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H_2O_2-Induced Proliferation of Primary Alveolar Epithelial Cells Is Mediated by MAP Kinases

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ABSTRACT

Exposure to supraphysiological oxygen concentrations during ventilatory oxygen therapy often causes tissue damage. Alveolar type II (AT II) cells are a major target for oxidant injury, and their ability to proliferate plays a critical role during the repair phase following injury. We hypothesized that reactive oxygen species (ROS), which are produced during hyperoxia, not only cause cellular damage, but may also play a role in the repair process by promoting AT II cell proliferation. We have tested the ability of ROS to induce proliferation in primary cultures of AT II cells by using a wide range of chronic and acute hydrogen peroxide (H_2O_2) exposures to mimic different types of oxidative stress. We found that chronic exposure to an extracellular flux of 10 μ*M* H_2O_2/h can significantly increase the intracellular concentration of oxidants, DNA synthesis, and cell proliferation. H_2O_2-induced AT II cell proliferation was preceded by activation of the mitogen-activated protein kinase ERK (extracellular signal-regulated kinase). Inhibition of ERK and p38 activation prevented H_2O_2-induced proliferation. These results show that changes in intracellular oxidant concentrations can modulate downstream signaling pathways controlling AT II cell proliferation. This mechanism could be important in the repair process following hyperoxia-induced injury. *Antioxid. Redox Signal.* 7, 6–13.

INTRODUCTION

Reactive oxygen species (ROS), which include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), and singlet oxygen ($^1O_2$), are by-products of electron transfer processes, and are therefore produced by the cell normal metabolism (14, 24). Accumulation of $O_2^{\bullet -}$ and H_2O_2 is prevented by specific detoxification systems: the antioxidant enzymes superoxide dismutases, catalase, and glutathione peroxidase (14). The balance between the production and detoxification rates determines the steady-state concentrations of ROS in each intracellular compartment, which under physiological conditions are kept below micromolar levels (4, 9).

The ability of H_2O_2 to regulate the cell cycle has been documented, and a variety of responses has been reported, including increase in cell proliferation (7), cell-cycle arrest (31), and apoptosis (44). The multiple responses of the lung to oxygen are a physiological paradigm of the variety of cellular responses to ROS *in vitro*. Exposure to supraphysiological oxygen concentrations, as occurs during ventilatory oxygen therapy, often causes tissue damage. In the lung, chronic exposure to oxygen leads to epithelial cell death, impaired water clearance (edema), and inflammation that ultimately results in morbidity and mortality (11). The alveolar epithelium is a major target for oxidant injury (38), and its repair following injury depends on the ability of its stem cells, the alveolar type II cells (AT II cells), to proliferate and differentiate (27). AT II cells also play a central role in the development of tolerance to hyperoxia in animals preexposed to mild and transient hyperoxia (12). ROS have been hypothesized to be critical mediators in the development of lung injury after hyperoxia, as well as in the adaptive and reparative responses of the pulmonary epithelium.

ROS regulates multiple cellular processes by activation of signal transduction pathways. Exposure of cells to extracellular H_2O_2 can activate transcription factors like nuclear factor-

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κB (41) and activator protein-1 (2), and stimulates the activity of protein kinases such as the mitogen-activated protein kinases (MAPKs) ERK (extracellular signal-regulated kinase), p38, and c-Jun N-terminal kinase (23). Although most studies used high, nonphysiological doses of H_2O_2 to obtain an effect, some recent articles showed that low doses of H_2O_2 could also activate kinases, at least in some cell types (32). A transient increase in the intracellular concentration of H_2O_2 can be observed following the activation of various cell-surface receptors by their ligands, including growth factor and cytokine receptors (33, 37), and this increase is necessary for downstream signal transduction (1, 43). In a previous report, we showed that scavenging of endogenous H_2O_2 by addition of extracellular catalase led to a decrease in primary AT II cell proliferation, and that normal proliferation is restored upon treatment with glucose/glucose oxidase (G/GO) (35). In the present study, we investigated the quantitative aspects of the promitogenic effect of H_2O_2 in AT II cells, a cell type of physiological relevance, to model the consequences of hyperoxia treatment. Because of the need to work with cells unaltered in the regulation of proliferation and to be closer to *in vivo* conditions, we decided to work with primary cultures of AT II cells. As the ability of extracellular H_2O_2 to trigger proliferation would depend on its capacity to modulate the intracellular level, we evaluated the effect of each treatment on intracellular H_2O_2 and measured the consequences on the cell cycle. Finally, we investigated what signaling pathways were activated by H_2O_2 treatment and necessary for H_2O_2-induced proliferation.

