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Redox Regulation of p38 MAPK Activation and Expression of ICAM-1 and Heme Oxygenase-1 in Human Alveolar Epithelial (A549) Cells

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ABSTRACT

We have explored the potential role of redox events in p38 mitogen-activated protein kinase (MAPK) activation and their relevance to the inducible expression of intercellular adhesion molecule-1 (ICAM-1) and heme oxygenase-1 (HO-1) in A549 cells. Tumor necrosis factor-α (TNFα) and hydrogen peroxide (H_2O_2) both activated p38, but only TNFα activated nuclear factor-κB (NF-κB). *N*-Acetyl-L-cysteine (20 m*M*) inhibited both H_2O_2- and TNFα-induced p38 phosphorylation (14 ± 7 and 37 ± 4% of control, respectively). The mitochondrial complex I and III inhibitors, rotenone and antimycin A, and allopurinol partially inhibited H_2O_2- but not TNFα-induced p38 activation. However, rotenone and antimycin A augmented intracellular oxidative stress measured by dichlorofluorescein fluorescence. TNFα, but not H_2O_2, induced ICAM-1 in A549 cells, which was attenuated by a proteasome inhibitor, but not by the p38 MAPK inhibitor SB203580. In contrast, hemin and hemoglobin, but neither TNFα nor H_2O_2, caused efficient HO-1 expression. However, hemin had no effect on p38 activation and SB203580 did not influence hemin-induced HO-1 protein expression. Collectively, these data suggest that p38 is a cytokine- and oxidative stress-responsive pathway in A549 cells. Whereas NF-κB appears crucial in ICAM-1 induction, p38 activation itself is not sufficient to confer HO-1 expression and may not be involved in HO-1 and ICAM-1 induction in A549 cells. *Antioxid. Redox Signal.* 7, 14–24.

INTRODUCTION

ALTHOUGH MANY COMPONENTS of the alveolar-capillary unit are involved in the orchestration of inflammation in acute lung injury, the prominent role played by the alveolar epithelium is now widely accepted (23, 38, 39). More than just a stage on which inflammatory processes are played out, the alveolar epithelium participates in virtually all aspects of inflammation, including initiation, amplification, down-regulation, and tissue repair. This central role involves responsiveness to a multitude of soluble and particulate proinflammatory factors and translation of these signals to adaptive responses. These include transcriptional changes leading either to proinflammatory gene expression or to up-regulation of genes that keep propagation of inflammation under control. A representative schema involves an insult to the alveolar-capillary unit inducing the local release of cytokines such as tumor necrosis factor-α (TNFα) from alveolar immune cells. TNFα then activates epithelial cells to produce chemokines and adhesion molecules to recruit neutrophils and monocytes/macrophages to the site of insult. Finally, these cells both perpetuate the inflammatory response and produce adaptive/survival factors to repair the insult.

Intercellular adhesion molecule-1 (ICAM-1) is a cell-surface glycoprotein that is a member of the immunoglobulin gene family. ICAM- 1 expression is variable among various epithe-

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lial cells in the lung both under basal conditions and in response to different stimuli (7). For instance, tracheal epithelial cells and alveolar type I cells exhibit constitutive ICAM-1, which is increased on exposure to TNFα or interleukin-1β (IL-1β). Interestingly, ICAM-1 expression is not constitutive on alveolar type II cells, but these cells show the greatest induction following different stimuli both *in vivo* and *in vitro*. Expression of ICAM-1 in the lung is of important physiological relevance, because neutralizing antibodies to ICAM-1 and lack of the ICAM-1 gene in some knockout models limit neutrophil accumulation and lung injury by hyperoxia or by bacterial lipopolysaccharide (7, 12, 13).

One of the crucial genes involved in epithelial adaptation, survival, and limitation of the inflammatory response is heme oxygenase (HO)-1 (5, 27, 32). The inducible HO-1 expression represents a ubiquitous stress response to stimulation by a large variety of chemical and physical stimuli. These include its physiological substrate heme, as well as heavy metals, ultraviolet radiation, oxidants, nitric oxide, thiol-reactive substances, and hyperoxic or hypoxic states. Furthermore, HO-1 responds to a number of cytokines, hormones, and related substances, including proinflammatory mediators such as TNFα, IL-1β, and bacterial lipopolysaccharide, the thrombopoietic cytokine IL-11, and growth factors such as platelet-derived growth factor and transforming growth factor-β_1 (26, 33, 45). The relevance of epithelial HO-1 induction in controlling lung inflammation is suggested by studies showing *in vivo* up-regulation of HO-1 in lung injury models and the efficacy of adenoviral expression of HO-1 in enhancing resolution of lung injury (17, 19).

One of the recent conceptual advances in our understanding of inflammatory gene regulation has been the recognition that the intracellular signal transduction pathways that activate crucial transcription factors frequently exhibit redox sensitivity (9, 10, 15, 28, 41). Hence, redox regulation of the nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) transcription factors has received much attention. Interestingly, there is important controversy regarding the role of oxidative stress and the influence of antioxidants on NF-κB activation in alveolar epithelial cells and the role of the proposed redox regulation of NF-κB in particular gene induction (6, 16, 18, 29–31). Regardless of the debate on redox aspects of NF-κB activation, redox regulation of alternative pathways is being increasingly considered. Among these, activation of the various subfamilies of the mitogen-activated protein kinase (MAPK) pathway has been shown to contribute to proinflammatory gene induction frequently in a redox-sensitive manner (41).

We have recently characterized NF-κB-mediated induction of ICAM-1 in the human alveolar type II-like adenocarcinoma cell line (A549), mainly in response to IL-1β (16). As oxidative stress did not activate NF-κB and antioxidants had no influence on cytokine-induced NF-κB activation, we have concluded that, under our experimental conditions, there was no evidence for redox sensitivity of this pathway. We now contrast redox regulation of TNFα-induced NF-κB activation to that of the MAPK pathway in A549 cells with particular focus on the p38 subfamily. In light of current concepts postulating the requirement of dual signal transduction pathways for efficient NF-κB-mediated gene induction, we wanted to determine the genetic consequences of these molecular events in pro- and antiinflammatory gene expression.

