.

Materials and Methods

Cell Culture, Plasmids, and Test Agents

A line of spontaneously transformed RLE alveolar type II cells was kindly provided by Dr. Kevin Driscoll (Procter & Gamble, Cincinnati, OH) and has been described elsewhere (32). RLE cells were propagated in Dulbecco's modified Eagle's medium (DMEM)/F12 containing penicillin, streptomycin, and 10% newborn bovine serum (NBS) (GIBCO BRL, Grand Island, NY). H_2O_2 was purchased from Sigma (St. Louis, MO). SIN-1 (Molecular Probes, Eugene, OR) spontaneously decomposes to generate $NO^{\cdot}$ and O_2^-, which react to form peroxynitrite ($ONOO^-$). This reaction has been verified by measurement of nitrotyrosines which are generated by $ONOO^-$ specifically (33). SIN-1 was dissolved in Hank's balanced salt solution and added to cultures immediately. In selected experiments, we used spermine [Z]-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio) butyl]-amino}-dia-

zen-1-ium-1,2-diolate (NONOate) as a selective generator of $NO^{\cdot}$ (33). Human recombinant TNF-α was purchased from Calbiochem (La Jolla, CA). MG132 was provided by Peptide Institute, Inc. (Osaka, Japan), and herbimycin A was purchased from GIBCO BRL. Plasmid 6 κB-tk-luc was kindly provided by Dr. Patrick Baeuerle (Tularik, Inc., San Francisco, CA). Dominant negative MEKK1, MEKK K-M, was provided by Dr. Bing Su (University of Texas, Houston, TX); and JNK 1 and 2 expression constructs (JNK1,2+/+), and glutathione-S-transferase (GST)-Jun were provided by Dr. Roger Davis (Howard Hughes Medical Institute, Worcester, MA). Plasmid PSV–β-gal (Promega, Madison, WI), which drives constitutive expression of β-galactosidase (β-gal), was used to control for variations in transfection efficiencies. Herbimycin A, MG132, and PD98059 were dissolved in dimethyl sulfoxide (DMSO) and used in cultures at a maximal concentration of 0.1% DMSO. DMSO was used as a vehicle control in cells not receiving inhibitors and did not affect the ability of oxidants to induce MAPK or NF-κB.

Transient Transfections

RLE cells at 50 to 80% confluency were trypsinized, suspended in complete medium, and electroporated using a Bio-Rad Gene Pulser electroporator at 240 V and a capacitance of 960 microfarads. This procedure resulted in 30 to 40% transfection efficiency as determined by transfection of green fluorescent protein and flow cytometric analysis (unpublished observations). Cells were incubated for 4 to 6 h, fresh medium was added, and cells were allowed to recover overnight. One hour before addition of test agents, the medium was switched to DMEM/F12 containing 1% NBS and agents were added for 8 to 16 h, at which time dishes were harvested for luciferase assays according to manufacturer's instructions (Promega). β-gal activities were measured using a Lumiglo assay system (Tropix, Bedford, MA). In all experiments, luciferase activities were normalized to β-gal activities and reported as luc/β-gal units. All assays were performed within 48 h after transfection, and experiments were repeated at least twice. In control experiments, RLE cells were transfected with the empty luciferase construct 36-Tk-Luc, which yielded 3-fold lower luciferase activities and resulted in $<$ 2-fold fluctuations in luciferase activities in response to TNF or oxidants, illustrating that the concentrations of oxidants used here do not interfere with luciferase activities (data not shown). Additionally, the use of another vector controlled by a thymidine kinase promoter (pRl-TK; Promega) to control for variations in transfection efficiencies did not detect alterations in luciferase activities in oxidant- or cytokine-treated cells (data not shown). Following some transfections, semiconfluent cells displayed some toxicity in response to 300 μM H_2O_2. Therefore, lower H_2O_2 concentrations were chosen to avoid toxic responses in these experiments. Despite variations in H_2O_2 concentrations between experiments, trends remained identical.

MAPK Assays

RLE cells were grown to 70% confluency and medium was switched to 1% NBS 1 h before addition of test agents. After selected time periods of exposure to ROS, RNS, or TNF, cells were transferred to ice, rinsed twice with cold phosphate-buffered saline (PBS), and lysed in 20 mM Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM sodium chloride, 2 mM ethylenediaminetetraacetic acid, 25 mM glycerophosphate, 1 mM orthovanadate, 2 mM pyrophosphate, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml leupeptin, 1 mM dithiothreitol (DTT), 10 mM sodium fluoride, and 20 μg/ml aprotinin. Lysates were cleared by centrifugation at 14,000 rpm, 4°C for 10 min. ERK-2, JNK-1, or p38 members of the MAPK family were immunoprecipitated using specific antibodies (SC 154, SC 474, and SC 535; Santa Cruz, Santa Cruz, CA) at 4°C for 90 min. Protein A agarose beads (GIBCO BRL) were added for 1 h, and precipitates were washed twice in lysis buffer and once in kinase buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N′*-ethane sulfonic acid (pH 7.4), 20 mM glycerophosphate, 20 mM magnesium chloride, 2 mM DTT, and 0.1 mM orthovanadate. ERK and p38 activities were assessed using myelo basic protein (MBP) as a substrate (GIBCO BRL), whereas GST-Jun was the substrate for JNK activities. Kinase reactions were performed in the presence of 10 μg of substrate and 0.5 μCi of γ^{32}P-adenosine triphosphate at 30°C for 30 min. Reactions were stopped by the addition of 2× Laemmli sample buffer, and samples were stored at −20°C. Samples were resolved on 15% polyacrylamide gels, gels were dried, and phosphorylation of substrate was examined by autoradiography. In addition, phosphorylation was quantitated on a phosphoimage analyzer (Bio-Rad, Hercules, CA). All experiments were performed with n = 2/exposure group and repeated at least twice.

