Journal of Cellular Biochemistry 92:502–513 (2004)

Mitogen-Activated Protein Kinases Modulate H_2O_2-Induced Apoptosis in Primary Rat Alveolar Epithelial Cells

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Abstract Increasing evidence suggests a role for apoptosis in the maintenance of the alveolar epithelium under normal and pathological conditions. However, the signaling pathways modulating alveolar type II (ATII) cell apoptosis remain poorly defined. Here we investigated the role of MAPKs as modulators of oxidant-mediated ATII cell apoptosis using in vitro models of H_2O_2-stress. H_2O_2, delivered either as a bolus or as a flux, lead to time- and concentration-dependent increases in ATII cells apoptosis. Increased apoptosis in primary rat ATII cells was detected at H_2O_2 concentrations and production rates in the physiological range (1 μM) and peaked at 100 μM H_2O_2. Immortalized rat lung epithelial cells (RLE), in contrast, required millimolar concentration of H_2O_2 for maximal responses. H_2O_2-induced apoptosis was preceded by rapid activation of all three classes of mitogen-activated protein kinases (MAPKs): ERK, JNK, and p38. Specific inhibition of JNK using antisense oligonucleotides and ERK and p38 using PD98059 or SB202190, respectively, indicated a pro-apoptotic role for JNK pathway and an anti-apoptotic role for ERK- and p38-initiated signaling events. Our data show that the balance between the activation of JNK, ERK, and p38 is a critical determinant of cell fate, suggesting that pharmacological interventions on the MAPK pathways may be useful in the treatment of oxidant-related lung injury. J. Cell. Biochem. 92: 502–513, 2004.

Key words: reactive oxygen species; hydrogen peroxide; alveolar type II cells; apoptosis; MAPKs

The lung is a primary target for oxygen toxicity because of its constant exposure to high oxygen levels and environmental oxidants. Reactive oxygen species (ROS: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^\bullet$); and singlet oxygen ($^1O_2$)) have been implicated as mediators of the lung injury associated to supplemental oxygen therapy [Kelly and Lubec, 1995] and to several environmental exposures [Gurgueira et al., 2002] and occupational lung diseases like silicosis and asbestosis [Ryrfeldt et al., 1993; Vallyathan and Shi, 1997].

The alveolar epithelium is a major target for hyperoxic injury, and its repair following injury depends on the ability of its stem cells, the alveolar type II cells (ATII cells), to proliferate and differentiate [Mason and Williams, 1997]. Alveolar type II cells also respond to oxidant stress with functional changes such as impairment of ATP [LaCagnin et al., 1990] and phospholipid [Holm et al., 1988] synthesis; and with adaptive responses including increases in the mRNA [Clerch and Massaro, 1993], protein [Crapo and McCord, 1976], and activity [Crapo and Tierney, 1974] of Mn and Cu–Zn superoxide dismutase, and increases in Na^+/K^+-ATPase function [González-Flecha et al., 1996].

ROS signaling may be relevant to the response of alveolar epithelial cells to hyperoxia. Sublethal increases in ROS modulate signal transduction pathways through altera-

Abbreviations used: ATII, alveolar type II cells; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase.

Grant sponsor: NIH; Grant number: RO1 HL68073; Grant sponsor: NIEHS; Grant number: P50 ES00002-38.

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Received 10 December 2003; Accepted 26 January 2004
DOI 10.1002/jcb.20070

tions in the intracellular redox status and increases in the oxidized/reduced ratio of signaling molecules [Finkel, 1998]. One example is the modulation of cell growth by ROS. Micromolar concentrations of H_2O_2 stimulate cell proliferation and the expression of growth-related gene products in certain cell types [Preston et al., 2001]. On the other hand, higher H_2O_2 concentrations induce significant increases in cellular apoptosis [Wood and Youle, 1994]. These observations have led to the hypothesis that regulation of the intracellular concentration of ROS is a key factor in cell fate decisions.

Apoptosis is a controlled type of cell death characterized by cell shrinkage, membrane blebbing, and DNA fragmentation. Apoptosis is the result of coordinated signaling pathways, which can be triggered by a variety of extracellular stimuli, such as radiation, cytokines, growth factor withdrawal, etc. In agreement with the postulate of mitochondria (one of the major intracellular sources of ROS) as central executioners of programmed cell death), oxidative stress is one of the most reproducible inducers of apoptosis. Induction of apoptosis by ROS in vitro has been reported in many cell lines, including human fibroblasts [Gansauge et al., 1997], human leukemia HL-60 cells [Matsura et al., 1999], polymorphonuclear neutrophils [Rollet-Labelle et al., 1998], and immortalized lung epithelial cells from rats (RLE) and humans (A549) [Janssen et al., 1997; Ye et al., 1999]. Increasing evidence suggests a role for apoptosis in the maintenance of the alveolar epithelium under normal and pathological conditions. Alveolar type II cells undergo apoptosis during normal development and maturation of the lungs [Nishino et al., 1999], and in association with acute lung injury [Bardales et al., 1996]. However, the signal transduction pathways modulating ATII cell apoptosis remain poorly defined.

Mitogen-activated protein kinases (MAPKs) are phosphorylated in response to a series of extracellular stimuli. There are at least three subfamilies of the MAPK superfamily: the extracellular signal-regulated protein kinase (ERK), the c-Jun N-terminal kinase/stress-activated kinase (JNK/SAPK), and the p38-MAPK. Activation of MAPKs leads to transactivation of transcription factors, such as c-Jun and other members of the AP-1 family, Elk-1, ATF-2, and NF-κB, culminating in a variety of cellular events including cell survival and proliferation [Zhang and Liu, 2002] or apoptosis [Cross et al., 2000]. Studies in several different cellular systems show that MAPKs can be activated by ROS [Chen et al., 1995; Guyton et al., 1996]. Furthermore, activation of the ERK and JNK pathways in immortalized lung mesothelial cells has been shown to modulate the apoptotic response of these cells to asbestos and H_2O_2. In this study, we used primary rat ATII cells to investigate the role of MAPKs as modulators of oxidant-mediated apoptosis in models of acute and chronic oxidative stress that mimic stresses of clinical and environmental relevance.

