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Effects of Hydrogen Peroxide on MAPK Activation, IL-8 Production and Cell Viability in Primary Cultures of Human Bronchial Epithelial Cells

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Abstract The airway epithelium is continuously exposed to inhaled oxidants, including airborne pollutants and cigarette smoke, which can exert harmful proinflammatory and cytotoxic effects. Therefore, the aim of our study was to investigate, in primary cultures of human bronchial epithelial cells (HBEC), the signal transduction pathways activated by increasing concentrations (0.25, 0.5, and 1 mM) of hydrogen peroxide (H_2O_2), as well as their effects on IL-8 production and cell viability. The reported results show that H_2O_2 elicited, in a concentration-dependent fashion, a remarkable increase in phosphorylation-dependent activation of mitogen-activated protein kinases (MAPKs), associated with a significant induction of IL-8 synthesis and a dramatically enhanced cell death. Pre-treatment of HBEC with MAPK inhibitors was able to significantly inhibit the effects of H_2O_2 on IL-8 secretion, and to effectively prevent cell death. Therefore, these findings suggest that MAPKs play a key role as molecular transducers of the airway epithelial injury triggered by oxidative stress, as well as potential pharmacologic targets for indirect antioxidant intervention. J. Cell. Biochem. 93: 142–152, 2004. © 2004 Wiley-Liss, Inc.

Key words: airway epithelial cells; oxidative stress; mitogen-activated protein kinases; interleukin-8; cell death

Because of its large surface area exposed to the external environment, the airway epithelium is highly susceptible to tissue injury caused by airborne pollutants like ozone, nitrogen dioxide, and sulfur dioxide, as well as by cigarette smoke [Halliwell and Gutteridge, 1989]. The latter, in particular, contains many oxidants and free radicals in both its gaseous and particulate phases [Pryor et al., 1983]. Furthermore, cigarette smoke is able to recruit within the air spaces inflammatory cells such as macrophages and neutrophils [MacNee et al., 1989], which once activated produce and release reactive oxygen species (ROS) such as hydroxyl radical (OH•) and superoxide anion ($O_2^{\bullet-}$), the latter being rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase [Thannickal and Fanburg, 2000]. ROS can also be released by the airway epithelium itself [Rochelle et al., 1998], that may directly stimulate the inflammatory cells thus contributing to propagate and amplify lung oxidative stress. At the molecular level, ROS exert their effects by activating transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which are responsible for the

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coordinated expression of several genes involved in inflammation, cell death, and proliferation, as well as cytoprotection and antioxidant defenses [Morcillo et al., 1999; Rahman and MacNee, 2000]. AP-1 and NF-κB are in turn regulated by complex signal transduction pathways mediated by mitogen-activated protein kinases (MAPKs), eukaryotic enzymes activated by environmental stresses, growth factors, and proinflammatory cytokines [Chang and Karin, 2001]. Three main MAPK subgroups are currently known, including c-Jun amino-terminal kinases (JNK), extracellular signal-regulated kinases (ERK), and p38 proteins, all of which share the common property of being activated via a multistep phosphorylation cascade that allows them to in turn phosphorylate several different substrates predominantly represented by transcription factors. With regard to MAPK phosphorylation, we have recently reported the stimulatory action of ROS (H_2O_2) and some proinflammatory cytokines (IL-1β, TNF-α) in pulmonary endothelial cells [Pelaia et al., 2001], as well as the MAPK-activating effect of transforming growth factor-β (TGF-β) in primary cultures of bronchial epithelial cells [Pelaia et al., 2003], respectively.

The oxidative injury triggered by both inhaled and locally generated ROS elicits an inflammatory response, which can profoundly impair the structural integrity and the biological properties of bronchial epithelium. As a consequence, the latter may no longer be able to optimally exert its important functions including protection of the internal airway milieu, ciliary activity, secretion of cytoprotective molecules such as defensins, and bi-directional interactions with the cellular elements of both innate and adaptive immune systems. Moreover, airborne pollutants and cigarette smoke can also induce the bronchial epithelium to acquire a proinflammatory phenotype, characterized by an increased production of autacoids, cytokines, and chemokines like interleukin-8 (IL-8) and RANTES (regulated upon activation, normal T-cell expressed and secreted) [Davies, 2001], which are powerful chemoattractants for neutrophils and eosinophils, respectively. Oxidant-induced phenotypic changes may thus significantly contribute to the key pathogenic role played by bronchial epithelial cells in inflammatory airway disorders such as asthma and chronic obstructive pulmonary disease (COPD) [Barnes, 1990, 2003; MacNee, 2001; MacNee and Rahman, 2001; Maselli et al., 2002; Crapo, 2003]. These considerations imply that a better understanding of the cellular and molecular mechanisms underlying the effects of oxidative stress on airway epithelium, could allow to gain further important information about the inflammatory events involved in COPD and asthma.

Therefore, we decided to study, in primary cultures of human bronchial epithelial cells (HBEC), the following aspects related to airway oxidative stress: (i) evaluation of the effects exerted by different concentrations of H_2O_2 on phosphorylation-dependent activation of MAPKs; (ii) assessment of IL-8 production in response to cell exposure to H_2O_2 and MAPK activation; (iii) investigation of the eventual relationships between H_2O_2 effects on MAPK activation and cell viability.

MATERIALS AND METHODS

Reagents

H_2O_2 was purchased from Sigma (St. Louis, MO). Anti-phospho-p38, anti-phospho-ERK1/2, and anti-phospho-JNK monoclonal antibodies were purchased from New England Biolabs (Beverly, MA); anti-(total)-p38, anti-(total)-ERK1/2, and anti-(total)-JNK polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-caspase-3 polyclonal antibody was purchased from StressGen Biotechnologies Corp. (Victoria, Canada). Propidium iodide (PI) was from Clontech Laboratories (Basel, Switzerland). MAPK inhibitors PD98059 and SB203580 were from Calbiochem (San Diego, CA); the MAPK inhibitor SP600125 was from Tocris Cookson, Inc. (Ellisville, MO).