Western Blotting of IκB-α

After time periods ranging from 15 min to 24 h of exposure to agents, cells were transferred to ice, washed twice in PBS, and lysed in buffer containing 20 mM Tris (pH 7.4), 150 mM sodium chloride, 1% nonidet P40, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and 20 μg/ml aprotinin. Lysates were incubated on ice for 30 min and centrifuged at 14,000 rpm, 4°C for 30 min, and 2× Laemmli sample buffer was added to supernatants before storage at −20°C. Protein concentrations were determined by the Bradford method, and 10-μg quantities per sample were resolved on 15% acrylamide gels, transferred to nitrocellulose (Schleicher & Shuell, Keene, NH), and kept in 5% milk in PBS overnight at 4°C. Filters were incubated with PBS containing 0.05% Tween-20 for 30 min and subsequently incubated with antibody directed against IκB-α (SC 371, 0.5 μg/ml; Santa Cruz) for 1 h. Filters were washed three times in PBS-Tween and incubated with a peroxidase-conjugated secondary antibody for 45 min. After three washes in PBS, conjugated peroxidase was visualized by enhanced chemiluminescence according to manufacturer's instructions.

Statistical Analysis

Results were analyzed by analysis of variance (ANOVA) using the Student–Newman–Keuls procedure to adjust for multiple comparisons. To evaluate whether synergistic responses occurred, interactions between treatment groups were evaluated via ANOVA.

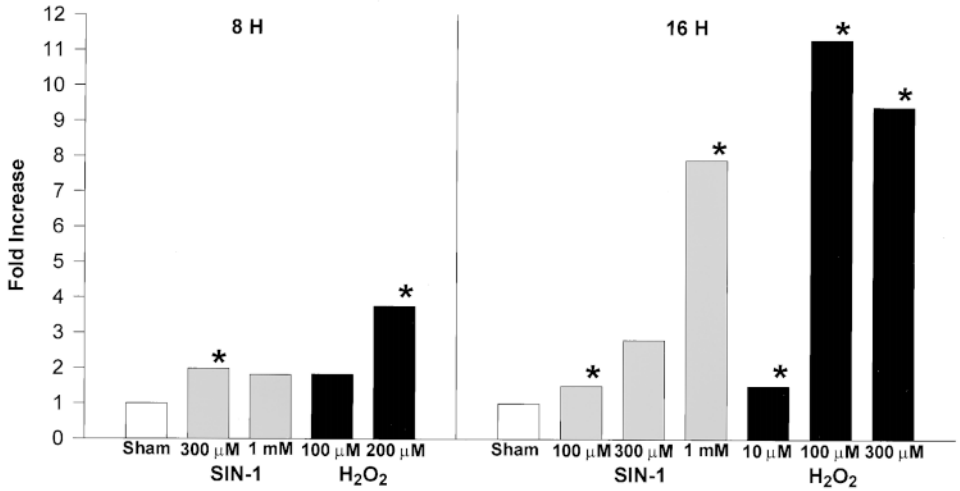

Figure 1. Activation of an NF-κB–dependent luciferase reporter vector in RLE cells exposed to H_2O_2 or SIN-1. RLE cells were transiently transfected with plasmid 6κB-tk-luc in the presence of plasmid PSV–β-gal. Cells were incubated with oxidants for 8 or 16 h and harvested for determination of luciferase and β-gal activities. Data show representative findings from five to 10 independent experiments and show fold increases compared with untreated controls. Statistics were performed on actual data. $*P <$ 0.05.

Results

We first examined whether ROS/RNS activated NF-κB in RLE cells. Transient transfection of a plasmid containing six consensus NF-κB sequences linked to a luciferase reporter construct illustrated the transactivation of an NF-κB–dependent reporter gene (Figure 1) by H_2O_2 or the reactive nitrogen generating species SIN-1. Increases in NF-κB reporter activity were apparent after 8 h of exposure to oxidants and were elevated more strikingly after 16 h. Because ROS/RNS and cytokines are generated concomitantly during pulmonary inflammation, we next determined patterns of NF-κB activation of cells following simultaneous exposure to oxidants or TNF-α. Results in Figure 2 demonstrate that oxidants and TNF-α synergized to cause transactivation of the NF-κB–directed luciferase reporter gene. This synergistic response suggests that distinct signaling pathways may be activated during the simultaneous exposure to oxidants and cytokines that may augment the activation of NF-κB.

Classically, degradation of IκB precedes the nuclear translocation of NF-κB complexes and transactivation of NF-κB–dependent gene expression. To determine whether exposure of RLE cells to ROS or RNS caused degradation of IκB, we performed time-course studies to examine IκB-α levels by Western blotting. As shown in Figure 3, IκB-α was degraded rapidly in RLE cells exposed to TNF, followed by its resynthesis and reappearance at later time points. In contrast, examination of IκB-α in RLE cells exposed to ROS or RNS for time periods ranging from 15 min to 24 h demonstrated lack of alterations of IκB-α levels (Figure 3). We next used MG132, a specific inhibitor of the proteosome pathway, to determine the involvement of proteosome-dependent degradation of IκB complexes in response to oxidants. As shown in Figure 4, pretreatment for 1 h with 0.5 μM MG132 abolished the TNF-mediated activation of NF-κB, whereas the ability of SIN-1 to induce NF-κB was not affected. These data suggest that proteosome-dependent pathways are not involved in the activation of NF-κB by oxidants. Importantly, the generic tyrosine kinase inhibitor herbimycin A abolished the NF-κB induction observed after SIN-1 exposure (Figure 4). Although some caution should be observed regarding the specificity of many tyrosine kinase inhibitors, our results suggest the involvement of a tyrosine kinase in the oxidant-mediated activation of NF-κB, consistent with observations by others (23).