MATERIALS AND METHODS

Reagents

Rat IgG, glucose oxidase, and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO). Elastase was from Worthington Biochemical Corp. (Lakewood, NJ). Dulbecco's modified Eagle's medium (DMEM), vitamins, and fetal bovine serum were from Gibco-BRL (Grand Island, NY). Acrylamide solution, ammonium persulfate, and TEMED were from Bio-Rad (Hercules, CA). PD98059 and SB202190 were from Calbiochem (San Diego, CA). All other chemicals were from Sigma Chemical Co. Antisense and scrambled sequence oligonucleotides were purchased from Operon (Alameda, CA).

Alveolar Type II Cell Isolation

Alveolar type II (ATII) cells were isolated from pathogen-free Sprague–Dawley rats weighing 180–220 g using the method of Dobbs [Dobbs, 1990] as described earlier [González-Flecha et al., 1996]. Rats were anesthetized with sodium pentobarbital (50 mg/kg body weight) and the heart and lungs were rapidly removed. Lungs were perfused via the pulmonary artery, lavaged and incubated with elastase solution (30 U/ml) for 20 min at 37°C. The tissue was then minced and filtered through sterile nylon filters of 140 and 20 μm mesh. ATII cells were purified by differential adherence to IgG-coated plates. They were then suspended in DMEM containing 10% fetal bovine serum, basal vitamin solution (BME, Gibco-BRL), 2 mM glutamine, 40 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin. Viability of the final preparation, assayed by exclusion of Trypan blue, was higher than 90%. Cells were used the day next to isolation. Purity

of the cells was ≥95% as assessed by Papanicolaou staining [Dobbs, 1990].

Cell Culture and H_2O_2 Treatments

Primary ATII cells were plated immediately after isolation in 96-well or 6-well tissue culture plates, at a density of 10^6 cells/cm^2. Cells were cultured in DMEM (supplemented as described above) in a humidified atmosphere of 5% CO_2–95% air at 37°C. After 24 h, non-adherent cells were removed by replacing the medium. Immortalized rat alveolar type II cells (RLE cells, [Driscoll et al., 1995], passage 43–46) were cultured at a density of 10^4 cell/cm^2 in DMEM/F12 containing 7% newborn calf serum, 2 mM glutamine, 40 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin.

We studied the dose-dependence and time-course of induction of apoptosis in two different in vitro models of oxidative stress. Acute and chronic stresses were simulated by treating the cells with boluses of H_2O_2 or with an H_2O_2-generating system, respectively. For the acute treatment, cells received H_2O_2 at initial concentrations ranging from 0.1 μM to 1 mM for primary ATII cells, and 1–5 mM for RLE cells. For the chronic treatment, glucose oxidase was added to the culture medium at concentrations that delivered controlled fluxes of H_2O_2 from 10^{-2} to 10^3 μM/h for primary ATII cells. The glucose concentration in the culture medium did not substantially change during incubations. H_2O_2 fluxes delivered by the glucose/glucose oxidase mixtures were calculated from the values of oxygen consumption measured using a Clark-type oxygen electrode [Estabrook, 1967]. Cell viability (estimated as LDH release) was not affected by the treatments. At the indicated times, adherent and non-adherent cells were harvested and assayed for apoptosis or MAPK activation as described below.

Apoptosis

Apoptosis was measured using two approaches: (i) by following the accumulation of mono- and oligo-nucleosomes in the cytoplasmatic fraction of cell lysates using a standard colorimetric ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN) [Ye et al., 1999], or (ii) by following increases in the sub-G_1 peak after cell cycle analysis. In the first case, cells were harvested and assayed for DNA fragmentation at different times using a Molecular Devices (Sunnyvale, CA) ν max scanning microplate reader. In the second case, apoptotic cells were identified as the sub-G_1 peak of the DNA content profiles after propidium iodide staining [Chen et al., 1996]. Briefly, cells were harvested by trypsinization, resuspended with 70% ethanol/PBS, and stored at 4°C until analysis. Cells were then stained with 50 μg/ml propidium iodide for 1 h in the presence of 40 μg/ml of RNAse A (DNAse-free). For the determinations of apoptosis, a total of 10^4 cells were counted. Measurements were carried out in a Coulter Flow Cytometer (Beckman Coulter, Fullerton, CA).

Immunodetection of Phosphorylated MAPKs

Cells lysates (50–100 μg protein/lane) were electrophoresed in SDS–12% polyacrylamide gels and electroblotted onto polyvinylidine difluoride membranes (NEN Life Science Products, Boston, MA). MAPKs activation was detected by using antibodies raised against their phosphorylated forms and visualized with the SuperSignal West Pico Chemiluminescent detection system (Pierce, Rockford, IL). Monoclonal antibodies against phosphorylated Tyr204 of ERK1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies against phosphorylated Thr183/Tyr185 of JNK and phosphorylated Thr180/Tyr182 of p38 were from Cell Signaling (Beverly, MA). Secondary antibodies were from Amersham (Piscataway, NJ) or Cell Signaling. Densitometry of the bands was performed using Bio-Rad GS-700 imaging densitometer. Equal loading was confirmed by Ponceau staining of membranes.

Inhibition of MAPKs

ERK and p38 phosphorylation was inhibited by treatment of the cells with 25 μM PD98059 or 50 μM SB202190, respectively [Wang et al., 1998], 2 h before the addition of H_2O_2. JNK activation was inhibited by using 20 mer antisense oligonucleotides directed against JNK1 and JNK2 (JNK1AS: 5′-CTC TCT GTA GGC CCG CTT GG-3′ and JNK2AS: 5′-GTC CGG GCC AGG CCA AAG TC-3′) [Potapova et al., 2000]. Cells were transfected with the antisense oligonucleotides using lipofectin (Life Technologies, Bethesda, MD) 6 h before the addition of H_2O_2. Antisense oligonucleotides with scrambled sequences were used as controls (JNK1ASscr: 5′-CTT TCC GTT GAA

CCC CTG GG-3′ and JNK2ASscr: 5′-GTG CGC GCG AGC CCG AAA TC-3′).

