ANTIOXIDANTS & REDOX SIGNALING
Volume 5, Number 6, 2003

Forum Original Research Communication

Redox Regulation of Reactive Oxygen Species-Induced p38 MAP Kinase Activation and Barrier Dysfunction in Lung Microvascular Endothelial Cells

PETER V. USATYUK,[1] SURYANARAYANA VEPA,[1] TONYA WATKINS,[1] DONGHONG HE,[1] NARASIMHAM L. PARINANDI,[2] and VISWANATHAN NATARAJAN[1]

ABSTRACT

Reactive oxygen species (ROS)-mediated compromise of endothelial barrier integrity has been implicated in a number of pulmonary disorders, including adult respiratory distress syndrome, pulmonary edema, and vasculitis. The mechanisms by which ROS increase endothelial permeability are unclear. We hypothesized that ROS-induced changes in cellular redox status (thiols) may contribute to endothelial barrier dysfunction. To test this hypothesis, we used *N*-acetylcysteine (NAC) and diamide to modulate intracellular levels of cellular glutathione (GSH) and investigated hydrogen peroxide (H_2O_2)-mediated mitogen-activated protein kinase (MAPK) activation and transendothelial electrical resistance (TER). Exposure of bovine lung microvascular endothelial cells (BLMVECs) to H_2O_2, in a dose- and time-dependent fashion, increased endothelial permeability. Pretreatment of BLMVECs with NAC (5 m*M*) for 1 h resulted in partial attenuation of H_2O_2-induced TER (a measure of increase in permeability) and GSH. Furthermore, treatment of BLMVECs with diamide, which is known to reduce the intracellular GSH, resulted in significant reduction in TER, which was prevented by NAC. To understand further the role of MAPKs in ROS-induced barrier dysfunction, we examined the role of extracellular signal-regulated kinase (ERK) and p38 MAPK on H_2O_2- and diamide-mediated permeability changes. Both H_2O_2 and diamide, in a dose-dependent manner, activated ERK and p38 MAPK in BLMVECs. However, SB203580, an inhibitor of p38 MAPK, but not PD98059, blocked H_2O_2- and diamide-induced TER. Also, NAC prevented H_2O_2- and diamide-induced p38 MAPK, but not ERK activation. These results suggest a role for redox regulation of p38 MAPK in ROS-dependent endothelial barrier dysfunction.
Antioxid. Redox Signal. 5, 723–730.

INTRODUCTION

REACTIVE OXYGEN SPECIES (ROS)-mediated endothelial cell (EC) injury has been implicated in a number of pulmonary disorders, including adult respiratory distress syndrome, pulmonary hypertension, and vasculitis (19, 32, 33). Molecular mechanisms underlying ROS-induced EC injury and permeability changes involve modulation of intracellular calcium, protein kinases, and phosphatases and cytoskeletal remodeling (5, 11, 19, 32, 33). Recent studies strongly suggest that ROS elicit specific EC responses via activation of mitogen-activated protein kinase (MAPK) signaling cascades (18–20, 22, 31, 33). At least four major groups of MAPKs, based on their dual threonine/tyrosine phosphorylation sites, have been characterized in mammalian cells, and extracellular stimuli elicit specific cellular responses through the relative activation of extracellular signal-regulated protein kinases (ERK1/2), big MAPK1, c-Jun N-terminal kinase (JNK), and p38 MAPK (3, 29). The MAPKs are major mediators of signal transducers from the plasma membrane through the cytoplasm to the nucleus of the cell (3, 29). Activation of ERK1/2 by growth factors and phorbol ester is robust

[1]Department of Medicine, Division of Pulmonary and Critical Care, Johns Hopkins University School of Medicine, Baltimore, MD.
[2]Department of Medicine, Ohio State University, Columbus, OH.

compared with JNK and p38 MAPK (31). On the contrary, ROS and hyperosmolar stress are potent stimuli for JNK and p38 MAPK in fibroblasts and ECs (21). However, in rat vascular smooth muscle cells, ROS stimulated big MAPK1, but not ERK1/2 (14). Thus, the specificity of activation of different classes of MAPKs by individual stimulus is dictated by specific agonist for each group of the kinase. One common substrate for the MAPKs is transcriptional factors such as nuclear factor kappa B and AP1, which may induce specific gene expression (12, 13, 20, 34).