To elucidate the initiation of signaling events by oxidants that culminate in the activation of NF-κB, we next examined the role of Ras. Ras appears to be a critical re-

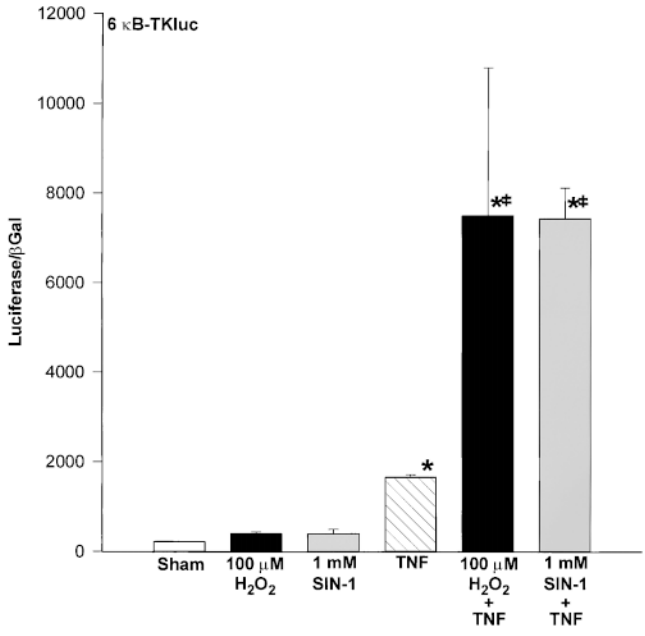

Figure 2. Synergistic activation of NF-κB–luciferase in RLE cells simultaneously exposed to ROS/RNS and TNF. Transiently transfected RLE cells were exposed to SIN-1 or H_2O_2, and TNF was added simultaneously. After 8 h of exposure, dishes were harvested for determination of luciferase and β-gal activities. Synergistic responses were confirmed by statistical analysis. $*P <$ 0.05, when compared with sham controls. $^{\ddagger}P < 0.05$, in comparison with single exposures of SIN-1, H_2O_2, or TNF (n = 2/treatment group).

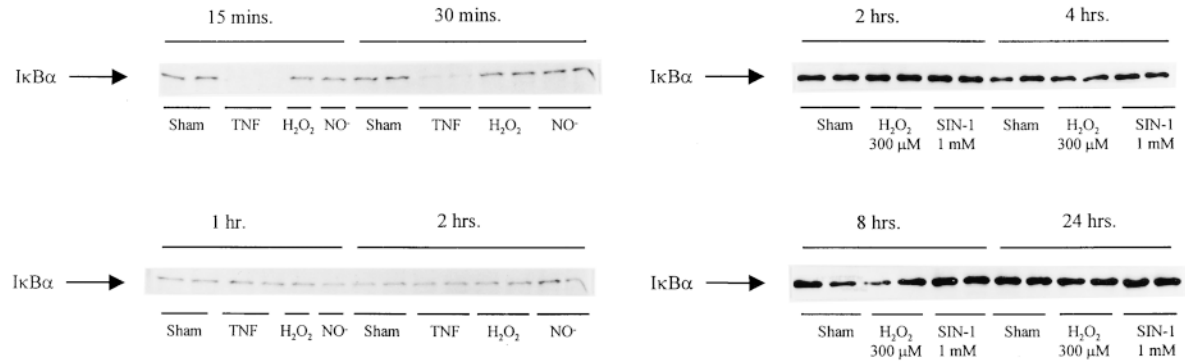

Figure 3. Western blotting of IκB-α in RLE cells exposed to 300 μM H_2O_2, 1 mM SIN-1, 300 μM spermine NONOate ($NO^·$), or 10 ng/ml TNF. Cells were exposed to agents for time periods ranging from 15 min to 2 h or 2 to 24 h, and lysates were prepared for Western blotting of IκB-α. Ten micrograms of cytoplasmic protein was applied per lane. Spermine NONOate was used here as another $NO^·$ donor (n = 2/treatment group).

dox sensor of oxidative stress that can be modified by ROS/RNS (25, 26). We transiently transfected a dominant negative Ras construct to determine its effects on the oxidant- or TNF-mediated activation of NF-κB. As shown in Figure 5, transient transfection of dominant negative Ras abolished the activation of NF-κB by H_2O_2 or SIN-1 (Figure 5B) but did not modify the TNF-mediated activation of NF-κB (Figure 5A), demonstrating that a Ras-dependent pathway is critical in the induction of NF-κB by oxidants but not by TNF in pulmonary epithelial cells.

Activation of MAPKs occur downstream of Ras and may play a role in the activation of NF-κB. We therefore determined whether MAPKs are activated in RLE cells after exposure to ROS or RNS and whether these proteins are involved in the activation of NF-κB by oxidants. Results in Figure 6 demonstrate the activation of ERK and JNK MAPK in RLE cells exposed to H_2O_2 or SIN-1. Small but significant increases in the activities of JNK and p38 MAPK were observed in RLE cells exposed to TNF. Examination of p38 revealed constitutive activity that was not enhanced by ROS or RNS at these time points examined. Transient transfection of a dominant negative MEKK1 construct ameliorated the oxidant-mediated activation of NF-κB (Figure 7). Similarly, dominant negative MEKK1 also ameliorated the TNF-mediated activation of NF-κB, as has been demonstrated by others (19). Overexpression of MEKK1 activated NF-κB in RLE cells and enhanced the TNF- or H_2O_2-mediated activation of NF-κB (Figure 7), suggesting that activation of this MAPKKK is important in the regulation of NF-κB activity in RLE cells. To verify that our MEKK vectors were expressed appropriately following transfection of RLE cells, we tested the constructs in JNK activity assays because it has been demonstrated that MEKK regulates the activity of JNK. For this purpose we used a wild-type hemagglutinin (HA)-tagged JNK construct, enabling us to selectively immunoprecipitate recombinant JNK from cells cotransfected with MEKK vectors. As expected, transfection of constitutively active MEKK1 activated JNK in RLE cells (Figure 8A). In a separate experiment, we demonstrated that dominant negative MEKK1 attenuated the H_2O_2-mediated activation of JNK (Figure 8B), thus confirming the appropriate function of these plasmids following transfection of RLE cells. Our data here suggest that JNK may be an important effector regulating NF-κB activity. To assess further the role of JNK in the activation of NF-κB, we employed JNK1,2+/+ to assess the modulation of NF-κB activity. As demonstrated in Figure 9, overexpression of JNK further enhanced NF-κB activity in RLE cells exposed to TNF or SIN-1. In summary, our findings suggest that MEKK1 and JNK are positive regulators of NF-κB activity. Our observations demonstrating the striking activation of JNK by ROS/RNS, and our findings that JNK overexpression enhances NF-κB transcriptional ability provide a

