Neutrophil elastase inhibition of cell cycle progression in airway epithelial cells in vitro is mediated by p27kip1

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Fischer BM, Zheng S, Fan R, Voynow JA. Neutrophil elastase inhibition of cell cycle progression in airway epithelial cells in vitro is mediated by p27kip1. Am J Physiol Lung Cell Mol Physiol 293: L762–L768, 2007. First published June 22, 2007; doi:10.1152/ajplung.00067.2007.—Neutrophil elastase (NE), a serine protease present in high concentrations in the airways of cystic fibrosis patients, injures the airway epithelium. We examined the epithelial response to NE-mediated proteolytic injury. We have previously reported that NE treatment of airway epithelial cells causes a marked decrease in epithelial DNA synthesis and proliferation. We hypothesized that NE inhibits DNA synthesis by arresting cell cycle progression. Progression through the cell cycle is positively regulated by cyclin complexes and negatively regulated by cyclin-dependent kinase inhibitors (CKI). To test whether NE arrests cell cycle progression, we treated normal human bronchial epithelial (NHBE) cells with NE (50 nM) or control vehicle for 24 h and assessed the effect of treatment on the cell cycle by flow cytometry. NE treatment resulted in G1 arrest. Arrest in G1 phase may be the result of CKI inhibition of the cyclin E complex; therefore, we evaluated whether NE upregulated CKI expression and/or affected the interaction of CKIs with the cyclin E complex. Following NE or control vehicle treatment, expression of p27kip1, a member of the Cip/Kip family, was evaluated. NE increased p27kip1 gene and protein expression. NE increased the coimmunoprecipitation of p27kip1 with cyclin E complex, suggesting that p27kip1 inhibited cyclin E complex activity. Our results demonstrate that p27 is regulated by NE and is critical for NE-induced cell cycle arrest.

MATERIALS AND METHODS

Normal human bronchial epithelial cell culture. Normal human bronchial epithelial cells (NHBE; Clonetics/Cambrex, Walkersville, MD) were cultured submerged and on collagen-coated Transwell Clear inserts (Corning Costar), as previously described, in serum-free, growth factor-supplemented bronchial epithelial base medium (Clonetics/Cambrex)-DMEM (1:1 ratio; Invitrogen, Carlsbad, CA; see Refs. 13, 14, and 24). NHBE cells were grown to confluence in six-well plates (Costar/Corning, Corning, NY) for RNase protection assays (RPA). NHBE cells were grown to confluence in 12-well plates (Costar/Corning) for flow cytometry assays and real-time PCR assays. For Western analyses and immunoprecipitation studies, NHBE cells were cultured in 60-mm tissue culture dishes to 100% confluence.

Cell cycle analysis by flow cytometry. Flow cytometry was used to assess DNA content as a surrogate measure for cell cycle phase. NHBE cells were synchronized in G0/G1 by incubation for 48 h in epidermal growth factor (EGF)-free and bovine pituitary extract

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(BPE)-free media (12). Growth factors (25 ng/ml EGF and 0.13 mg/ml BPE) were added to initiate cell cycling, and at the same time cells were treated with NE (50 nM, 22–24 h) or control vehicle (50 μM sodium acetate, pH 5, 100 μM sodium chloride). We have previously reported that this noncytotoxic concentration of NE decreases airway epithelial DNA synthesis and proliferation (12, 13). At the end of the treatment period, cells were collected by trypsinization and resuspended in Hanks’ balanced salt solution (Invitrogen). For fixation, while the cells were vortexing, ice-cold 100% ethanol was added dropwise to reach a final concentration of 75% ethanol. Cells in ethanol were stored at 20°C overnight. Fixed cells were pelleted by centrifugation and resuspended in 500 μl of PBS containing 25 μg/ml propidium iodide (Molecular Probes/Invitrogen, Eugene, OR) and 100 μg of RNase A (Sigma, St. Louis, MO). Fixed and stained cells were then analyzed by flow cytometry for DNA content.