MATERIALS AND METHODS

Materials and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, L-glutamine, penicillin–streptomycin solution, tyrpsin-EDTA solution, phosphate-buffered saline (PBS; 10×), *N*-acetyl-L-cysteine (NAC), dithiothreitol, phenylmethylsulfonyl fluoride, aprotinin, pepstatin, anti-β-tubulin monoclonal antibody (clone TUB 2.1), and anti-activated (diphosphorylated) p38 monoclonal antibody (clone p38-TY) were all obtained from Sigma–Aldrich Chemical Co. (Dorset, U.K.). Rabbit polyclonal anti-HO-1 was from Bioquote Ltd. (York, U.K.). Tween 20, 2-mercaptoethanol, and 30% (vol/vol) hydrogen peroxide (H_2O_2) were from Merck (Middlesex, U.K.). IκBα and NF-κB p65 polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal NF-κB p65 antibody (clone 20) was from Transduction Laboratories. Anti-ICAM-1 monoclonal antibody (clone 6.5B5) and horseradish peroxidase-conjugated secondary antibodies were from Dako (Cambridgeshire, U.K.). Hybond C Super nitrocellulose membrane, ECL™ detection kit, and Hyperfilm were all from Amersham–Pharmacia (Amersham, U.K.). IL-1β, TNFα, and leupeptin were all obtained from Roche Diagnostics (Lewis, U.K.). All tissue culture plastics (TPP) were from Helena Biosciences (Sunderland, U.K.).

Experimental design and procedure

Initial experiments focused on TNFα- and H_2O_2-induced activation of p38 MAPK and contrasted these events to those of NF-κB. To establish redox regulation of these events, the primary emphasis of the next series of experiments was to explore the influence of the widely used antioxidant and glutathione precursor NAC on these signaling pathways. The underlying hypothesis was that p38 MAPK activation exhibits NAC sensitivity. As intracellular oxidative stress has been implicated both in the action of TNFα and in the amplification of H_2O_2-induced intracellular responses (2), we explored the potential contribution of various intracellular oxidant-generating pathways, including NADPH oxidase, xanthine oxidase, and mitochondrial electron transport chain, to inflammatory mediator-induced p38 activation by utilization of their well known pharmacological inhibitors. We expected to see that these inhibitors would attenuate TNFα- and H_2O_2-induced p38 activation. To substantiate these pharmacological experiments, the effect of inhibitors on intracellular oxidative stress was monitored by fluorescence measurements. Finally, the genetic influence of these signaling events on up-regulation of ICAM-1 and HO-1 was studied in TNFα- and H_2O_2-stimulated A549 cells. It was reasoned that p38 would be important in ICAM and HO-1 induction. Supporting experiments would be temporal association between p38 activation by cytokines and H_2O_2 and attenuation of gene induction by specific p38 inhibitors.

Cell culture

A549 cells were grown in DMEM containing 10% heat inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 m*M* L-glutamine. Cells were maintained at

37°C in humid conditions with 5% CO_2 and seeded into 24-well plates when confluent.

Cells were incubated in media containing no additive for 24 h prior to the start of each experiment. Cells were incubated at 37°C with either medium (control), various antioxidants, or MAPK inhibitors for 30–60 min prior to the addition of TNFα or H_2O_2 at the indicated concentration for the indicated time. Potential cytotoxic effects of treatments were monitored using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay that relies on the ability of viable cell mitochondria dehydrogenase to cleave the tetrazolium ring from MTT to yield purple formazan crystals, which can be solubilized and quantified spectrophotometrically. After incubation, cells were harvested for western blotting (IκBα, p38, HO-1), flow cytometric analysis (ICAM-1), or immunocytochemistry (NF-κB nuclear translocation).

Western blotting

Total cell lysis was obtained using 1% sodium dodecyl sulfate lysis buffer as previously described (16). The samples were assayed for protein content using the Pierce bicinchoninic acid protein assay kit and then stored at −20°C in aliquots.

All protein samples were resolved on NuPage 10% Tris-Bis gel plates and transferred to Hybond C nitrocellulose membrane. After nonspecific binding was blocked with 3% nonfat dry milk solution in PBS containing 0.05% Tween 20, the membranes were incubated for 60 min at room temperature with primary antibodies or mouse monoclonal anti-β-tubulin. Membranes were then washed with PBS-Tween followed by 60 min of incubation with relevant horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized using an enhanced chemiluminesence detection kit. Optical density analysis of protein bands was performed using a Bio-Rad GS710 Imaging densitometer and Quantity One software package.

Measurement of ICAM-1 expression

Cells were washed in PBS, trypsinized, and washed in PBS containing 20% fetal calf serum. After a 30-min incubation with either anti-ICAM-1 antibody (1:50) or IgG1 isotype control (MOPC 21, 10 μg/ml), cells were washed in PBS and incubated for a further 30 min with fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody. Cells were washed in PBS and resuspended in 0.5 ml of PBS, and fluorescence was detected by flow cytometry using a Coulter XL measuring log fluorescence intensity. Gates were set at 2% on isotype control.

Immunocytochemistry

Cells were fixed and permeabilized in ice-cold acetone for 10 min and allowed to air dry. Slides were rinsed in fresh Tris-buffered saline (TBS) and incubated with monoclonal anti-NF-κB p65 (Santa Cruz) for 1 h at room temperature in a humidified atmosphere. Slides were washed in TBS and incubated for 30 min with secondary antibody (Envision, Dako). Slides were washed again in TBS and incubated for 5 min with diaminobenzidine (Sigma Fast). After washing in TBS, slides were rinsed in cold running water and the cell nuclei counterstained with Meyers hematoxylin. Cells were then dehydrated through graded alcohol, cleared, and mounted.

Fluorescent measurement of intracellular oxidation

This method is based on the internalization of the nonfluorescent compound 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) by the cells, cleavage of the diacetate group by intracellular esterases, resulting in the formation of 2',7'-dichlorodihydrofluorescein (DCFH) and the oxidation of DCFH upon oxidative stress, generating the highly fluorescent compound 2',7'-dichlorofluorescein (DCF) (44). A549 cells grown in 96-multiwell plates were washed with phenol red-free DMEM and incubated with 5 μ*M* DCFH-DA for 30 min. After medium change, cells were preincubated with NAC, rotenone, antimycin A, or vehicle for 30 min followed by addition of increasing concentrations of H_2O_2 and TNFα. Fluorescence intensities (excitation/emission wavelength of 480/530) were measured at baseline, at the end of the preincubation period, and 5, 15, and 30 min after stimulation.