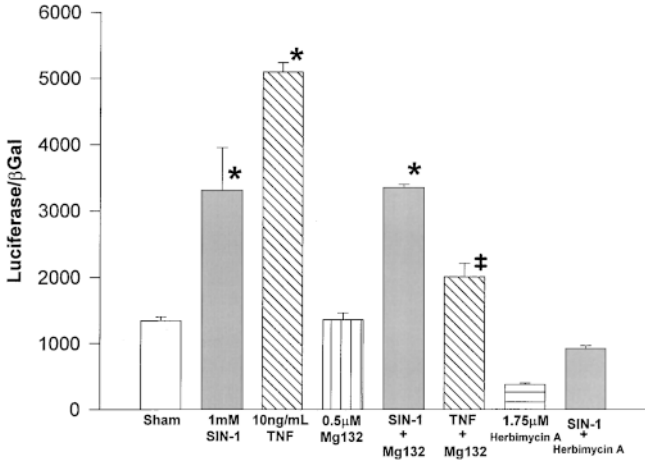

Figure 4. Effects of the proteosome inhibitor MG132 or the tyrosine kinase inhibitor herbimycin A on the activation of NF-κB luciferase in RLE cells exposed to SIN-1 or TNF. RLE cells were transiently transfected with plasmid 6κB-tk-luc and exposed to agents for 16 h. Cells were preincubated with MG132 or with herbimycin A for 1 h before the addition of agents. DMSO was present in all cultures at a concentration of 0.1% which did not affect NF-κB activities. Luciferase activities shown on the y-axis are normalized to β-gal. *$P < 0.05$, in comparison with sham controls. ‡$P < 0.05$, in comparison with TNF-exposed cells (n = 2/treatment group).

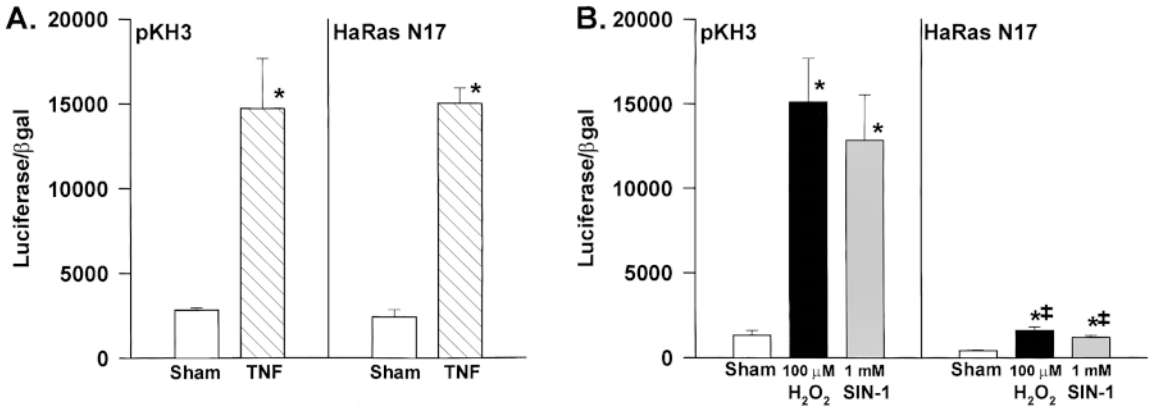

Figure 5. Effects of dominant negative Ras (HaRas N17) on the activation of NF-κB by TNF (*A*) or oxidants (*B*). RLE cells were transfected with control vector (pKH-3) or dominant negative Ras in the presence of plasmids 6κB-tk-luc and PSV–β-gal. Transfected cells were exposed to TNF or oxidants for 16 h, and luciferase and β-gal activities were measured as described. $^{*}P < 0.05$ in comparison with sham controls. $^{\ddagger}P < 0.05$ in comparison with pKH3 vector controls ($n = 2$/treatment group).

plausible explanation for the cooperativity of ROS/RNS and TNF in the activation of this transcription factor.

Use of a chemical inhibitor to modify the ERK–MAPK pathway further documented effects on NF-κB activity. We exposed RLE cells to the MEK inhibitor PD98059 and verified its ability to block ERK MAPK. As shown in Figure 10A, exposure to 20 μM PD98059 diminished the H_2O_2-mediated activation of ERK, whereas activities of JNK or

