Mitogen-activated protein kinases mediate peroxynitrite-induced cell death in human bronchial epithelial cells

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Submitted 6 June 2002; accepted in final form 13 February 2003

Peroxynitrite (ONOO⁻) is a potent oxidant generated by the rapid nonenzymatic interaction between nitric oxide (NO⁻) and the superoxide anion radical (O₂⁻), both of which are generated during inflammatory stress (10). Overproduction of ONOO⁻ causes oxidative damage to proteins, lipids, DNA, and carbohydrates (6). One of the major protein modifications induced by ONOO⁻ is the nitration of tyrosine residues on a variety of proteins to form the stable product 3-nitrotyrosine that may lead to a change of protein or enzyme function (8, 11, 44). Moreover, 3-nitrotyrosine formation has been found in the lungs, bronchoalveolar lavage fluid, and exhaled breath taken from patients with acute respiratory distress syndrome and asthma (16, 19, 25, 38, 41). However, the functional outcome of ONOO⁻ on human respiratory tract cells is so far unknown. Recent evidence suggests that ONOO⁻ can induce apoptosis in pulmonary cells, but the mechanism of action has not yet been identified (12).

The mitogen-activated protein kinase (MAPK) pathways are reported to be involved in signaling pathways induced by NO⁻ and O₂⁻ (13, 20). MAPKs are activated in response to mitogenic stimuli and environmental stresses and can influence diverse cell functions such as proliferation, cell cycle arrest, differentiation, and apoptosis (5, 27, 45). Three distinct MAPKs have been identified in mammalian cells: extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK)/stress-activated protein kinase, and p38 MAPK. MAPKs are activated upon phosphorylation on both tyrosine and threonine residues by the dual-function kinase MAPKKK or mitogen-activated extracellular-regulated protein kinase kinase (MEK). Recently, it has been reported that MAPKs are activated in various cell lines by the administration of peroxynitrite (21, 40, 48). These results suggest that MAPKs may also mediate signal transduction pathways induced by ONOO⁻ leading to the modulation of gene expression.

The present study examined the effects of 3-morpholinosydnonimine (5,10-diamino-3-morpholinosydnonimine, SIN-1), a peroxynitrite generator, on the human bronchial epithelial cell line BEAS-2B, were examined. SIN-1 exposure resulted in cell death in a time- and dose-dependent manner. Depletion of intracellular glutathione increased the vulnerability of the cells. Pretreatment with Mn(III)tetrakis(N-methyl-4-pyridyl)porphyrin (MnTMPyP) or hydroxocobalamin (HC), O₂⁻ and NO⁻ scavengers, respectively, reduced significantly SIN-1-induced cell death (18.66 ± 3.57 vs. 77.01 ± 14.07 or 82.20 ± 9.64, % cell viability SIN-1 vs. MnTMPyP or HC). Moreover, the mitogen-activated protein kinases (MAPK) p44/42 (ERK), p38, and p54/46 (JNK) were also activated in a time- and concentration-dependent manner. PD-98059 and SB-239063, specific inhibitors of ERK and p38 MAPK pathways, failed to protect cells against 1 mM SIN-1. However, PD-98059 partially inhibited (60% cell survival) SIN-1 effects at 0.25 mM, and this was increased with the inclusion of SB-239063. Therefore, MAPKs may mediate signal transduction pathways induced by peroxynitrite in lung epithelial cells leading to cell death.

BEAS-2B cells; superoxide; nitric oxide; nitrosative stress; 3-morpholinosydnonimine

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ERK, p38, and JNK were purchased from New England Biolabs (Hitchin, UK). Monoclonal antinitrotyrosine antibody was purchased from Calbiochem (La Jolla, CA). Enhanced chemiluminescence (ECL) reagent and Hybond-ECL nitrocellulose were obtained from Amersham (Little Chalfont, UK). 4–12% Bis-Tris SDS-PAGE gels and buffers were purchased from Novex (San Diego, CA). t-Buthionine-[S,R]-sulfoximine (BSO), hydroxocobalamin (vitamin B12a), and other reagents were purchased from Sigma Chemical (Poole, UK). Trans-1-(4-hydroxycyclohexyl)-1-(4-fluorophenyl)-5-(2-methoxypryridimidin-4-yl)imidazol (SB-239063) was a kind gift from GlaxoSmithKline Laboratories (Stevenage, UK).