Primary Cultures of HBEC

HBEC were obtained from fresh surgical specimens taken from patients who underwent either lobectomy or pneumonectomy for lung cancer at "V. Monaldi" University Hospital (Naples, Italy). Lung segments away from and free of the tumor were used. Bronchial mucosal biopsy samples were dissected from the underlying tissues and soaked in 0.1% protease solution (Type XIV; *Streptomyces griseus*, Sigma) overnight at 4°C [Gruenert et al., 1990; Kelsen et al., 1992]. The following day, samples were flushed with Eagle's minimum essential

medium containing 10% fetal calf serum (FCS); the resulting suspension was filtered through a 100-μm sterile Nitex mesh to remove mucus, and centrifuged for 5 min at 1,500*g*. Bronchial epithelial cells were then harvested and cultured at 37°C, 5% CO_2 in Bronchial Epithelial Growth Medium (BEGM; Clonetics, San Diego, CA) with added antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin; Sigma) and Fungizone (1 μg/ml; Gibco-BRL, Gaithersburg, MD). For assays, cells (passage 3 or 4) were seeded into 24-well trays (1 ml BEGM/well containing 5×10^4 cells/ml) and cultured until approximately 80% confluent. The medium was then replaced by 1 ml/well of Bronchial Epithelial Basal Medium (BEBM; Clonetics) containing 1% of insulin, transferrin, and sodium selenite (ITS) media supplement for 24 h to render the cells quiescent. The medium was then replaced with 1 ml/well of BEBM/ITS, and cells were exposed for 2 h to different concentrations of H_2O_2 (0.25, 0.5, and 1 mM). After 2 h, the medium was removed and cells were processed for protein extraction and immunoblotting.

Protein Extraction and Immunoblot Analysis

For Western blotting, HBEC were grown to confluence and, following stimulation with H_2O_2, lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM NaF, 10% glycerol, 4 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 50 mM HEPES, pH 7.4, plus PPIM, 25 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Protein extracts were then separated on a 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia, Little Chalfont, UK). Immunoblotting was performed using anti-phospho-p38, anti-phospho-ERK1/2, and anti-phospho-JNK monoclonal antibodies. After being "stripped," the membranes were re-probed with polyclonal antibodies against total (phosphorylated and unphosphorylated) p38, ERK1/2, and JNK. Blots were also incubated with a polyclonal antibody directed against both inactive pro-caspase-3 and active caspase-3, a common enzymatic marker of apoptosis [Nicholson and Thornberry, 1997]. Antibody binding was visualized by enhanced chemiluminescence (ECL-Plus; Amersham Pharmacia). These experiments were performed in triplicate.

IL-8 Release

HBEC were exposed to three different concentrations of H_2O_2 (0.25, 0.5, and 1 mM) for 4 and 8 h, respectively, in the presence or absence of a pharmacological pre-treatment with a mixture of the MAPK inhibitors PD98059 (40 μM), SB203580 (1 μM), and SP600125 (20 μM), initiated 12 h before cell exposure to H_2O_2. Culture supernatants were collected and assayed for IL-8 by ELISA using a commercially available kit (Peli-Kine kit; Eurogenetics (Hampton, UK); sensitivity limit, 1 pg/ml), according to manufacturer's protocol. These experiments were performed in triplicate.

Cell Viability

In the presence or absence of a pharmacological pre-treatment with the above-mentioned concentrations of MAPK inhibitors, used individually as well as in combination, cell death was detected 6 h after removal of the stimulus (i.e., 8 h after the initial addition of H_2O_2) by fluorescence microscopy using PI staining. The percentage of dead cells was evaluated by fluorescence-activated cell sorting (FACS) analysis (FACSCalibur) using annexin-V (An-V) staining. These experiments were performed in triplicate.

Statistical Analysis

All data are expressed as mean ± standard error (SEM). Statistical evaluation of the results was performed by analysis of variance (ANOVA). Differences identified by ANOVA were pinpointed by unpaired Student's *t*-test. The threshold of statistical significance was set at $P < 0.05$.

RESULTS

Effects of H_2O_2 on MAPK Phosphorylation

Evaluation of the results obtained in three independent sets of experiments showed that exposure of primary cultures of HBEC for 2 h to H_2O_2 induced a concentration-dependent increase in the phosphorylation pattern of JNK, ERK1/2, and p38 (Fig. 1). Since the monoclonal antibodies (anti-phospho-JNK, anti-phospho-ERK1/2, and anti-phospho-p38) used in this study specifically recognize the phosphorylated,