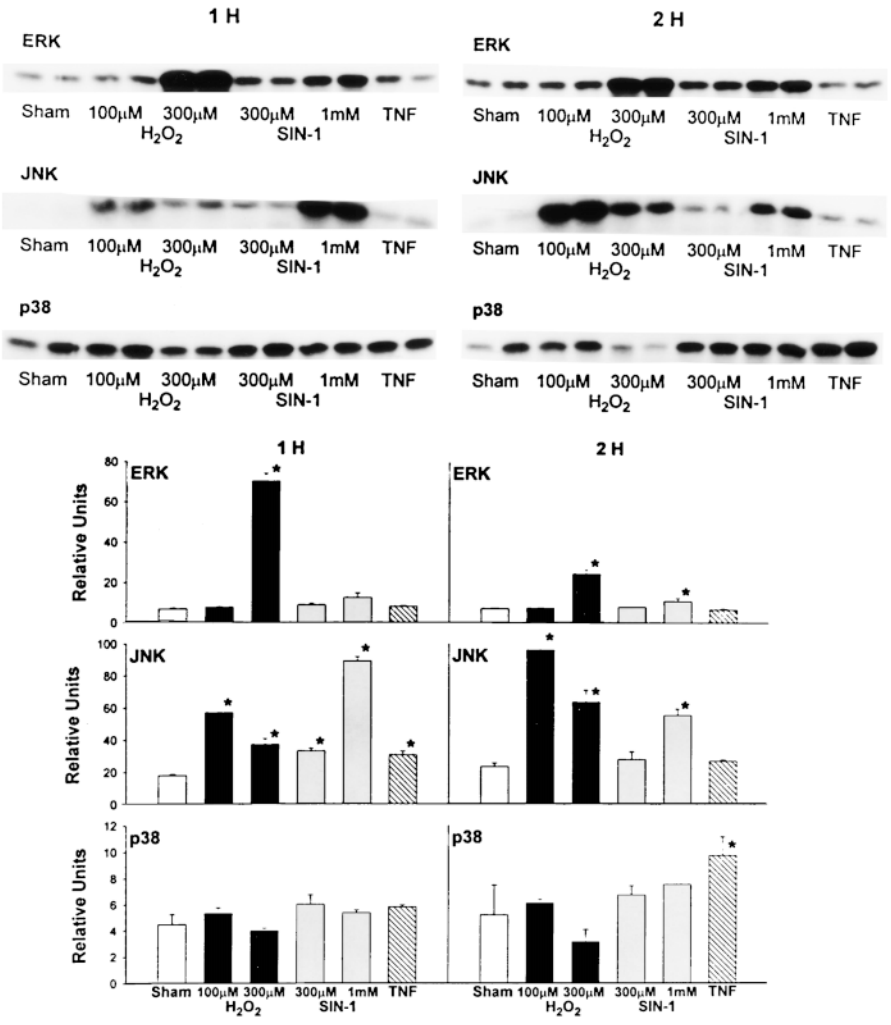

Figure 6. Activation of MAPKs by ROS or RNS. Cells were exposed to H_2O_2 or SIN-1 for 1 or 2 h, and ERK, JNK, or p38 MAPK proteins were immunoprecipitated for MAPK activities. MBP was used as a substrate for ERK or p38, whereas GST-Jun was used to determine JNK activity. Quantitated results, obtained after phosphoimage analysis, are expressed as relative units. $^{*}P < 0.05$ in comparison with sham controls ($n = 2$/treatment group).

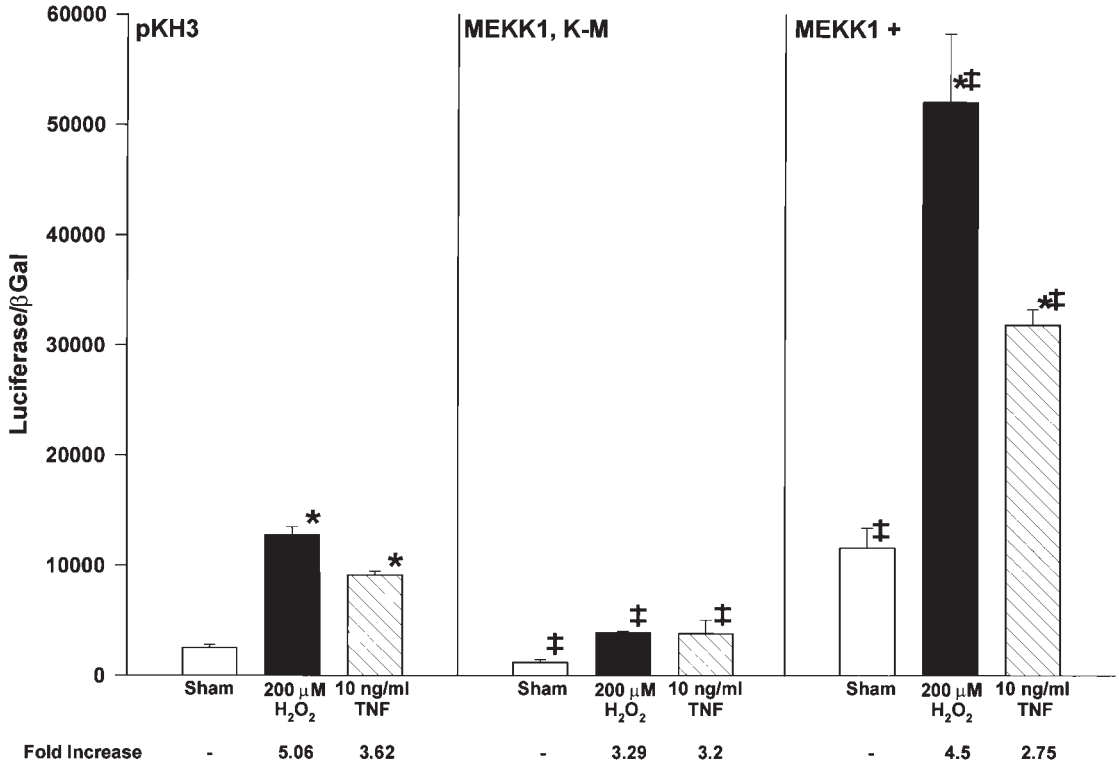

Figure 7. Modulation of NF-κB luciferase activity in RLE cells transfected with a constitutively active MEKK1 construct (MEKK1+) or dominant negative MEKK1 (MEKK1 K-M). Cells were transfected with these constructs in the presence of plasmids 6κB-tk-luc and PSV–β-gal, and exposed to agents for 16 h. Luciferase activities shown on the *y*-axis are normalized to β-gal activities. $^*P < 0.05$, in comparison with sham controls. $^‡P < 0.05$, in comparison with the pKH3 vector control (n = 2/treatment group).

p38 were not affected. We next evaluated the effect of PD98-059 on NF-κB activation. Results in Figure 10B demonstrate the dose-dependent activation of NF-κB after exposure to PD98059 and an enhancement of the TNF-mediated activation of NF-κB following pre-exposure to this MEK inhibitor. These data suggest that the MEK–ERK pathway negatively regulates NF-κB. Our results also indicate that various members of the MAPK family can regulate NF-κB differently.

