

# Differential induction of *c-fos*, *c-jun*, and apoptosis in lung epithelial cells exposed to ROS or RNS

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**Janssen, Yvonne M. W., Sadis Matalon, and Brooke T. Mossman.** Differential induction of *c-fos*, *c-jun*, and apoptosis in lung epithelial cells exposed to ROS or RNS. *Am. J. Physiol.* 273 (Lung Cell. Mol. Physiol. 17): L789–L796, 1997.—Reactive oxygen (ROS) or nitrogen (RNS) species can affect epithelial cells to cause acute damage and an array of pulmonary diseases. The goal of this study was to determine patterns of early response gene expression and functional end points of exposure to nitric oxide (NO·), H<sub>2</sub>O<sub>2</sub>, or peroxynitrite (ONOO<sup>−</sup>) in a line of rat lung epithelial (RLE) cells. Our focus was on *c-fos* and *c-jun* protooncogenes, as these genes play an important role in proliferation or apoptosis, possible end points of exposure to reactive metabolites in lung. Our data demonstrate that NO· generated by spermine 1,3-propanediamine *N*-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazinol-butyl] or *S*-nitroso-*N*-acetylpenicillamine as well as H<sub>2</sub>O<sub>2</sub> cause increased *c-fos* and *c-jun* mRNA levels, nuclear proteins, and complexes binding the activator protein-1 recognition sequence in RLE cells. These agents also lead to apoptosis and increased membrane permeability. In contrast, exogenously administered ONOO<sup>−</sup> or 3-morpholinosydnonimine do not induce protooncogenes or apoptosis in RLE cells despite nitration of tyrosines. We conclude that ROS and RNS can elicit distinct molecular and phenotypic responses in a target cell of pulmonary disease.

nitric oxide; peroxynitrite; hydrogen peroxide; lung epithelium; reactive oxygen species; reactive nitrogen species

EPITHELIAL CELLS of the lung are exposed to a variety of free radical species both after inhalation of oxidant gases and other pollutants and as a consequence of inflammation (6, 11, 12, 26). The chemistry of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is complex and involves the formation of a number of intermediate species that may interact differently with biological targets (2). Formation of ROS and RNS and their reactions with target cells of disease are thought to be linked to the initiation of a number of pulmonary disorders, including pulmonary fibrosis, acute respiratory distress syndrome, bronchiolitis, asthma, and lung cancer (6, 11, 12, 15).

The identity of reactive species causing alterations in gene expression and biological responses in target cells of disease in the lung and other organs still remains uncertain. Likely candidates include H<sub>2</sub>O<sub>2</sub>, nitric oxide (NO·), and peroxynitrite (ONOO<sup>−</sup>), which is formed from the rapid reaction of superoxide (O<sub>2</sub><sup>·−</sup>) with NO·. ONOO<sup>−</sup> is an extremely potent oxidant that can cause lipid peroxidation, DNA damage, and alterations of protein function in vitro (2, 15). However, the effects of ROS and RNS on early response gene expression and phenotypic consequences in pulmonary target cells of disease and other cell types have not been investigated.

One goal of these studies was to determine whether *c-fos* and *c-jun* protooncogenes are activated selectively by these reactive species in rat lung type II alveolar epithelial (RLE) cells. Although we and others have shown the rapid induction of *c-fos* and *c-jun* in other cell types after exposure to H<sub>2</sub>O<sub>2</sub> or NO· (14, 17, 22, 23), nothing is known about induction of early response protooncogenes by ONOO<sup>−</sup> or the phenotypic consequences of gene expression by ONOO<sup>−</sup>. We therefore used a number of chemical generators of NO·, ONOO<sup>−</sup>, or H<sub>2</sub>O<sub>2</sub> to determine whether these oxidants could activate these molecular events and apoptosis in an RLE cell line. We also wanted to determine whether responses were unique to the type of reactive species encountered or general phenomena observed in oxidative stress.

The *c-fos* and *c-jun* genes encode proteins that can dimerize to form homodimeric (Jun/Jun) and heterodimeric (Fos-Jun) complexes of the activator protein (AP)-1 family, accessory transcription factors that interact with DNA regulatory sequences known as 12-*O*-tetradecanoylphorbol-13-acetate response elements or AP-1 sites (1). In this study, we comparatively measured *c-fos* and *c-jun* mRNA levels, Fos and Jun nuclear proteins, and increases in AP-1 DNA binding activity in RLE cells exposed to NO·, H<sub>2</sub>O<sub>2</sub>, or ONOO<sup>−</sup>. In addition, because early response gene transactivation has been linked to the development of apoptosis, a unique type of programmed cell injury, in other cell types (24), we measured apoptosis and membrane damage as biological consequences of exposure to RNS or ROS in epithelial cells of the lung.

Our results show that exogenously administered NO· or H<sub>2</sub>O<sub>2</sub>, but not ONOO<sup>−</sup>, causes increases in *c-fos* and *c-jun* gene and protein expression, AP-1 DNA binding activity, apoptosis, and membrane permeability. Our results also demonstrate that, despite protein tyrosine nitration, no effects were observed with ONOO<sup>−</sup>.

## EXPERIMENTAL PROCEDURES

**Cell culture and exposure to test agents.** A rat lung type II epithelial cell line that retains its normal differentiated features and near-diploid karyotype (RLE-6TN) was propagated as described previously (8). Cells were grown to confluency, and medium was switched to 1% serum containing medium for 24 h before addition of test agents.

Synthetic ONOO<sup>−</sup> was manufactured by the reaction of H<sub>2</sub>O<sub>2</sub> with NaNO<sub>2</sub>, and excess H<sub>2</sub>O<sub>2</sub> was removed by treatment with MnO<sub>2</sub> according to procedures described elsewhere. 3-Morpholinosydnonimine (SIN-1; Molecular Probes, Eugene, OR) was used as a continuous generating system for ONOO<sup>−</sup> as this compound undergoes a base-catalyzed reaction leading to the simultaneous release of O<sub>2</sub><sup>·−</sup> and NO·, which rapidly react to form ONOO<sup>−</sup> (2). Spermine

1-3-propanediamine *N*-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl] (NONOate) (Cayman Chemical, Ann Arbor, MI) was used as an NO $\cdot$  donor. Alternatively, *S*-nitroso-*N*-acetylpenicillamine (SNAP; Molecular Probes) in the presence of L-cysteine (100  $\mu$ M; GIBCO) and SIN-1 in the presence of superoxide dismutase (SOD, 300 U/ml; Calbiochem, San Diego, CA) were used to confirm results obtained with spermine NONOate. All NO $\cdot$  generators were dissolved in Hanks' balanced salt solution (HBSS) and were added to cell cultures immediately. H $_2$ O $_2$  was purchased from Sigma (St. Louis, MO). Cells were exposed to these agents in 1% serum containing medium employing a range of concentrations that varied from 50  $\mu$ M to 1 mM. As a reagent control for ONOO $^-$ , cells were exposed to inactivated ONOO $^-$  after a 24-h incubation at room temperature. ONOO $^-$  was quantitated at 302 nm using an extinction coefficient of 1,670 M $^{-1}$ ·cm $^{-1}$ .