MATERIALS AND METHODS

Cell isolation and culture

AT II cells were isolated from pathogen-free Sprague–Dawley rats weighing 180–220 g by using the method of Dobbs (13) as described (22). In brief, the lungs were perfused via the pulmonary artery, lavaged, and incubated with elastase solution (60 U/lung) for 20 min at 37°C. The tissue was then minced and filtered through sterile filters of 140 μm and 20 μm nylon mesh. The AT II cells were purified by differential adherence to IgG-coated plates. Typically, we obtained 20 million cells per lung, with viability higher than 90%. The cells were plated (1.5×10^6 cells/cm^2) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), vitamins, 2 m*M* glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 40 mg/ml gentamicin. The cells were cultured at 37°C in the presence of 5% CO_2 and used 24 h after plating.

Cell proliferation and apoptosis

Cells to be assayed for DNA synthesis were coincubated with bromodeoxyuridine (BrdU) during the treatment. Forty-eight hours after treatment, cells were harvested and assayed for BrdU incorporation using an ELISA detection kit (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.). Cell-cycle progression and apoptosis were determined by propidium iodide staining and analysis with a flow cytometer (Beckman Coulter, Fullerton, CA, U.S.A.), according to standard protocols.

ROS measurements

The steady-state H_2O_2 concentration was measured as described previously (5). In brief, adherent AT II cells were washed twice and incubated in H_2O_2-free phosphate-buffered saline (PBS). The intracellular concentration of H_2O_2 was estimated from the concentration in the incubation medium after equilibration of the intracellular and extracellular concentrations (5). The H_2O_2 concentration in the incubation medium was measured using horseradish peroxidase (HRP) and scopoletin. H_2O_2 concentration was calculated from the catalase-inhibitable intensity of fluorescence after reaction of HRP-H_2O_2 with scopoletin.

This method was also adapted to measure the rate of H_2O_2 release. The cells were harvested with trypsin, resuspended in serum-free Hanks' balanced salt solution (HBSS) at the desired density, and mixed with an equal volume of reaction solution (0.1 *M* buffer KP_i, pH 7.4, 5.6 U/ml HRP, 2 μ*M* scopoletin). Fluorescence decrease (excitation at 380 nm, emission at 460 nm) was followed for 3 min. The rates of H_2O_2 release were calculated from the initial slopes of the H_2O_2 concentration versus time plots.

Changes in intracellular ROS concentrations were assessed with a method adapted from Bass *et al.* (3). In brief, AT II cells were plated in a 96-well plate, grown overnight, and loaded for 30 min with 20 μ*M* 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA). The cells were then rinsed three times with HBSS and kept in HBSS, with or without 10% FBS. H_2O_2 or glucose oxidase was then added, and the accumulation of 2',7'-dichlorofluorescein (DCF) was followed for up to 4 h. DCF fluorescence (excitation at 485 nm, emission at 535 nm) was measured in a plate reader (SpectraFluor Plus, Tecan). The maximum rate of increase in fluorescence intensity was calculated for each treatment and is expressed relative to untreated control.