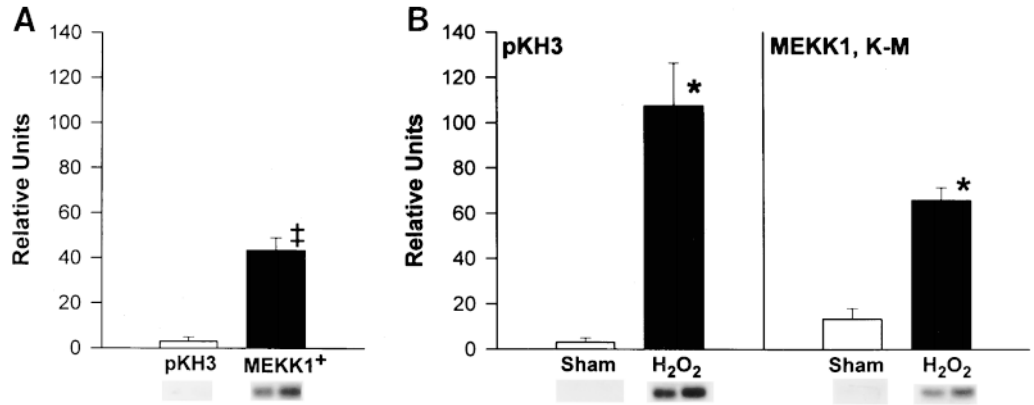

Figure 8. Modulation of JNK activities in RLE cells transfected with MEKK constructs. In these experiments, cells were cotransfected with a HA-conjugated wild-type JNK construct to assess the activity of JNK in transfected cells only. (*A*) Overexpression of MEKK1+ activates JNK activity. At 24 h after transfection, lysates were prepared, recombinant JNK was immunoprecipitated with anti-HA antibody (Boehringer; Clone 12CA5), and JNK activity was determined as described in MATERIALS AND METHODS. $^‡P < 0.05$, in comparison with the pKH3 vector control (n = 2/treatment group). (*B*) Overexpression of MEKK1 K-M diminishes the induction of JNK by H_2O_2. At 24 h after transfection, cells were exposed to 300 μM H_2O_2 for 1 h, and lysates were prepared for immunoprecipitation of HA–JNK and evaluation of kinase activity using GST-Jun as a substrate. $^*P < 0.05$, in comparison with sham controls (n = 2/treatment group). Note the decreased activation of JNK by H_2O_2 in cells transfected with MEKK1 K-M compared with the pKH3 vector control.

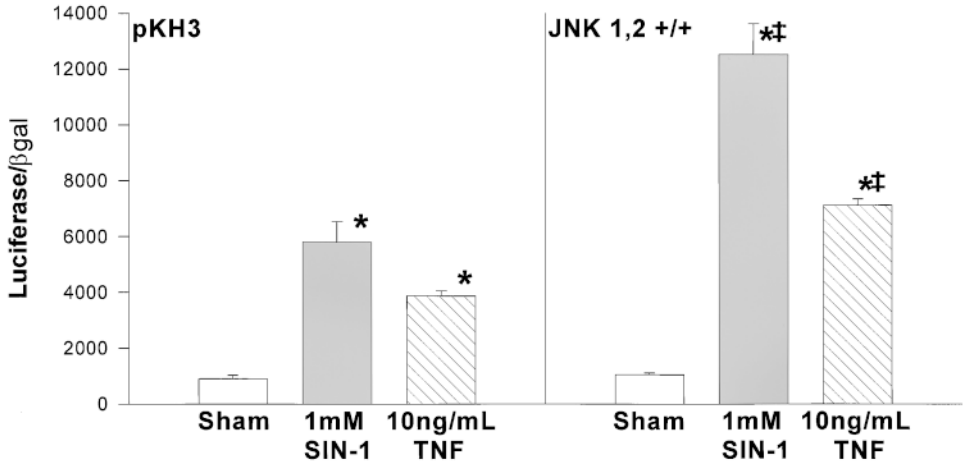

Figure 9. Enhancement of NF-κB activation following transfection of JNK1,2+/+. RLE cells were cotransfected with plasmids 6 κB-tk-luc and PSV–β-gal, exposed to SIN-1 or TNF for 16 h, and harvested for luciferase and β-gal activities. $*P < 0.05$, in comparison with sham controls. $‡P < 0.05$, in comparison with the pKH3 vector control (n = 2/treatment group).

Discussion

The induction of NF-κB is thought to be critical in the regulation of inflammatory responses (2, 15). For instance, the production of chemokines and other immune-modulatory factors observed in lung after inhalation of environmental pollutants and other inflammatory agents is mediated in part by NF-κB (2, 11, 15, 31). Exposure of pulmonary target cells to inflammatory agents or pulmonary irritants that cause inflammation also causes the induction of NF-κB (6, 7). These agents frequently induce oxidative stress or activate the release of oxidants from macrophages, neutrophils, and other immune cells that are recruited to sites of injury (1, 14). In fact, many studies have reported that oxidative stress is important in the activation of NF-κB and elicitation of inflammatory cytokines by these agents. Enhanced oxidant production has been documented in mitochondria of cells exposed to cytokines (34), suggesting multiple interactions. Antioxidants or metal chelators often prevent activation of NF-κB by cytokines, phorbol esters, and other inducers, suggesting that a redox-sensitive step occurs in the pathway of NF-κB activation (16).