**Measurement of NO $\cdot$  in RLE cell medium after exposure to NO $\cdot$ -generating systems.** Evolution of NO $\cdot$  in the medium by spermine NONOate, SNAP plus L-cysteine (100  $\mu$ M), or SIN-1 in presence of SOD (300 U/ml) was measured in HBSS with an ISO-NO electrochemical probe (WPI) connected to a chart recorder as described elsewhere (16). The ISO-NO meter was calibrated by the addition of a NO $\cdot$ -saturated (1.8 mM) solution.

**Isolation of RNA and Northern blot analyses.** At selected time periods after exposure to ONOO $^-$ , NO $\cdot$ -generating systems, or H $_2$ O $_2$ , cells were harvested for extraction of total RNA. RNA was electrophoresed on 3-(*N*-morpholino)propane-sulfonic acid formaldehyde gels and was transferred onto nitrocellulose. Blots were hybridized with random primed [ $^{32}$ P]cDNA probes (28). cDNAs encoding *c-fos* and *c-jun* were obtained from R. Gaynor at the University of California at Los Angeles. Blots were washed in standard sodium citrate (28) and were exposed to film (NEF 496; NEN, Boston, MA). Quantitation was performed using a PhosphoImage Analyzer (Bio-Rad, Hercules, CA).

**Western blotting of *c-Fos* and *c-Jun* proteins.** Nuclear extracts prepared from RLE cells, as described previously, were diluted in 2 $\times$  Laemmli sample buffer (28, 29). Samples (5  $\mu$ g/lane) were electrophoresed on a 15% polyacrylamide gel and were electroblotted onto nitrocellulose (Ellard Instrumentation, Seattle, WA) according to standard procedures (28). Blots were incubated overnight at 4°C in tris(hydroxymethyl)-aminomethane (Tris)-buffered salt (TBS) solution containing 5% nonfat milk. Membranes were then washed with TBS containing 0.05% Tween 20 for 30 min followed by incubation with *c-Fos* or *c-Jun* antibodies for 1 h (0.5  $\mu$ g/ml; Santa Cruz Biotechnology, Santa Cruz, CA). Blots were washed and then were incubated with peroxidase-conjugated secondary antibody (0.3  $\mu$ g/ml; Jackson Immunoresearch Laboratories, West Grove, PA). Proteins were visualized by enhanced chemiluminescence (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

**Electrophoretic mobility shift analyses.** To determine binding of AP-1 transcription factor complexes to the AP-1 consensus DNA sequence (Promega, Madison, WI), nuclear extracts from RLE cells treated with ONOO $^-$ , NO $\cdot$ -generating systems, or H $_2$ O $_2$  were prepared according to procedures by Staal et al. (29). Four micrograms of nuclear protein were applied per lane and were electrophoresed on 4% polyacrylamide gels in 0.25 $\times$  Tris-borate-EDTA buffer at 100 V for 2 h. Gels were dried and exposed to film at room temperature. Afterward, the shifted bands containing the AP-1 complex were quantitated by phosphoimage analysis.

**Measurement of cell cycle distribution and apoptosis.** Flow cytometry was performed on cell cultures exposed to ONOO $^-$ , NO $\cdot$ -generating systems, or H $_2$ O $_2$  for 24, 48, or 72 h. Total cells (attached plus floating) were harvested by brief trypsin-

ization and were resuspended in 3.75 mM sodium citrate, pH 7.0, 0.1% Triton X-100, 32  $\mu$ g/ml ribonuclease A, and 50  $\mu$ g/ml propidium iodide (Sigma Chemical) by methods described previously (3). Ten thousand gated events per group per experiment in duplicate were evaluated to determine the percentage of cells in G $_0$ /G $_1$ , S, and G $_2$ /M phases of the cell cycle and those exhibiting a hypodiploid DNA content characteristic of apoptosis (7).

To also visualize the occurrence of DNA laddering, which is characteristic of apoptosis, genomic DNA was extracted from cells exposed to ONOO $^-$ , NO $\cdot$ -generating systems, or H $_2$ O $_2$  for 24 h (5). Thirty micrograms of DNA were loaded on a 1.6% agarose gel containing ethidium bromide and were electrophoresed in Tris-borate-EDTA buffer at 20 V overnight. Gels were then photographed under ultraviolet light.

**Assessment of membrane permeability.** To determine whether test agents caused membrane damage, the fluorescent dye Sytox (Molecular Probes), which selectively enters cells with permeable membranes, was used. After 24 h of exposure to agents, cells were trypsinized from dishes and incubated in 10 nM Sytox in HBSS (GIBCO), and ten thousand cells per group were analyzed by flow cytometry (Coulter EPICS Elite, Miami, FL). Numbers of fluorescent cells correlated with numbers staining positively using the trypan blue exclusion technique to assess viability (data not shown).

**Immunofluorescence of nitrotyrosine residues.** RLE cells were grown on glass coverslips and were treated with test agents in 1% serum-containing medium as described before. After 4 h of exposure, cells were washed two times with phosphate-buffered saline (PBS) and were fixed in 100% methanol (−20°C) for 5 min. After three washes with PBS (5 min each), cells were permeabilized for 20 min in PBS containing 0.1% Triton X-100. Sections were washed in PBS containing 1% bovine serum albumin and were incubated for 1 h with a rabbit polyclonal anti-nitrotyrosine antibody (2  $\mu$ g/ml PBS; Upstate Biotechnology, Lake Placid, NY), washed with PBS, and incubated with a lissamine-rhodamine-conjugated goat anti-rabbit secondary antibody (20  $\mu$ g/ml; Jackson Immunoresearch Laboratories) for 1 h. Coverslips were mounted onto slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA) for analysis by confocal microscopy (Bio-Rad).