Statistical Analysis

The values and the bars in the figures indicate the mean value of 3–4 experiments ± the standard error of the mean (SEM). Data were analyzed statistically by factorial analysis of variance (ANOVA) followed by Fisher's test for comparison among means. Statistical analyses were performed using Graph Pad Instat software for PC or Statview 4.5 software for Macintosh.

RESULTS

H_2O_2 Stress Induces Alveolar Type II Apoptosis in a Time- and Dose-Dependent Manner

We have shown in previous studies in prokaryotic cells that transcriptional responses to chronic or acute H_2O_2 stresses are dramatically different [González-Flecha and Demple, 1995]. We therefore studied the effect of both chronic and acute exposures to H_2O_2 on the rate of DNA fragmentation in ATII cells.

H_2O_2 given either as a bolus or as an increased flux induced significant increases in DNA fragmentation in primary ATII cells (Fig. 1). The apoptotic response showed a trend of linearity in the range of 1–100 μM for the acute administration (Fig. 1A), and 10 nM/h to 5 mM/h for the chronic treatment (Fig. 1B). The time-course of the apoptotic response was different for acute and chronic exposures. Acute treatments with 100 μM H_2O_2 induced DNA fragmentation shortly after addition of H_2O_2 and reached maximal values at 6 h. On the other hand, chronic administration of 50 μM H_2O_2/h showed significant increases in DNA fragmentation at times longer than 8 h, with maximal responses 12 h after addition of glucose oxidase (Fig. 1C). Similar results were observed when apoptosis was measured by flow cytometric analysis of the cell DNA content using propidium iodide staining (Fig. 1D). Untreated cells showed a low percentage of apoptotic cells (~7%, Fig. 1D). The sub-G_1 peak increased after addition of 200 μM H_2O_2 to the culture, reaching a maximum value approximately threefold higher than control at 10 h (Fig. 1D).

In order to study possible differences in apoptotic responses between immortalized and primary rat epithelial cells, we used RLE cells exposed to H_2O_2 using the acute oxidative stress model. RLE cells were treated with 0–5 mM H_2O_2 and assayed for DNA fragmentation 12 h after treatment, the time for maximal responses (Fig. 2). Our results show that the apoptotic response to H_2O_2-stress in RLE cells differs from that in primary cultures in intensity and dose required for maximal effects. H_2O_2 concentrations below 200 μM, which gave maximal responses in primary ATII cells, failed to induce DNA fragmentation in this cell line, whereas H_2O_2 concentrations in the millimolar range led to greater than fourfold increases in the percentage of DNA fragmentation with respect to untreated cells (Fig. 2).

H_2O_2-Induced Apoptosis Is Preceded by MAPKs Activation

We investigated the potential activation of JNK, ERK, and p38 in H_2O_2-treated ATII cells. Activation of JNK was measured by immunoblotting using antibodies directed against the p46 and p54 isoforms of JNK dually phosphorylated at threonine 183 and tyrosine 185. ERK activation was measured using antibodies against the p42 and p44 isoforms of ERK phosphorylated at Tyr204. Finally, activation of p38 was evaluated using antibodies against Thr183/Tyr185-phosphorylated p38.

Treatment of ATII cells with 200 μM H_2O_2 stimulated phosphorylation of all three subfamilies of MAPKs (Fig. 3). However, the half-lives of the phosphorylated forms of JNK1/2, ERK1/2, and p38 were remarkably different. JNK phosphorylation was observed as early as 2 min after addition of H_2O_2 with maximal values at ~5 min and returned to almost control values within the first hour (Fig. 3, upper panel). Phosphorylated ERK1/2 showed significant increases 30 min after treatment and remained elevated for up to 6 h (Fig. 3, middle panel). Finally, phosphorylated p38 peaked 10 min after addition of H_2O_2 and returned to control values in less than half an hour (Fig. 3, lower panel).

Inhibition of JNK Phosphorylation Prevents H_2O_2-Induced Apoptosis

To determine whether MAPKs activation was essential for H_2O_2-induced apoptosis, we sequentially inhibited activation of each subfamily. JNKs activation was blocked using antisense oligonucleotides directed against the JNK1 and JNK2 sequences [Potapova et al., 2000]. ATII cells were transfected with anti-

JNK1 and anti-JNK2 oligonucleotides or with oligonucleotides with scrambled sequences using lipofectin (control) 6 h prior to the addition of H_2O_2. The 6-h transfection time was chosen in preliminary experiments as the minimal time required for efficient internalization of the oligonucleotides. Figure 4 shows that anti-JNK oligonucleotides effectively prevented H_2O_2-induced JNK1/2 phosphorylation and apoptosis. Control values are higher in this case due to the transfection. Transfection of cells with the control scrambled sequences did not significantly alter basal levels of apoptosis or their response to H_2O_2 (data not shown). The observed inhibition of H_2O_2-induced apoptosis by blockage of JNK activation suggests that this event is necessary to initiate apoptosis induced by H_2O_2 in ATII cells.