Western blot analysis

Cell lysates were prepared and their protein content analyzed according to standard western-blot procedures. Immunoreactive bands were visualized using enhanced chemiluminescence (Pierce, Rockford, IL, U.S.A.) and BioMax films (Kodak, New Haven, CT, U.S.A.). The densitometric analysis of the films was performed on a Macintosh computer (Apple, Cupertino, CA, U.S.A.) using the public domain NIH Image program. The values measured for the phosphorylated forms of ERK (pERK) were normalized to the nonphosphorylated forms (ERK). The primary antibodies (diluted 1:1,000) were from Cell Signaling (Beverly, MA, U.S.A.) (anti-phospho-p38) and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) (anti-phospho-ERK1/2 and anti-ERK1/2). Secondary antibodies from the same providers were diluted 1:10,000.

Cell treatments

Glucose oxidase was dissolved in PBS just before addition to the culture medium. Glucose oxidase was present in the medium throughout the treatment. The kinase inhibitors (Calbiochem, San Diego, CA, U.S.A.) were dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 50 m*M*. Two hours before treatment with glucose oxidase, the cells were

exposed to the inhibitor or vehicle (DMSO). The kinase inhibitors were used at a final concentration of 25 μ*M*, corresponding to 0.05% DMSO (vol/vol).

Chemicals

All reagents were from Sigma except were indicated.

Statistical analysis

Data are reported as means ± SEM. Data were analyzed statistically by factorial analysis of variance followed by Dunnett's or Fisher's test for comparison of the means, using the Statview 4.5 software (Abacus Concepts, Berkeley, CA, U.S.A.).

RESULTS

Intracellular H_2O_2 concentration in primary AT II cells subjected to chronic H_2O_2 stress

The intracellular steady-state concentration of H_2O_2 in prokaryotic and eukaryotic cells results from the balance between H_2O_2 production and H_2O_2-scavenging systems (19). To characterize our experimental system, we first determined the rates of production and steady-state concentration of H_2O_2 in primary AT II cells under standard culture conditions.

The rate of H_2O_2 production was estimated from the rate of release of H_2O_2 into initially H_2O_2-free medium, which was measured using the HRP/scopoletin method. The measurements were performed in serum-free medium due to the strong fluorescence of FBS. We have previously shown that the rate of H_2O_2 release measured in these conditions depends on cell density (16); therefore, we tested three different cell densities: 5×10^3, 5×10^4, and 5×10^5 cells/ml. The rate of H_2O_2 release increased linearly with the cell density from 5×10^3 to 5×10^4 cells/ml (Table 1). The corresponding endogenous production rate, calculated from the rate of H_2O_2 release at 5×10^4 cells/ml (0.23 ± 0.10 μ*M*/h), was 0.08 nmol/min × 10^6 cells. The linear response was lost at higher cell densities. It is likely that as the cell density increases, the global detoxification capability of the system will increase, mostly due to an increase in the total catalase. As described for bacterial cells (34), the rate of H_2O_2 elimination by catalase is faster than the rate of production; therefore, when the cell density reaches a threshold, H_2O_2 concentration in the medium cannot be further increased. The intracellular H_2O_2 concentration under standard culture conditions (adherent cells, medium supplemented with 10% FBS) was 0.10 ± 0.02 μ*M*, measured as described in Materials and Methods.

We then modeled chronic oxidative stress by treating the AT II cell cultures with the H_2O_2-generating system, G/GO. The glucose oxidase concentration was calculated to deliver 10 μ*M* H_2O_2/h and the actual H_2O_2 flux in cell cultures was determined as before (Table 1). The net H_2O_2 rate of release in AT II cell cultures decreased with increased cell densities, probably due to the cumulative effects of cellular H_2O_2-scavenging systems and reactions with the cell membrane. For a cell density of 5×10^4 cells/ml, the H_2O_2 release rate was 8.68 ± 0.09 μ*M*/h, ~40 times higher than the release rate measured without G/GO (Table 1).