Cell culture. The transformed human bronchial epithelial cell line BEAS-2B was grown in K-SFM supplemented with 25 mg of BPE and 2.5 μg of EGF. Cells were cultured at 37°C in a humidified atmosphere containing 5% (vol/vol) CO2 in air. Cells were growth factor starved for 24 h before treatment with SIN-1 and/or MnTMPyP (1–20 μM), hydroxocobalamin (0.1–1 mM), PD-98059 (10–100 μM), and SB-239063 (0.1–10 μM). In some experimental conditions, BSO, an inhibitor of glutathione (GSH) synthesis, was supplemented to media (1 mM) 24 h before exposure to the stipulated reagents.

Cell viability. Cell viability was quantified by CCK-8 assay according to the manufacturer’s instructions. In brief, cells were seeded in 96-well plates and then incubated in K-SFM for 24 h with or without 1 mM BSO. Cells were treated with reagents from 8 to 48 h in complete media or K-SFM, and 10 μl of CCK-8 reagent were added to each well. Plates were incubated from 30 min to 2 h at 37°C, and the difference in absorbance between 450 and 600 nm was measured as an indicator of cell viability. Control cells were treated in the same way with diluents when necessary, and the value of different absorbance was defined as 100% survival.

Measurement of ONOO− generation. ONOO− generation from SIN-1 was measured essentially by the method of Haddad et al. (14), whereby production of ONOO− was assessed by oxidation of dihydrorhodamine 123 to rhodamine, and absorbance was measured at 500 nm.

Western blotting. Cells treated with reagents in six-well plates were scraped into 1 ml of Hanks’ balanced salt solution and centrifuged at 1,000 g for 5 min. The cell pellets were lysed with 10 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 1% (vol/vol) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin (lysis buffer) for 30 min in ice. The lysed cells were then centrifuged at 16,000 g for 15 min. The protein concentration of the resulting supernatant was determined by the Bio-Rad protein assay system according to the manufacturer’s instructions.

Equal amounts of protein (20–80 μg) were resolved with 4–12% precast Bis-Tris gels. After transferring the proteins onto a nitrocellulose membrane, we blocked the membranes with 5% (wt/vol) nonfat milk in phosphate-buffered saline solution containing 0.1% (vol/vol) Tween 20 for 1 h at room temperature. The blot was then incubated with antibodies specific for either phosphorylated or total ERK, p38, and JNK (1/1,000 dilution). The membrane was then treated with appropriate secondary antibody conjugated with horseradish peroxidase and visualized by chemiluminescence with ECL. In some experiments, we probed blots for nitrotyrosine for Western blotting.

In vitro MAPK assay. The in vitro activity of ERK and p38 was measured with a p44/42 or p38 MAPK assay kit (New England Biolabs). Cellular proteins (40 μg) were incubated with either immobilized phospho-p44/42 MAPK (Thr202/Tyr204) monoclonal antibody or immobilized phospho-p38 MAPK (Thr180/Tyr182) monoclonal antibody overnight at 4°C to precipitate ERK or p38, respectively. After washing the pellet, we performed an in vitro kinase reaction at 30°C for 30 min in kinase buffer [25 mM Tris pH 7.5, 5 mM β-glycerolphosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na2VO4, and 10 mM MgCl2] containing 200 μM ATP and either glutathione-S-transferase (GST)-Elk-1 (307–428) (substrate for ERK) or GST-activating transcription factor (ATF)-2 (19–96) (substrate for p38) fusion proteins. The reaction was terminated by the addition of 3× SDS sample buffer [187.5 mM Tris·HCl pH 6.8, 6.8% (wt/vol) SDS, 30% (vol/vol) glycerol, 150 mM DTT, and 0.3% (wt/vol) bromphenol blue]. The samples were then boiled for 3 min, and the proteins were resolved with a 4–12% (wt/vol) Bis-Tris gel. Transfer of proteins onto nitrocellulose, membrane blocking, and antibody incubations were as described above with antibodies specific for phosphorylated Elk-1 (Ser383) or phosphorylated ATF-2 (Thr71) (1/1,000).