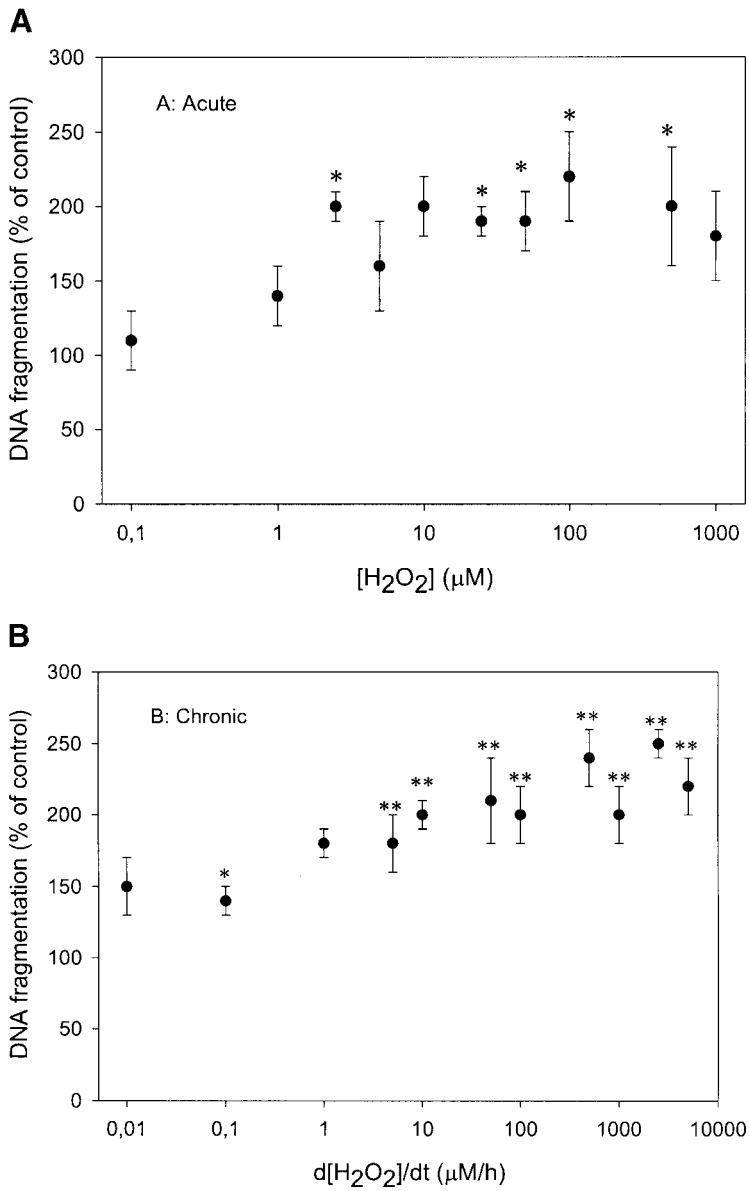

Fig. 1. Apoptosis in ATII cells subjected to H_2O_2 stress. **A–C**: DNA fragmentation; (**D**) Percentage of sub-G_1 cells. A: Acute exposure. ATII cells were treated with boluses of H_2O_2 for 6 h. B: Chronic exposure. ATII cells were treated for 12 h with defined external fluxes of H_2O_2 provided by the glucose/glucose oxidase system. C: Time-course of apoptosis in ATII cells subjected in vitro to H_2O_2 stress. ATII cells were treated with 50 μM/h H_2O_2 or with 100 μM H_2O_2 for the times indicated. After exposure, DNA fragmentation was measured as described in "Materials and Methods." Bars represent the mean of 4–6 determinations ± SEM. D: Time-course of apoptosis in ATII cells subjected in vitro to H_2O_2 stress. Cells were treated with 200 μM H_2O_2 and apoptosis was determined by flow cytometry analysis, scoring the number of cells with sub-G_1 DNA content. Results are representative of three experiments. $^*P < 0.05$, $^{**}P < 0.01$ vs. controls (untreated cells).

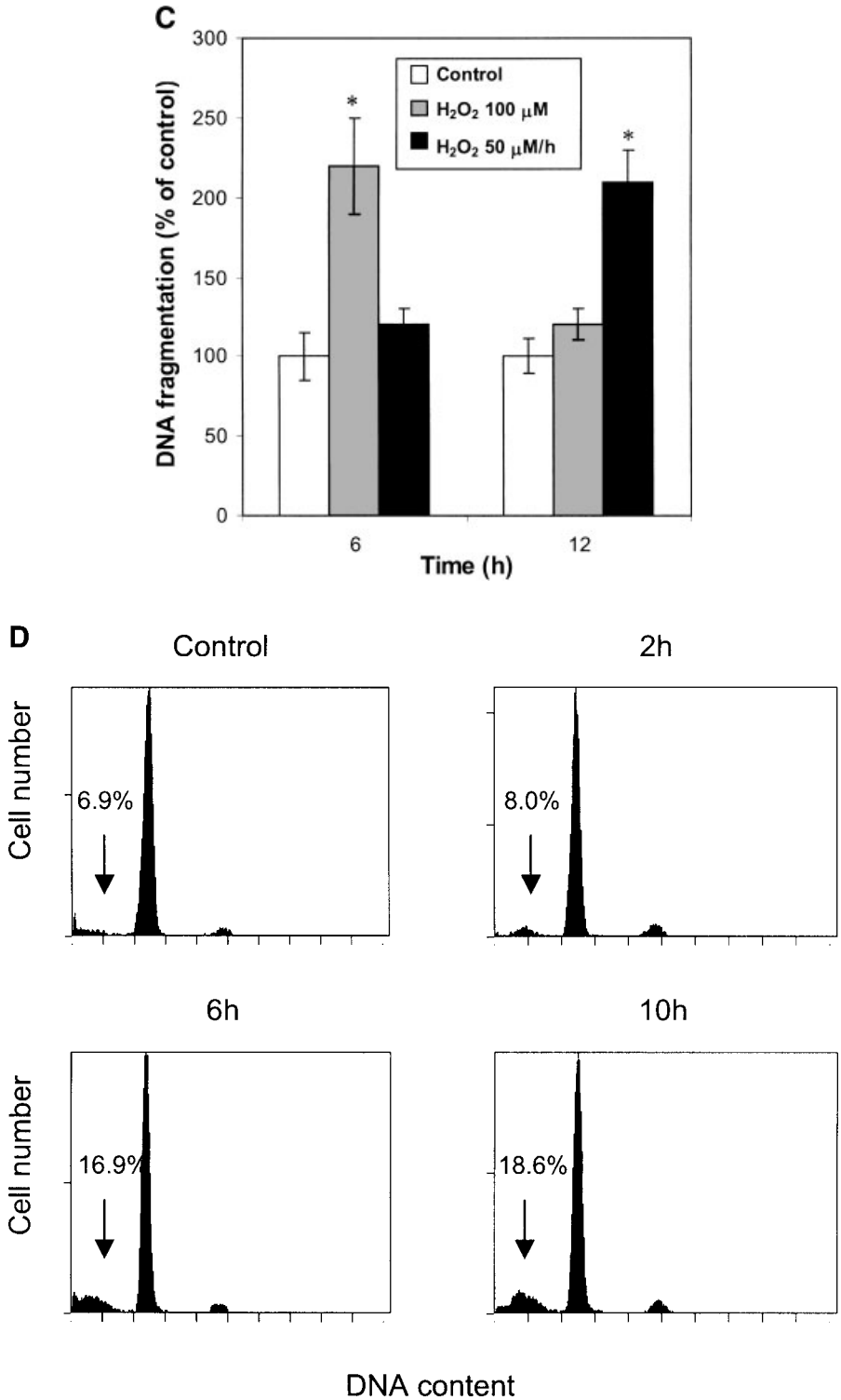

Fig. 1. (*Continued*)