To verify that exposure to extracellular H_2O_2 had an effect on intracellular ROS concentration, we used the intracellular fluorescent probe 2',7'-dichlorodihydrofluorescin (DCFH). Glucose oxidase concentrations were chosen to deliver fluxes of 0–50 μ*M* H_2O_2/h (Fig. 1). The intracellular concentration of ROS was significantly affected by extracellular H_2O_2 fluxes (Fig. 1). DCFH oxidation in G/GO-treated cells increased linearly over a period of ~4 h and then reached a plateau. Maximal increases of around four-fold in intracellular ROS were observed with fluxes higher than 10 μ*M* H_2O_2/h (Fig. 1). To confirm that the same effect was observable in standard culture conditions, we performed the experiment in medium supplemented with 10% FBS. Addition of FBS to the medium attenuated the effect of exogenous H_2O_2 on the intracellular ROS concentrations, probably due to scavenging of H_2O_2 by serum components (Fig. 1). Nonetheless, AT II cells treated with >10 μ*M* H_2O_2/h showed significant three-fold increases in intracellular ROS. In contrast, cells treated with boluses of H_2O_2 did not show any changes in their intracellular concentration (data not shown).

To determine if growth factors were able to modulate the intracellular ROS concentration in our system, we treated AT II cells with 100 ng/ml acidic fibroblast growth factor (aFGF)

TABLE 1. MEASUREMENT OF H_2O_2 RELEASE RATES IN AT II CELLS IN SUSPENSION

	H_2O_2 release rate (μM/h)	
	Endogenous	*Glucose oxidase*
No cells	0.00 ± 0.04	10.95 ± 0.22
5×10^3 cells/ml	0.02 ± 0.07	9.15 ± 0.25
5×10^4 cells/ml	0.23 ± 0.10	8.68 ± 0.09
5×10^5 cells/ml	0.28 ± 0.09	7.09 ± 0.26

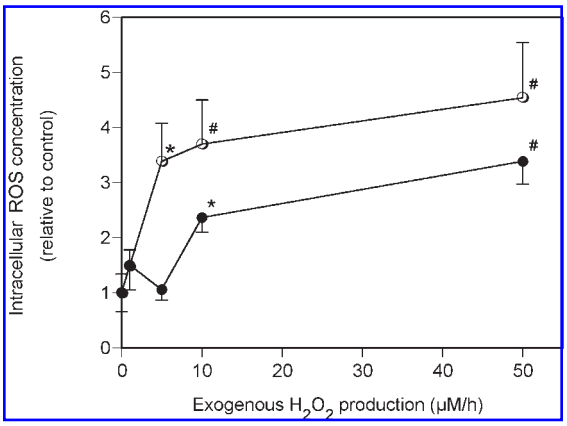

FIG. 1. Modulation of intracellular ROS concentration by G/GO. Adherent primary AT II cells were exposed to a range of H_2O_2 fluxes produced by the G/GO system in the absence (○) or presence (●) of 10% FBS. Intracellular ROS concentration was measured using DCFH-DA. *$p < 0.05$, #$p < 0.01$ versus untreated cells. Data represent means ± SEM of at least three independent experiments.

in the presence of 10% FBS. We measured a two-fold (2.13 ± 0.45, $p < 0.05$ versus control) increase in intracellular ROS concentration under these conditions.

H_2O_2-induced proliferation in primary AT II cells

We then sought to test the hypothesis that cell proliferation can be modulated by subtle changes in the intracellular ROS concentration. We have previously shown that transcriptional responses to chronic or acute H_2O_2 stress are dramatically different and that these differences can be ascribed to different effects on intracellular H_2O_2 (18). We therefore tested the effect of both chronic and acute H_2O_2 stress on AT II cell proliferation, measured as increases in their rate of DNA synthesis and progression throughout the cell cycle. All the experiments were performed in the presence of 10% FBS.