Statistical analysis. Data are presented as the means ± SE for n determinations. Differences were analyzed by one-way ANOVA or Mann-Whitney’s tests as appropriate. P < 0.05 was considered significant.

RESULTS

Effects of SIN-1 on BEAS-2B cell viability. The addition of 1 mM SIN-1 reduced the viability of BEAS-2B cells in a time- and dose-dependent manner, as determined by CCK-8 assay. Twenty-four-hour treatment of BEAS-2B cells with 1 mM SIN-1 led to a significant reduction in cell viability to ~50% of control levels and could be reduced further after 48 h to 30% (Fig. 1A). To determine whether SIN-1-induced cell death involved oxidative mechanisms, we pretreated cells with 1 mM BSO, an inhibitor of the GSH synthesis, for 24 h. This treatment enhanced the SIN-1-mediated loss of cell viability such that at 8 h post-1 mM SIN-1 exposure, only ~17% of control cells remained viable (Fig. 1A). The pretreatment of BEAS-2B cells with 1 mM BSO alone for 24 h was not cytotoxic (data not shown). To further examine this effect of SIN-1, we exposed cells to increasing concentrations of SIN-1 for 8 h in the absence or presence of growth factors. Concentrations of SIN-1 of 0.25–1 mM in K-SFM supplemented with growth factors (Fig. 1B) and 0.25–1 mM in K-SFM (Fig. 1B) elicited a significant reduction in cell viability.

To determine the specificity of action of SIN-1, we examined the effects of MnTMPyP and hydroxocobalamin, and O2− and NO• scavengers, respectively, on SIN-1-induced cytotoxicity (Fig. 1, C and D). Pretreatment for 30 min with MnTMPyP (1–20 μM) or hydroxocobalamin (0.1–1 mM) before 8 h of SIN-1 exposure dose dependently inhibited the effect of SIN-1, suggesting that this effect on cell viability was not mediated by either NO• or O2−, but via the action of ONOO−.

ONOO− has a very short half-life; however, the effects of SIN-1 on cell viability were not observed until 8 h of exposure (Fig. 1). To determine whether this could be a direct effect of ONOO− at this time point, we measured the production of ONOO− from the dissociation of SIN-1. Dissociation of SIN-1 (0.5 mM) to produce ONOO− reached a peak at 1–2 h but had decreased markedly by 8 h (Fig. 2A). At 24 h, the level of ONOO− decreased significantly, consistent with the observed time course of cell death.
ONOO\(^{-}\) had returned to that of baseline (Fig. 2A). These data indicate that the effect of SIN-1 on cell viability is most likely not a direct effect of ONOO\(^{-}\) but that exposure to ONOO\(^{-}\) initiates a cascade of response, leading over time, to cell death. We addressed the possibility that the presence of cells could alter dissociation of SIN-1 by performing the same experiment in the presence of BEAS-2B cells that had been pretreated in the absence or presence of BSO. The presence of cellular superoxide dismutase did not alter the kinetics of ONOO\(^{-}\) formation (Fig. 2A). Moreover, addition of hydroxocobalamin significantly inhibited ONOO\(^{-}\) formation (0.5 mM SIN-1, 5.3 ± 0.7 \(\mu\)M rhodamine; 0.5 mM SIN-1 + 1 mM hydroxocobalamin, 0.3 ± 0.3 \(\mu\)M rhodamine; \(n = 3\)). Similar experiments could not be performed with MnTMPyP as the inhibitor interfered with absorbance at 500 nm.

In an attempt to investigate the mechanism by which ONOO\(^{-}\) mediates cell death in BEAS-2B cells, we investigated the possibility that nitration of tyrosine residues of proteins regulates this process. Treatment of BEAS-2B cells with SIN-1 (1 mM) for up to 24 h (Fig. 2B) did not alter the amount of nitrotyrosine adducts, therefore it is unlikely that nitration of tyrosine residues is the mechanism by which SIN-1 mediates cell death.