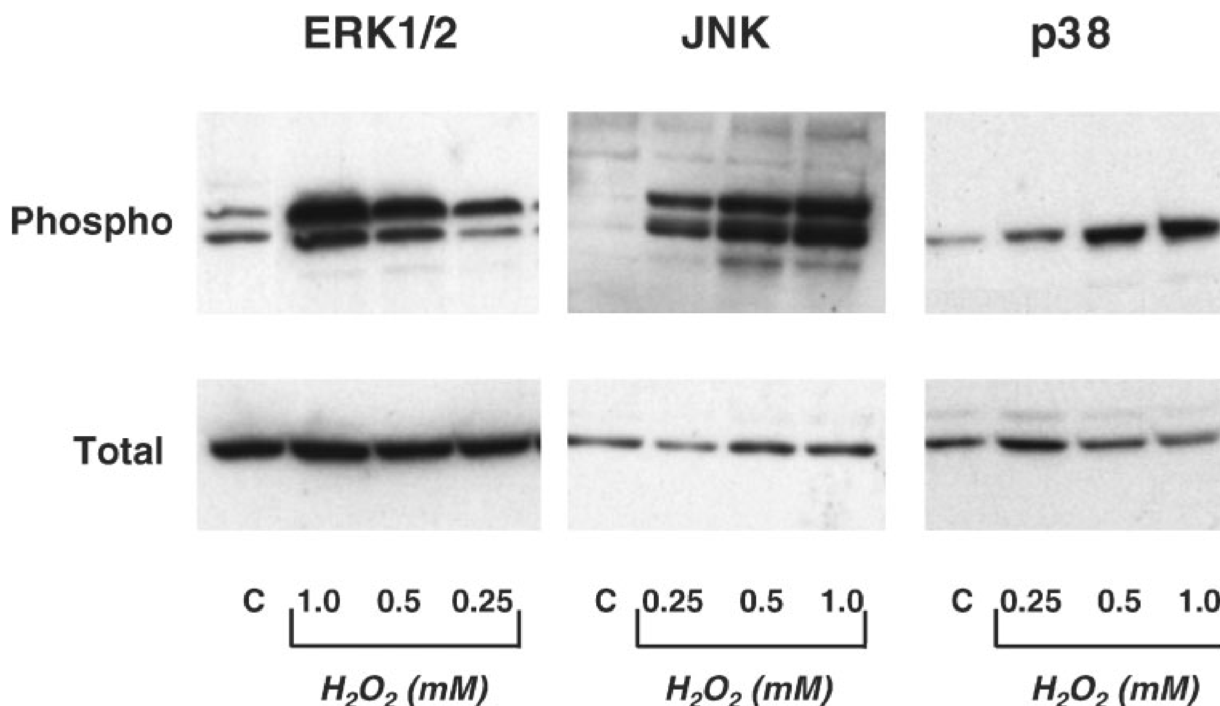


Fig. 1. H_2O_2-induced phosphorylation of ERK1/2, JNK, and p38. A 2 h incubation of primary cultures of HBEC with H_2O_2 (0.25, 0.5, and 1 mM) induced a concentration-dependent increase in the amount of phosphorylated ERK1/2, JNK, and p38. H_2O_2 did not affect the total expression of these MAPK subgroups. Phosphorylation of ERK1/2, JNK, and p38 was detected by immunoblotting using anti-phospho-ERK1/2, anti-phospho-JNK, and anti-phospho-p38 monoclonal antibodies, respectively. The total expression of these enzymes was detected by anti-total-ERK1/2, anti-total-JNK, and anti-total-p38 polyclonal antibodies, respectively. C, untreated control.

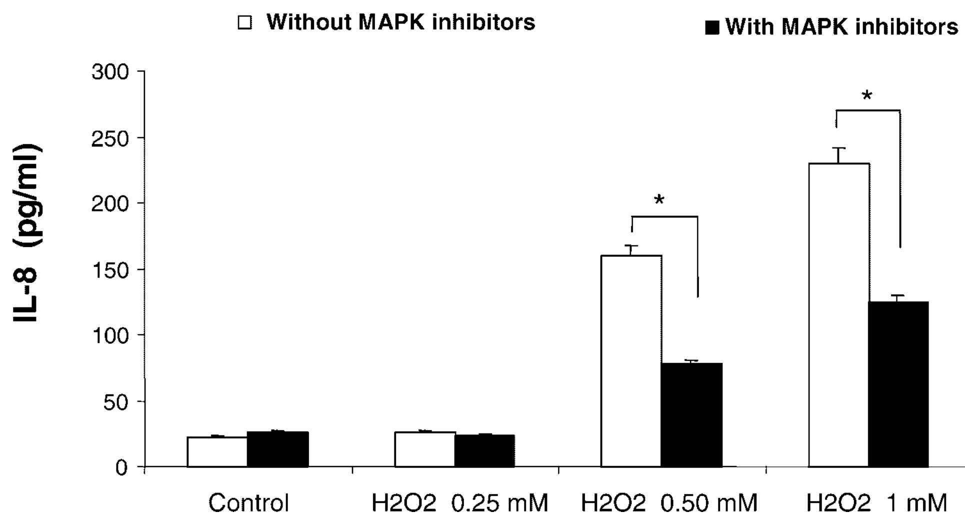
active forms of MAPKs, the observed stimulated phosphorylation of these enzymes can be considered as a reliable marker of their activation elicited by H_2O_2. H_2O_2 exerted its effects uniquely on phosphorylation-dependent activation of MAPKs, without affecting their total expression, as shown by the unchanged binding patterns of the anti-(total)MAPK polyclonal antibodies (Fig. 1).

Effects of H_2O_2 and MAPK Inhibitors on IL-8 Production

Overnight exposure of HBEC to a mixture of specific inhibitors of JNK (SP600125, 20 μM), MEK-ERK1/2 (PD98059, 40 μM), and p38 (SB203580, 1 μM), respectively, had no effect on IL-8 release into culture supernatants. IL-8 secretion was found to be significantly enhanced, in a concentration-dependent fashion, after 4 and especially after 8 h of exposure to H_2O_2 (Fig. 2). This IL-8 increase was significantly ($P < 0.01$), though not completely, inhibited by pre-treatment with the above-mentioned MAPK inhibitors (Fig. 2).

Effects of H_2O_2 on HBEC Viability and Caspase-3 Activation

At the concentration of 0.5 mM, H_2O_2 dramatically enhanced cell death, as shown by the marked increase in PI staining visualized by fluorescence microscopy (Fig. 3). Light microscope morphology evidenced that control cells had a polygonal shape and maintained confluence (Fig. 3). Otherwise, dead cells appeared to be rounded and granulated, and were found to be detached from the bottom of the well and characterized by loosened cell–cell contacts. The effect of H_2O_2 on cell viability was associated with a partial conversion of the inactive pro-caspase-3 into the active (cleaved) caspase-3, as demonstrated by Western blotting (Fig. 4), although the untreated control exhibited a relatively high amount of active caspase-3 possibly due to protease activity after cell lysis. Pre-treatment with MAPK inhibitors used in combination was able to exert an effective protection against H_2O_2-induced cell death (Fig. 3). Furthermore, each MAPK inhibitor



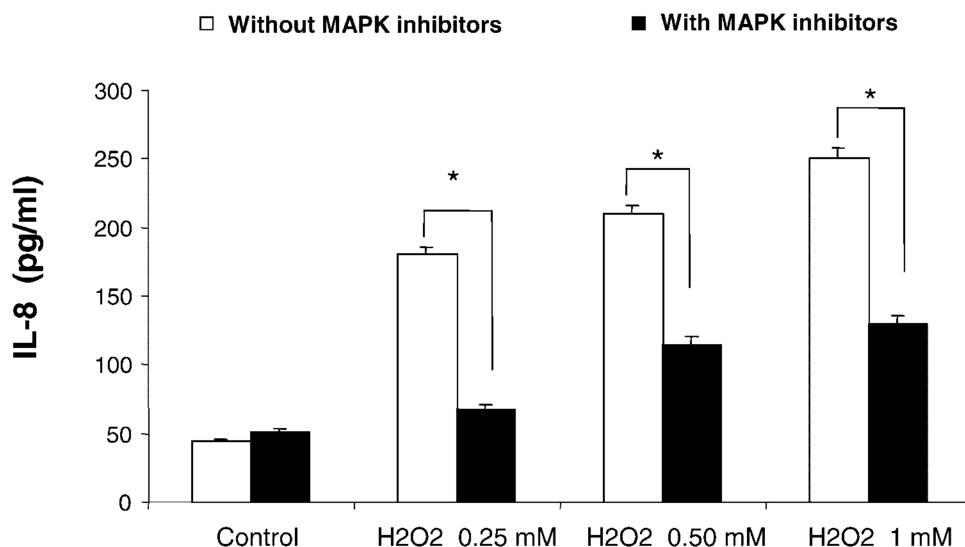


Fig. 2. H_2O_2-induced release of IL-8. IL-8 levels detected by ELISA in HBEC culture supernatants after 4 h (**upper panel**) and 8 h (**lower panel**) of cell exposure to increasing concentrations of H_2O_2 (0.25, 0.5, 1 mM), in the presence (black columns) or absence (white columns) of overnight pre-treatment with a mixture of MAPK inhibitors (PD98059, 40 μM; SP600125, 20 μM; SB203580, 1 μM). All data are expressed as mean ± standard error (SEM). *$P < 0.01$.

used individually prevented the H_2O_2-dependent increase in the percentage of dead cells, as shown by FACS analysis using An-V staining (Fig. 5).