**Statistical analysis.** Results were evaluated by one-way analysis of variance using the Student-Newman-Keuls procedure for adjustment of multiple comparisons. Trend analysis was performed to assess the dosage dependence of responses.

## RESULTS

**Production of NO $\cdot$  in medium.** The maximal concentrations of NO $\cdot$  measured over a period of 2 h were 15  $\mu$ M for 1 mM spermine NONOate and 30  $\mu$ M for 1 mM SNAP in the presence of 100  $\mu$ M L-cysteine. One millimolar SIN-1 in the presence of 300 U/ml SOD yielded ~0.8–1  $\mu$ M NO $\cdot$  similar to values reported previously (16), whereas no NO $\cdot$  could be measured in the absence of SOD. It is of importance to note that the rate of NO $\cdot$  formation in medium differs between these compounds. The maximal NO $\cdot$  concentrations were found after 10 min of exposure to SNAP plus L-cysteine, which decreased by 50% after 20 min and disappeared after 2 h. However, spermine NONOate and SIN-1 plus SOD released constant levels of NO $\cdot$  over a time period encompassing ~2 h, which disappeared after 4 h of exposure. The difference in kinetics in NO $\cdot$  generation

over time may explain the differences in magnitude of responses observed in RLE cells exposed to  $\text{NO}\cdot$  generators (see below).

$\text{H}_2\text{O}_2$  and  $\text{NO}\cdot$ , but not  $\text{ONOO}^-$ , cause increased *c-fos* and *c-jun* mRNA levels, protein, and AP-1 DNA binding activity. Because the *fos* and *jun* gene families appear to regulate a number of cellular events, including proliferation and apoptosis, we first determined whether various  $\text{NO}\cdot$ -generating systems,  $\text{ONOO}^-$ , or  $\text{H}_2\text{O}_2$  could induce *c-fos* and *c-jun* and their protein products. Figure 1A demonstrates increases in steady-state mRNA levels of *c-fos* and *c-jun* after 2 h of exposure of RLE cells to  $\text{H}_2\text{O}_2$  or spermine NONOate. With these agents, increases in mRNA levels appeared after 1 h of exposure, were maximal at 2 h, and decreased after 4 h of exposure (data not shown). Exposure to  $\text{ONOO}^-$  (150  $\mu\text{M}$ –1 mM) did not alter mRNA expression of *c-fos* or *c-jun* at any time point. To avoid the possibility that components in newborn bovine serum may have scavenged  $\text{ONOO}^-$ , we also examined *c-fos* and *c-jun* expression in cells after exposure to  $\text{ONOO}^-$  in serum-free medium and obtained similar negative results (data

not shown). Figure 1B shows *c-jun* mRNA levels after 2-h exposures to a range of  $\text{NO}\cdot$  generators. Like spermine NONOate, SNAP, in the presence of L-cysteine, caused statistically significant increases in expression of *c-jun*. The  $\text{ONOO}^-$  generator SIN-1 did not affect *c-jun* mRNA, similar to findings with synthesized  $\text{ONOO}^-$ . However, SIN-1, in the presence of SOD, a protocol generating  $\text{NO}\cdot$  and  $\text{H}_2\text{O}_2$ , augmented *c-jun* mRNA levels ( $P < 0.05$ ).

We next determined whether increases in mRNA levels were accompanied by increases in nuclear c-Fos and c-Jun proteins. As shown in Fig. 2, increases in c-Fos were observed after 4 or 8 h of exposure to  $\text{H}_2\text{O}_2$ , and c-Jun was increased at all time points. Similarly, c-Jun levels were elevated at all time points examined after exposure to spermine NONOate, whereas c-Fos was increased after 8 h. Similar to results obtained by Northern blotting,  $\text{ONOO}^-$  failed to induce elevations of c-Fos and c-Jun.

We next performed electrophoretic mobility shift assays to confirm binding of nuclear AP-1 transcription factor complexes to the consensus AP-1 DNA sequence.