**Time course effects of SIN-1 on MAPK activation.** To examine whether SIN-1 could mediate MAPK activities in BEAS-2B cells, we immunoblotted whole cell lysates using antibodies against phospho-ERK, phospho-p38, or phospho-JNK. SIN-1 treatment in BSO-pretreated BEAS-2B cells activated all three MAPK subgroups; however, the kinetics of the responses were different. The level of phosphorylated ERK began to increase 1 h following 1 mM SIN-1 treatment, reached a maximum at 8 h, then persisted at 24 h (Fig. 3A). This result was confirmed by in vitro ERK kinase assay using GST-Elk-1 as a substrate of phospho-ERK and
revealed by immunoblotting with phospho-Elk-1 antibody. Similarly, the phosphorylation of p38 also increased after 1-h treatment with 1 mM SIN-1, reached a maximum at 4 h, then decreased to the basal level after 8 h (Fig. 3B). An in vitro p38 kinase assay using GST-ATF-2 as a substrate of phospho-p38 and phospho-ATF-2 antibody to measure p38 activity demonstrated a similar kinetic profile. In contrast, the phosphorylation of JNK was delayed compared with ERK and p38, starting 2 h after SIN-1 treatment, before reaching a maximum at 4 h, then returning to the basal level after 8 h (Fig. 3C). Phosphorylated ERK, JNK, and p38 did not change in control cells during the 24-h time course (data not shown). Similar experiments were performed on cells that had not been pre-treated with BSO; under these conditions there was no activation of the MAPK pathways (data not shown). This strongly suggests that these observations are dependent on oxidant stress of the epithelial cells.

Dose-dependent effects of SIN-1 on MAPK activation. Having determined the time course of MAPK activation with SIN-1, we performed experiments using the appropriate time points to determine whether this effect was concentration dependent. ERK phosphorylation and activity increased dose dependently following 2 h of treatment of BEAS-2B cells with SIN-1 (Fig. 4A). Likewise, phosphorylation of p38, p38 kinase activity, and phosphorylation of JNK in the presence of SIN-1 was also increased dose dependently (Fig. 4B and C). We determined the possibility that activation of MAPK was not due to ONOO− but to the alternative product of the reaction, SIN-1C, by exposing cells to media that had contained SIN-1 for 24 h. At this time point, ONOO− is no longer generated; however, this medium did not alter the levels of phosphorylated ERK (data not shown).

Effects of NO− and O2• scavengers on SIN-1-induced MAPK activation. To determine whether the MAPK activation was specific to the effect of ONOO− and not NO− and/or O2•, we treated BEAS-2B cells with MnTMPyP or hydroxocobalamin in presence of 1 mM SIN-1 and then examined MAPK activities. Both MnTMPyP (10 μM) and hydroxocobalamin (500 μM) inhibited SIN-1-induced ERK phosphorylation (Fig. 5A) and SIN-1-induced p38 and JNK phosphorylation together with p38 kinase activity (Fig. 5, B and C). These data suggest that the effects of SIN-1 on MAPK activity are mediated via the generation of ONOO− and are not due to the effects of NO− or O2•.

Effects of MAPK pathway inhibitors on SIN-1-induced MAPK activation. To determine whether SIN-1-induced ERK activity was a MEK-dependent mechanism, we cultured BEAS-2B cells in the presence of PD-98059, an inhibitor of MEK. Cells were treated with 50 μM PD-98059 30 min before exposure with 1 mM SIN-1 for 2 h. This treatment inhibited partially SIN-1-induced ERK activity as determined by phosphorylation immunoblotting and in vitro kinase assay (Fig. 6A). Furthermore, a selective inhibitor of p38, SB-239063, was also able to inhibit SIN-1-induced p38 activity (Fig. 6B).

Effects of MAPK pathway inhibitors on SIN-1-induced cell death. Because SIN-1 could activate the MAPK pathways, the effects of MAPK inhibitors on the SIN-1-induced cell death were examined. PD-98059 and SB-239063 failed to inhibit SIN-1 (1 mM)-induced cell death (Fig. 7, A and B), suggesting that the effects of SIN-1 on the MAPK pathways might be unrelated to