Inhibition of ERK and p38 Activation Increases H_2O_2-Induced Apoptosis

ERK and p38 phosphorylation was inhibited using the specific functional inhibitors PD98059 and SB202190, respectively [Bhat and Zhang, 1999]. Pretreatment of ATII cells with the MEK inhibitor PD98059 effectively prevented ERK1/2 phosphorylation after H_2O_2 treatment (Fig. 5B). When assayed for apoptosis, PD98059-treated

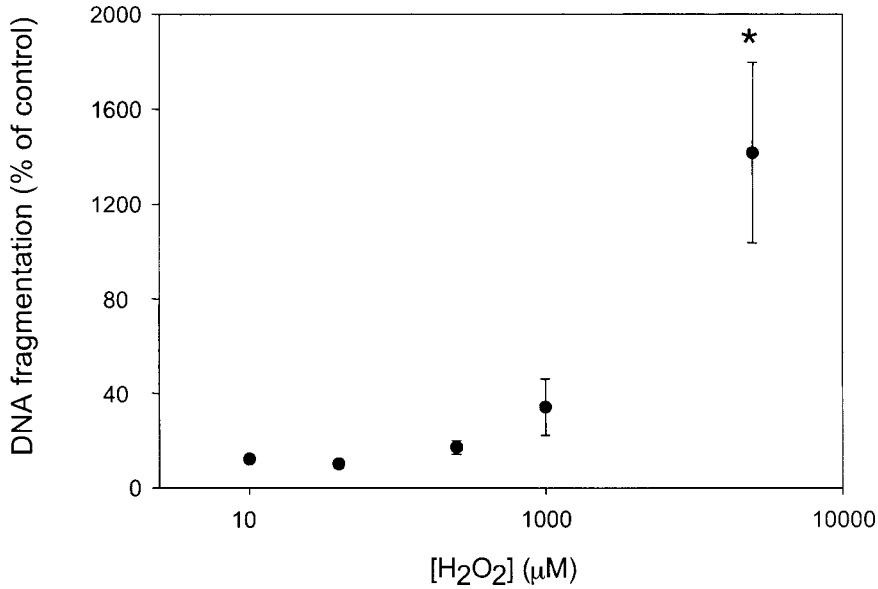

Fig. 2. DNA fragmentation in RLE cells subjected to H_2O_2 stress. RLE cells (passage 43–46) were treated with 0–5 mM H_2O_2 and assayed for DNA fragmentation 12 h after treatment. Bars represent the mean of 4–6 determinations ± SEM. *$P < 0.01$ vs. control (untreated cells).

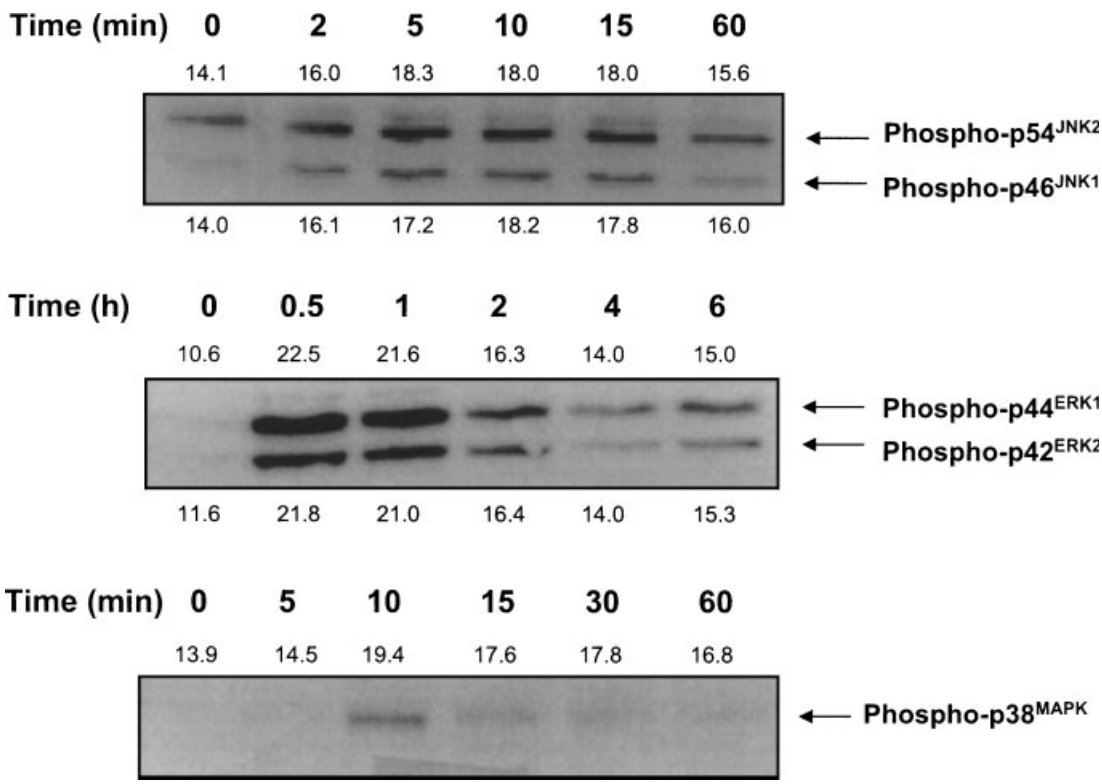

Fig. 3. MAPKs activation in H_2O_2-treated ATII cells. Primary ATII cells were treated with 200 μM H_2O_2 and harvested at the indicated times. The activity of JNK, p38, or ERK was assayed by Western blot using antibodies against the phosphorylated forms of each protein, as described in the ''Materials and Methods.'' The numbers just above the lanes are relative densitometry values for the bands. The results are representative of 2–3 experiments.