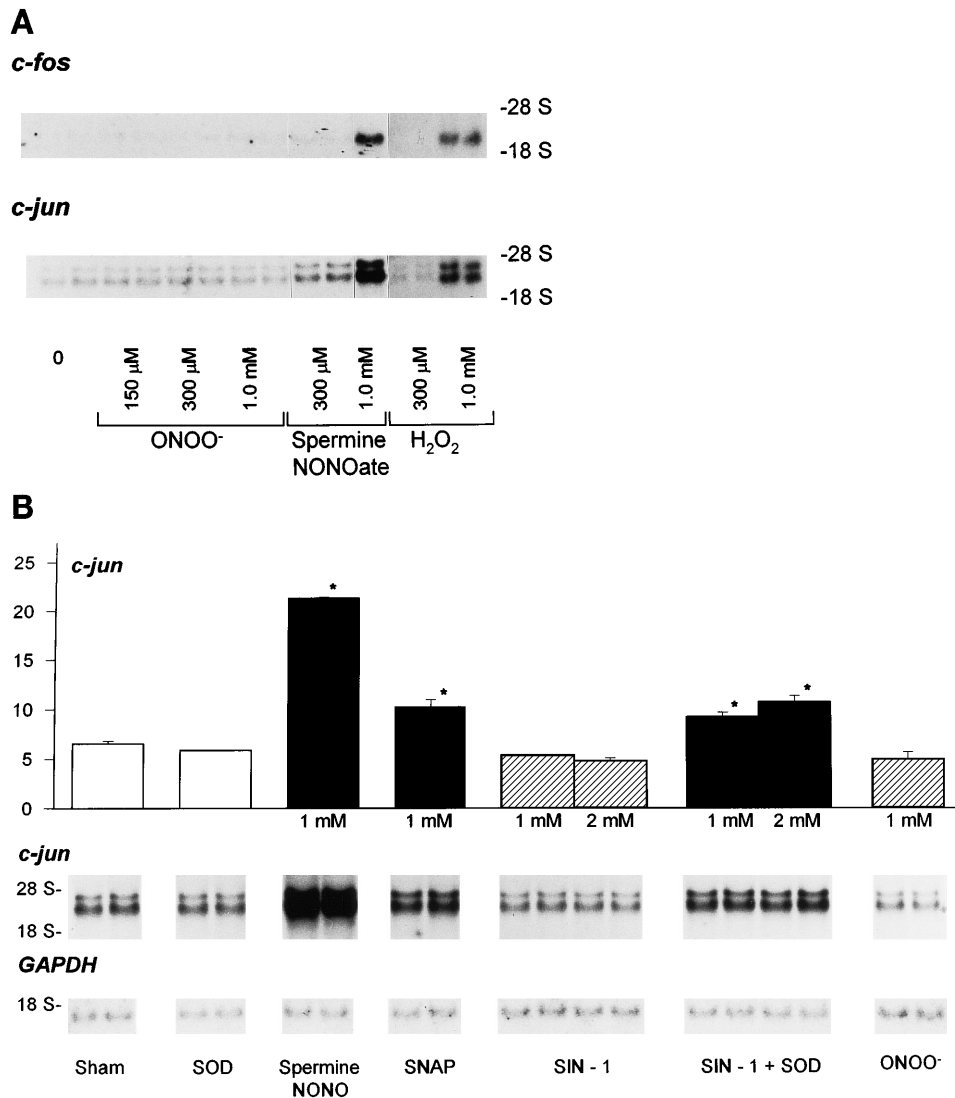
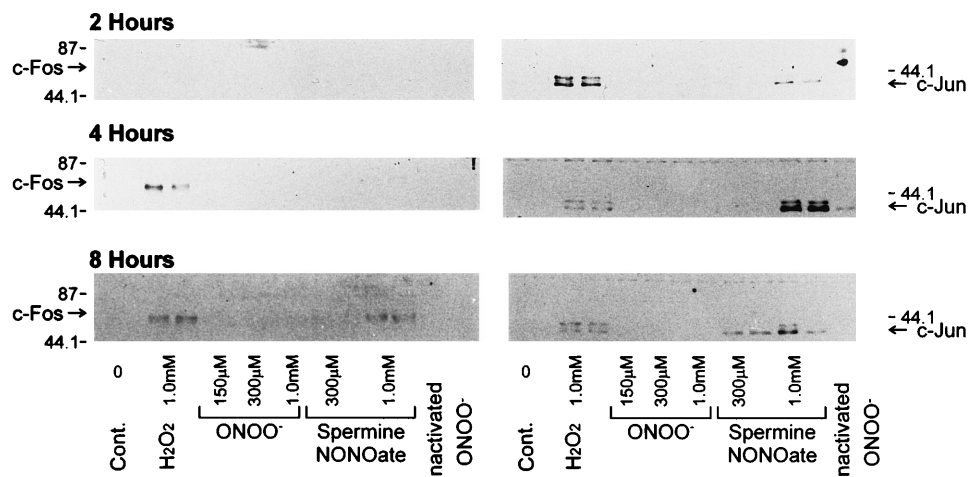


Fig. 1. A: steady-state mRNA levels of *c-fos* and *c-jun* protooncogenes in rat lung epithelial (RLE) cells exposed to  $\text{H}_2\text{O}_2$ , spermine 1,3-propanediamine *N*-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl] (NONOate), or peroxynitrite ( $\text{ONOO}^-$ ). RLE cells were exposed to agents for 2 h, and RNA was extracted for Northern blot analyses. Increases in gene expression were apparent in cells exposed to  $\text{H}_2\text{O}_2$  and spermine NONOate [ $P < 0.05$ , analysis of variance (ANOVA)] but not  $\text{ONOO}^-$ . B: *c-jun* mRNA levels in RLE cells exposed to spermine NONOate, *S*-nitroso-*N*-acetylpenicillamine (SNAP) plus L-cysteine (100  $\mu\text{M}$ ), SIN-1 in the absence or presence of superoxide dismutase (SOD; 300 U/ml), or  $\text{ONOO}^-$  for 2 h. Graph shows quantitative data ( $n = 2/\text{group}$ ) by phosphoimage analysis. Values on y-axis are relative units. \* $P < 0.05$  by ANOVA. Representative Northern blot of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown to confirm homogeneity of RNA content in blots. SIN, 3-morpholininosydnonimine.



Fig. 2. *c-Fos* and *c-Jun* protein levels in RLE cells after 2, 4, or 8 h of exposure to spermine NONOate,  $H_2O_2$ , or  $ONOO^-$ . Nuclear extracts were prepared, and 5  $\mu$ g protein/lane were used for Western blots. One 39-kDa band is detected by the *c-Jun* antibody, whereas one 72-kDa species is visible after reaction with the *c-Fos* antibody. Molecular size markers are indicated. Cont, control.



Increases in AP-1 DNA binding activity occurred after 2, 4, or 8 h of exposure of RLE cells to  $H_2O_2$  or spermine NONOate (1 mM;  $P < 0.05$ ) but not to  $ONOO^-$  (Fig. 3). In additional experiments, the  $NO\cdot$  donors spermine