\[^{3}H\]thymidine incorporation. As a model for quiescent cells, NHBE were cultured in ALI. After 2 wk of ALI culture, cells were washed, and the medium was replaced with serum-free defined medium without EGF and BPE for 16–24 h before the addition of NE. Cells were treated with a noncytotoxic concentration of NE (500 nM, 1 h; Elastin Products, Owensville, MO; see Ref. 14), at both the apical and basolateral surfaces and rinsed, and the medium was replaced with serum-free EGF- and BPE-free defined medium. Cells were then followed over time (chase, 22–72 h) for \[^{3}H\]thymidine incorporation as a marker of DNA synthesis and proliferation. After the treatment period, cells were incubated in EGF-free and BPE-free medium with \[^{3}H\]thymidine (5 μCi/ml; Amersham Biosciences/GE Healthcare, Piscataway, NJ). At 22, 48, and 72 h posttreatment (chase period), \[^{3}H\]thymidine incorporation analysis was performed as previously described, with cells being solubilized in 1% SDS + 0.2 M NaOH, and samples were corrected for protein concentration (DC Protein assay, Bio-Rad, Hercules, CA; see Refs. 10 and 12).

Real-time RT-PCR analysis for p27 mRNA expression. NHBE were treated with NE (50 nM, 4–16 h) or control vehicle, then RNA was collected with Trizol, and p27 gene expression was analyzed by quantitative real-time RT-PCR. A commercially available primer-probe set was used that comes as a ready-to-use 20× mix: Taqman Assays-On-Demand for p27-Hs00153277_m1 (Applied Biosystems). Real-time RT-PCR was performed with an ABI Prism 7500 Sequence Detection System by a one-step method. Duplicates of each RNA sample (150 ng/well) were loaded in 96-well plates with Multiscribe RT enzyme, RNase inhibitor, TaqMan Master Mix (Applied Biosystems), and a p27 specific primer-probe set. PCR conditions are as follows: reverse transcription, 50°C for 30 min, initial denaturation at 95°C for 10 min, then 40 cycles at 95°C for 15 s; 60°C for 1 min. As controls, real-time PCR was also performed without RT and without RNA template in the reaction mix. After determination of the threshold cycle (Ct) for each sample, the relative amount of p27 mRNA was evaluated by the comparative Ct method (ΔΔCt): amplification of p27 was first normalized to the 18S rRNA amplification (18s primer-probe set from Applied Biosystems) in the same sample (ΔCt = p27 gene Ct − 18S Ct) and then each ΔCt was normalized to the control, nontargeting small-interfering RNA (siRNA) ΔCt value (sample ΔCt − control siRNA ΔCt = ΔΔCt; see Ref. 6). Relative mRNA values were calculated by 2^−ΔΔCt.

Preparation of lysates for Western analyses and immunoprecipitations. NHBE, submerged or ALI, were treated with NE (submerged: 50 nM, 4–24 h; ALI: 500 nM, 1 h) or control vehicle, rinsed with ice-cold PBS, and scraped in a 1× phosphate inhibitor cocktail (Cocktail no. 2, Sigma). Cells were pelleted by centrifugation and resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 10 μg/ml leupeptin, 40 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml antipain, 10 μg/ml chymostatin, 10 μg/ml benzamidine, and 1× phosphate inhibitor cocktail (all chemicals from Sigma)). Lysates were passed through a 25-gauge needle to break up large aggregates of cells and then incubated on ice for a total of 1 h, with vortexing every 20 min. The lysates were clarified by centrifugation (13,000 revolutions/min, 4°C, 10 min). Total protein concentrations were determined by Bio-Rad DC Protein Assay.