Data analysis

Data are presented as either representative figures or as means ± SEM of at least three independent experiments. Graphs of densitometric analysis of western blots are expressed as percentage of untreated control values and, where appropriate, normalized to β-tubulin levels. Flow cytometry data are expressed as the percentage of cells that were ICAM-1-positive. Statistical analysis was performed using one-way analysis of variance with Student–Newman–Keuls or Dunnett post hoc tests where appropriate. A p value < 0.05 was taken as statistically significant.

RESULTS

Influence of inflammatory stimuli on intracellular signal transduction

As we have shown before, TNF stimulation of A549 resulted in a time- and concentration-dependent activation of NF-κB (16). Figure 1a shows that IκBα levels are rapidly depleted following TNFα (10 ng/ml) exposure, thus leaving only a fraction of IκBα levels following 30 min of cytokine treatment. TNFα-induced IκBα depletion was associated with increased nuclear NF-κB p65 as demonstrated by western blot analysis of nuclear proteins (2,040.4 ± 1,083.7% of control levels) and immunoperoxidase staining of A549 cells on chamber slides (Fig. 1b and c). These events were regulated by the proteasome pathway, because the proteasome inhibitor MG132 inhibited TNFα-induced IκBα degradation, causing accumulation of the higher-molecular-weight phosphorylated and ubiquitinated forms of the protein (Fig. 1a). Associated with inhibition of IκBα degradation, MG132 reduced TNFα-induced accumulation of NF-κB p65 in the cell nuclei (Fig. 1d). In contrast to TNFα and IL-1β, reagent H_2O_2 did not deplete IκBα and had no influence on nuclear translocation of NF-κB p65 (data not shown) (16).

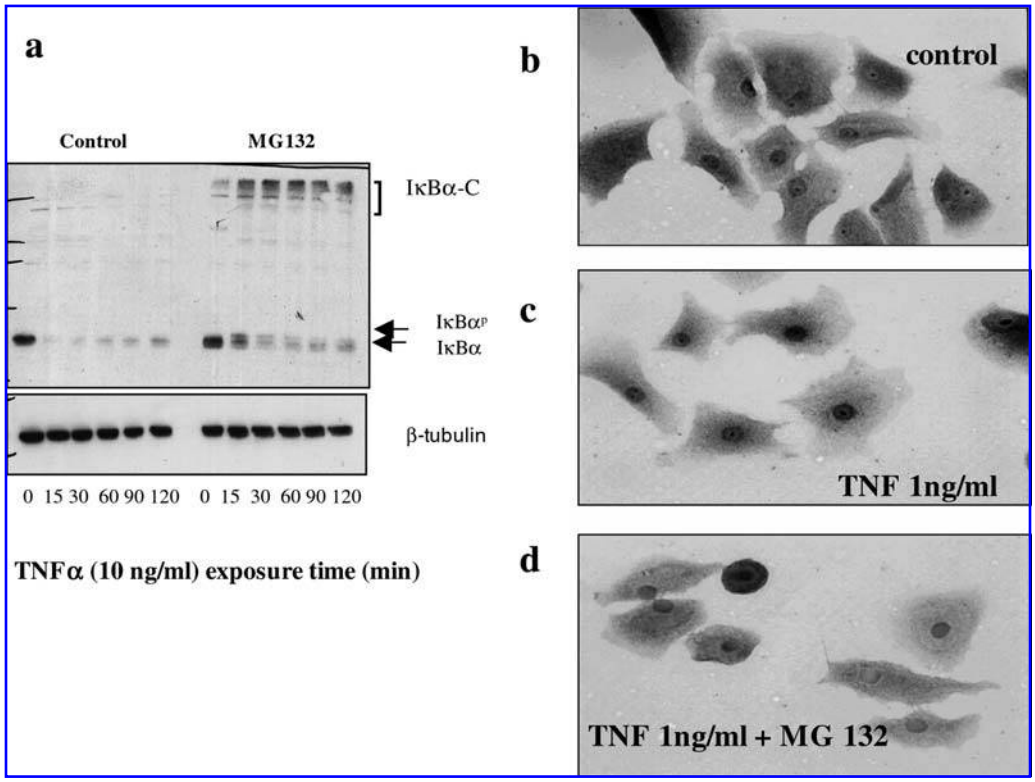

FIG. 1. Influence of TNFα and the proteasome inhibitor on activation of the NF-κB pathway in A549 cells. (**a**) Representative western blot demonstrating the time course of TNFα-induced changes in IκBα levels in the absence and presence of MG132 (10 μ*M*). IκBα^P depicts phosphorylated, whereas IκBα-C shows high-molecular-weight, presumably polyubiquitinylated forms of IκBα. (**b–d**) Immunoperoxidase staining demonstrating the cellular localization of NF-κB p65 under resting (b) and TNFα-stimulated (60 min) conditions in the absence (c) and presence of 10 μ*M* MG132 (d).

Similarly to activation of the NF-κB pathway, TNFα also induced double phosphorylation of the p38 MAPK in A549 cells (Fig. 2a). This was evident within 10 min of TNF exposure and lasted for >60 min. The response was also concentration-dependent in the range of 0.1–10 ng/ml (data not shown). In contrast to the lack of effect on the NF-κB pathway, H_2O_2 (100–1,000 μ*M*) caused activation of the p38 MAPK in A549 cells (Fig. 2b).

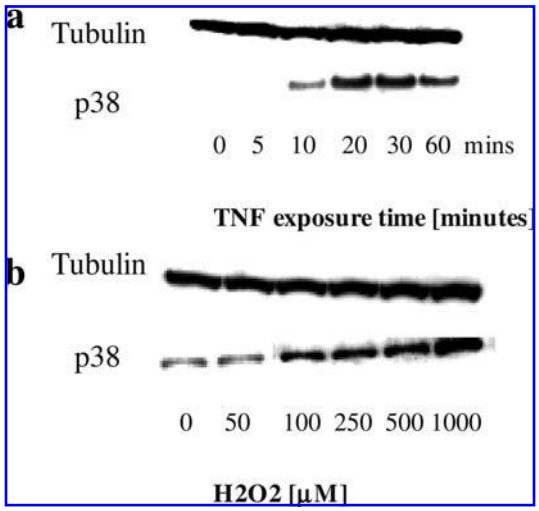

FIG. 2. Influence of TNF and H_2O_2 on phosphorylation of p38 MAPK in A549 cells. Representative western blots show time course of TNF-induced p38 activation (**a**) and concentration-dependent effects of H_2O_2 following 30-min incubations (**b**). Tubulin control is provided for the same representative gels.