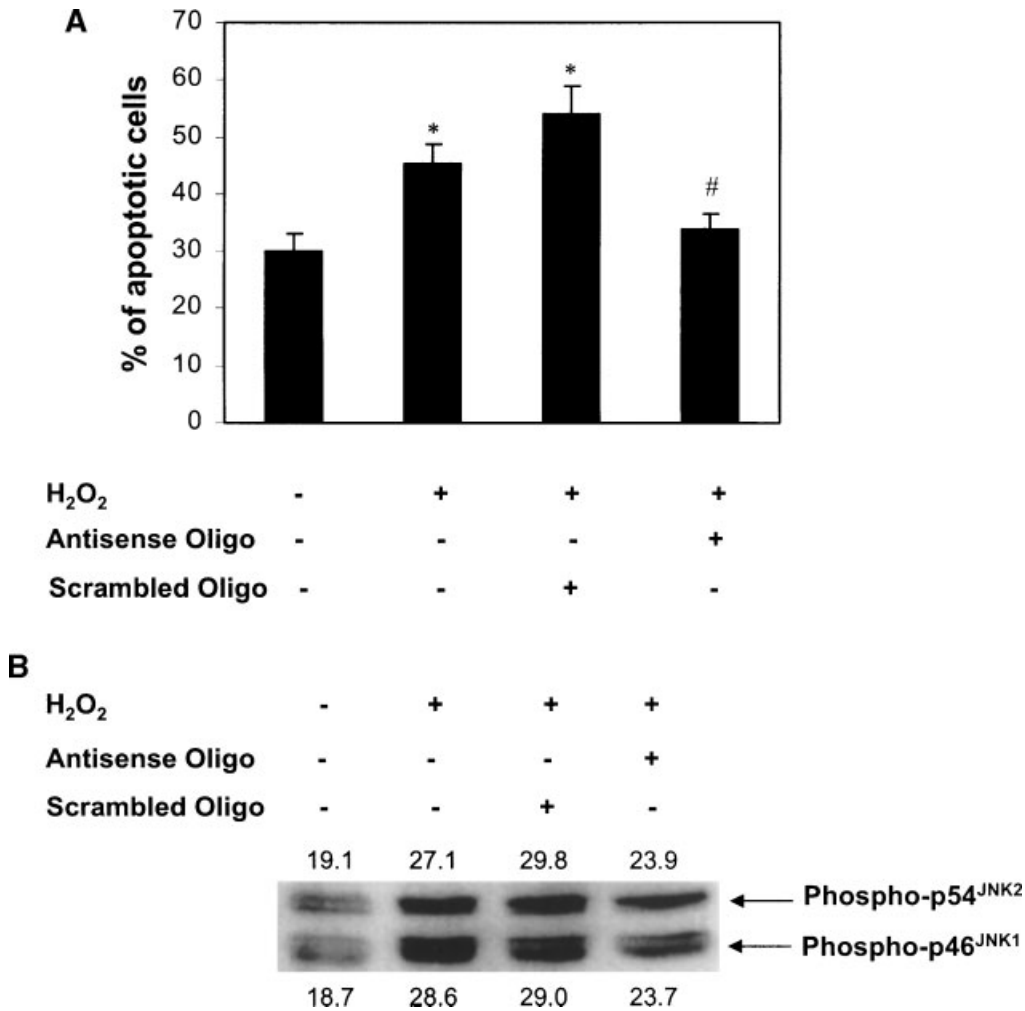

Fig. 4. Effects of JNK inhibition on H_2O_2-mediated apoptosis. **A**: Effects of JNK inhibition on the induction of apoptosis by H_2O_2. ATII cells were treated with antisense or scrambled sequence oligonucleotides 6 h prior to H_2O_2 treatment. Six hours after H_2O_2-treatment (200 μM), cells were harvested and analyzed by flow cytometry. Bars represent the mean of four determinations ± SEM. $*P < 0.02$ vs. control (untreated cells); $\#P < 0.01$ vs. H_2O_2-treated cells. **B**: Effect of JNK inhibition on H_2O_2-dependent JNK phosphorylation. Whole cell lysates were collected 5 min after H_2O_2-treatment and the level of phosphorylated JNK was determined by immunoblotting as described in ''Materials and Methods.'' The numbers above and below the lanes are relative densitometry values for the bands. The blot is representative for three independent experiments.

cells showed an enhanced susceptibility to H_2O_2 (Fig. 5A), indicating that ERK activation triggers programs related to cell survival essential for H_2O_2 resistance. PD98059-treated cells did not show differences in their basal levels of apoptosis.

Finally, pretreatment of primary ATII cells with the p38 inhibitor SB202190 significantly decreased p38 phosphorylation after H_2O_2 treatment, although did not completely abolish it (Fig. 6B). As in the case of ERK inhibition, SB202190-treated ATII cells showed a higher sensitivity to H_2O_2 killing than control cells (Fig. 6A) suggesting that p38 also triggers cell survival pathways. SB202190 treatment alone led to small increases in the number of apoptotic cells, probably indicating a particularly significant role for p38 in the normal metabolism of this cell type.