Primary AT II cells treated with boluses of H_2O_2 (acute exposure) in a range of concentrations from 0.1 μ*M* to 1 m*M* showed no significant increases in the incorporation of BrdU into DNA (data not shown). In contrast, primary cultures of AT II cells treated with the H_2O_2-generating system G/GO showed an increase in BrdU incorporation (Fig. 2). The response was biphasic, showing increased DNA synthesis in cells treated with 0.01–5 μ*M* H_2O_2/h, and inhibition of DNA synthesis at rates higher than 100 μ*M* H_2O_2/h. The maximum response (~40% above control values) was observed for fluxes of 1–10 μ*M* H_2O_2/h. Figure 3 summarizes the maximal effects of acute and chronic exposures to H_2O_2. The mitogenic effect exerted by 1 μ*M* H_2O_2/h was almost equivalent to the one produced by 100 ng/ml aFGF, one of the strongest mitogens for primary AT II cells in culture.

To confirm that the H_2O_2-induced increase in DNA synthesis was associated with cell proliferation, we analyzed the progression throughout the cell cycle in primary AT II cells treated with G/GO (Table 2). As compared with the untreated controls, primary AT II cells treated with 10 μ*M* H_2O_2/h showed

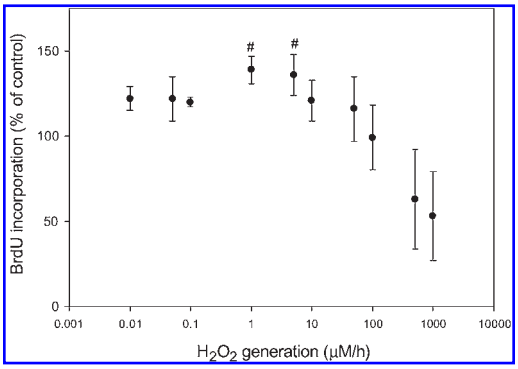

FIG. 2. DNA synthesis in response to chronic H_2O_2 exposure. AT II cells were treated with a defined external flux of H_2O_2 provided by the G/GO system. Glucose oxidase was present in the culture medium throughout the experiment. Forty-eight hours after addition of glucose oxidase, BrdU incorporation was measured as described in Materials and Methods. BrdU values for the untreated controls were used as reference (100%). Data represent the means of four to six determinations ± SEM. #$p < 0.01$ versus untreated cells.

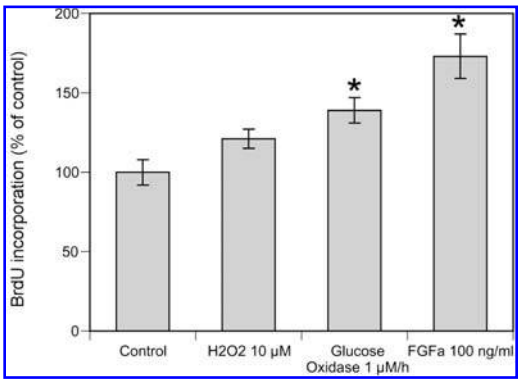

FIG. 3. Comparison of the effect of different stimuli on DNA synthesis in AT II cells. AT II cells were treated for 48 h with a bolus of H_2O_2 (10 μ*M*), a defined external flux of H_2O_2 (1 μ*M*/h) provided by the G/GO system, or aFGF (100 ng/ml). After the exposure time, BrdU incorporation was measured as described in Materials and Methods. Columns represent the means of four to six determinations ± SEM. *$p < 0.01$ compared with the control (untreated cells).

a significantly lower percentage of cells in G_o/G_1 phases and an increased number of cells in the S and G_2/M phases of the cell cycle. Strikingly, the effect of H_2O_2 on cell-cycle progression was equivalent to the one exerted by aFGF (Table 2). Lower fluxes of H_2O_2 did not show significant effect on cell proliferation. Treatment with <10 μ*M* H_2O_2/h led to no change in the number of apoptotic AT II cells (10 μ*M* H_2O_2/h: 10.0 ± 1.5% apoptotic cells; control: 10.2 ± 1.5%). On the other hand, fluxes of >10 μ*M* H_2O_2/h strongly increased AT II cell apoptosis (25 μ*M* H_2O_2/h: 17.6 ± 2.8% apoptotic cells, $p < 0.01$ versus 10 μ*M* H_2O_2/h and control).