Western analyses for \(^{2}p27\) protein expression. Cell lysates (20–30 μg) were separated by SDS-PAGE on a 12% gel (Bio-Rad) and transferred to nitrocellulose. Membranes were blocked overnight in 5% nonfat milk in Tris-buffered saline-Tween buffer (TBST: 20 mM Tris, 500 mM NaCl, and 0.1% Tween 20; all chemicals from Sigma) and then probed with a monoclonal antibody for human \(^{2}p27\) (p27, 1:150; clone 57; Pharmingen/Biosciences) in TBST plus 5% nonfat milk for 1 h at room temperature. After being washed, membranes were incubated with a sheep anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5,000; Amersham Biosciences) in TBST plus milk for 1 h at room temperature. Antibody complexes were visualized by enhanced chemiluminescence (ECL plus; Amersham Biosciences) and autoradiography and then digitalized with a Nikon digital camera and ACT-1 software (Nikon).

Immunoprecipitation of cyclin E complex. Cyclin E complexes were immunoprecipitated to evaluate the interaction of p27 CKI with cyclin E. Binding of p27 to cyclin E is indicative of inhibition of cell cycle progression. Cell lysates were collected as described above. Immunoprecipitation was performed as described by Frey et al. (15). Briefly, lysates were first precleared by incubating cell lysates (100 μg) with rabbit IgG (5 μg; Santa Cruz Biotechnology, Santa Cruz, CA) plus 20 μl of protein A/G agarose beads (A/G agarose Plus beads; Santa Cruz Biotechnology) and incubated for 30 min at 4°C. Beads were collected by centrifugation and the supernatant saved. Precleared lysates were then incubated with 2 μg of rabbit antihuman cyclin E antibody (Santa Cruz Biotechnology) overnight at 4°C. Antibody-antigen complexes were captured with protein A/G agarose beads (Santa Cruz Biotechnology) for 1 h at 4°C, collected by centrifugation, washed in lysis buffer, boiled for 2 min in SDS-PAGE loading buffer (4×, 5 μl) and 15 μl lysis buffer, and then loaded on a 12% gel (normal human bronchial epithelial; Bio-Rad). After transfer, Western analysis for p27 protein was performed as described above. As a negative control, normal rabbit IgG was substituted for the immunoprecipitation antibody. To demonstrate that there were equivalent levels of cyclin E available for immunoprecipitation, cell lysates were also evaluated for cyclin E protein expression by Western analysis using the same rabbit anti-human cyclin E antibody (1:200) that was used for immunoprecipitation. Similar to p27 Western analyses, membranes were stripped and reprobed for β-actin. HeLa cell lysates were used as a positive control for the Western blot. Cyclin E protein expression was normalized to β-actin expression.

Statistical analyses. Analysis of data, except cyclin E immunoblotting data, was performed using the Kruskal-Wallis one-way nonparametric ANOVA and post hoc comparisons by the Wilcoxon rank sum test (44). Cyclin E immunoblotting data were analyzed by a Wilcoxon rank sum test. Differences were considered significant at P < 0.05.

RESULTS

**NE induced G1 cell cycle arrest in NHBE cells in vitro.** To assess the effects of NE treatment of NHBE cells on the cell cycle, the cells were initially synchronized in G0/G1. As shown in the histogram in Fig. 1A, after culturing NHBE cells submerged for 48 h in serum-free, EGF-free, and BPE-free defined
medium, 83.1 ± 0.3% (mean ± SE, n = 18) of the cells were in G0/G1. To begin cell cycling, NHBE cells were then switched to medium with growth factors, and then cells were treated with control vehicle or NE (50 nM) for 8, 16, and 24 h (Fig. 1B). NE treatment for 8 h had no significant cell cycle alterations. However, a 16-h treatment period resulted in a small decrease in the percent of cells in S phase (control 5%, NE 3%) but no significant change in the percent of cells in G0/G1 or G2/M. In contrast, NE treatment for 24 h resulted in G0/G1 cell cycle arrest (Fig. 1B), with increased percent of cells in G0/G1 and decreased percent of cells in S and G2/M phases.