In endothelium, ROS, such as hydrogen peroxide (H_2O_2) and diperoxovanadate, activate ERK1/2, JNK, and p38 MAPK (18, 22). The stress-induced stimulation of MAPKs is regulated by intracellular glutathione (GSH)/oxidized glutathione (GSSG) levels, suggesting a role for redox status in modulating MAPK activity (11, 12, 33). As ROS alter EC permeability to macromolecules, we hypothesized that redox changes in ECs regulate ROS-dependent MAPK activation and barrier dysfunction. Here we show that H_2O_2-induced barrier dysfunction in bovine lung microvascular ECs (BLMVECs) is dependent on p38 MAPK, but not ERK1/2. Furthermore, a decrease in intracellular levels of GSH induced by either H_2O_2 or diamide regulates p38 MAPK activation and EC barrier function. These alterations by intracellular redox imbalance were blocked by pretreatment of the cells with *N*-acetylcysteine (NAC).

MATERIALS AND METHODS

Reagents

BLMVECs were purchased from Clonetics (San Diego, CA, U.S.A.). H_2O_2, NAC, diamide, EC growth factor, minimum essential medium (MEM), fetal bovine serum, and nonessential amino acids were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PD98059 and SB203580 were purchased from Calbiochem (La Jolla, CA, U.S.A.). Polyclonal ERK1/2, p38 MAPK, phospho-specific ERK1/2, and p38 MAPK antibodies were procured from Cell Signaling (Boston, MA, U.S.A.). Secondary anti-rabbit or anti-mouse Ig (H+L) horseradish peroxidase conjugates were obtained from Bio-Rad (Hercules, CA, U.S.A.). Enhanced chemiluminescence kit was from Amersham (Arlington Heights, IL, U.S.A.). Polyvinylidene difluoride immobilon–P transfer membrane was purchased from Millipore, U.K.

Cell culture

BLMVECs were cultured in MEM supplemented with 10% fetal bovine serum, antibiotics, and growth factors as described previously (28). Confluent cells in T-75 cm^2 flasks were trypsinized and subcultured in 35-mm dishes; the cells showed cobblestone morphology and stained positive for factor VIII-related antigen. All experiments were carried out between 5 and 9 passages.

Measurement of EC permeability by albumin flux

Albumin permeability across EC monolayers was performed as previously described (30). In brief, the system consisted of two compartments, upper (luminal) and lower (abluminal), which were separated by a polycarbonate microporemembrane filter (Nuclepore, Pleasanton, CA, U.S.A.). Medium 199 with 25 m*M* HEPES was used in both compartments. BLMVECs grown on polycarbonate membranes were pretreated for 1 h with PD98059 (25 μ*M*) or SB203580 (25 μ*M*) followed by addition of H_2O_2 (100 μ*M*). Clearance of albumin (4% final concentration) coupled to Evans blue dye across cell monolayers was determined for 2 h. Transendothelial cell albumin transport was determined by measuring the absorbance of Evans blue dye in abluminal chamber samples at 620 nm in a spectrophotometer (Vmax Multiplate Reader, Molecular Devices, Menlo Park, CA, U.S.A.).

Measurement of transendothelial electrical resistance (TER) as an index of barrier dysfunction

BLMVECs were seeded on gelatin-coated gold electrodes (eight wells, one electrode per well) to ~95% confluence as described earlier (30). Electrodes were placed into an electrical cell-substrate impedance sensing system (ECIS; Applied Biophysics Inc., Troy, NY, U.S.A.) incubator for 1 h to stabilize basal electrical resistance before pretreatment with NAC or MAPK inhibitors or H_2O_2 as indicated. The total endothelial electrical resistance, as measured across the EC monolayers, was determined by the combined resistance between the basal and/or cell matrix adhesion (8, 9). Measurements were done in triplicate and expressed as normalized resistance (means ± SD) for each of the treatments.

Quantification of endothelial GSH and GSSG

BLMVEC GSH and GSSG levels were determined by earlier published procedures using sulfosalicylic acid-dependent color reaction with GSH in cell lysates (24). Absorbance at 412 nm against appropriate blanks was carried out on a Beckman DU-650 spectrophotometer.