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Fig. 3. Time course effect of SIN-1 on MAPK activation. BEAS-2B cells were incubated for 24 h in K-SFM with 1 mM BSO and subsequently treated with 1 mM SIN-1 for the indicated time. A: cell lysates were analyzed by immunoblotting with antibodies against phosphorylated (p) or total ERK or immunoprecipitated (IP) using an antiphospho-ERK and activity demonstrated using glutathione S-transferase (GST)-Elk-1 as substrate followed by immunoblotting with an antiphospho-Elk-1 antibody. B: cell lysates were analyzed by immunoblotting with antibodies against phosphorylated or total p38 MAPK or immunoprecipitated using an antiphospho-p38 MAPK and activity demonstrated using GST-activating transcription factor (ATF)-2 as substrate followed by immunoblotting with an antiphospho-ATF-2 antibody. C: cell lysates were analyzed by immunoblotting with antibodies against phosphorylated or total JNK. Shown are representatives of at least 3 independent experiments.
the effects on cell survival. To confirm whether this was in fact the case, we repeated the experiment using 0.25 mM SIN-1 for 8 h. Under these experimental conditions, PD-98059 significantly attenuated SIN-1-induced cell death (60% cell survival at the concentration of 25 μM), whereas SB-239063 had no effect (Fig. 7, C and D), suggesting that this effect of SIN-1 is mediated via ERK and not p38. However, when both these inhibitors were used together, SB-239063 potentiated the effects of PD-98059 on SIN-1-induced cell death with cell viability (Fig. 8A). These data suggested an interaction between the ERK and p38 MAPK pathways. To examine this possibility further, we treated cells with PD-98059 and determined p38

Fig. 4. Concentration effects of SIN-1 on MAPK activation. BEAS-2B cells were incubated for 24 h in K-SFM with 1 mM BSO then treated with various concentrations of SIN-1 for 2 h in the case of ERK measurements and 4 h for p38 and JNK measurements. A: cell lysates were analyzed by immunoblotting with antibodies against phosphorylated or total ERK or immunoprecipitated using antiphospho-ERK and using GST-Elk-1 as substrate; activity was determined by immunoblotting with an antiphospho-Elk-1 antibody. B: cell lysates were analyzed by immunoblotting with antibodies against phosphorylated or total p38 MAPK or immunoprecipitated using antiphospho-p38 MAPK and using GST-ATF-2 as substrate; activity was determined by immunoblotting with antiphospho-ATF-2 antibody. C: cell lysates were analyzed by immunoblotting with antibodies against phosphorylated or total JNK. Shown are representatives of 3 independent experiments.

Fig. 5. Effects of nitric oxide (NO)· and superoxide anion (O2·−) scavengers on SIN-1-induced MAPK activation. BEAS-2B cells were incubated for 24 h in K-SFM with 1 mM BSO then treated for 30 min with 10 μM MnTMPyP or 0.5 mM HC before exposure to 1 mM SIN-1 for 2 h in the case of ERK measurements and 4 h for p38 and JNK measurements. A: cell lysates were analyzed by immunoblotting with antibodies against phosphorylated or total ERK or immunoprecipitated using antiphospho-ERK and using GST-Elk-1 as substrate; activity was determined by immunoblotting with an antiphospho-Elk-1 antibody. B: cell lysates were analyzed by immunoblotting with antibodies against phosphorylated or total p38 MAPK or immunoprecipitated using antiphospho-p38 MAPK and using GST-ATF-2 as substrate; activity was determined by immunoblotting with antiphospho-ATF-2 antibody. C: cell lysates were analyzed by immunoblotting with antibodies against phosphorylated or total JNK. Shown are representatives of 3 independent experiments.
MAPK phosphorylation after 4-h SIN-1 exposure. However, PD-98059 failed to inhibit SIN-1-induced p38 phosphorylation (Fig. 8B). In contrast, when cells were treated with the p38 inhibitor SB-239063 in the presence of SIN-1, ERK activation was reduced, suggesting cross talk between these pathways (Fig. 8C).

**DISCUSSION**

Several lines of evidence suggest a role for ONOO\(^-\) in the pathophysiology of chronic inflammatory diseases, including asthma and COPD (35, 38). The present study examined the effects of SIN-1, a ONOO\(^-\) generator, on proliferation of human bronchial epithelial cells. This study reports that SIN-1 exposure led to a dramatic cell death. Furthermore, it appeared that this effect was associated with the activation of the three major subgroups of MAPK pathways: ERK, JNK, and p38 MAPK. This was further supported by data that showed that inhibition of ERK and p38 MAPK pathways could reduce the cell death induced by SIN-1.