DISCUSSION

The airway epithelium is continuously exposed to both exogenous and endogenous oxidants, including air pollutants and cigarette smoke or generated by activated inflammatory cells through mitochondrial electron transport, respectively. Therefore, by exposing primary cultures of HBEC to increasing concentrations of H_2O_2, we tried to mimic in vitro a micro-environment characterized by a high oxidative burden to which the airway epithelium can be

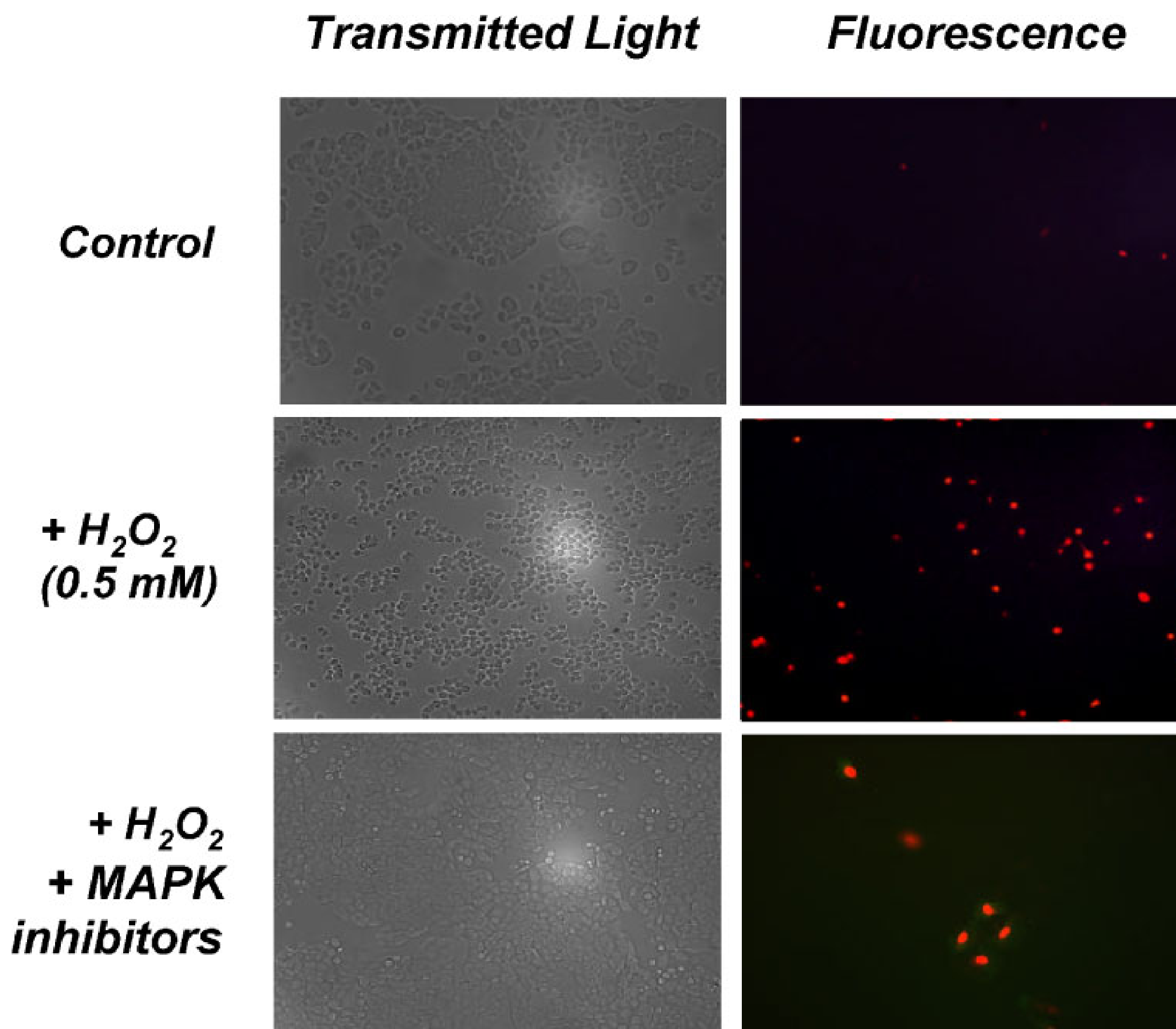


Fig. 3. H_2O_2-induced cell death. **Left panel**: Light microscope morphology of primary cultures of HBEC exposed to 0.5 mM H_2O_2, in the absence or presence of an overnight pre-treatment with a mixture of MAPK inhibitors. **Right panel**: Propidium iodide (PI) staining, performed 6 h after removal of H_2O_2, (i.e., 8 h after addition of 0.5 mM H_2O_2), shows a high number of dead cells. Overnight pre-treatment with a mixture of MAPK inhibitors was able to prevent cell death. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

really subjected. Our results clearly indicate that H_2O_2 dramatically enhanced cell death and also significantly stimulated the synthesis of IL-8, a chemokine responsible for neutrophil recruitment and activation [Biggioni et al., 1984]. Both these effects resulted to be dependent on signal transduction pathways mediated by MAPKs. This implies that H_2O_2 is able to exert, via MAPK activation, a proinflammatory and cytotoxic action on bronchial epithelial cells. On the other hand, MAPKs are known to be actively involved in the cellular responses to several different types of environmental stress [Karin, 1998]. Indeed, a phosphorylation-dependent activation of p38 and JNK has also been demonstrated in hyperosmolarity-stimulated HBEC [Hashimoto et al., 1999]. In particular, it is likely that MAPK phosphorylation induced by exogenous and endogenous oxidants, as well as by other environmental stresses, is responsible for the activation of transcription factors such as NF-κB, AP-1, and Elk-1. The latter may in turn activate proinflammatory genes encoding cytokines, chemokines, and adhesion molecules. For example, hyperosmolar stress is able to stimulate, via p38 and JNK activation, IL-8 and RANTES production by HBEC [Furuichi et al., 2002].