Modulation of intracellular GSH

Lung microvascular EC GSH levels were modified by treating with thiol-depleting or thiol-generating agents. Diamide, a potent cell-permeable oxidizing agent, preferentially oxidizes low-molecular-weight free thiols (GSH) and protein-SH groups and induces formation of disulfide bonds and disulfide cross-links in proteins (24), whereas buthionine sulfoximine (BSO) is a specific and irreversible inhibitor of γ-glutamylcysteine synthase. These inhibitors cause a marked decrease in total intracellular GSH pool by different action. NAC, on the contrary, provides cysteine to cells, thereby enhancing intracellular GSH (4, 38). Thiol depletion in BLMVECs was carried out by exposing cells to varying concentrations of diamide for 1 or 2 h. Cells were treated with NAC (5 m*M*) for 1 h and washed with MEM before exposure to H_2O_2.

Treatment of cells with agents, preparation of cell lysates, and western blotting

BLMVECs grown on 100-mm dishes to ~95% confluence were pretreated with NAC, diamide, or MAPK inhibitors in

MEM for specified time periods, as indicated before stimulation with H_2O_2 or other agents. The cells were washed once in ice-cold phosphate-bufferedsaline containing 1 m*M* orthovanadate, scraped into 0.5 ml of lysis buffer (20 m*M* Tris-HCl buffer, pH 7.4, containing 0.5% deoxycholate, 0.5% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% Nonidet P-40, 1 m*M* sodium orthovanadate, and protease inhibitor cocktail). The samples were sonicated three times for 15 s each with a probe sonicator and centrifuged at 5,000 *g* for 5 min at 4°C. An aliquot of the supernatant was used for total protein determination by the BCA protein assay (Pierce Chemicals). Another aliquot of the supernatant was mixed with 6× Laemmli buffer to give a final protein concentration of 1 μg/μl and was subjected to SDS– polyacrylamide gel electrophoresis (PAGE) on 10% gels. After SDS-PAGE, the proteins were transferred to Immobilon-P membranes by electroblotting, blocked for 1 h with TBST (Tris-buffered saline with 0.1% Tween 20) containing 5% bovine serum albumin and incubated for 18–24 h at 4°C with either ERK1/2 (1:2,000 dilution), p38 MAPK (1:3,000 dilution), phospho-ERK1/2 or phospho-p38 MAPK (1:1,000 dilution), or phospho-ATF-2 (ATF-2, activating transcription factor-2) (1:1,000 dilution) antibodies. The membranes were washed at least three times with TBST, followed by incubation with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (1:2,000 dilution) for 2–4 h at room temperature, and the blots were developed with enhanced chemiluminescence. Densitometry of the blots was carried out with a scanner and quantified by Image analyzer.

Statistical analysis

All values are expressed as means ± SD from triplicate samples and three independent experiments. Data were subjected to one-way ANOVA, and pairwise multiple comparisons were done by Dunnett's method. A p value of <0.05 was considered significant.

RESULTS

H_2O_2 decreases TER

Measurement of TER generated across EC monolayers under normal and stimulated conditions serves as a reliable and sensitive method to determine barrier function (8, 9). To determine the effect of H_2O_2 on barrier function, BLMVECs grown on gold electrodes were stimulated with varying concentrations of H_2O_2, which decreased TER in a dose-dependent fashion (vehicle, resistance normalized to 1; 25 μ*M* H_2O_2, 0.88 ± 0.2; 50 μ*M* H_2O_2, 0.64 ± 0.19; 100 μ*M* H_2O_2, 0.51 ± 0.29 after 1 h of treatment) (Fig. 1). At lower doses of H_2O_2, the decrease in TER was partially reversible with prolonged time periods (Fig. 1). These results show that H_2O_2, in a dose- and time-dependent fashion, causes barrier dysfunction in lung microvascular EC monolayers.

Redox imbalance contributes to H_2O_2-induced barrier dysfunction

As ROS alter redox status of ECs (19, 32, 33, 38), we investigated the role of cellular GSH on barrier dysfunction.

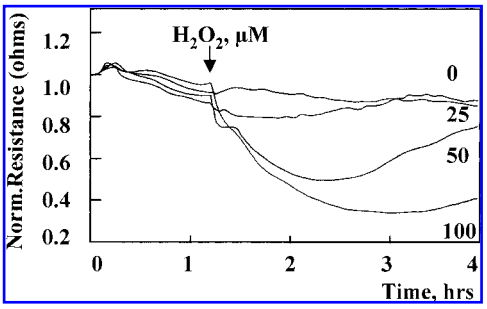

FIG. 1. H_2O_2 induces endothelial barrier dysfunction. BLMVECs grown on gold microelectrodes to ~95% confluence were challenged with varying concentrations of H_2O_2, TER was measured as described in Materials and Methods. Shown is a representative tracing from three independent experiments in duplicate.