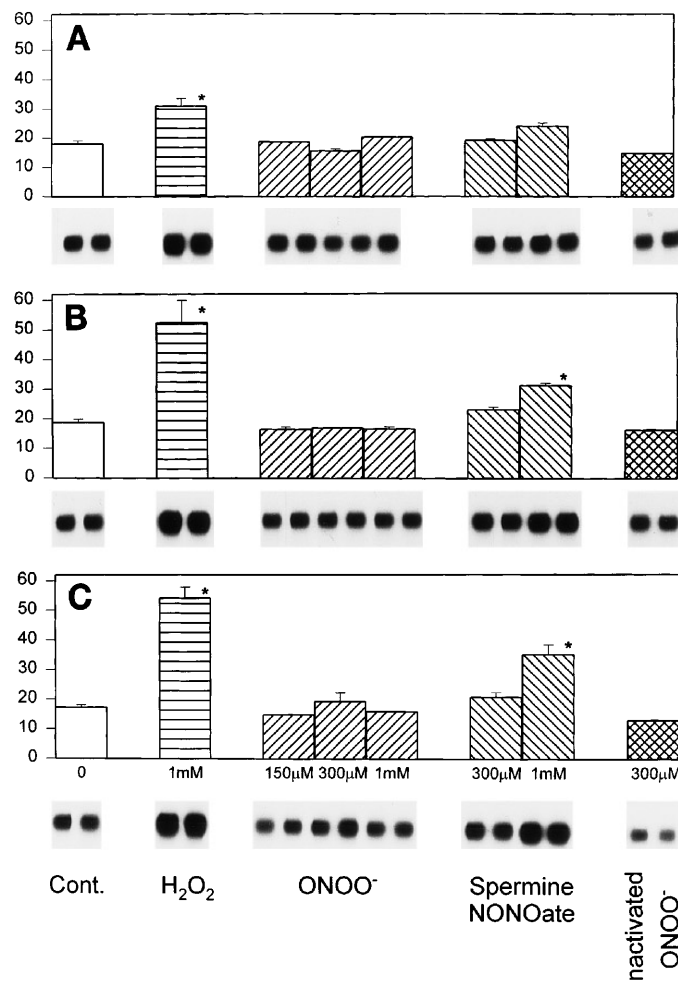


Fig. 3. Electrophoretic mobility shift assays (EMSA) evaluating binding of nuclear proteins to the activator protein (AP)-1 consensus DNA sequence. RLE cells were exposed to  $H_2O_2$ , spermine NONOate, or  $ONOO^-$  for 2 (A), 4 (B), or 8 (C) h before preparation of nuclear extracts. Graphs show quantitation of shifted bands by phosphorimage analysis. Values on y-axis are relative units.  $*P < 0.05$  by ANOVA;  $n = 2$ /treatment group.

NONOate and SNAP caused increases in AP-1 to DNA binding activity (Fig. 4), whereas  $ONOO^-$  and the  $ONOO^-$  generator SIN-1 revealed no changes. SIN-1, in the presence of SOD, caused significant ( $P < 0.05$ ) increases in AP-1 DNA binding activity.

Results in concert (Figs. 1–4) show that  $NO\cdot$  or  $H_2O_2$ , but not  $ONOO^-$ , induce *c-fos* and *c-jun* gene and protein expression, which corresponds with binding of their protein complexes to the consensus AP-1 DNA sequence.

**Differential induction of apoptosis and membrane permeability by  $H_2O_2$ ,  $NO\cdot$ , and  $ONOO^-$ .** Because early response protooncogenes may be important in the activation of the apoptotic pathway (24), we next determined whether exposure to  $NO\cdot$ -generating systems,  $ONOO^-$ , or  $H_2O_2$  induced apoptosis or increases in membrane permeability. We first used flow cytometry to determine the fractions of RLE cells in different phases of the cell cycle. After a 24-h exposure to spermine NONOate, SNAP, SIN-1 in the presence of SOD, or  $H_2O_2$ , a significant ( $P < 0.05$ ) percentage of cells exhibiting a hypodiploid DNA content indicative of apoptosis was apparent (Fig. 5A). Numbers of apoptotic cells increased over a 72-h period (data not shown). RLE cells exposed to  $ONOO^-$  did not display alter-

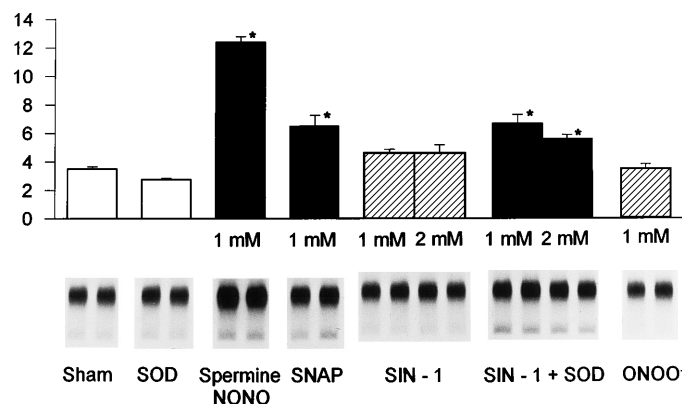


Fig. 4. EMSA determining binding of nuclear protein complexes to the AP-1 consensus DNA sequence. RLE cells were exposed to a battery of agents generating nitric oxide ( $NO\cdot$ ) or  $ONOO^-$  for 6 h at which time nuclear extracts were prepared for assays. Data in graph are expressed as relative units after quantitation by phosphorimage analysis.  $*P < 0.05$  by ANOVA;  $n = 2$ /treatment group.

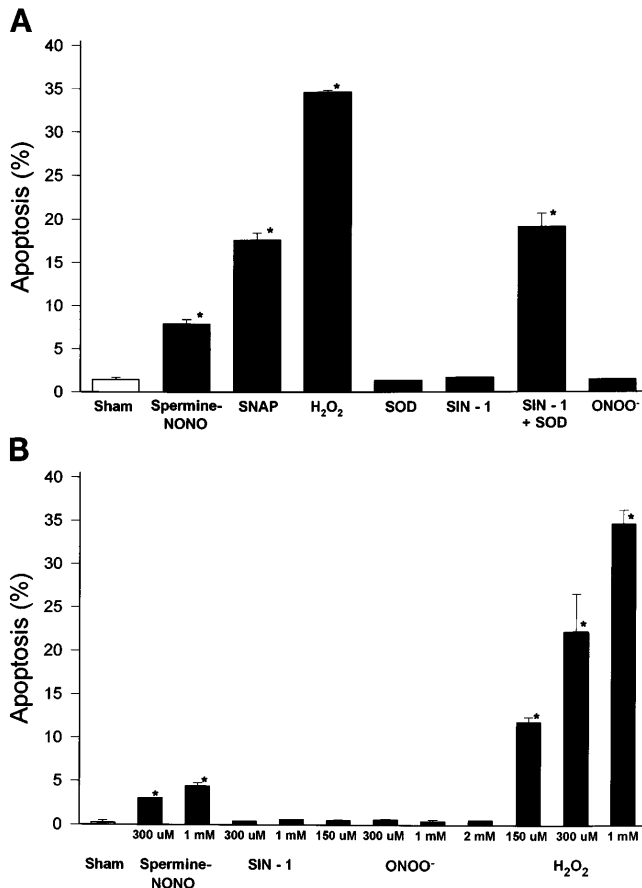


Fig. 5. A: quantitation of apoptosis as measured by flow cytometry after exposure of RLE cells for 24 h to spermine NONOate (1 mM), SNAP plus L-cysteine (1 mM), H<sub>2</sub>O<sub>2</sub> (1 mM), SOD (300 U/ml), SIN-1 (1 mM), SIN-1 (1 mM) in the presence of SOD (300 U/ml), or ONOO<sup>-</sup> (1 mM). B: dose-dependent increases in apoptosis in RLE cells exposed to spermine NONOate or H<sub>2</sub>O<sub>2</sub> for 24 h. Dosage dependence was confirmed by trend analysis. \**P* < 0.05 by ANOVA; *n* = 2/treatment group.