With regard to the stimulatory effect of H_2O_2 on IL-8 secretion by HBEC, several molecular

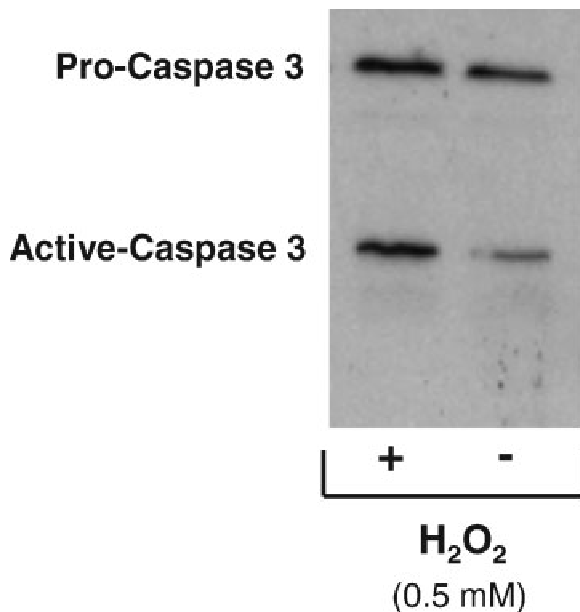


Fig. 4. Expression of both inactive and active forms of caspase-3. Immunoblot obtained using a polyclonal antibody against both inactive (pro-caspase-3) and active (cleaved) caspase-3, in the presence or absence of HBEC exposure to 0.5 mM H_2O_2.

mechanisms may be involved. In the present study, we found very low basal levels of IL-8 synthesized by HBEC, not affected by MAPK inhibitors. Conversely, the latter significantly inhibited the concentration-dependent stimulatory effect of H_2O_2 on IL-8 production. In fact, the increased IL-8 levels observed in cell culture supernatants appeared to be closely related to MAPK phosphorylation, which was also elicited by H_2O_2 in a concentration-dependent fashion. H_2O_2-induced IL-8 release, as well as the relative inhibitory effect of the mixture of SB203580, PD98059, and SP600125, were detected after 4 h of HBEC exposure to H_2O_2, but resulted to be significantly more evident after 8 h. This temporal pattern might suggest the involvement of complex, time-requiring events mediated by MAPKs and responsible for the inducible expression of the *IL-8* gene. The latter is transcriptionally regulated by NF-κB and AP-1 binding to their respective consensus DNA sequences located within the gene promoter [Hoffmann et al., 2002]. Activation of AP-1 and NF-κB is at least in part controlled by MAPKs, also in HBEC [Li et al., 2002; Zhou et al., 2003]. With regard to MAPK-dependent, transcriptional regulation of *IL-8* gene, a crucial role is played by the influences of oxidative stress on chromatin remodeling. In particular, it has been shown in pulmonary alveolar epithelial cells that H_2O_2 is able to stimulate the enzymatic activity of histone acetyl transferases (HATs) [Rahman et al., 2001]. As a consequence, the enhanced acetylation of the basic lysine residues of nucleosome core histones H3 and H4 neutralizes histone positive charges, thus markedly reducing their electrostatic interactions with negatively charged DNA [Grunstein, 1997; Cheung et al., 2000]. The subsequent DNA unwinding around nucleosomes facilitates NF-κB and AP-1 binding to their cognate promoter sites in target genes, which otherwise result to be hardly accessible because of the tight DNA supercoiling [Rahman, 2003]. MAPKs exert a key function in mediating oxidant-induced histone acetylation, which is responsible for the increased expression of proinflammatory cytokines and chemokines. Indeed, oxidative stress-dependent activation of ERK and JNK has been found to be associated with an enhanced HAT activity of co-activator macromolecular complexes such as CBP/p300 and ATF-2 [Rahman, 2002]. Furthermore, p38 promotes the so-called H3 phosphoacetylation, consisting of the p38-catalyzed phosphorylation of a specific serine residue (Ser10) of histone H3, which facilitates its interactions with HATs [Saccani et al., 2002]. The subsequent acetylation of Lys14 located within the H3 amino-terminal tail results in a remarkable increase in gene transcription. In particular, H3 phosphoacetylation leads to an increased recruitment of NF-κB to its binding sites present in the IL-8 gene promoter [Saccani et al., 2002]. H_2O_2 is also able to induce, in a time-dependent manner, the acetylation of histone H4 and the closely related synthesis of IL-8 by both bronchial and alveolar epithelial cells [Gilmour et al., 2003; Tomita et al., 2003]. In other cell types such as alveolar macrophages, it has also been shown that H_2O_2 and cigarette smoke can stimulate IL-8 secretion by inhibiting the activity of histone deacetylase (HDAC) enzymes [Ito et al., 2001]. HDACs indeed repress gene transcription by deacetylating core histones, thus enhancing chromatin condensation and DNA supercoiling [Ayer, 1999]. In addition to these transcriptional mechanisms, MAPKs affect IL-8 synthesis also at the post-transcriptional level. For example, p38 exerts a stabilizing function on IL-8 mRNA, which is mediated by the p38-dependent kinase MAPKAP-K2 and requires the presence of AU-rich nucleotide sequences within the mRNA