Because exposure to ROS/RNS and cytokines can occur concomitantly during inflammation, we examined the pathways employed by extracellular oxidants or inflammatory cytokines that lead to the induction of NF-κB. Our findings demonstrate that ROS/RNS can synergize with TNF to activate NF-κB. A number of plausible explanations exist for these synergistic responses. Here, we describe the ability of oxidants to initiate signaling to NF-κB via Ras. Previously, Ras was shown to be activated by H_2O_2 or $NO^{\cdot}$ (25, 26) and it was shown that this is important in the activation of downstream cascades. In fact, activation of MAPKs occurs in response to these agents (Figure 6), findings in line with observations by others (28, 29, 35). Cross-talk between pathways leading to activation of NF-κB and MAPKs has been illustrated by numerous reports demonstrating that agents that activate JNK also activate NF-κB (19). MEKK1, the upstream activator of JNK, also activates IκB kinase (19), suggesting a point of convergence. Recent data report that following activation of TNF receptors, the adapter molecule TRAF-2 is the bifurcation point that leads to activation either of JNK via RIP or of NF-κB via NIK (36).

Our data also suggest that MAPKs can regulate NF-κB in lung epithelium. Inhibition of the MEK–ERK pathway activates NF-κB, whereas the MEKK–JNK pathways can enhance the activation of NF-κB by oxidants or TNF. These data are in line with multiple observations that show the involvement of MAPKs in the activation of NF-κB in different systems. For instance, studies have shown that: (*1*) p38 appears to be involved in the activation of NF-κB–regulated genes in response to cytokine exposure (37, 38); (*2*) overexpression of ERK can activate NF-κB (39); (*3*) a physical association exists between the c-Rel protein of the NF-κB family and JNK-1 (40); and (*4*) p38 and ERK are required in the TNF-mediated activation of the NF-κB regulated gene, IL-6 (41). In our model employing RLE cells, activities of p38 are mostly constitutive at the time periods during which agents were investigated. Moreover, the negative role of the MEK–ERK pathway in the regulation of NF-κB activity described here is in contrast to these findings (39, 41) and suggests that MAPKs may regulate NF-κB uniquely in different cell types. It is important to document further the contribution of the different MAPKs to the activation of NF-κB in different cell types to obtain a clearer understanding of the involvement of these signaling cascades in inflammatory diseases.

The lack of degradation of IκB-α via a proteosome-dependent pathway in response to oxidants is an intriguing observation. However, recent reports have shown that tyrosine phosphorylation of IκB-α in models of pervanadate or hypoxia–reoxygenation results in its dissociation but does not involve degradation through the proteosome pathway (23, 42). Our observations fit these findings and illustrate the involvement of a different pathway of NF-κB activation by oxidants. Our results employing the tyrosine kinase inhibitor herbimycin A demonstrated that the activation of the NF-κB–luciferase reporter gene is abolished in response to oxidant exposure (Figure 4), illustrating a tyrosine kinase–dependent step in the activation of NF-κB.

Oxidants can interact with cell surface receptors to cause conformational changes or dimerization that may facilitate growth factor or cytokine receptor signaling. For

