Proteinase-Activated Receptor-1 Mediates Elastase-Induced Apoptosis of Human Lung Epithelial Cells

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Apoptosis of distal lung epithelial cells plays a pivotal role in the pathogenesis of acute lung injury. In this context, proteinases, either circulating or leukocyte-derived, may contribute to epithelial apoptosis and lung injury. We hypothesized that apoptosis of lung epithelial cells induced by leukocyte elastase is mediated via the proteinase activated receptor (PAR)-1. Leukocyte elastase, thrombin, and PAR-1–activating peptide, but not the control peptide, induced apoptosis in human airway and alveolar epithelial cells as assessed by increases in cytoplasmic histone-associated DNA fragments and TUNEL staining. These effects were largely prevented by a specific PAR-1 antagonist and by short interfering RNA directed against PAR-1. To ascertain the mechanism of epithelial apoptosis, we determined that PAR-1AP, thrombin, and leukocyte elastase dissipated mitochondrial membrane potential, induced translocation of cytochrome *c* to the cytosol, enhanced cleavage of caspase-9 and caspase-3, and led to JNK activation and Akt inhibition. In concert, these observations provide strong evidence that leukocyte elastase mediates apoptosis of human lung epithelial cells through PAR-1–dependent modulation of the intrinsic apoptotic pathway via alterations in mitochondrial permeability and by modulation of JNK and Akt.

Keywords: acute lung injury; proteinases; inflammation; signal transduction

Activated neutrophils can release proteinases such as elastase and other proinflammatory molecules that contribute to the pathogenesis of the inflammatory tissue injury seen in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (1, 2). In support of this notion, leukocyte elastase levels are increased in serum and bronchoalveolar lavage (BAL) fluid in clinical studies and animal models of ALI/ARDS (3–5). We have recently reported that leukocyte elastase released by activated neutrophils induces apoptosis of gastrointestinal epithelial cells in an *in vitro* model (6, 7). These observations are relevant to the pathogenesis of both acute (ALI/ARDS) and chronic (emphysema) lung injury, where inappropriate apoptosis of lung epithelial cells may contribute to the pathogenesis of pulmonary dysfunction (8–13).

Apoptosis, or programmed cell death, is fundamental to physiologic processes such as embryogenesis and in the remodeling of tissues that occurs during normal repair processes (9). Cells dying from apoptosis tend to be disposed of in a noninflammatory manner, whereas death by necrosis is often associated with the activation of proinflammatory pathways in the surrounding environment. The signaling pathways leading to apoptosis can generally be divided into the extrinsic (surface receptor–mediated) and intrinsic (mitochondrial-dependent) pathways (14). The former pathway is triggered following ligand binding to "death" receptors of the TNF superfamily such as TNFR1, Fas, and TRAIL receptors (15). In contrast, the intrinsic pathway can be executed independently of death receptors and involves alteration of mitochondrial permeability (16). Apoptotic pathways can be modulated by various inflammatory molecules and elucidation of these mechanisms may lead to novel therapeutic interventions that inhibit key steps in the apoptosis pathways.

In addition to leukocyte-derived proteases, serum proteases, and in particular thrombin, may play an important role in ALI/ARDS. Indeed, thrombin has been implicated in the pathogenesis of a diverse array of inflammatory diseases, such as inflammatory bowel disease, ventilator-associated pneumonia, and myocardial injury (17–19). The importance of the coagulation cascade in the setting of ALI/ARDS is reinforced by studies revealing fibrin and microthrombi in the lungs of these patients, increased procoagulant activity in the early exudative phase of lung injury, and increased BAL fibrinopeptide A levels (20, 21).

Thrombin can influence cell function through a group of cell surface receptors known as proteinase-activated receptors (PARs). These are G protein–coupled receptors that are activated by cleavage of their extracellular N-terminus by proteinases (22, 23). This cleavage creates a neo-amino terminus or "tethered ligand" that binds to and activates the receptor. There are four known PARs, and all are expressed by human lung epithelial cells (24). Although thrombin activates PAR-1, -3, and -4, PAR-1 is considered the archetypal receptor for thrombin. Synthetic peptides such as S(T)FLLR-NH$_2$, which mimic the tethered ligand sequence of PAR-1, can function independently of receptor cleavage (23, 25). PAR activation has been linked to a number of responses including platelet aggregation, vasodilation, vasoconstriction, increased vascular permeability, increased intestinal permeability, granulocyte chemotaxis, and intestinal epithelial apoptosis (26–28). Although generally proinflammatory, there is evidence that PAR-2 activation may attenuate

pulmonary inflammation (29, 30) Our knowledge of the effects of PAR activation on lung epithelial function is incomplete.

In the current study, we focused on the role of PAR-1 in the regulation of lung epithelial apoptosis. We report that leukocyte elastase, through PAR-1 activation, promotes epithelial apoptosis via a mitochondrial-dependent pathway. We also examined the signal transduction pathways downstream of PAR-1 modulated by elastase and other receptor agonists. In the present study, we report that elastase modulates the activity of Akt and the amino-terminal c-Jun kinase (JNK) and thus regulates apoptosis.