Influence of NAC on cytokine-induced NF-κB activation

As we have demonstrated before in the context of IL-1-induced signaling in A549 cells (16), the widely used antioxidant and glutathione precursor NAC (20 m*M*) had no significant effect on TNFα-induced IκBα depletion in these cells. For instance, IκBα levels were reduced to 13 ± 5% and 17 ± 5% of control cells following 15 min of TNFα (10 ng/ml) treatment in the presence and absence of NAC, respectively (Fig. 3). Furthermore, this antioxidant failed to significantly affect the resynthesis of IκBα following TNFα-induced depletion. In the presence of TNFα alone, IκBα levels returned to 78 ± 23% of control cells 2 h after TNFα exposure, and a similar tendency (58 ± 12% of control) was observed in cells treated with TNFα in the presence of NAC (Fig. 3). In addition to effects on IκB kinetics, NAC also failed to influence NF-κB nuclear translocation.

In contrast to its inability to modulate the NF-κB pathway, NAC pretreatment significantly attenuated both H_2O_2- and TNFα-induced p38 activation (14.1 ± 6.7% and 37.5 ± 3.6% of control, respectively; Fig. 4).

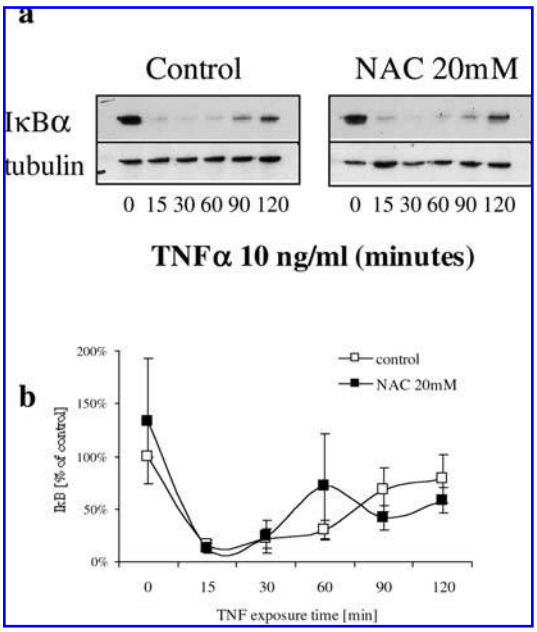

FIG. 3. Influence of the antioxidant NAC (20 m*M*) on cytokine-induced NF-κB activation. Representative western blots (**a**) and summary graphs of the optical density analysis of western blots (**b**) demonstrate the time course of IκBα depletion and resynthesis in A549 cells in response to TNFα (10 ng/ml) treatment in the presence and absence of 20 m*M* NAC.

Influence of inhibitors of oxidant-generating pathways on p38 activation

To explore further the role of cytosolic and mitochondrial redox-sensitive events in MAPK activation, we have investigated the influence of antioxidants, mitochondrial electron transport chain inhibitors, and NADPH oxidase and xanthine oxidase inhibitors, as a pharmacological tool, on H_2O_2- and TNFα-induced p38 activation. The mitochondrial complex I and III inhibitors, rotenone and antimycin A, inhibited the H_2O_2 response (45 ± 25 and 20 ± 10% of control). In some experiments, antimycin A appeared to abolish completely the H_2O_2-induced p38 phosphorylation, and it also had a tendency to affect TNFα action (51 ± 13% of control, Figs. 4 and 5). Allopurinol partially inhibited H_2O_2- but not TNFα-induced p38 activation (71 ± 11% of control for H_2O_2), and the flavoprotein reductase inhibitor, diphenyliodonium, had no effect on p38 phosphorylation by either stimulus (Fig. 4).

Influence of antioxidants on H_2O_2- and TNFα-induced intracellular oxidative stress

The increase in baseline DCF fluorescence over 30 min was significantly augmented by increasing concentrations of H_2O_2 (10–1,000 μ*M*, Fig. 5a), confirming ongoing intracellular oxidative stress. Incubation with NAC reduced both baseline DCF fluorescence and spontaneous increase over 30 min. In addition, the H_2O_2-induced increase in DCF fluorescence was eliminated in the presence of NAC (Fig. 5b versus 5a). In contrast, TNFα had no stimulatory effect on DCF fluorescence under identical conditions (Fig. 5c). We have tested the influence of mitochondrial inhibitors on baseline and H_2O_2- and TNFα-induced oxidative stress. Both antimycin A and rotenone appeared to potentiate DCF fluorescence (Fig. 5d).

Relative roles of NF-κB and p38 activation on gene expression in A549 cells

To elucidate the role of these signal transduction pathways on ICAM-1 and HO-1 gene expression, we first evaluated the ability of TNFα and H_2O_2 to activate these genes, and we then