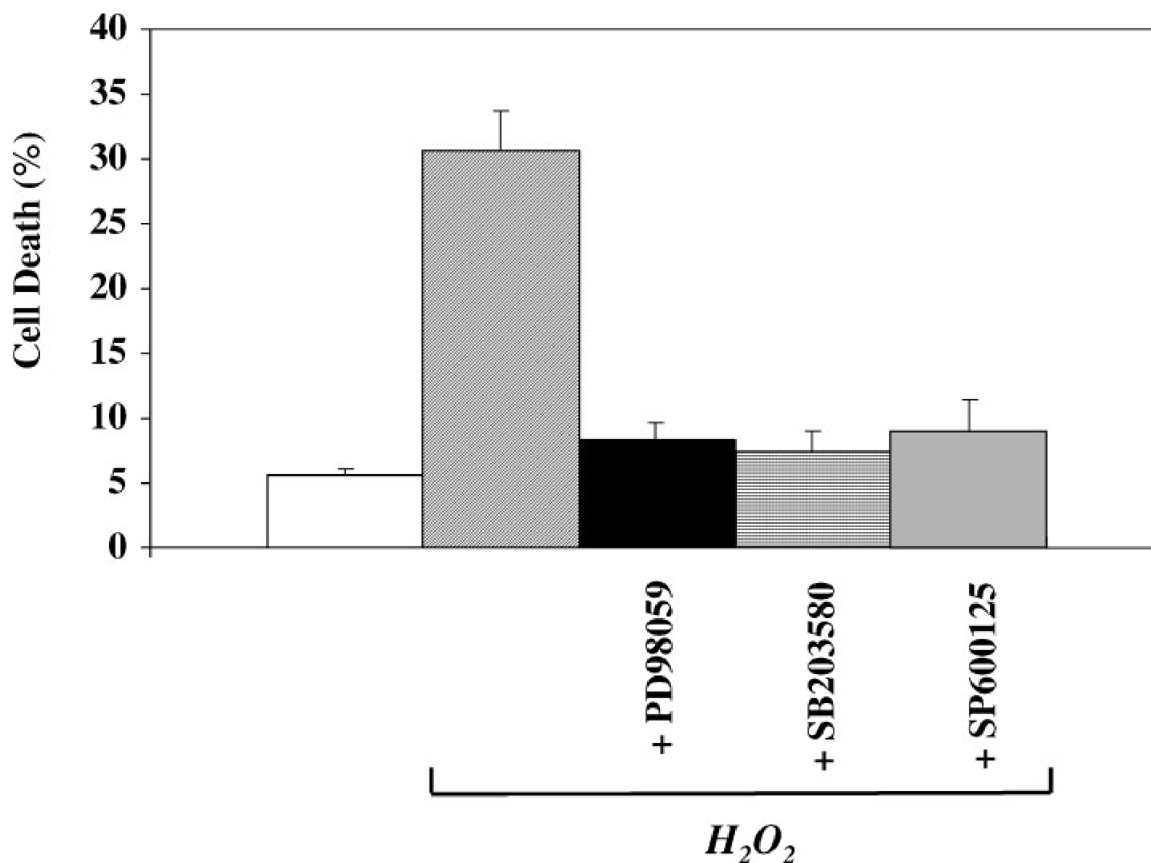


Fig. 5. H_2O_2-induced cell death is prevented by MAPK inhibitors. FACS analysis of HBEC stained with annexin-V (An-V) in the absence or presence of a pre-treatment with each of MAPK inhibitors PD98059 (40 μM), SB203580 (1 μM), and SP600125 (20 μM). Pre-incubation (for 12 h) with a specific MEK/ERK1/2 (PD98059), p38 (SB203580), or JNK (SP600125) inhibitor was able to prevent cell death induced by exposure to H_2O_2 (0.5 mM). Histograms represent the percentage of An-V-positive cells, determined in three independent experiments. All data are expressed as mean $\pm$ SEM.

3′-untranslated region [Winzen et al., 1999]. Although MAPKs can tightly control IL-8 expression, our results suggest that the blockade of all three MAPK circuits reduces, without however abolishing completely, the stimulatory action of H_2O_2 on IL-8 production. The latter may thereby also be due, at least in part, to MAPK-independent signal transduction pathways activated by H_2O_2 in HBEC. In this regard, it has been recently observed in airway epithelial cells that a key role in NF-κB-dependent activity of IL-8 promoter is played by specific isoforms of protein kinase C (PKC) such as PKC-delta [Page et al., 2003].

In any case, the close relationship linking oxidative stress and IL-8 secretion may be extremely important for the pathogenesis of asthma and COPD. In fact, H_2O_2 concentration is significantly greater in the exhaled breath of asthmatic patients with respect to normal individuals [Emelyanov et al., 2001]. Furthermore, bronchial epithelial cells from asthmatics are characterized, when compared to normal controls, by higher expression levels of IL-8 in basal conditions as well as after exposure to environmental pollutants [Devalia et al., 1999]. These observations might contribute to explain the association of asthma, documented by some epidemiological studies, with air pollution and a low dietary intake of antioxidants [Hatch, 1995; Sheppard et al., 1999]. Increased IL-8 levels are also detectable in the sputum obtained from COPD patients [Beeh et al., 2003], who also present high concentrations of exhaled H_2O_2, especially during disease exacerbations [Dekhuijzen et al., 1996]. Therefore, it is likely that MAPK activation plays a central role in the proinflammatory action exerted by oxidative stress on the airway epithelium of asthmatic and COPD patients.

In addition to inducing IL-8 release, in the present study H_2O_2 also dramatically increased

the amount of HBEC exhibiting morphologic and fluorescence features of dead cells. Cell death was mediated by activation of all three MAPK pathways, and indeed specific inhibitors of each, used either individually or as a cocktail, were capable of preventing H_2O_2-induced cytotoxicity. These findings imply that each MAPK module contributed to determine cell death. The latter was at least in part due to apoptotic mechanisms, as demonstrated by both FACS analysis and partial activation of caspase-3. This is indeed a cysteinyl protease acting as a common effector pathway for the apoptotic processes originating at both cell membrane and mitochondrial levels [O'Sullivan et al., 2003]. In our experimental model, however, the lack of a complete caspase-3 activation might suggest the involvement also of cell death mechanisms other than apoptosis, such as necrosis. This is quite consistent with very recent studies showing that cigarette smoke can induce necrosis of alveolar endothelial and epithelial cells, being also able to inhibit caspase-3 activation and apoptosis [Wickenden et al., 2003]. On the other hand, it has been reported that caspases may be either activated or inhibited by H_2O_2, depending on its concentrations as well as on the cellular context [Hampton and Orrenius, 1997; Lee and Shacter, 2000; Borutaite and Brown, 2001]. In particular, the inhibitory action of H_2O_2 might be mediated by its effect on caspase cysteine residues, which can be directly targeted by oxidants. Moreover, other studies performed on endothelial cells have demonstrated that MAPK activation can result in either stimulation of, or protection against oxidant-induced apoptosis, depending on the different isoforms of p21 Ras proteins activated by H_2O_2. In particular, ERK1/2 activation driven by Harvey-Ras (Ha-Ras) enhanced H_2O_2-induced apoptosis, whereas an anti-apoptotic effect was detected when ERK1/2 activity was stimulated by Kirsten-Ras (Ki-Ras) [Cuda et al., 2002]. These observations imply that both susceptibility and resistance to apoptosis elicited by oxidative stress may be determined, at least in part, by the Ras isoform predominantly expressed by a given tissue.