NE treatment resulted in decreased S phase activity in quiescent NHBE cells. NHBE cells grown for 2 wk in ALI cultures were used as a model of quiescent cells. To assess the effects of NE treatment on quiescent cells, NHBE cells were treated with NE (500 nM, 1 h) and then followed over time for \[^{3}H\]thymidine incorporation, a marker for DNA synthesis indicative of S phase activity. As shown in Fig. 2A, NE treatment resulted in an initial significant decrease in \[^{3}H\]thymidine incorporation at 24 h that did not significantly increase or return to control levels within 72 h (Fig. 2B). These results suggest that NE treatment resulted in a decrease in S phase activity corresponding to decreased cell cycle progression and decreased proliferation.

NE increased Cip/Kip CKI mRNA expression and p27\(^{kip1}\) protein expression before G1 cell cycle arrest. To investigate the mechanisms of NE-induced cell cycle arrest, NE-induced changes in p27 mRNA expression were assessed with real-time PCR. NE induced an increase in p27 mRNA expression over time, with a significant increase at 16 h of NE treatment (Fig. 3). Western analysis for p27 protein was performed to determine the effect of NE treatment on p27 protein expression. NE induced an increase in p27 protein expression over time, with a significant increase at 16 h of NE treatment that persisted until 24 h of treatment (Fig. 4). In addition, in
quiescent NHBE grown in ALI culture, NE treatment significantly increased p27 protein expression after 1 h (Fig. 5). Collectively, these results demonstrate that p27 protein expression increased before the occurrence of G1 cell cycle arrest. We then evaluated whether the increase in p27 expression affected p27 association with the cyclin E complex, its target for cell cycle arrest.

**p27Kip1 CKI mediates NE-induced cell cycle arrest in NHBE cells.** To evaluate if NE-induced G1 arrest was a result of p27Kip1 binding to the cyclin E complex, cyclin E immunoprecipitations and subsequent p27 immunoblots were performed on NHBE cell lysates from cells treated with NE or control vehicle. Following NE exposure, there was increased p27 coimmunoprecipitation with cyclin E (Fig. 6A). There was no evidence of p27 binding when rabbit IgG was used in place of the cyclin E immunoprecipitation antibody. In addition, in response to NE treatment, there were no significant changes to total cyclin E levels (Fig. 6B). These results suggest that, after NE exposure, p27 binding to the cyclin E complex increased, consistent with inhibition of the cyclin E complex activity.

These results indicate that p27 plays a critical role in mediating NE-induced alterations in cell cycle progression.

**DISCUSSION**

We have previously reported that NE alters airway epithelial homeostasis by significantly reducing DNA synthesis and...
vascular smooth muscle, fibroblasts, and cancer cells. Most of the evidence presented here and in the literature suggests that p27 serves an important function in mediating cell cycle arrest and regulation of apoptosis in response to inflammatory injury. 

p27 is a multifaceted protein important in many fundamental cellular processes such as cellular proliferation and differentiation as well as apoptosis (reviewed in Ref. 42). For example, p27 mediates transforming growth factor (TGF)-β or contact inhibition-induced G1 cell cycle arrest (34, 35). Differentiation or differentiation-associated cell cycle arrest is regulated by p27 in a variety of cell types, including luteal cells (50), osteoblasts (9), and keratinocytes (20). p27 has also been reported to have an anti-apoptotic role; p27 is required for inhibition of proliferation and apoptosis following inflammatory injury of renal epithelial cells (31). We have shown that NE treatment of airway epithelial cells reduces proliferation (12), and in this report, we present evidence that p27 is a key molecule regulating NE-induced cell cycle arrest. Collectively, the evidence presented here and in the literature suggests that p27 serves an important function in mediating cell cycle arrest and regulation of apoptosis in response to inflammatory injury.