**ONOO\(^-\)-inducible cell death has been demonstrated in neuroblastoma cells where SIN-1 treatment led to cytotoxicity after 24-h exposure by apoptosis measured with flow cytometric analysis and the activation of**

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**Fig. 6. Effects of MAPK pathway inhibitors on SIN-1-induced MAPK activation.** A: BEAS-2B cells were incubated for 24 h in K-SFM with 1 mM BSO then treated for 30 min with 50 \(\mu\)M PD-98059 before exposure to 1 mM SIN-1 for 2 h. Cell lysates were analyzed by immunoblotting with antibodies against phosphorylated or total ERK or immunoprecipitated using antiphospho-ERK and using GST-Elk-1 as substrate; activity was determined by immunoblotting with an antiphospho-Elk-1 antibody or treated for 30 min with 10 \(\mu\)M SB-239063 before exposure to 1 mM SIN-1 for 4 h (B). Cell lysates were analyzed by immunoprecipitation using antiphospho-p38 MAPK and using GST-ATF-2 as substrate; activity was determined by immunoblotting with antiphospho-ATF-2 antibody. Shown are representatives of 3 independent experiments.

**Fig. 7. Effects of MAPK pathway inhibitors on SIN-1-induced cell death.** BEAS-2B cells were incubated for 24 h in K-SFM with 1 mM BSO then treated for 30 min with various concentrations of PD-98059 (A) or SB-239063 (B) before treatment with 1 mM SIN-1 (solid bars) or without SIN-1 (open bars) for 8 h. Alternatively, cells were incubated for 24 h in K-SFM with 1 mM BSO then with various concentrations of PD-98059 (C) or SB-239063 (D) in K-SFM for 30 min before 8-h exposure to 0.25 mM SIN-1. Cell viability was determined with the difference absorbance of CCK-8 as described in MATERIALS AND METHODS. Values represent means ± SE of 3 experiments each performed in triplicate and are expressed as percentage of untreated control cells. *\(P < 0.05\), **\(P < 0.01\), and ***\(P < 0.001\).
Similar effects of ONOO− have also been observed in HL-60 and neuronal cells (28, 42). In the lung, cell death induced by ONOO− has been demonstrated in rat type II epithelial cells, bovine pulmonary artery endothelial cells, and rat pulmonary myofibroblasts, but not in human bronchial epithelial cells (12, 33, 47). This study showed that SIN-1-induced cell death occurred through an oxidative pathway, since inhibition of GSH synthesis accelerated cell death. Recently, an increased vulnerability of neuroblastoma SH-SY5Y cells with SIN-1 exposure after depletion in GSH by BSO pretreatment was reported (31). This may be important since GSH depletion has been linked to the pathophysiology of many airway diseases, including idiopathic pulmonary fibrosis, acute respiratory distress syndrome, bronchopulmonary dysplasia, and cystic fibrosis (3, 4, 34, 39). SIN-1 is thought to release simultaneously NO• and O2•−, thus forming ONOO−. MnTMPyP, a scavenger of O2•−, and hydroxocobalamin, a scavenger of NO• (2, 9), almost completely reduced cell death induced by SIN-1. These results suggested that it is ONOO−, but not O2•− or NO•, that caused these deleterious effects. The mechanism of these ONOO−-mediated events is unclear, since ONOO− production via SIN-1 is back to baseline levels at 8 h. This suggests that ONOO− is causing a cascade of events leading to cell death at 8 h. ONOO− may lead to nitration of tyrosine residues of proteins and this may alter phosphotyrosine-dependent signaling. However, not only can ONOO− prevent phosphorylation of tyrosine residues by nitration, but also such covalent modification of tyrosine residues may induce phosphorylation. Such a mechanism has been reported for src tyrosine kinases, Akt kinase, and EGF receptor in some cell types (24, 29, 47). Furthermore, ONOO− can activate MAPK pathways in a variety of cells such as neuronal cells, polymorphonuclear leukocytes, liver epithelial cells, or fibroblasts (1, 21, 26, 40). The data presented in this study demonstrate for the first time that SIN-1 can activate the three major subgroups of MAPKs in human bronchial epithelial cells in both a time- and concentration-dependent manner. Moreover, the inhibition of activation of MAPKs induced by SIN-1 with either MnTMPyP or hydroxocobalamin treatments suggests the involvement of ONOO− in this response. It is unlikely that this is a direct effect of ONOO− on MAPK, since the effects on activity are not evident for hours following stimulation. This suggests that ONOO− may be mediating upstream effectors in the activation pathway; nevertheless, it is ONOO− and not NO• or O2•− that mediates this effect. There were no differences in the levels of nitrotyrosine adducts in cells following treatment with SIN-1; therefore, an alternative mechanism.
possibly nitrosothiol formation, may be responsible for the effects observed in this study. Alternative mechanisms of ONOO\textsuperscript{–}-mediated cell death have been suggested, including disruption of mitochondrial function (46), which can lead to release of cytochrome c and activation of the caspase pathway (37). Such mitochondrial disruption can also alter ATP levels in the cell, contributing to cell death (14). Recently, it has been suggested that lipid peroxidation via the action of ONOO\textsuperscript{–} may also be contributing to cell death in pulmonary epithelial cells (17).