H_2O_2-induced proliferation is controlled by ERK and p38

Growth signals and stress responses are known to be largely mediated by members of the MAPK family (10). ERK and p38 have also been shown to be activated by ROS (6, 23). To determine if ERK and p38 were essential for H_2O_2-induced proliferation of AT II cells, we used inhibitors specific for each kinase.

H_2O_2-induced proliferation was totally abolished in cells pretreated with either the ERK pathway inhibitor PD98059 or

TABLE 2. CELL CYCLE ANALYSIS OF AT II CELLS TREATED WITH G/GO

Treatment	*G_0/G_1 phase*	*S phase*	*G_2/M phase*
Control	92.1 ± 0.5	3.6 ± 0.3	3.1 ± 0.4
GO, 1 μ*M* H_2O_2/h	90.5 ± 0.7	4.0 ± 0.4	4.1 ± 0.4
GO, 10 μ*M* H_2O_2/h	87.3 ± 1.7*	5.0 ± 0.7‡	5.4 ± 0.4*
aFGF 100 ng/ml	87.9 ± 1.6*	6.1 ± 0.7*	4.5 ± 0.7‡

Data are means ± SEM of at least six independent determinations.

*$p < 0.05$, ‡$p < 0.1$ compared with control (untreated) cells.

the p38 kinase inhibitor SB202190 (Fig. 4). Neither PD98059 nor SB202190 alone showed significant effects on untreated cells (Fig. 4). These results indicate that disruption of either the ERK or the p38 pathway suppresses the proliferative effect of H_2O_2. Both pathways are necessary, but not sufficient, for H_2O_2-induced proliferation.

To confirm that ERK and p38 were involved in the control of H_2O_2-induced proliferation, we tested their level of activation during G/GO treatment. Cellular extracts were collected at different times and assayed for activation of MAPKs using monoclonal antibodies targeted against the phosphorylated forms of ERK1/2 and p38. Phosphorylated p38 was undetectable both in untreated controls and in H_2O_2-treated cells, indicating that if any activation occurred, it remained below the detection limit. On the other hand, chronic H_2O_2 stress induced a rapid and transient increase in phosphorylated ERK in primary AT II cells (Fig. 5). pERK increases were maximum 2–5 min after exposure to H_2O_2. The level of phosphorylation returned to control values after 10 min, and remained at control levels for at least 24 h (data not shown).

DISCUSSION

An increasing number of studies show that H_2O_2 can regulate a variety of cellular functions, including proliferation, differentiation, and more generally gene expression (24). However, quantitative data on the basal concentrations of H_2O_2 and the thresholds for signaling and toxicity are limited. In this study, we have quantified the metabolic production of H_2O_2 in primary cultures of AT II cells and studied the effect of either chronic or acute H_2O_2 stress on cell proliferation, cell apoptosis, and signaling. Our data show that the steady-state concentration of H_2O_2 in rat primary AT II cells in culture is ~0.10 μ*M*. Two-fold increases in intracellular ROS concentration were sufficient to induce cell proliferation.

The intracellular concentration of H_2O_2 reported here is comparable to values measured in rat hepatocytes (0.1 μ*M*) (15), rat kidney (0.08 μ*M*) (21), and rat liver (0.09 μ*M*) (20), and lower than the one previously measured in freshly isolated AT II cells (0.6 μ*M*) (22). This difference between fresh suspensions versus adherent cells is in agreement with previous findings by Kinnula *et al.,* who reported a rapid decrease of H_2O_2 release by AT II cells in the first 12 h following isolation (29).