Our primary cultures of HBEC seem to be much more sensitive to H_2O_2, in terms of both cytotoxicity and MAPK activation, than pulmonary endothelial cells exposed by us in a previous study to higher concentrations (2 mM) of H_2O_2 [Pelaia et al., 2001]. The sources of airway epithelial cell cultures are also very important in relation to the effects of oxidative stress. Indeed, bronchial epithelial cells obtained from asthmatic patients were found to be more susceptible, with respect to those derived from normal subjects, to H_2O_2-induced apoptosis [Bucchieri et al., 2002]. Overall, the biological behavior of the airway epithelium depends not only on the peculiar features of its cells, but also on the specific type of stimulus. For instance, we have recently reported that normal HBEC undergo a remarkable MAPK-dependent apoptosis and a complete caspase-3 activation when exposed to TGF-β1 [Pelaia et al., 2003], thus suggesting that the full implementation of an apoptotic program can exclusively be triggered by appropriate stimuli acting within particular cell contexts. Our current findings, obtained in the same HBEC cultures, are consistent with the induction by H_2O_2 of molecular events responsible for an epithelial damage, which may only in part culminate in apoptosis. Hence, because oxidative stress plays a crucial pathophysiologic role in COPD [Barnes, 1990], it could be argued that oxidant-induced cytotoxicity precedes the occurrence of squamous metaplasia, which might be the result of an epithelial repair response.

In conclusion, we herein demonstrate that H_2O_2 elicits, in primary cultures of HBEC, a cytotoxic effect mediated by phosphorylation-dependent activation of MAPKs. On the other hand, the latter are also responsible for cell death of HBEC caused by other agents such as peroxynitrite [Nabeyrat et al., 2003], which originates from the reaction of superoxide anion with nitric oxide. Moreover, MAPKs are also involved in the inflammatory changes produced by H_2O_2, and exemplified in our study by the enhanced airway epithelial synthesis of IL-8. Therefore, especially in the presence of lowered antioxidant defenses, oxidant-activated MAPKs could stimulate the expression of several genes encoding cytokines and chemokines implicated in bronchial inflammation. However, the specific molecular sensors of oxidative stress, as well as the precise cascade of biochemical events leading to oxidant-mediated MAPK phosphorylation, are still not well defined. For all these reasons, a remarkable interest surrounds the experimental investigations aimed at further elucidating the

cellular mechanisms underlying the effects of oxidative stress in chronic inflammatory airway diseases. Such studies acquire a significant relevance also for their potential therapeutic impact, in that they may contribute to identify pharmacological targets suitable for the development of new antioxidant treatments.

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REFERENCES

Ayer DE. 1999. Histone deacetylases: Transcriptional repression with SINers and NuRDs. Trends Cell Biol 9: 193–198.

Barnes PJ. 1990. Reactive oxygen species and airway inflammation. Free Radic Biol Med 9:235–243.

Barnes PJ. 2003. New concepts in chronic obstructive pulmonary disease. Annu Rev Med 54:113–129.

Beeh KM, Kornmann O, Buhl R, Culpitt SV, Giembycz MA, Barnes PJ. 2003. Neutrophil chemotactic activity of sputum from patients with COPD: Role of interleukin 8 and leukotriene B4. Chest 123:1240–1247.

Biggioni M, Dewald B, Moser B. 1984. Interleukin-8 and related chemotactic cytokines-CXC and CC chemokines. Adv Immunol 55:97–179.

Borutaite V, Brown GC. 2001. Caspases are reversibly inactivated by hydrogen peroxide. FEBS Lett 500:114–118.

Bucchieri F, Puddicombe SM, Lordan JL, Richter A, Buchanan D, Wilson SJ, Ward J, Zummo G, Howarth PH, Djukanovich R, Holgate ST, Davies DE. 2002. Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. Am J Respir Cell Mol Biol 27: 179–185.

Chang L, Karin M. 2001. Mammalian MAP kinase signalling cascades. Nature 410:37–40.

Cheung P, Allis CD, Sassone-Corsi P. 2000. Signaling to chromatin through histone modifications. Cell 103:263–271.

Crapo JD. 2003. Oxidative stress as an initiator of cytokine release and cell damage. Eur Respir J 22(Suppl 44):4s–6s.

Cuda G, Paternò R, Ceravolo R, Candigliota M, Perrotti N, Perticone F, Faniello MC, Schepis F, Ruocco A, Mele E, Cassano S, Bifulco M, Santillo M, Avvedimento VE. 2002. Protection of human endothelial cells from oxidative stress: Role of Ras-ERK1/2 signaling. Circulation 105: 968–974.

Davies DE. 2001. The bronchial epithelium: Translating gene and environment interactions in asthma. Curr Opin Allergy Clin Immunol 1:67–71.

Dekhuijzen PN, Aben KK, Dekker I, Aarts LP, Wielders PL, van Herwaarden CL, Bast A. 1996. Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease. Am J Respir Crit Care Med 154:813–816.

Devalia JL, Bayram H, Abdelaziz MM, Sapsford RJ, Davies RJ. 1999. Differences between cytokine release from bronchial epithelial cells of asthmatic patients and non-asthmatic subjects: Effects of exposure to diesel exhaust particles. Int Arch Allergy Immunol 118:437–439.

Emelyanov A, Fedoseev G, Abulimity A, Rudinski K, Fedulov A, Karabanov A, Barnes PJ. 2001. Elevated concentrations of exhaled hydrogen peroxide in asthmatic patients. Chest 120:1136–1139.

Furuichi S, Hashimoto S, Gon Y, Matsumoto K, Horie T. 2002. p38 mitogen-activated protein kinase and c-Jun-NH2-terminal kinase regulate interleukin-8 and RANTES production in hyperosmolarity-stimulated human bronchial epithelial cells. Respirology 7:193–200.

Gilmour PS, Rahman I, Donaldson K, MacNee W. 2003. Histone acetylation regulates epithelial IL-8 release mediated by oxidative stress from environmental particles. Am J Physiol Lung Cell Mol Physiol 284:L533–L540.

Gruenert DC, Basbaum CB, Widdicombe JH. 1990. Long-term culture of normal and cystic fibrosis epithelial cells grown under serum-free conditions. In Vitro Cell Dev Biol 26:411–418.

Grunstein M. 1997. Histone acetylation in chromatin structure and transcription. Nature 389:349–352.

Halliwell B, Gutteridge JMC. 1989. Free radicals in biology and medicine. Oxford: Oxford University Press. p 543.

Hampton MB, Orrenius S. 1997. Dual regulation of caspase activity by hydrogen peroxide: Implications for apoptosis. FEBS Lett 414:552–556.

Hashimoto S, Matsumoto K, Gon Y, Nakayama T, Takeshita I, Horie T. 1999. Hyperosmolarity-induced interleukin-8 expression in human bronchial epithelial cells through p38 mitogen-activated protein kinase. Am J Respir Crit Care Med 159:634–640.