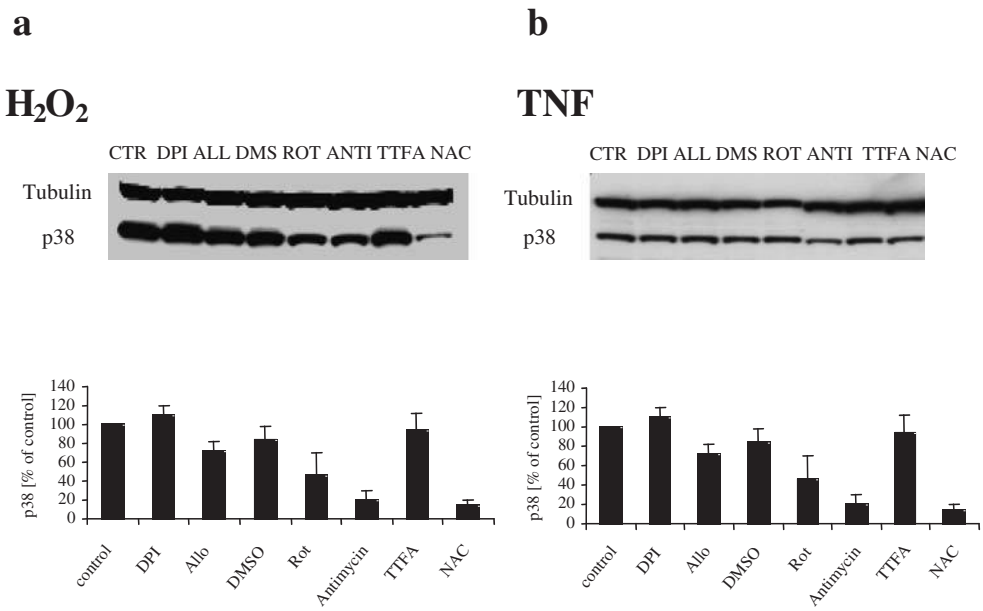

FIG. 4. Effects of antioxidants and redox modulators on H_2O_2- and TNFα-induced p38 phosphorylation in A549 cells. Representative and summary data depict the influence of vehicle [dimethyl sulfoxide (DMSO)], diphenyliodonium (DPI, 10 μ*M*), allopurinol (Allo, 10 μ*M*), rotenone (Rot 10 μ*M*), antimycin A (Anti, 10 μ*M*), thenoyltrifluoroacetone (TTFA, 10 μ*M*), and NAC (20 m*M*) on H_2O_2 (1,000 μ*M*)- (**a**) and TNFα (10 ng/ml)-induced (**b**) p38 activation.

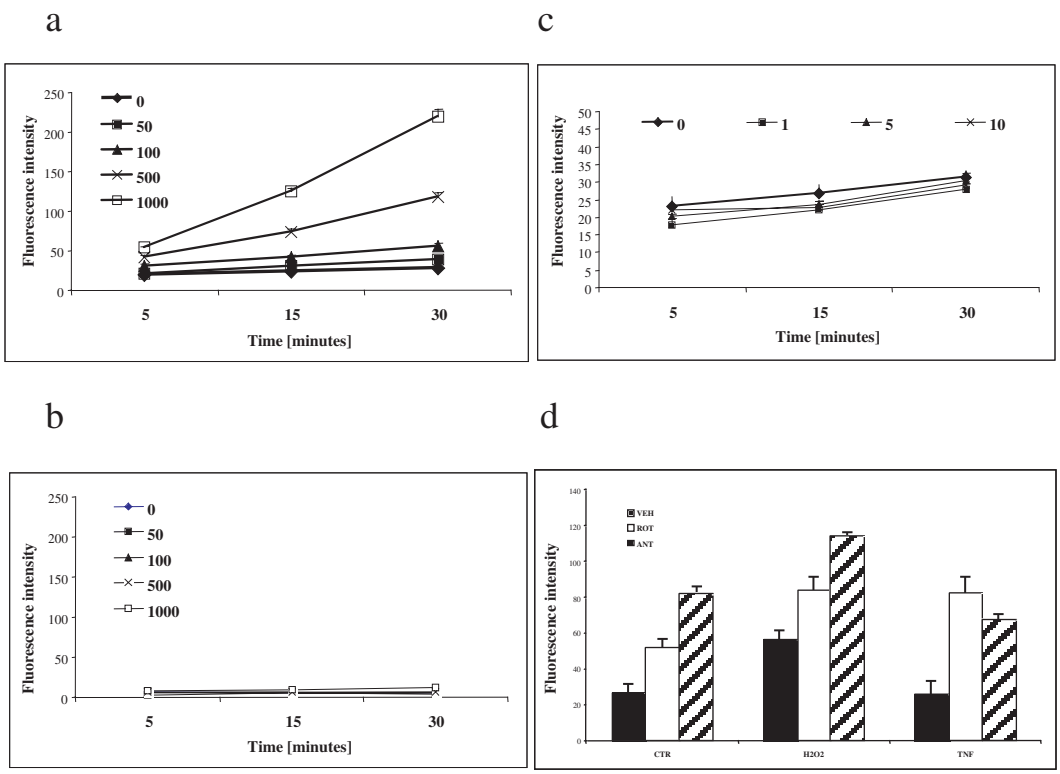

FIG. 5. Effects of NAC (20 m*M*) and mitochondrial inhibitors on H_2O_2- and TNFα-induced intracellular oxidative stress in A549 cells. Graphs depict time- (5–30 min) and concentration- (50–1,000 μ*M*) dependent effects of H_2O_2 on DCF fluorescence intensities in the absence (**a**) and presence (**b**) of NAC. (**c**) Increasing concentrations of TNF (1–10 ng/ml) had no effect on DCF fluorescence. (**d**) Influence of rotenone (ROT 10 μ*M*) and antimycin A (ANT, 10 μ*M*) on DCF fluorescence under basal and TNF- and H_2O_2-stimulated conditions.

explored the influence of NF-κB and p38 inhibitors on gene expression. TNFα, but not H_2O_2, was able to induce ICAM-1 expression in A549 cells following 18 h of stimulation. MG132 significantly attenuated TNFα-induced ICAM-1 induction (Fig. 6a). SB203580, a relatively specific inhibitor of p38 MAPK, however, had no statistically significant influence on TNF-induced ICAM-1 expression over a wide range of TNFα concentrations (Fig. 6b). Although NAC had no influence on NF-κB activation in short-term incubations, it was interesting to explore the influence of NAC on ICAM-1 expression over a longer time course. However, these long incubations with NAC resulted in significant cytotoxicity (Fig. 7b), which prevented assessment of NAC sensitivity of ICAM-1 induction.

There was a low level of constitutive HO-1 expression in A549 cells. The cytokines TNFα and IL-1β failed to stimulate HO-1 expression further in these cells (Fig. 8a). However, hemin, a classical activator of HO-1, increased steady-state levels of HO-1 within the same time frame and identical conditions (444 ± 103% of control). This was mimicked by similar concentrations of hemoglobin (253 ± 80% of control). In the concentration range of 10–500 μ*M*, H_2O_2 did not produce a statistically significant increase in HO-1 expression (69–145% of control). This lack of response was not due to cytotoxicity as only long-term (18 h) treatment with 1 m*M* H_2O_2 exhibited reduction in MTT conversion (Fig 7).