DISCUSSION

Lung epithelial cells are major targets for oxidant injury. The survival of functional ATII cells is critical to ensure efficient repair of the damaged alveolar surface after injury. Depending on the environmental signals, ATII cells can remain quiescent or proceed toward prolifera-

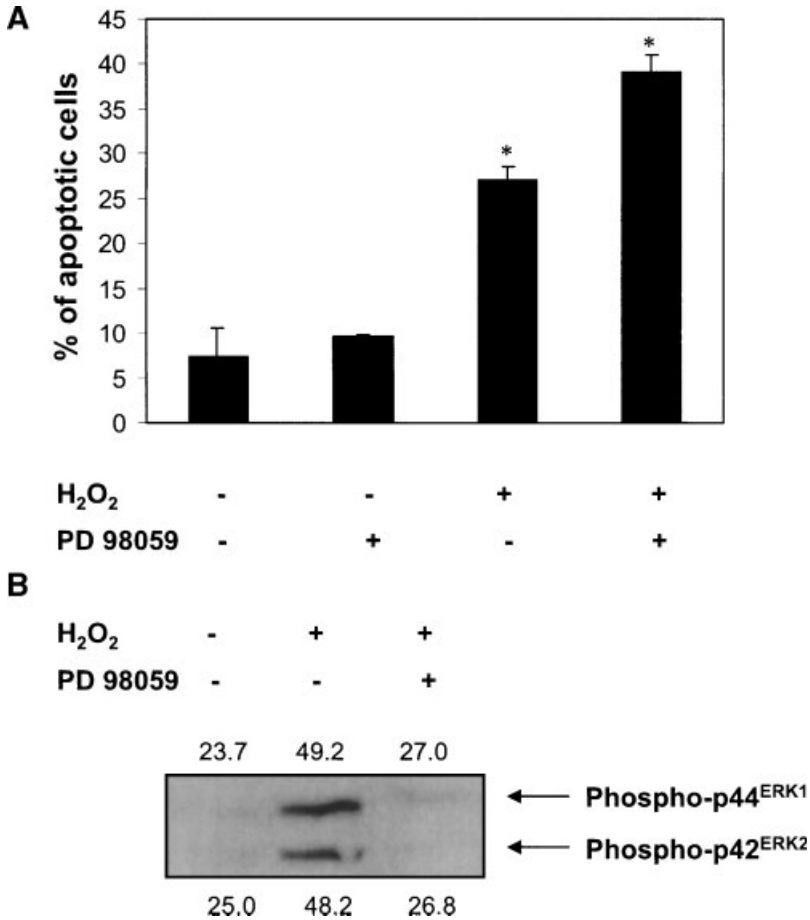

Fig. 5. Effects of ERK inhibition on H_2O_2-mediated apoptosis. **A**: Effects of ERK inhibition on the induction of apoptosis by H_2O_2. ATII cells were treated with 25 μM PD98059 2 h prior to H_2O_2 treatment. Six hours after H_2O_2-treatment (200 μM), cells were harvested and analyzed by flow cytometry. Bars represent the mean of three determinations ± SEM. $^*P < 0.01$ vs. control (untreated cells). **B**: Effect of ERK inhibition on H_2O_2-dependent ERK phosphorylation. Whole cell lysates were collected 1 h after H_2O_2-treatment and the level of phosphorylated ERK was determined by immunoblotting as described in ''Materials and Methods.'' The numbers above and below the lanes are relative densitometry values for the bands. The blot is representative for three independent experiments.

tion or apoptosis. Apoptosis may be important for the resolution or promotion of carcinogenesis or other proliferative diseases [Manning and Patierno, 1996] but the signaling pathways modulating apoptotic changes in ATII cells are undefined. Our results show that both acute and chronic exposure to H_2O_2 lead to significant increases in DNA fragmentation and in the percentage of apoptotic ATII cells. We found that H_2O_2-induced apoptosis in primary ATII cells is regulated through signaling pathways that involve early phosphorylation of MAPKs and that the net-balance between the activated forms of each specific MAPK influences the fate of ATII cells.

Oxidative stress occurs in association with a wide variety of situations of clinical and environmental relevance. Acute oxidative insults, such as short-term exposures to supra-physiological concentrations of oxygen (hyperoxia) or other inhaled oxidants, produce transient increases in the intracellular concentrations of ROS. Chronic exposures, in contrast, produce steady increases in the rates of production and steady-state concentrations of ROS, which are maintained for longer times [Evelson and González-Flecha, 2000]. The wide range of concentrations of H_2O_2 used in this study allows us to integrate our data with previous reports in immortalized cell lines using high concentrations of H_2O_2, and show for the first time the relevance of this response at H_2O_2 concentrations that might occur in vivo. Our data show that primary cultures of ATII cells are exquisitely sensitive to H_2O_2-induced apoptosis showing significant increases in DNA