Because SIN-1 could activate the MAPK pathways, inhibitors of both ERK or p38 MAPK pathways were investigated to determine whether MAPK were responsible for this observation. Neither PD-98059, an inhibitor of MEK (7), nor SB-239063, a selective inhibitor of p38 (43), had any effect on cell survival after treatment with 1 mM SIN-1. These data suggest that there was no interaction between these two effects of SIN-1 in epithelial cells. The possibility that 1 mM SIN-1 is a supramaximal concentration to test these inhibitors was addressed by use of a lower concentration of SIN-1. Treatment of BEAS-2B cells with 0.25 mM SIN-1 led to a cytotoxicity of 60% compared with control cells after 8-h exposure. The MEK inhibitor PD-98059 partially enhanced cell survival, whereas SB-239063, a p38 MAPK inhibitor, was not able to protect cells against SIN-1 exposure. These results suggest that the ERK pathway is as least in part involved in SIN-1-induced cell death. In most systems studied, the ERK pathway is activated in response to mitogenic factors and is generally poorly stimulated by stress stimuli. However, the protective effect of PD-98059 against ONOO\textsuperscript{–} treatment has been reported in neuroblastoma cells and pulmonary myofibroblasts, indicating that the ERK pathway is able to transduce cell death signals of ONOO\textsuperscript{–} exposure (32, 36, 47). This has been confirmed in this study of human bronchial epithelial cells. Moreover, treatment of BEAS-2B cells with both PD-98059 and SB-239063 inhibited SIN-1-induced cell death, leading to >80% of cell survival. This is of interest because it provides evidence of an interaction between the ERK and p38 MAPK pathways in the response to SIN-1 in human bronchial epithelial cells. In addition, we show a decrease of phosphorylated ERK induced by SIN-1 with the inhibition of p38 MAPK, suggesting that ERK activation by SIN-1 was partially mediated by p38 MAPK. Recently, Houlston et al. (18) reported cross talk between ERK and p38 MAPK pathways, where SB-203580 treatment could enhance IL-1\textalpha-induced ERK but reduced throbmin-stimulated ERK pathway in human umbilical vein endothelial cells, suggesting a differential signal up-stream ERK pathway. A similar mechanism may be responsible for this observed result.

In conclusion, this study demonstrates that ONOO\textsuperscript{–} can affect MAPK signaling pathways in human bronchial epithelial cells and that their inhibition could protect cells against the oxidative stress induced by ONOO\textsuperscript{–}. Such stresses could occur in the inflamed airway due to the induction of inducible nitric oxide synthase, which could produce NO, in association with an increase in inflammatory cells, such as neutrophils and macrophages, which could produce superoxide. This is the case in diseases such as asthma and COPD, where exhaled levels of NO are increased and there is an increase in inflammatory cells in the airway (15, 22, 23, 30). This effect may be relevant in inflammatory airway diseases such as asthma, where the airway epithelium may be exposed to ONOO\textsuperscript{–}, which may then in turn lead to the epithelial damage and loss, which is characteristic of asthma, thus prolonging the cycle of inflammation observed in such diseases.

This work was supported by grants from the National Asthma Campaign, UK, and Pharmacia.

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