Treatment of BLMVECs with H_2O_2 (100 μ*M*) decreased intracellular GSH by ~20% compared with control cells (vehicle, 4.1 ± 0.15 μg/mg of protein; H_2O_2, 3.2 ± 0.1 μg/mg of protein), and preincubation of cells with NAC enhanced cellular GSH in the absence or presence of H_2O_2 (NAC alone, 11.6 ± 0.4 μg/mg of protein; NAC + H_2O_2, 9.1 ± 0.3 μg/mg of protein) (Fig. 2). To determine the relative contribution of cellular GSH levels on barrier function, BLMVECs were pretreated with 5 m*M* NAC before exposure to H_2O_2. As shown in Fig. 3, NAC prevented the H_2O_2–induced changes in TER in a time-dependent manner. These data suggest a role for cellular GSH in regulating ROS-mediated barrier dysfunction in ECs.

Depletion of intracellular GSH by diamide causes EC barrier dysfunction

To establish further a direct correlation between changes in cellular GSH and barrier dysfunction, we evaluated the effect of diamide, an agent that oxidizes GSH to GSSG and –SH

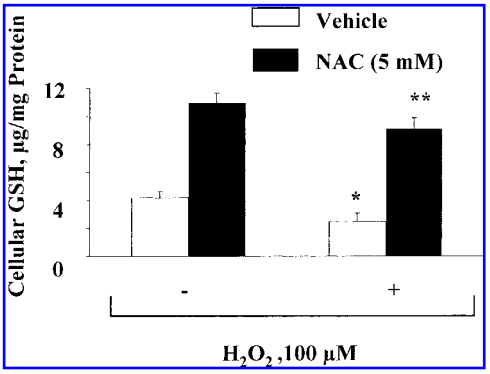

FIG. 2. NAC prevented H_2O_2-induced reduction in cellular GSH. BLMVECs were pretreated with NAC (5 m*M*) for 1 h and then challenged with H_2O_2 (100 μ*M*) for 1 h. GSH level in cell lysates was determined as described in Materials and Methods. n = 3. *Significantly different from vehicle ($p <$ 0.05); **significantly different from NAC treatment ($p < 0.05$).

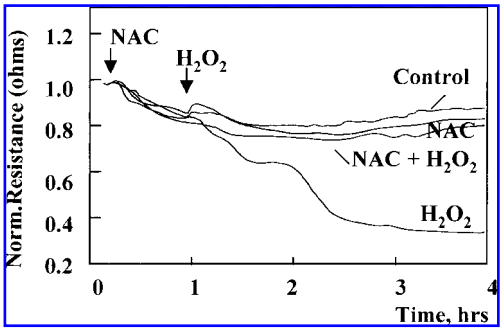

FIG. 3. NAC attenuates H_2O_2-induced endothelial barrier dysfunction. BLMVECs grown on gold microelectrodes to ~95% confluence were pretreated with NAC (5 m*M*) for 1 h and then challenged with H_2O_2 (100 μ*M*), and TER was measured as described in Materials and Methods. Shown is a representative tracing from three independent experiments in duplicate.

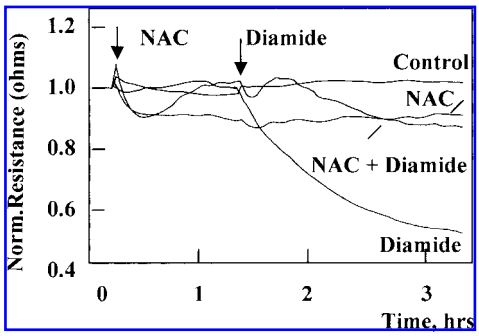

FIG. 5. NAC attenuates diamide-induced endothelial barrier dysfunction. BLMVECs grown on gold microelectrodes to ~95% confluence were pretreated with NAC (5 m*M*) for 1 h and then were challenged with diamide (100 μ*M*), and TER was measured as described in Materials and Methods. Shown is a representative tracing from three independent experiments in duplicate.