Additional experiments were performed to define further the role of p38 activation in hemin-induced HO-1 activation. First, the influence of the positive stimulus hemin on p38 was explored. As shown in Fig. 9a, H_2O_2, but not hemin, activated

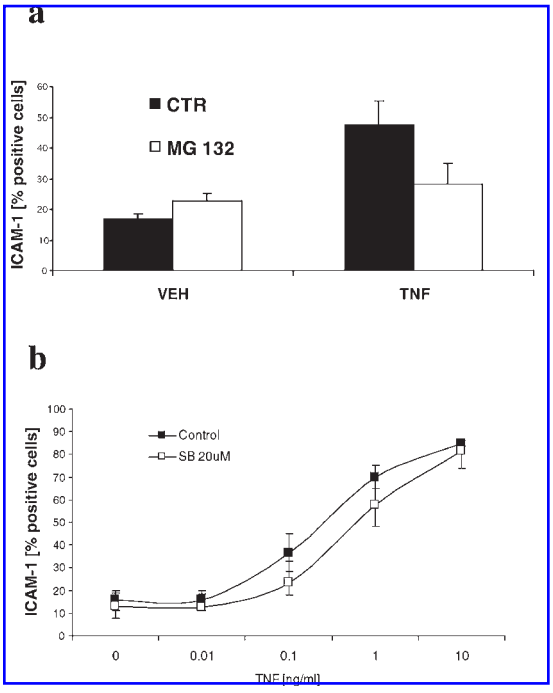

FIG. 6. Influence of NF-κB and p38 MAPK inhibitors on TNFα-induced ICAM-1 expression in A549 cells. (**a**) Effects of MG132 (10 μ*M*) on TNFα (1 ng/ml)-induced ICAM-1 induction. (**b**) Influence of SB203580 (10 μ*M*) on ICAM-1 expression induced by increasing concentrations of TNFα.

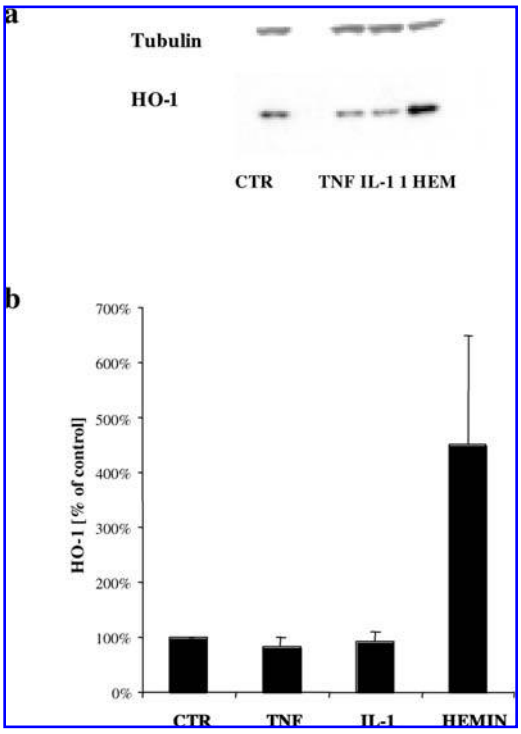

FIG. 7. Influence of cytokines and hemin on HO-1 expression in A549 cells. (**a**) Representative western blot demonstrating HO-1 protein levels following 18 h of treatment with TNFα (10 ng/ml), IL-1 (50 U/ml), and hemin (100 μ*M*, as positive control). (**b**) Summary data of HO-1 western blots normalized to β-tubulin levels.

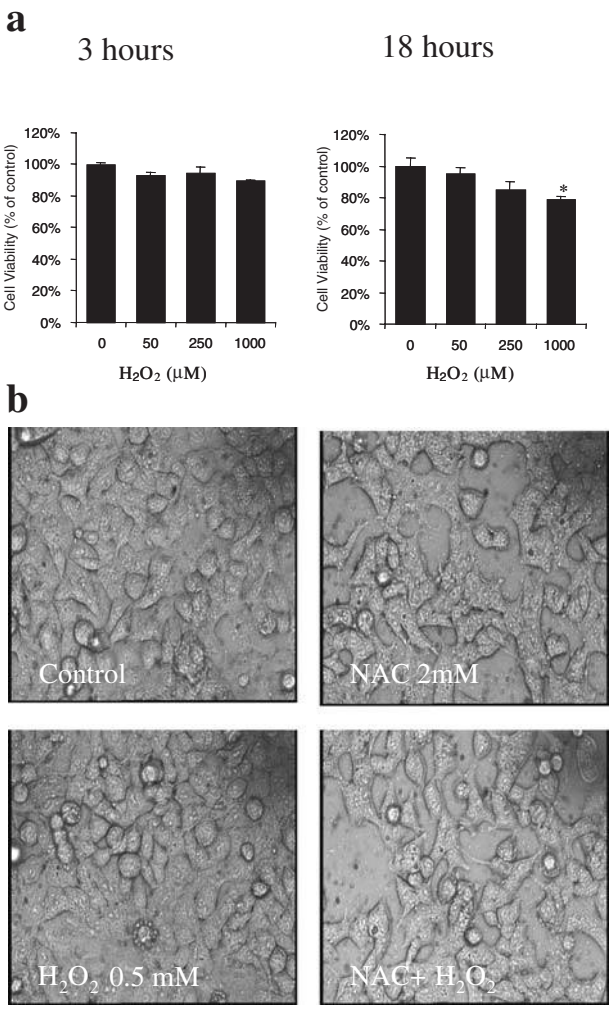

FIG. 8. Influence of H_2O_2 and NAC (20 m*M*) on cell viability. (**a**) Summary data of MTT conversion following 3 and 18 h of treatment of A549 cells with increasing concentrations of H_2O_2. (**b**) Morphological changes as observed with phase-contrast microscopy in cells treated for 18 h with NAC, but not with H_2O_2, at the indicated concentrations.

p38 MAPK following A549 cell stimulation. In addition, the p38 MAPK inhibitor SB203580 did not influence basal and hemin-induced HO-1 expression (Fig. 9b and c).

DISCUSSION

We have explored the redox regulation of p38 MAPK and contrasted it to NF-κB activation in cytokine- and H_2O_2-stimulated human alveolar epithelial cells. We found that these inflammatory stimuli activated these pathways differentially. Firstly, although TNFα activated both signal transduction events, only activation of the p38 pathway exhibited redox sensitivity for inhibition by the antioxidant NAC. Secondly, H_2O_2 only activated the p38 pathway in an NAC-sensitive manner. In addition, our study further explores the relative roles of p38 and NF-κB activation in ICAM-1 and HO-1 induction. The results suggest that activation of p38 is not sufficient for and is unlikely to contribute to activation of either gene, whereas NF-κB is crucial in ICAM-1 expression in A549 cells.