p27 is expressed in the airway epithelium, airway and vascular smooth muscle, fibroblasts, and cancer cells. Most of the information about the regulation of p27 expression is from cancer models and smooth muscle cells, with only limited information about its regulation in airway epithelium. Interferon-γ inhibits bronchial epithelial and airway smooth muscle proliferation by upregulating p27 expression, decreasing cyclin E and CDK2 activity, and thus decreasing retinoblastoma protein phosphorylation (3, 48). In a balloon angioplasty model, vascular smooth muscle growth arrest is regulated by Sp1-mediated transcriptional upregulation of p27 (4). In small cell lung cancer cells, Raf-1-mediated mitogen-activated protein kinase (MAPK) activation induces p27 upregulation and cell cycle arrest (39). The mechanism of NE-regulated p27 expression is not yet known. However, potential mechanisms of regulation include transcription and/or posttranscriptional regulation via MAPK by NE (19) or by NE-generated ROS (1, 5, 14). In addition, TGF may also function in an autocrine/paracrine manner to upregulate p21 and p27 expression. NE has been reported to trigger release or secretion of TGF-β from epithelial cells, endothelial cells, and airway smooth muscle cells (26, 47). Our own unpublished observations and results in published reports demonstrate that TGF-β will increase p21 and p27 expression in epithelial cells (40, 51, 53). Future studies may evaluate if TGF-β mediates NE-induced p27 expression and cell cycle arrest in airway epithelial cells.

In addition to p27, RPA results revealed both p21 and p57 are also upregulated (11). In response to ROS treatment, p21 has been shown to inhibit cyclin-CDK2 activity in alveolar epithelial cells (40, 51, 53). Future studies may evaluate if TGF-β mediates NE-induced p27 expression and cell cycle arrest in airway epithelial cells. In this report, we have focused on p27 as a key regulator of cell cycle arrest in response to NE treatment.
regulator of NE-induced cell cycle arrest; however, NE may increase protein expression of p21 and p57, resulting in an additive or synergistic effect on cell cycle arrest.

In diseases such as CF, CB, and asthma, the tissues of the airways are repeatedly bombarded by a variety of inflammatory mediators, including cytokines, proteases, and ROS. In patients with asthma compared with control individuals, there is increased expression of Cip/Kip mitotic inhibitor p21 in the airway epithelium that increases with severity of disease (36). In addition, these investigators demonstrate that exogenously administered ROS upregulate p21 expression in vitro (36). Similarly, alveolar macrophages from smokers exhibit increased p21 expression (49). Other inflammation-associated lung disorders, such as idiopathic pulmonary fibrosis, have been reported to have an association between p21 expression and DNA strand breaks (25). In addition, overexpression of p21 in a mouse model of idiopathic pulmonary fibrosis reduced the fibrotic response (22). These studies demonstrate that, in these chronic inflammatory airway diseases, there are cycles of injury and repair with corresponding increased expression of the CKIs.

When cells are in cell cycle arrest, they are “sitting on the fence” between heading toward apoptosis vs. continuing on in the cell cycle. Several proteases have been reported to induce apoptosis. Mast cell chymase has been reported to induce apoptosis of vascular smooth muscle cells, as evidenced by appearance of cells in sub-G1 by flow cytometry, DNA laddering, and apoptosis-associated microscopic cellular morphology changes (27). Using similar techniques, other investigators have demonstrated that neutrophil-derived serine proteases, proteinase 3 and NE, will induce apoptosis of endothelial cells (52). Furthermore, NE induces apoptosis of epithelial cells in vitro (17, 46). The factors controlling cell fate (apoptosis vs. cell cycle entry) following cell cycle arrest are not known and are an important area of future investigation.

We (14) and others (5) have previously reported that NE induces oxidative stress in airway epithelial cells. Oxidative airway injury like hyperoxia also induces DNA damage with increased 8-oxoguanine staining as well as DNA strand breaks (41). Hyperoxia induces G1 cell cycle arrest by upregulating p21 expression (38). It is this arrest that serves to protect the cell from DNA damage and subsequent cell death (37). Furthermore, during neonatal lung development, it is the p21-mediated cell cycle regulation that helps to maintain lung architecture following hyperoxic exposure (29). We speculate that NE induces G1 cell cycle arrest as a protective mechanism to permit time for repair from oxidative/inflammatory injury.

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