groups in proteins to –S-S- linkage (20). Exposure of BLMVECs to diamide (100 μ*M*) for 1 h decreased cellular GSH content by 40% compared with control cells (vehicle, 3.8 ± 0.2 μg/mg of protein; diamide, 2.3 ± 0.1 μg/mg of protein) (Fig. 4). Pretreating the cells with NAC (5 m*M*) for 1 h elevated GSH levels in both control and diamide-challenged cells by 2.5-fold and 1.8-fold, respectively, compared with cells not treated with NAC (NAC, 9.5 ± 0.3 μg/mg of protein; NAC + diamide, 6.9 ± 0.2 μg/mg of protein) (Fig. 4). Next we investigated the effect of diamide and NAC plus diamide on EC barrier function. Diamide (25–100 μ*M*) treatment of BLMVECs decreased TER, an index of monolayer permeability (Fig. 5). This effect of diamide on TER was partially prevented by pretreatment of cells with NAC (5 m*M*), which elevated intracellular GSH content (Fig. 4). These results demonstrate that depletion of cellular GSH by a chemical agent such as diamide resulted in EC barrier dysfunction similar to that of H_2O_2, which was reversed by NAC, suggesting redox regulation of permeability changes in microvascular ECs.

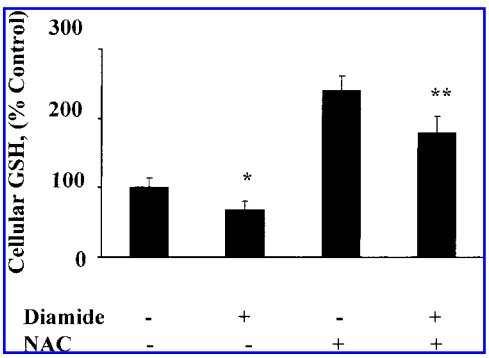

FIG. 4. NAC prevents diamide-induced reduction in cellular GSH. BLMVECs were pretreated with NAC (5 m*M*) for 1 h and then challenged with diamide (100 μ*M*) for 1 h. GSH level in cell lysates was determined as described in Materials and Methods. $n = 3$. *Significantly different from vehicle ($p < 0.05$); **significantly different from NAC treatment ($p < 0.05$).

H_2O_2 and diamide activate ERK and p38 MAPK in microvascular ECs

To investigate signal transduction pathways regulating barrier dysfunction, BLMVECs were treated with H_2O_2 or diamide, and total cell lysates were analyzed by western blotting for phosphorylation of ERK and p38 MAPK with phospho-specific antibodies. As shown in Fig. 6, both H_2O_2 and diamide, in a dose-dependent manner, enhanced phosphorylation of threonine/tyrosine residues of ERK1/2 and p38 MAPK.

H_2O_2- and diamide-induced barrier dysfunction requires p38 MAPK activation

To elucidate further the role of MAPKs in mediating H_2O_2- and diamide–induced barrier dysfunction, we used selective and known pharmacological inhibitors of ERK1/2 and p38 MAPK. As shown in Fig. 7, pretreatment of BLMVECs with PD98059 (25 μ*M*), a specific inhibitor of MEK1/2 (6), did not affect H_2O_2-induced increase in albumin flux. In contrast, pretreatment of ECs with SB203580 (25 μ*M*), a selective blocker of p38 MAPK (17), partially prevented H_2O_2- or diamide-induced permeability changes as measured by enhanced albumin flux across the ECs (Fig. 7) or TER (Fig. 8). The effect of SB203580 on H_2O_2- or diamide-induced barrier dysfunction was time-dependent (Fig. 8). In parallel experiments, the effect on SB203580 on H_2O_2- or diamide-induced p38 MAPK activation was examined. As shown in Fig. 9, SB203580 attenuated p38 MAPK activity as determined by phosphorylation of ATF-2 and had no effect phosphorylation of p38 MAPK because SB203580 directly binds to p38 MAPK and alters its kinase activity (17). These data suggest that p38 MAPK activation is part of the signaling cascade involved in H_2O_2- or diamide-induced barrier dysfunction.