Although redox regulation of gene expression in general has received much recent attention, the detailed mechanisms of redox processes and their relevance to particular gene expression in lung cells remain controversial (6, 16, 18, 29–31). Despite the fact that cytokine- and oxidative stress-induced activation of both the NF-κB and MAPK pathways has been described in human alveolar epithelial cells, our data provide new information on these issues.

It has emerged that NF-κB is one of the primary redox-sensitive transcription factors responsible for inflammatory gene induction (36, 37). This has been largely based on the ability of oxidants to activate this signaling pathway and antioxidants to attenuate NF-κB nuclear translocation, DNA binding, and transactivation. Despite this substantial evidence of NF-κB being the redox switch of inflammatory gene induction, the puzzle regarding redox regulation of NF-κB appears to be far from complete. In particular, a closer analysis of available data highlights major inconsistencies of this paradigm. These include a substantial volume of data against oxidative stress being the universal activator of NF-κB, cell type- and stimulus-dependent responses, reproducibility, and lack of conclusive demonstration of the molecular substrate of redox sensitivity (3, 14, 20, 22).

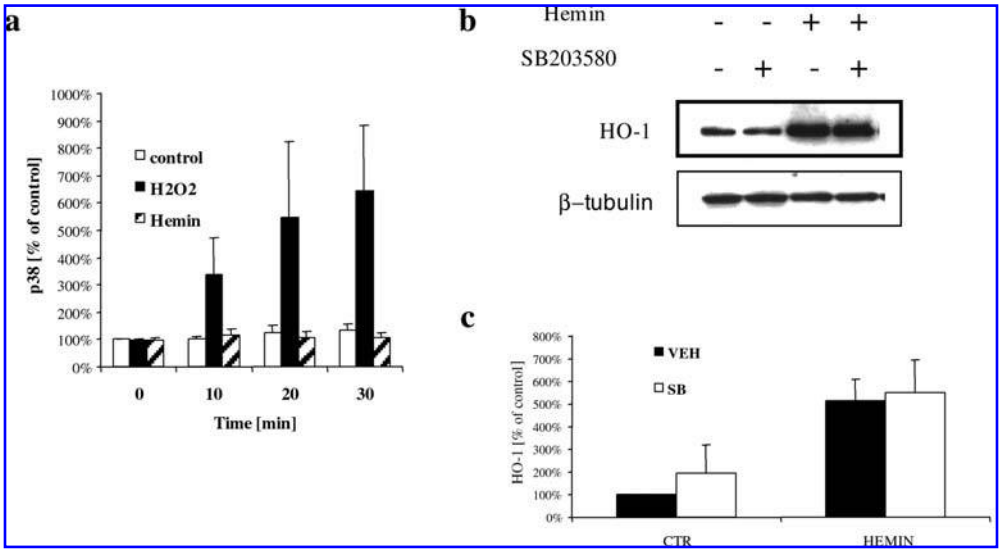

FIG. 9. Role of p38 in hemin-induced HO-1 expression in A549 cells. (**a**) Summary data comparing the effects of H_2O_2 and hemin on time-dependent p38 activation. (**b**) Representative western blot demonstrating HO-1 protein levels in A549 cells, following 18 h of treatment with hemin (100 μ*M*) in the presence and absence of SB203580 (10 μ*M*). (**c**) Summary graph demonstrating optical density analysis of HO-1 western blots normalized to β-tubulin levels.

These controversies are pertinent to signal transduction in A549 cells. Rahman's group has repeatedly demonstrated H_2O_2-induced NF-κB DNA binding, reporter activity and related IL-8 transcription, which was sensitive to distinct thiol-containing antioxidants (1, 28–30). Similarly, adenovirus has been shown to activate NF-κB and gene transcription in A549 cells in an oxidative stress-dependent manner (25). However, others have failed to demonstrate oxidant-induced NF-κB activation in these cells. For instance, Das *et al.* showed no NF-κB DNA binding following exposure to superoxide-generating systems or to reagent H_2O_2 (6). Furthermore, Lakshminarayan *et al.* showed the inability of oxidative stress to activate NF-κB-mediated ICAM-1 expression, whereas the same stimulus readily induced AP-1-mediated IL-8 release in these cells (18, 31). Our own experience regarding oxidative stress-mediated NF-κB activation and endogenous gene induction highlights cell and stimulus specificity of this response. In particular, NAC inhibited TNFα- but not IL-1β-induced E-selectin expression in human pulmonary artery endothelial cells (16, 21). The current study provides relevant information to this issue by demonstrating identical kinetics of IκBα depletion in the presence and absence of NAC in A549 cells. In addition, it appears that NAC had no effect on an endogenous NF-κB-mediated gene induction, namely, IκBα itself, as kinetics of rapid IκBα replenishment, an NF-κB-mediated event, was not altered by NAC. However, the unexpected cytotoxicity of NAC in long-term experiments (which has been reported in other human systems) (42) prevented us from fully investigating the influence of NAC on ICAM-1 expression in A549 cells.

Thus, further studies are needed to resolve the apparent controversy regarding redox regulation of NF-κB in human alveolar epithelial cells. It remains to be determined whether the conflicting results are due to selection of different subclones of the A549 cell line or specific culture conditions and experimental technique. Conclusive identification of the putative redox step in NF-κB activation in these cells would be a major step forward in this debate.

In addition to particular molecular steps within the NF-κB activation pathway, current concepts of inflammatory gene induction offer multiple alternative mechanisms to confer redox sensitivity of particular gene expression. It has been demonstrated that NF-κB nuclear translocation alone may not be a sufficient mechanism to explain inflammatory gene induction. In particular, NF-κB nuclear translocation is usually completed in less than an hour in most systems; however, induction of a cluster of NF-κB-regulated genes requires several hours. Natoli's group came to the fascinating conclusion that the overall presence of NF-κB in the nucleus does not correlate with binding of the transcription factor at individual promoters (34, 35). They have suggested that additional active mechanisms involving acetylation and phosphorylation of histone proteins are required to direct the otherwise available NF-κB to the promoter of selective groups of inflammatory genes.