Hatch GE. 1995. Asthma, inhaled oxidant, and dietary antioxidants. Am J Clin Nutr 61:625S–630S.

Hoffmann E, Dittrich-Breiholz O, Holtmann H, Kracht M. 2002. Multiple control of interleukin-8 gene expression. J Leukoc Biol 72:847–855.

Ito K, Lim S, Caramori G, Chung KF, Barnes PJ, Adcock IM. 2001. Cigarette smoking reduces histone deacetylase 2 expression, and inhibits glucocorticoid actions in alveolar macrophages. FASEB J 15:1110–1112.

Karin M. 1998. Mitogen-activated protein kinase cascades as regulators of stress responses. Ann NY Acad Sci 851: 139–146.

Kelsen SG, Mardini IA, Zhou S, Benovic JL, Higgins NC. 1992. A technique to harvest viable tracheobronchial epithelial cells from living human donors. Am J Respir Cell Mol Biol 7:66–72.

Lee YJ, Shacter E. 2000. Hydrogen peroxide inhibits activation, not activity, of cellular caspase-3 in vivo. Free Radic Biol Med 29:684–692.

Li J, Kartha S, Iasvovskaia S, Tan A, Bhat RK, Manaligod JM, Page K, Brasier AR, Hershenson MB. 2002. Regulation of human airway epithelial cell IL-8 expression by MAP kinases. Am J Physiol Lung Cell Mol Physiol 283: L690–L699.

MacNee W. 2001. Oxidative stress and lung inflammation in airways disease. Eur J Pharmacol 429:195–207.
MacNee W, Rahman I. 2001. Is oxidative stress central to the pathogenesis of chronic obstructive pulmonary disease? Trends Mol Med 7:55–62.
MacNee W, Wiggs BB, Berzberg AS, Hogg JC. 1989. The effect of cigarette smoking on neutrophil kinetics in human lungs. N Engl J Med 321:924–928.
Maselli R, Grembiale RD, Pelaia G, Cuda G. 2002. Oxidative stress and lung diseases. Monaldi Arch Chest Dis 57:180–181.
Morcillo EJ, Estrela J, Cortijo J. 1999. Oxidative stress and pulmonary inflammation: Pharmacological intervention with antioxidants. Pharmacol Res 40:393–404.
Nabeyrat E, Jones GE, Fenwick PS, Barnes PJ, Donnelly LE. 2003. Mitogen-activated protein kinases mediate peroxynitrite-induced cell death in human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 284:L1112–L1120.
Nicholson DW, Thornberry NA. 1997. Caspases: Killer proteases. Trends Biochem Sci 22:299–306.
O'Sullivan MP, Tyner JW, Holtzman MJ. 2003. Apoptosis in the airways: Another balancing act in the epithelial program. Am J Respir Cell Mol Biol 29:3–7.
Page K, Li J, Zhou L, Iasvovskaia S, Corbit KC, Soh JW, Weinstein IB, Brasier AR, Lin A, Hershenson MB. 2003. Regulation of airway epithelial cell NF-κB-dependent gene expression by protein kinase C delta. J Immunol 170:5681–5689.
Pelaia G, Cuda G, Vatrella A, Grembiale RD, De Sarro GB, Maselli R, Costanzo FS, Avvedimento VE, Rotiroti D, Marsico SA. 2001. Effects of glucocorticoids on activation of c-Jun N-terminal, extracellular signal-regulated, and p38 MAP kinases in human pulmonary endothelial cells. Biochem Pharmacol 62:1719–1724.
Pelaia G, Cuda G, Vatrella A, Grembiale RD, Fratto D, Tagliaferri P, Maselli R, Costanzo FS, Marsico SA. 2003. Effects of transforming growth factor-β and budesonide on mitogen-activated protein kinase activation and apoptosis in airway epithelial cells. Am J Respir Cell Mol Biol 29:12–18.
Pryor WA, Prier DG, Church DF. 1983. Electron-spin resonance study of mainstream and sidestream cigarette smoke: Nature of the free radicals in gasphase smoke and in cigarette tar. Environ Health Perspect 47:345–355.
Rahman I. 2002. Oxidative stress, transcription factors, and chromatin remodelling in lung inflammation. Biochem Pharmacol 64:935–942.
Rahman I. 2003. Oxidative stress, chromatin remodeling, and gene transcription in inflammation and chronic lung diseases. J Biochem Mol Biol 36:95–109.
Rahman I, MacNee W. 2000. Oxidative stress and regulation of glutathione in lung inflammation. Eur Respir J 16:534–554.
Rahman I, Gilmour PS, Jimenez LA, MacNee W. 2001. Oxidative stress induces histone acetylation in alveolar epithelial cells (A549). Am J Respir Crit Care Med 163: A61.
Rochelle LG, Fischer BM, Adler KB. 1998. Concurrent production of reactive oxygen and nitrogen species by airway epithelial cells in vitro. Free Radic Biol Med 24: 863–868.
Saccani S, Pantano S, Natoli G. 2002. p38-marking of inflammatory genes for increased NF-κB recruitment. Nature Immunol 3:69–75.
Sheppard L, Levy D, Norris G, Larson TV, Koenig JQ. 1999. Effects of ambient air pollution on nonelderly asthma hospital admission in Seattle, Washington, 1987–1994. Epidemiology 10:23–30.
Thannickal VJ, Fanburg BL. 2000. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol 279:L1005–L1028.
Tomita K, Barnes PJ, Adcock IM. 2003. The effect of oxidative stress on histone acetylation and IL-8 release. Biochem Biophys Res Commun 301:572–577.
Wickenden JA, Clarke MC, Rossi AG, Rahman I, Faux SP, Donaldson K, MacNee W. 2003. Cigarette smoke prevents apoptosis through inhibition of caspase-3 activation and induces necrosis. Am J Respir Cell Mol Biol 29: 562–570.
Winzen R, Kracht M, Ritter B, Wilhelm A, Chen C-Y A, Shyu A-B, Muller M, Gaestel M, Resch K, Holtmann H. 1999. The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. EMBO J 18:4969–4980.
Zhou L, Tan A, Iasvovskaia S, Li J, Lin A, Hershenson MB. 2003. Ras and mitogen-activated protein kinase kinase kinase-1 coregulate activator protein-1- and nuclear factor-κB-mediated gene expression in airway epithelial cells. Am J Respir Cell Mol Biol 28:762–769.