NAC attenuates H_2O_2- and diamide-induced p38 MAPK activation

To determine further if activation of p38 MAPK is under redox regulation in lung microvascular ECs, we studied the

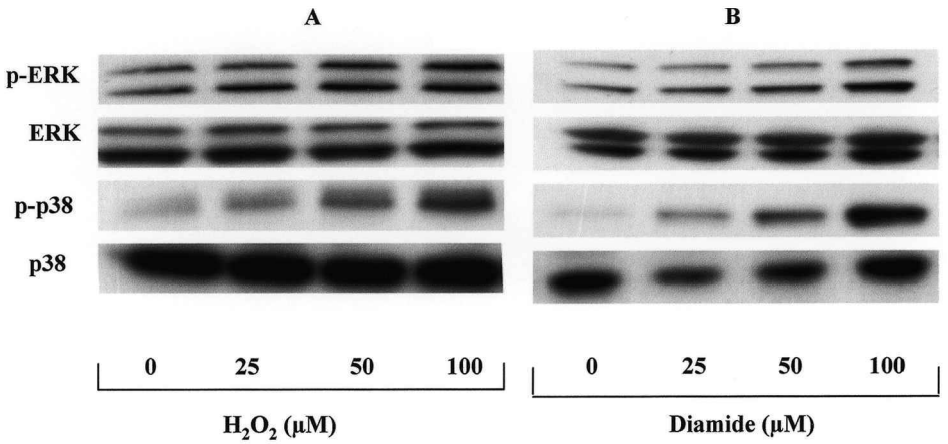

FIG. 6. Effects of H_2O_2 and diamide on phosphorylation of MAPKs. BLMVECs grown to confluence in 100-mm dishes were treated with different concentrations of (**A**) H_2O_2 or (**B**) diamide for 1 h. Cell lysates (20–40 μg of protein) were subjected to 10% SDS-PAGE and probed with anti-phospho-ERK or phospho-p38, or total ERK and p38 antibodies as described under Materials and Methods.

effect of NAC on H_2O_2- and diamide-induced MAPK phosphorylation. As shown in Fig. 10, pretreatment of BLMVECs with NAC (5 m*M*) for 1 h attenuated the H_2O_2- or diamide-induced p38 MAPK phosphorylation and ATF-2 phosphorylation. These results indicate that NAC negatively regulated p38 MAPK activation by H_2O_2 or diamide by increasing intracellular levels of GSH.

DISCUSSION

In the present study, we examined signal transduction pathways that regulate barrier dysfunction by ROS in lung microvascular ECs. The results show that (a) H_2O_2-induced EC barrier dysfunction is dependent on depletion of intracellular GSH levels; (b) H_2O_2-induced barrier dysfunction is regulated by the p38 MAPK signaling pathway; (c) diamide, an agent that oxidizes GSH, also altered EC permeability via p38 MAPK; and (d) NAC prevented H_2O_2- and diamide-induced p38 MAPK activation and barrier dysfunction. These results indicate that redox regulation by intracellular GSH plays a crucial role in ROS-mediated activation of MAPK and barrier dysfunction in lung microvascular ECs.

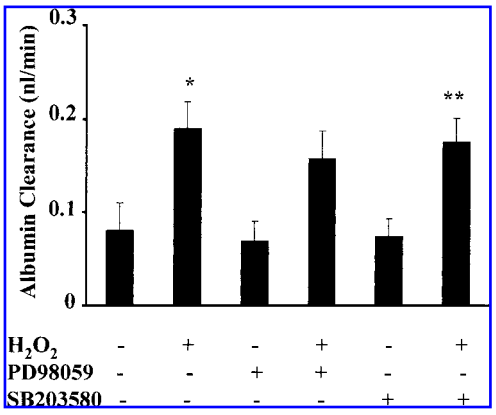

FIG. 7. Effect of MAPK inhibitors on H_2O_2-induced EC permeability changes. BLMVECs grown on polycarbonate membranes were pretreated for 1 h with PD98059 (25 μ*M*) or SB203580 (25 μ*M*), followed by addition of H_2O_2 (100 μ*M*). Clearance of albumin coupled to Evans blue dye across cell monolayers was determined for 2 h. Values are means ± SE of three independent experiments. *Significantly different from vehicle ($p < 0.05$); **significantly different from H_2O_2 treatment ($p < 0.005$).