In addition, the NF-κB transcription factor (particularly the p65 subunit) is required to undergo specific phosphorylation, presumably by a dual MAPK pathway involving MSK-1 (mitogen- and stress-activated protein kinase-1), for effective building of enhanceosome and DNA transactivation (43). Thus, conceptually, the present model of inflammatory gene induction advocates the crucial importance of a dual signaling pathway. On the one hand, the first stimulus is required to activate the NF-κB pathway resulting in nuclear translocation. On the other hand, the same stimulus or cooperating additional factors activate parallel signal transduction pathways that determine chromatin events and effective recruitment of NF-κB to the individual promoter (11, 35). As both histone acetylation by histone acetyltransferases and histone phosphorylation by the postulated p38 MAPK cascade are greatly influenced by

oxidative stress, one could hypothesize that redox sensitivity of inflammatory gene induction might be better explained by these novel mechanisms than by direct redox modulation of the NF-κB pathway (8, 22, 40).

To elucidate better the potential redox regulation of the p38 MAPK in A549 cells, we have investigated p38 activation in response to both TNFα and oxidative stress. Similarly to other systems including lung endothelial cells, both stimuli activated p38 in a concentration- and time-dependent manner. NAC nearly completely abolished H_2O_2-induced p38 activation, and it also attenuated (to a lesser degree) TNFα-induced p38 MAPK activation. The latter mechanism is particularly interesting as under identical conditions the effect of TNFα on NF-κB was not affected. As oxidant-induced activation of p38 was sensitive to NAC, these data would be compatible with an explanation of TNFα also producing oxidative stress, which is modulated by NAC. Our attempt to provide experimental proof for this hypothesis was not successful. In particular, monitoring oxidative stress by DCF fluorescence demonstrated no increased intracellular oxidation of the fluorescent dye in the presence of TNFα, whereas this was evident in response to H_2O_2. Interestingly, others also failed to demonstrate increased intracellular oxidative stress by TNFα in A549 cells. However, there is biochemical evidence that TNFα depletes nearly 50% of intracellular reduced glutathione and increases oxidized glutathione within 1 h of exposure (29).

There are multiple membrane, cytosolic, and mitochondrial mechanisms whereby TNFα and reagent H_2O_2 can alter cellular homeostasis and produce intracellular oxidants. Previous studies have identified the NADPH oxidase system, xanthine oxidase, and the mitochondrial electron transport chain as potential targets of these stimuli and mediators of cellular responses. In our studies, several mitochondrial complex inhibitors appeared to modulate p38 phosphorylation. These findings are interesting, because previous studies have suggested the importance of intracellular amplification mechanisms in oxidant-induced cellular responses. In particular, intact mitochondrial electron chain transport was essential in H_2O_2-induced signaling in myocytes (2). Importantly, the influence of mitochondrial inhibitors on p38 activation seemed to be specific for complex III and, to a lesser degree, complex I inhibitors, because thenoyltrifluoroacetone, a complex II inhibitor, remained without effect following both TNFα and H_2O_2 stimulation. However, we could not confirm that antimycin A and rotenone inhibited intracellular oxidative stress. In fact, both inhibitors augmented DCF fluorescence under both baseline and stimulated conditions. The effect of antimycin is likely related to increased superoxide anion production following inhibition of complex III. Similarly, rotenone might increase oxidative stress by promoting electron flow through the FMN-complex I site (44). However, more precise experiments are required to dissect these issues and to elucidate the exact role of mitochondrial pathways in MAPK activation in A549 cells.

Regardless of the redox-sensitive activation of p38 MAPK, its functional role seems to be limited in A549 cells in the context of TNFα- and oxidative stress-related ICAM-1 and HO-1 expression. Our pharmacological data indicate that NF-κB, but not p38 MAPK, activation is indispensable for ICAM-1 expression in these cells. These data appear to corroborate previous studies regarding molecular mechanisms of ICAM-1 promoter regulation in A549 cells (4, 7, 31).

On the basis of literature evidence for the role of p38 MAPK in HO-1 induction, our working hypothesis was that redox regulation of p38 confers HO-1 induction by TNFα and oxidative stress in A549 cells. However, the obtained data suggest that p38 regulation can be dissociated from HO-1 induction. Firstly, prominent activators of p38, including TNFα, H_2O_2, and IL-1, did not result in detectable HO-1 protein expression within the time frame and conditions of the experiments. Although the reasons for this are not clear, constitutive expression of HO-1 in A549 cells might be a confounding factor. However, classic inducers of HO-1, including hemoglobin and hemin, produced substantial up-regulation of HO-1 under identical conditions. Nevertheless, the positive stimulus failed to induce early p38 activation and a specific inhibitor of p38 MAPK had no effect on hemin-induced protein expression. Altogether, these data suggest that p38 alone is insufficient to confer HO-1 gene induction and p38 activation does not contribute to HO-1 induction by hemin, a classic HO-1 inducer. It thus appears that p38 MAPK does not cooperate with other transcriptional events, such as inactivation of the transcriptional repressor bach1, during hemin-induced HO-1 induction. However, it remains possible that p38 plays some role in HO-1 activation in these cells in response to other classes of inducing agents.

Interestingly, despite the generally positive correlation between p38 MAPK and HO-1 activation in the literature, similar dissociation between MAPK and HO-1 gene expression is not unprecedented (26, 33, 45). For instance, Ryter *et al.* found that MAPK inhibitors, in fact, activated HO-1 in vascular cells, particularly during hypoxic conditions (33). In addition, Masuya and colleagues showed that HO-1 expression by various inducers in HeLa cells was not suppressed by MAPK inhibitors (24). Thus, our conclusion is in agreement with that of Ryter *et al.* advocating the tissue- and inducer-specific nature of the MAPK dependency of HO-1 induction (33).

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ABBREVIATIONS

AP-1, activator protein-1; DCF, 2',7'-dichlorofluorescein; DCFH, 2',7'-dichlorodihydrofluorescein; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; H_2O_2, hydrogen peroxide; HO-1, heme oxygenase-1; ICAM-1, Intercellular adhesion molecule-1; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide; NAC, *N*-acetyl-L-cysteine; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TNFα, tumor necrosis factor-α.

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