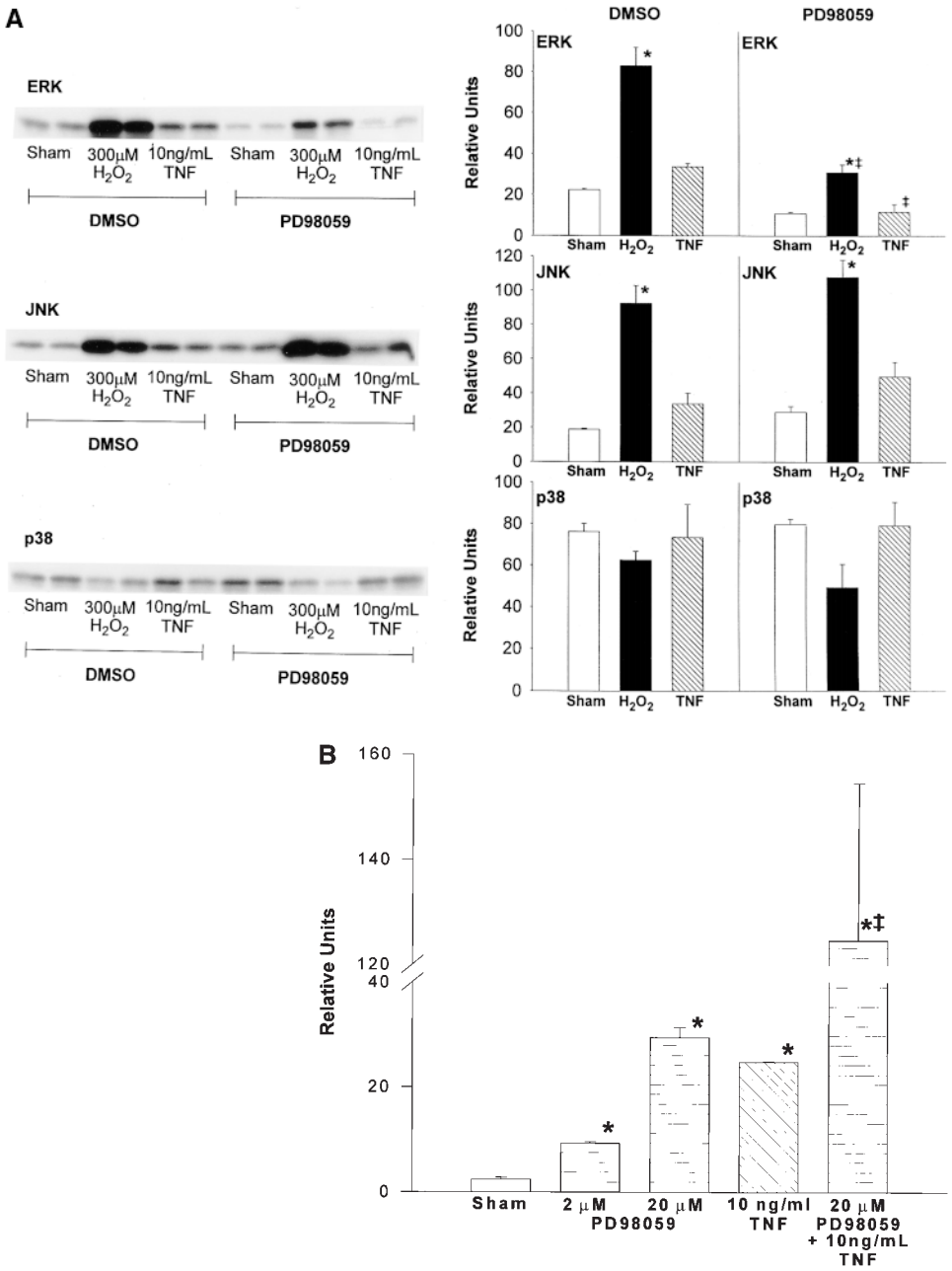

Figure 10. (*A*) Specificity of PD98059. RLE cells were incubated with 20 μM of PD98059 for 1 h and subsequently exposed to H_2O_2 or TNF for 1 h, and MAPK activity assays were performed as described in MATERIALS AND METHODS. Results from MAPK activities were quantitated by phosphoimage analysis. $*P < 0.05$, in comparison with sham controls. $^{\ddagger}P < 0.05$, in comparison with the DMSO vehicle control. Note the inhibition of ERK activity induced after H_2O_2 exposure by PD98059 and the lack of effects on JNK or p38 activities. (*B*) Activation of NF-κB in RLE cells exposed to the MEK inhibitor PD98059. RLE cells transiently transfected with 6 κB-tk-luc were incubated with PD98059 and evaluated for luciferase activity 16 h after exposure. In selected experiments, cells were pre-exposed to 20 μM PD98059 for 1 h and then exposed to TNF for 16 h. Note the augmentation of NF-κB activity following pre-exposure to PD98059. $*P < 0.05$, in comparison with sham controls. $^{\ddagger}P < 0.05$, in comparison with TNF only ($n = 2$/treatment group).

instance, others have shown that thiol-reactive mercury or ultraviolet radiation causes cross-linking of receptors or receptor clustering that activates downstream signaling cascades (43, 44). It is likely that H_2O_2 or SIN-1, which generates the reactive $ONOO^-$ molecule, may trigger NF-κB signaling pathways via similar mechanisms. At present we have no information on modification of TNF receptors by oxidants, which would offer an alternative explanation for the cooperative activation of NF-κB by oxidants and TNF.

SIN-1 and H_2O_2 are oxidants with different reactivities but they activate NF-κB via similar pathways, as shown in the present study. These findings are surprising given our previous observations that H_2O_2 causes apoptosis in RLE cells, whereas SIN-1 did not cause apoptosis under conditions used here (33). Interestingly, a striking activation of ERK by H_2O_2 was observed, in contrast to minor increases after exposure to SIN-1. Our data demonstrating a negative role of ERK in the activation of NF-κB is consistent with findings that, at higher concentrations of H_2O_2 or after longer exposure times, NF-κB activity decreases and apoptosis ensues, in contrast to the response in SIN-1–exposed cells (data not shown). Therefore, the activation of NF-κB by oxidants may allow cell survival, consistent with findings by others (3–5).

Our present data describing the cooperation between TNF and ROS/RNS–dependent pathways leading to activation of NF-κB are summarized in Figure 11. ROS/RNS signaling is initiated via a Ras-dependent pathway that results in activation of MEKK1, which activates JNK MAPK. The TNF-mediated activation of IKK results in

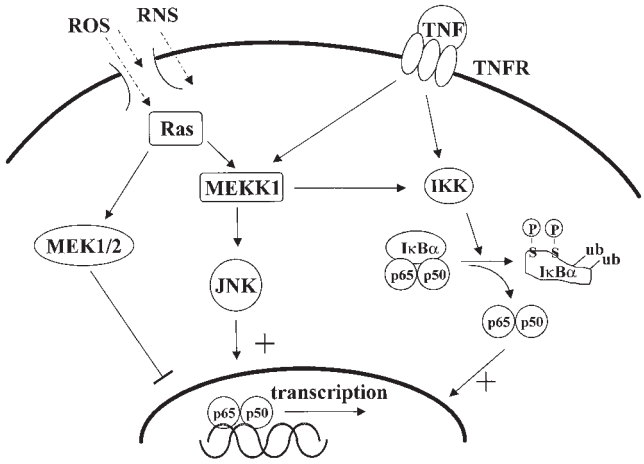

Figure 11. Model of activation of NF-κB in RLE cells exposed to TNF or oxidants. Multiple pathways are activated in response to ROS/RNS or TNF exposure that culminate in the synergistic activation of NF-κB. The MEK–ERK pathway appears to regulate NF-κB negatively, whereas the MEKK1–JNK pathway positively regulates activation of NF-κB. MEKK1 could be the point of convergence at which ROS/RNS and TNF synergize to activate NF-κB. The simultaneous occurrence of ROS/RNS and cytokines during inflammation suggests that multiple pathways are activated to induce transcription of NF-κB–regulated genes and may contribute to injury.

the activation of NF-κB via degradation of IκB, allowing translocation of NF-κB to the nucleus. Additionally, binding of TNF to its receptor activates TRAF-2 (36), which can activate JNK. Our data here suggest that MEKK/JNK are a point of convergence at which ROS/RNS and TNF cooperate in the activation of NF-κB. This diagram provides a working model of how ROS/RNS and cytokines can cooperate to potentially aggravate inflammatory diseases associated with exposure to these reactive agents and cytokines.

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