The mechanisms of ROS-induced permeability changes in ECs have not been completely defined. Earlier studies suggest that ROS-mediated permeability changes in ECs involve increase of intracellular calcium, activation of protein kinase C, Src kinase, and myosin light chain kinase (5, 11, 30, 33, 35). A novel finding of the present study is the role of p38 MAPK activation regulating ROS-induced barrier dysfunction in lung microvascular ECs. Furthermore, NAC pretreatment blocked p38 MAPK, but not ERK1/2 phosphorylation and barrier dysfunction, implicating redox regulation of p38 MAPK and endothelial permeability changes by intracellular GSH levels modulated by oxidative stress. p38 MAPK belongs to a subfamily of mammalian MAPKs that also includes ERK1/2, JNK/stress-activated protein kinases, and ERK5. Activation of p38 MAPK by stimuli is mediated by phosphorylation of threonine/tyrosine residues catalyzed by distinct and dual specific serine/threonine MAPKs (MKK3, MKK4, and MMK6), which in turn are phosphorylated by upstream MKK kinases (3). Our study is consistent with the earlier reports that showed a link between depletion of cellular GSH, modulation of MAPKs, and altered cytokine expression and secretion (26). Tumor necrosis factor-α induced p38 MAPK activation, and p38 MAPK-mediated interleukin-8 secretion in human pulmonary vascular ECs was attenuated by NAC, indicating redox regulation of the tumor necrosis factor-α effect (27). Similarly, thiol depletion in lung fibroblasts induced oxidant accumulation and apoptosis (1). Thiol depletion in lung fibroblasts also induced leukotriene synthesis and p38 MAPK phosphorylation, which were essential for

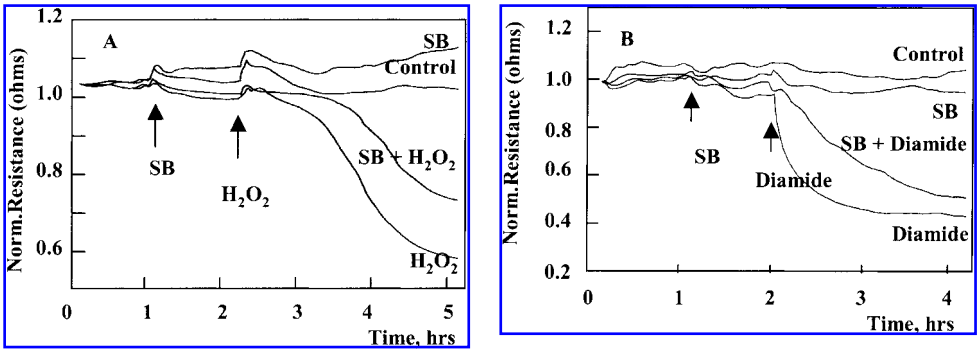

FIG. 8. MAPK inhibitor, SB203580, attenuates H_2O_2- and diamide-induced endothelial barrier dysfunction. BLMVECs grown on gold microelectrodes to ~95% confluence were pretreated with SB203580 (25 μ*M*) for 1 h and then challenged with (**A**) H_2O_2 (100 μ*M*) or (**B**) diamide (100 μ*M*) and TER was measured as described in Materials and Methods. Shown is a representative tracing from three independent experiments in duplicate.

induction of apoptosis (1). We have demonstrated earlier a role for intracellular thiols in ROS-induced phospholipase D (PLD) activation in bovine pulmonary artery ECs, which was confirmed by depletion of intracellular GSH by diamide and BSO. In these studies, NAC not only reversed ROS-induced PLD activation, but also attenuated diamide-mediated PLD stimulation and tyrosine phosphorylation of proteins (24). The ability of NAC to inhibit different MAPKs is varied. In rat cardiac fibroblasts, NAC and nitric oxide blocked angiotensin II-mediated activation of ERK1/2, consistent with redox regulation of ERK (36). In the present study, although H_2O_2 and diamide enhanced phosphorylation of ERK1/2, and p38 MAPK, pretreatment of BLMVECs with NAC blocked p38 MAPK, but not ERK 1/2, with both treatments. These results further suggest that in BLMVECs the MAPKs are differently regulated by redox status of the cell.

How p38 activation by ROS regulates endothelial barrier function is unclear. Possible mechanism(s) include the involvement of heat shock protein (HSP) 27, cytoskeleton, focal adhesion plaques, and adherens junction proteins. HSP 27 is closely associated with the regulation of actin polymerization (15). Phosphorylation of HSP 27 is mediated by the mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK-2), an immediate downstream target of p38 MAPK (23). As ROS-mediated activation of p38 MAPK involves MKK3/6 and MAPK phosphatases-1 (2), it should be of interest to determine which of these enzymes is redox-regulated. In addition to HSP 27, p38 MAPK may be involved in phosphorylation of caveolin-1 at tyrosine 14 residues via Src kinase. Phosphorylation of caveolin-1 is known to regulate changes in cell shape and reorganization of cytoskeleton (16). Focal adhesions are major cellular sites of nonreceptor tyrosine kinase-mediated signal transduction, and as phospho-caveolin-1 is localized in close proximity to focal adhesions, the signaling pathway of p38 MAPK → cSrc → phospho-cave-