ations in normal cell cycle distributions or increases in apoptosis at any time point. We next exposed cells to various concentrations of agents to confirm these observations. As demonstrated in Fig. 5B, exposure to spermine NONOate or H<sub>2</sub>O<sub>2</sub> caused dosage-dependent increases in apoptosis, whereas synthetic ONOO<sup>-</sup> or the

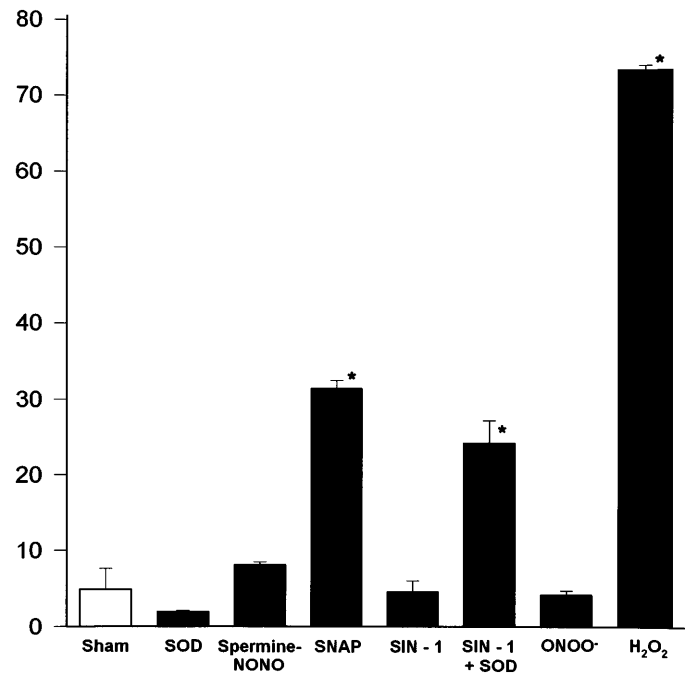


Fig. 7. Evaluation of membrane permeability by the Sytox method in RLE cells exposed to the NO<sup>-</sup>-generating systems, spermine NONOate (1 mM), SNAP (1 mM) in presence of 100  $\mu$ M L-cysteine, SIN-1 (1 mM) in presence or absence SOD (300 U/ml), ONOO<sup>-</sup> (1 mM), or H<sub>2</sub>O<sub>2</sub> (1 mM) for 24 h. Data are expressed as the percentage of Sytox positive cells. \**P* < 0.05 by ANOVA; *n* = 2/treatment group.

ONOO<sup>-</sup> generator SIN-1 did not. As shown in Fig. 6, DNA ladders appeared in RLE cells after exposure to spermine NONOate, SNAP, or H<sub>2</sub>O<sub>2</sub>. No evidence of DNA degradation was present in RLE cells exposed to SIN-1. Similar to results by flow cytometry, SIN-1, in the presence of SOD, caused the typical laddering pattern that is characteristic of apoptosis. Although synthesized ONOO<sup>-</sup> did not cause discrete laddering of DNA, some smearing was evident, which may be indicative of nonspecific cleavage of base pairs. In support of data obtained by flow cytometry and DNA laddering, assessment of nuclear morphology using 4',6-diamidino-2-phenylindole showed characteristic apoptotic bodies in RLE cells after exposure to H<sub>2</sub>O<sub>2</sub> or

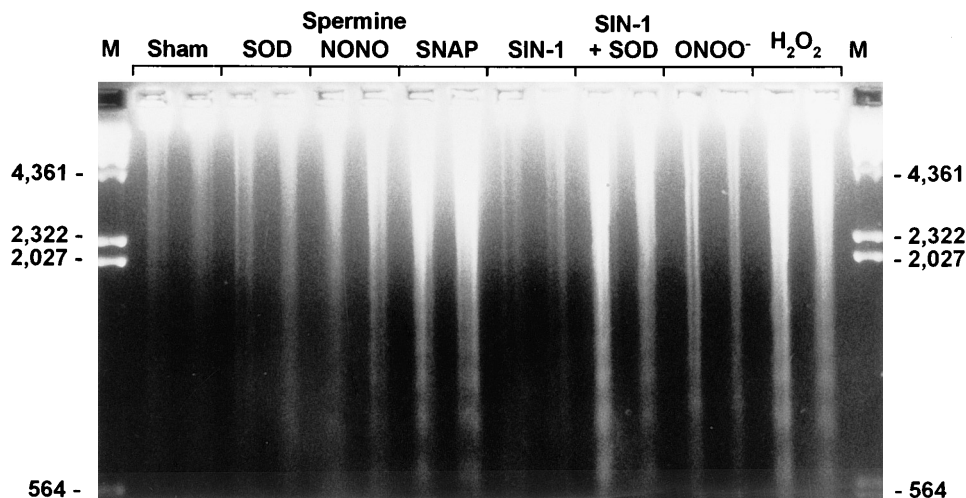


Fig. 6. Confirmation of apoptosis by DNA laddering in RLE cells after 24 h of exposure to spermine NONOate (1 mM), SNAP plus L-cysteine (1 mM and 100  $\mu$ M, respectively), SIN-1 (1 mM) in presence of 300 U/ml SOD, or H<sub>2</sub>O<sub>2</sub> (300  $\mu$ M). Molecular size markers (M) are indicated.