AT II cells treated with the H_2O_2-generating system G/GO showed significant increases in the rate of H_2O_2 production (Table 1). An increase in intracellular ROS concentration was also observed using DCFH as a detection system (Fig. 1). Although DCFH is not specific for H_2O_2 (36), several studies show that the increase in DCFH fluorescence associated with extracellular stimuli was inhibitable by catalase and therefore due mainly to H_2O_2 (1, 39). In our model, we followed increases in DCFH oxidation after treatment with an extracellular source of H_2O_2 (glucose oxidase); therefore, most likely the increase in intracellular ROS observed in Fig. 1 was due to G/GO-generated H_2O_2 that has diffused into cells and escaped metabolism by intracellular catalase and glutathione peroxidase. Interestingly, cells treated with fluxes of H_2O_2 ~40-fold higher than their metabolic rates of H_2O_2 release showed only an ap-

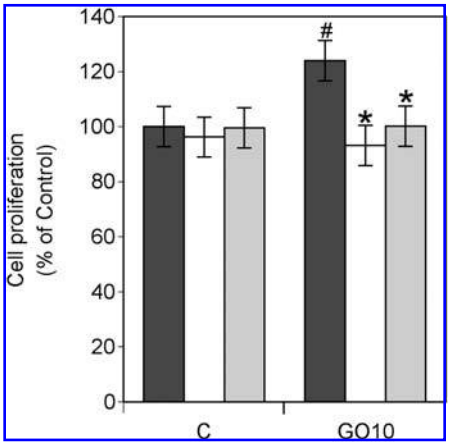

FIG. 4. Effect of MAPK inhibition on H_2O_2-induced proliferation. AT II cells were treated with DMSO (dark gray columns), the ERK pathway inhibitor PD98059 (white columns), or the p38 inhibitor SB202190 (light gray columns) 2 h prior to G/GO treatment (10 μ*M* H_2O_2/h). Twenty-four hours after addition of glucose oxidase, the number of proliferating cells (G_2/M and S phases) was assessed by flow cytometry. Proliferation is expressed as fold increase with respect to control, untreated cells. #$p < 0.01$ compared with non G/GO-treated cells; *$p < 0.05$ compared with G/GO-treated cells without kinase inhibitors. Data represent the means ± SEM of at least three independent experiments.

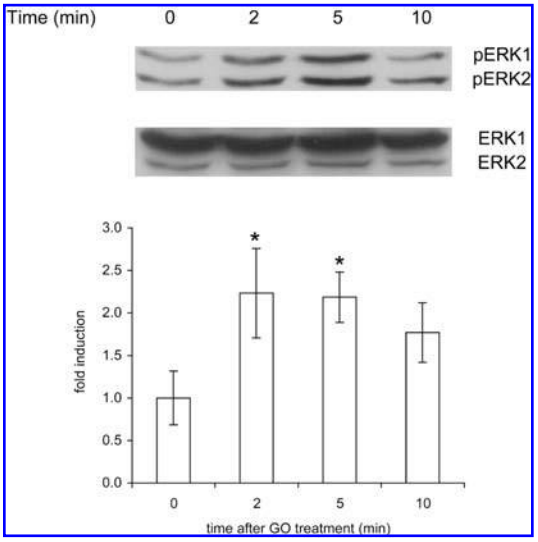

FIG. 5. Activation of ERK by H_2O_2. AT II cells were treated with a flux of H_2O_2 (10 μ*M*/h) produced by the G/GO system. At the indicated times, cells were harvested and assayed for phosphorylated and nonphosphorylated ERK by western blot analysis as described in Materials and Methods. The level of activation of the phosphorylated form of ERK was quantified by densitometry and normalized to the nonphosphorylated form. Data represent the means ± SEM of at least five independent experiments. *$p < 0.05$ compared with untreated cells.

proximately three-fold increase in their intracellular ROS levels (Fig. 1). The difference between extracellular and intracellular H_2O_2 could be due to partial detoxification by intracellular catalase, as previously shown by Seaver and Imlay (42), and is consistent with our finding that treatment with boluses of H_2O_2 did not change the intracellular ROS concentration.