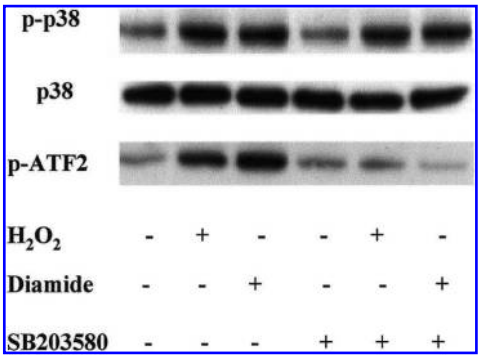

FIG. 9. MAPK inhibitor, SB203580, attenuates H_2O_2- and diamide-induced p38 MAPK activity. BLMVECs grown to confluence in 100-mm dishes were pretreated with SB203580 (25 μ*M*) for 1 h and then challenged with H_2O_2 (100 μ*M*) or diamide (100 μ*M*) for 1 h. Cell lysates (40 μg of protein) were subjected to 10% SDS-PAGE and probed with phospho-p38 MAPKs, total p38 MAPKs, or phospho-ATF-2 antibodies as described under Materials and Methods.

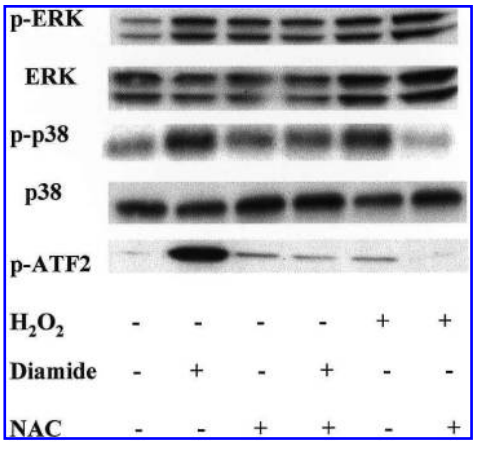

FIG. 10. NAC attenuates H_2O_2- and diamide-induced p38 MAPK activation. BLMVECs grown to confluence in 100-mm dishes were pretreated with NAC (5 m*M*) for 1 h and then challenged with H_2O_2 (100 μ*M*) or diamide (100 μ*M*) for 1 h. Cell lysates (20–40 μg of protein) were subjected to 10% SDS-PAGE and probed with phospho-ERK or total ERK, and phospho-p38 MAPK or phospho-ATF-2 antibodies as described under Materials and Methods.

olin-1 in part may be involved in ROS-induced cell shape, cytoskeletal reorganization, and barrier disruption. Experiments are currently under way to elucidate further the mechanism(s) of p38 MAPK in ROS-induced reorganization of actin cytoskeleton and barrier dysfunction.

In summary, we have shown that H_2O_2 treatment of lung microvascular ECs results in the activation of p38 MAPK and permeability changes as measured by transendothelial resistance measurement. Increased phosphorylation of p38 MAPK and barrier dysfunction by H_2O_2 were prevented by pretreatment of cells with NAC, an agent that modulates intracellular levels of GSH and cellular thiols. Furthermore, diamide mimicked the action of H_2O_2 in reducing intracellular GSH, increasing p38 MAPK phosphorylation, and decreasing endothelial electrical resistance, demonstrating an important role for redox regulation of barrier function in the endothelium.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants HL 57260 and HL 69909 to V.N.

ABBREVIATIONS

ATF-2, activating transcription factor-2; BLMVEC, bovine lung microvascular endothelial cell; BSO, buthionine sulfoximine; EC, endothelial cell; ERK, extracellular signal-regulated kinase; GSH, glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; HSP, heat shock protein; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEM, minimal essential medium; NAC, *N*-acetylcysteine; PAGE, polyacrylamide gel electrophoresis; PLD, phospholipase D; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline with Tween 20; TER, transendothelial electrical resistance.

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Address reprint requests to:
Dr. V. Natarajan, Ph.D.
Department of Medicine
Division of Pulmonary and Critical Care Medicine
Johns Hopkins University School of Medicine
Mason F. Lord Building,
Center Tower, Room 683
5200 Eastern Avenue
Baltimore, MD 21224

E-mail: vnataraj@jhmi.edu

Received for publication July 11, 2003; accepted August 1, 2003.

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