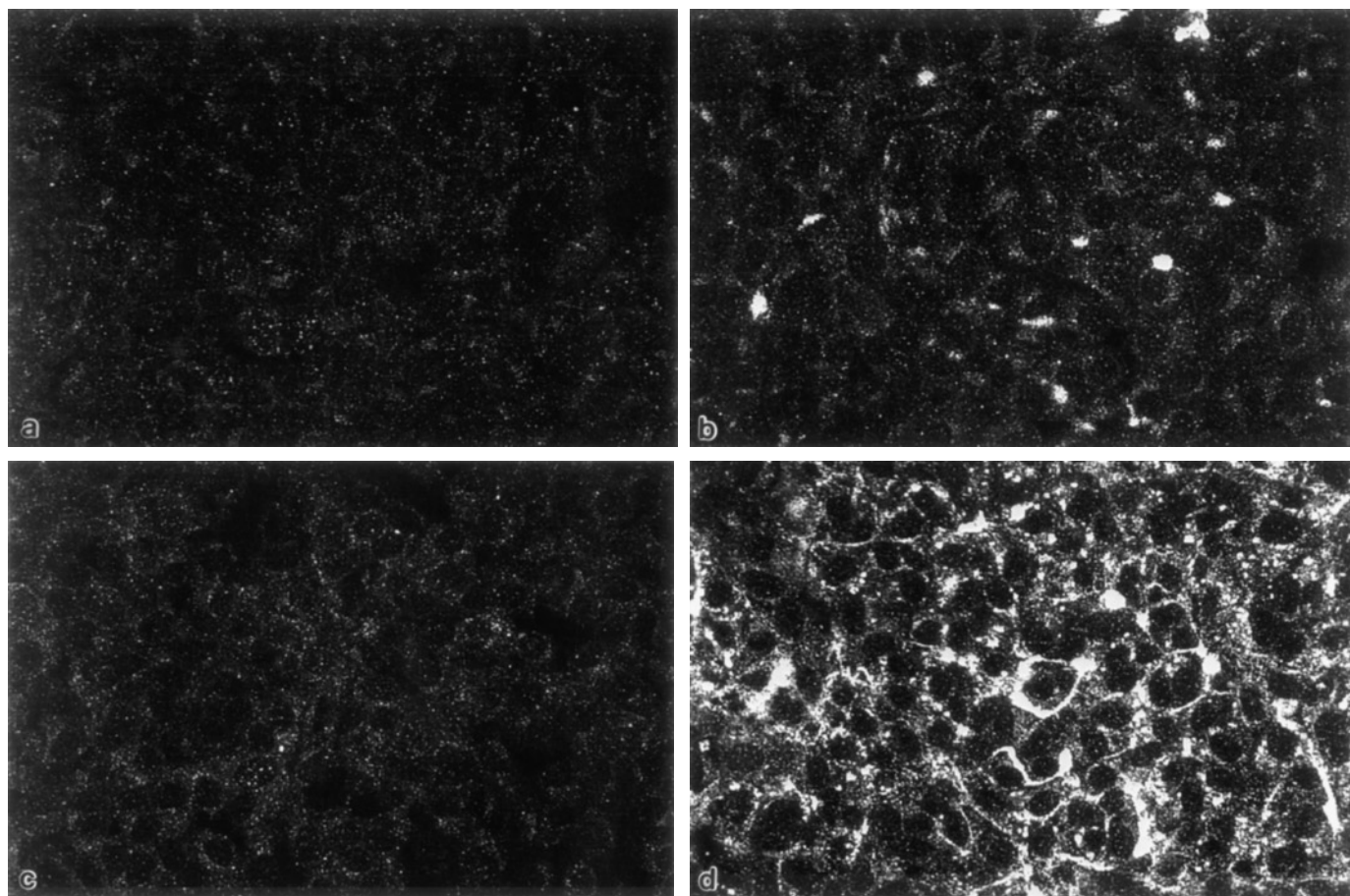


Fig. 8. Visualization of nitrotyrosine residues in sham control RLE cells (a) or after 4 h of exposure to SIN-1 (1 mM; b), SIN-1 (1 mM) in the presence of SOD (300 U/ml; c), or  $\text{ONOO}^-$  (1 mM; d). No significant immunoreactivity was observed after exposure to  $\text{H}_2\text{O}_2$ , spermine NONOate, or SNAP (not shown). Incubation of antibody with an excess of nitrotyrosine abolished the immunoreactivity observed after  $\text{ONOO}^-$  exposure (data not shown).

$\text{NO}\cdot$ -generating systems, but not  $\text{ONOO}^-$  (data not shown).

To determine the extent of membrane damage triggered by compounds 24 h postexposure, we used the cell-impermeant probe Sytox, which becomes fluorescent after uptake by cells. Figure 7 shows results from these Sytox uptake experiments. Addition of SNAP, SIN-1 plus SOD, or  $\text{H}_2\text{O}_2$  caused significant ( $P < 0.05$ ) increases in percentages of Sytox-positive cells, whereas synthesized  $\text{ONOO}^-$  or SIN-1 showed no elevations compared with sham controls. Interestingly, increased membrane permeability was not observed with spermine NONOate, an agent causing striking increases in apoptosis.

**Protein nitrotyrosine formation in RLE cells exposed to  $\text{ONOO}^-$ .** Formation of nitrotyrosine residues is a footprint of exposure to  $\text{ONOO}^-$  and an indicator of reactivity of  $\text{ONOO}^-$  with cells. As shown in Fig. 8, extensive nitration of tyrosine residues occurred after 4 h of exposure of RLE cells to SIN-1 (b) or  $\text{ONOO}^-$  (d) but not after exposure to SIN-1 in presence of SOD (c). Interestingly, patterns of protein tyrosine nitration after exposure to  $\text{ONOO}^-$  are consistent with reactivity at the cell membrane, as is expected due to the reactivity of  $\text{ONOO}^-$ . Neither  $\text{H}_2\text{O}_2$ , spermine NONOate, nor

SNAP caused nitration of tyrosines (not shown). Furthermore, addition of a 10-fold excess of nitrotyrosine abolished the immunofluorescence, indicating the specificity of the reaction (not shown). These results demonstrate that the lack of effect of  $\text{ONOO}^-$  in the induction of *c-fos* and *c-jun* protooncogenes, apoptosis, and membrane permeability is not due to its lack of reactivity with RLE cells.