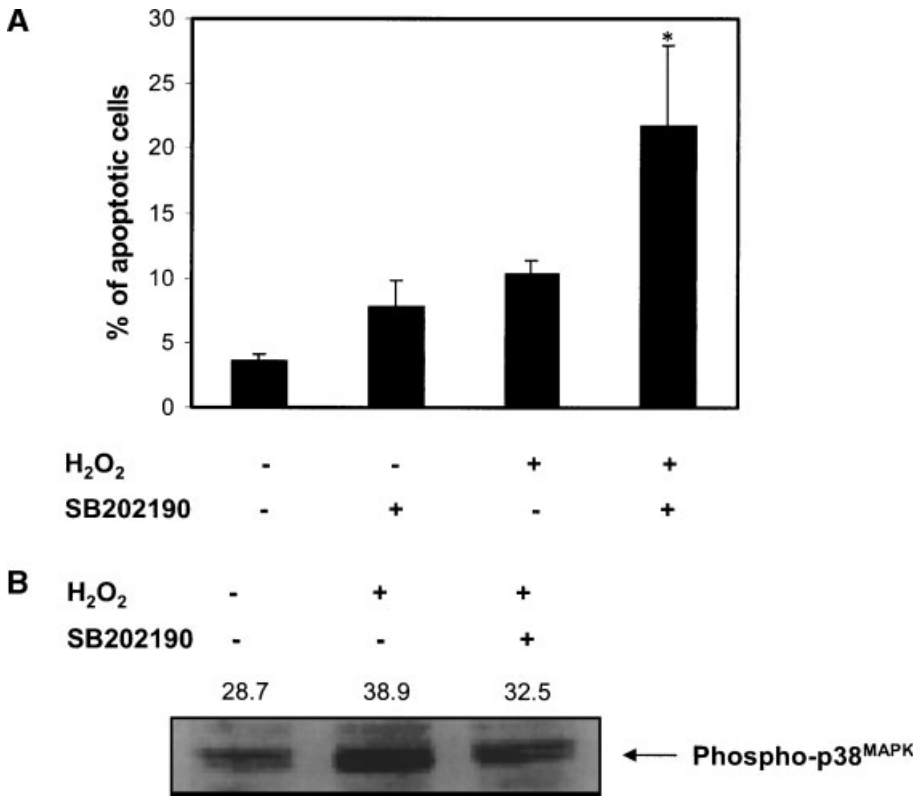

Fig. 6. Effects of p38 inhibition on H_2O_2-mediated apoptosis. **A**: Effects of p38 inhibition on the induction of apoptosis by H_2O_2. ATII cells were treated with 50 μM SB202190 2 h prior to H_2O_2 treatment. Six hours after H_2O_2-treatment (200 μM), cells were harvested and analyzed by flow cytometry. Bars represent the mean of three determinations ± SEM. *$P < 0.04$ vs. H_2O_2-treated cells. **B**: Effect of p38 inhibition on H_2O_2-dependent p38 phosphorylation. Whole cell lysates were collected 10 min after H_2O_2-treatment and the level of phosphorylated p38 was determined by immunoblotting as described in ''Materials and Methods.'' The numbers above the lanes are relative densitometry values for the bands. The blot is representative for three independent experiments.

fragmentation for H_2O_2 concentrations as low as 1 μM or H_2O_2 production rates of 1 μM/h. Strikingly, these values are in the same range than those measured in non-stimulated freshly-isolated ATII cells (0.6 and 1 μM/h, respectively [González-Flecha et al., 1996]), indicating that these responses can occur at physiological concentrations of oxidants.

In contrast, immortalized ATII cells (RLE cells) were insensitive to H_2O_2-induced apoptosis at H_2O_2 concentrations <500 μM H_2O_2. Furthermore, in agreement with previous observations in this [Janssen et al., 1997] and other immortalized cells lines [Wang et al., 1998; Bhat and Zhang, 1999], when treated with >500 μM H_2O_2 RLE cells showed a much stronger increase in DNA fragmentation. The differences in sensitivity to H_2O_2 in primary vs. immortalized ATII cells do not seem to be attributable to differences in their activity of H_2O_2-detoxifying enzymes. Catalase activity is only 1.5-fold higher in RLE cells (2.6 ± 0.2 pmol/mg protein; primary ATII cells: 1.7 ± 0.2 pmol/mg protein) [González-Flecha et al., 1996] and they require a 10-fold higher H_2O_2 concentration to induce maximal apoptosis. This evidence, which points out to a major alteration in the cell cycle control in RLE cells, suggests some caution when interpreting studies on the mechanisms of apoptosis in immortalized cells lines.

MAPK signal transduction pathways are activated in response to various agents such as growth factors, phorbol esters, ionizing radiation, and oxidants. We show here that H_2O_2 stress activates MAPK signaling pathways in primary ATII cells. Our results show that H_2O_2, at concentrations that cause significant DNA fragmentation and increases in the number of apoptotic ATII cells, induced activation of JNK1/2, ERK1/2, and p38. Activation of MAPKs by H_2O_2 was observed within the first few minutes and declined over a 1–6-h period. Blockage of MAPKs activation using specific

inhibitors or antisense oligonucleotides showed that the activation of the JNK pathway may be required to trigger the apoptotic program. On the opposite, chemical inhibition of ERK and p38 led to an increase in apoptosis, suggesting they are activated in order to inhibit H_2O_2 killing.

Our data show that phosphorylated JNKs levels are upregulated in response to concentrations of H_2O_2 that induce apoptosis in ATII cells. JNK activation by H_2O_2 precedes significant accumulation of apoptotic cells and its prevention, using antisense oligonucleotides, abolished H_2O_2-induced apoptosis. These results demonstrate that the JNK pathway is causally associated with the induction of apoptosis by H_2O_2. This finding is consistent with the role of JNKs in the cellular responses to environmental stresses known to act through oxidant mechanisms such as UV radiation, osmotic shock, and pro-inflammatory cytokines [Lo et al., 1996; Rosette and Karin, 1996].

In addition to the increase in JNK activity, H_2O_2 treatment also increased the phosphorylation of ERK and p38. The ERK pathway has been primarily implicated in cell proliferation and differentiation. Previous studies in ATII cells isolated from rats exposed in vivo to hyperoxia showed that ERK activation protects ATII cells from hyperoxic damage [Buckley et al., 1999]. Furthermore, inhibition of either ERK or p38 in an oligodendrocyte cell line was shown to increase H_2O_2-induced apoptosis [Bhat and Zhang, 1999]. Consistently, our results show that inhibition of ERK or p38 activation leads to increases in H_2O_2-dependent apoptosis in ATII cells indicating that these pathways trigger anti-apoptotic signaling. Nevertheless, in our model, transient exposure of ATII cells to 100 μM H_2O_2 led to significant increases in the percentage of apoptotic cells, indicating that the net balance between the activation of the JNK, ERK, and p38 signaling determines susceptibility to apoptosis in this cell type. Similar observations have been reported for HeLa cells [Wang et al., 1998], oligodendrocyte cell lines [Bhat and Zhang, 1999], and cardiac muscle cell lines [Turner et al., 1998], suggesting that the effects observed here are not unique to alveolar epithelial cells.

Apoptosis has been recently shown to be a major pathway for the resolution of ATII cells after acute lung injury [Bardales et al., 1996]. We show here that MAPK signal transduction pathways directly regulate ATII cells apoptosis in response to oxidative stress. Our data suggest that the balance between the activation of the JNK pathway, on one side, and the ERK and p38 pathways, on the other, may influence cell fate. Additionally, pharmacological interventions to alter MAPKs pathways may be potentially useful in the treatment of oxidant-related lung injury.

ACKNOWLEDGMENTS

We thank Ms. A. Imrich and Dr. Yao Yu Ning for their assistance in the determinations of apoptosis by flow cytometry. We are grateful to all our colleagues in the Physiology Program for insightful discussions. B.G.F. acknowledges a Fellowship in Pulmonary Medicine from the Francis Families Foundation. H.C. acknowledges a fellowship from FAPESP (Brazil).

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