It is therefore not surprising that no increase was found in DNA synthesis in cells treated with boluses of H_2O_2. Cell proliferation could only be achieved by exposing the cells to a constant flux of H_2O_2 generated by the G/GO system (Fig. 2 and Table 2). The differences in the proliferative responses to boluses versus continuous production of H_2O_2 are in agreement with previous reports in primary bovine aortic endothelial cells (40). We determined that the treatment with G/GO at a concentration able to induce proliferation resulted in an approximately two-fold increase in intracellular ROS (Fig. 1). Twofold increases in H_2O_2 were reported to be enough to trigger transcriptional responses in prokaryotic cells (17) and were also observed in human carcinoma cells (A431) treated with epidermal growth factor (1), in human adipocytes stimulated with insulin (30), and in murine epidermal cells treated with phorbol esters (39). In our system, treatment of AT II cells with aFGF also resulted in a twofold increase in intracellular ROS, and both aFGF and G/GO treatments were able to promote AT II cell proliferation (Table 2). Taken together, these data indicate that treatment with an extracellular source of H_2O_2 increases intracellular ROS in a way similar to growth factors, and that such increase results in cell proliferation.

Of note, although treatment with growth factors leads to transient increases in intracellular H_2O_2, it has been shown that prolonged and continuous exposure to growth factors is required to commit cells to the cell cycle. Furthermore, Jones and Kazlauskas have shown that the requirement for prolonged stimulation by growth factors can be replaced with two short pulses of mitogen a few hours apart (26). These data are consistent with our findings of a lack of mitogenic effects with H_2O_2 boluses and the need for continuous generation of H_2O_2. In this context, continuous stimulation by growth factors would trigger several activation events. Likewise, only continuous exposure to H_2O_2 would be able to trigger the multiple signaling events required for proliferation.

Our data show that the range of H_2O_2 fluxes able to induce AT II cells to proliferate is very narrow. H_2O_2 fluxes lower than 1 μM/h had no significant effects on proliferation, and fluxes higher than 10 μM/h induced apoptosis. Indeed, the range of increase in intracellular ROS for the induction of proliferation was two- to threefold, whereas increases higher than 3.4-fold led to significant AT II cell apoptosis.

ERK activation is known to be required for growth factor-dependent mitogenic signaling (10). The p38 kinase is most frequently activated in response to stress and inflammatory signals, but it also plays a role in the regulation of cell proliferation. The outcome of its activation is dependent on the cellular context, antagonizing or collaborating with ERK (10). Here we show that in AT II cells both ERK and p38 kinases are necessary for H_2O_2-induced cell proliferation. These findings are consistent with reports showing that both ERK and p38 activation promote cell proliferation in vascular smooth muscle cells and erythroid progenitor cells in response to growth factors (28, 45). Interestingly, the requirement for p38 in ROS-induced proliferation has also been reported in a Chinese hamster lung fibroblasts cell line (V79), although in this cell type no activation of ERK was observed (25).

It is worth noting that H_2O_2 boluses in the minimolar range do have the ability to activate MAPKs in this cell type (8). However, the main outcome of such an acute and more severe stress is apoptosis instead of cell proliferation (8).

Proliferation of AT II cells is essential for the maintenance of the lung epithelium and for repair after oxidant injury. Understanding the effect of ROS on cell growth in this cell type is therefore particularly relevant. We show here that subtle variations in intracellular ROS concentration are necessary and sufficient to modulate primary AT II cell proliferation, and that this regulation proceeds through the ERK and p38 pathways. More studies are under way to determine if these pathways are activated in the lung *in vivo* during hyperoxia or excessive inflammation, two scenarios in which the lung epithelium is subjected to increased levels of ROS.

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ABBREVIATIONS

aFGF, acidic fibroblast growth factor; AT II cells, alveolar type II cells; BrDU, bromodeoxyuridine; DCF, 2',7'-dichlorofluorescein; DCFH, 2',7'-dichlorodihydrofluorescin; DCFH-DA, 2',7'-dichlorodihydrofluorescin diacetate; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; G/GO, glucose/glucose oxidase; HBSS, Hanks' balanced salt solution; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; $O_2^{\bullet -}$, superoxide anion; PBS, phosphate-buffered saline; ROS, reactive oxygen species.

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