## DISCUSSION

Studies here indicate that both  $\text{H}_2\text{O}_2$  and  $\text{NO}\cdot$  serve as important intracellular signaling molecules to activate protooncogene expression and transcription factors. Our focus here was on *c-fos* and *c-jun*, since protein products of these protooncogenes are thought to play a role in cell differentiation, transformation, proliferation, and apoptosis (1, 24, 25). These events may be critical to the development of pulmonary disease associated with exposure to reactive metabolites. Although others have demonstrated activation of mitogen-activated protein kinases by  $\text{H}_2\text{O}_2$  (13, 25) and  $\text{NO}\cdot$  (20), suggesting that early response genes may be downstream targets, we demonstrate here that  $\text{H}_2\text{O}_2$  and a variety of  $\text{NO}\cdot$ -generating systems activate *c-fos* and



*c-jun* protooncogenes, as evidenced by increased mRNA expression, nuclear proteins, and AP-1 DNA binding activity. Furthermore,  $\text{H}_2\text{O}_2$  or  $\text{NO}\cdot$  also lead to the development of apoptosis, as defined by flow cytometry, DNA laddering, and morphological criteria for apoptosis.

Increased mRNA levels of *fos* and *jun* family members and activation of AP-1-dependent gene expression occurs in other cell types after exposure to  $\text{NO}\cdot$  or  $\text{H}_2\text{O}_2$  (14, 17, 22, 23). In addition,  $\text{NO}\cdot$ -generating systems (4, 18) and  $\text{H}_2\text{O}_2$  (3) cause apoptosis in a variety of cell types, including pancreatic  $\beta$ -cells and cortical and mesothelial cells. Although our studies do not provide a direct link between early response gene activation and apoptosis, the time frame of events and our negative results using  $\text{ONOO}^-$  suggest a possible association. Present studies that involve overexpression of c-Fos or c-Jun in RLE cells may verify the association of these protooncogenes with apoptosis.

$\text{NO}\cdot$  can react with a number of substrates that may dramatically alter its reactivity and toxic potential. For instance, reaction with heme-containing proteins may alter their function (2), whereas reaction with thiol moieties in airways may increase the half-life of  $\text{NO}\cdot$  by providing a continuous source of  $\text{NO}\cdot$  release from nitrosothiols (12). At high concentrations,  $\text{NO}\cdot$  reacts with  $\text{O}_2$  to form  $\text{NO}_2\cdot$  (2). At the peak concentrations of  $\text{NO}\cdot$  generated here, small levels of  $\text{NO}_2\cdot$  may have been formed and may have accounted for some of the observed effects. Because  $\text{NO}_2\cdot$  is also a nitrating agent, the lack of nitrotyrosine immunofluorescence after exposure to  $\text{NO}\cdot$  generators (not shown) indicates that  $\text{NO}_2\cdot$  levels were minimal.

The reaction of  $\text{NO}\cdot$  with  $\text{O}_2\cdot^-$  that generates the potent oxidant,  $\text{ONOO}^-$ , is extremely rapid, and, in situations where elevated  $\text{NO}\cdot$  concentrations exist,  $\text{NO}\cdot$  outcompetes SOD for reaction with  $\text{O}_2\cdot^-$  (2). In inflammatory conditions or ischemia-reperfusion,  $\text{O}_2\cdot^-$  and  $\text{NO}\cdot$  can be generated simultaneously from different cell types (26), thus providing substrates for  $\text{ONOO}^-$  generation.  $\text{ONOO}^-$ , in its physiological, protonated form, also is extremely reactive and decomposes into a number of reactive metabolites that may involve  $\text{NO}_2\cdot$  and  $\text{OH}\cdot$  radicals (2), which may contribute to oxidative DNA modification (31), alterations in protein function (30), or lipid peroxidation (27). Thus the overall reaction chemistry of RNS and ROS and the critical reactive species that ultimately damage lung cells are extremely complex.

Exogenous administration of  $\text{ONOO}^-$  at a range of concentrations (150  $\mu\text{M}$ –1 mM) causing nitration of tyrosines (Fig. 8) did not lead to alterations in early response gene expression or apoptosis in RLE cells when assessed by flow cytometry, DNA laddering, and nuclear staining techniques. The lack of increased membrane permeability by  $\text{ONOO}^-$  confirmed these findings and further substantiated its inability to induce injury in this cell type. The use of SIN-1 as another exogenous generating system of  $\text{ONOO}^-$  supported these findings. However, incubation of SIN-1 in the presence of SOD to yield  $\text{NO}\cdot$  and  $\text{H}_2\text{O}_2$  caused

protooncogene induction, apoptosis, and membrane damage, suggesting that  $\text{NO}\cdot$  and  $\text{H}_2\text{O}_2$ , but not  $\text{ONOO}^-$ , induce signaling events that lead to these phenotypic end points. These findings, however, contrast with other studies showing apoptosis in PC-12 cells (9), cerebrocortical cultures (4), and HL-60 cells (21) exposed to  $\text{ONOO}^-$ . However, in support of our findings, a recent study demonstrates that  $\text{ONOO}^-$  generated by SIN-1 does not cause cytotoxicity, as measured by lactic dehydrogenase release in a human ovarian cancer cell line (10). In contrast, SIN-1 plus SOD resulted in enhanced toxicity, suggesting a role for  $\text{NO}\cdot$  and  $\text{H}_2\text{O}_2$  in the cytotoxic effect (10). This work and the lack of responsiveness of RLE cells to  $\text{ONOO}^-$  is surprising given the reactivity of this species (2). However, a number of possible explanations exist. First, signaling cascades that lead to activation of *c-fos* and *c-jun* and consequent apoptosis may be inactivated by  $\text{ONOO}^-$ . For example, it was demonstrated recently that nitration of tyrosine residues by  $\text{ONOO}^-$  may block phosphorylation (19), thereby impairing tyrosine kinase signaling cascades that may be required for protooncogene activation or apoptosis. Alternatively, externally administered  $\text{ONOO}^-$  may react with cell surface components, as is indicated by patterns of tyrosine nitration, whereas  $\text{NO}\cdot$  or  $\text{H}_2\text{O}_2$  can traverse the cell membrane to elicit protooncogene expression. However, sensitive transient transfection assays in RLE cells to measure activation of gene transcription by nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) demonstrate that  $\text{ONOO}^-$  activates NF- $\kappa\text{B}$ -dependent gene expression (unpublished observations).

In conclusion, our findings demonstrate that  $\text{NO}\cdot$  or  $\text{H}_2\text{O}_2$  causes increased early response gene expression resulting in apoptosis in lung epithelial cells. In contrast,  $\text{ONOO}^-$  did not cause these responses. However, the lack of gene expression and apoptosis observed here with  $\text{ONOO}^-$  occurred in the presence of nitrotyrosine residues, suggesting that their formation per se cannot be equated with functional ramifications examined here.

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