MATERIALS AND METHODS

Cell Culture

Primary human small airway epithelial (HSAE) cells (Cambrex, Walkersville, MD) were grown in Small Airway Cell Basal Medium (SABM) supplemented with growth factors and antibiotics according to the manufacturer's instructions. BEAS-2B, a human bronchial epithelial cell line, was provided by Dr. Reen Wu (University of California, Davis). Cells were grown in 1:1 mix of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 Nutrient Mix (DMEM/F12) (GIBCO/BRL, Grand Island, NY) supplemented with the following: 10 μg/ml of human recombinant insulin, 25 ng/ml of recombinant human epidermal growth factor, 5 μg/ml of transferrin, 2% (vol/vol) penicillin-streptomycin (all from GIBCO/BRL, Grand Island, NY) and 0.1 μM of hydrocortisone (Sigma, St. Louis, MO). Primary human alveolar type II cells were isolated as described (31) and grown in DMEM with 10% fetal calf serum. A549 cells (American Type Tissue Collection, Gaithersburg, MD), neoplastic lung cells with features of alveolar type II cells, were grown in DMEM with 10% fetal bovine serum. Epithelial cells were seeded on tissue culture plastic plates coated with human type VI collagen (Sigma), and grown to confluence at 37°C in 5% CO_2.
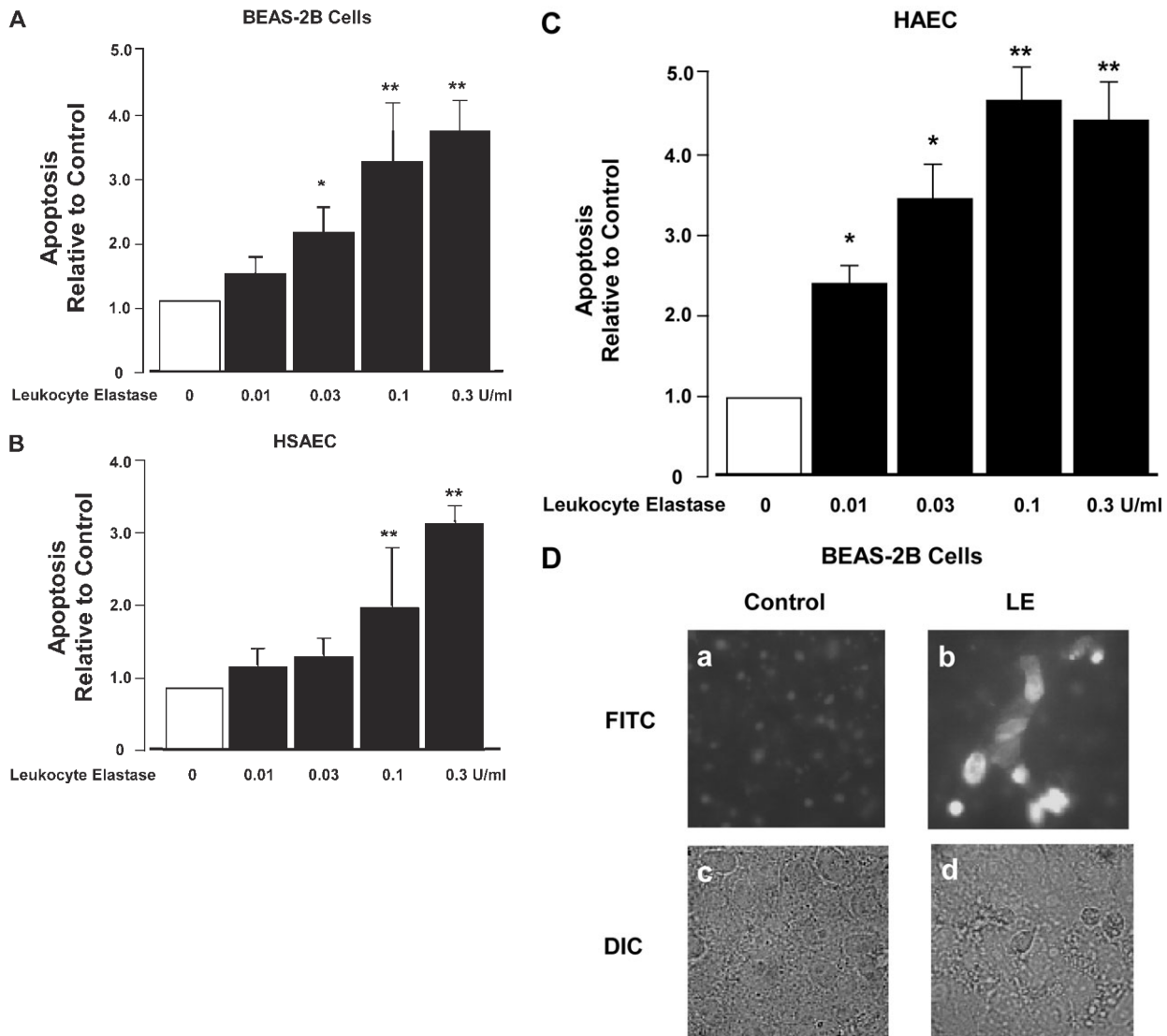
Figure 1. Leukocyte elastase (LE) induces lung epithelial apoptosis in a dose-dependent manner. Cell death detection assay (A–C) and TUNEL staining (D–F) were used for the detection of apoptosis. Three different lung epithelial cell types BEAS-2B (A and D), human small airway epithelial (HSAE) cells (B and E), and primary human alveolar type II (HAEC) cells (C and F) were used. In experiments with HSAE cells, many cells lifted off the coverslip during washing. Therefore, we collected cells in the supernatant and applied them to slides using a cytocentrifuge. The graphs (A–C) represent the absorbance values relative to control (buffer only). Values are mean ± SD; *P < 0.05 and **P < 0.01 compared with control. (A) n = 10; (B and C) n = 4. For TUNEL labeling (D–F), apoptotic cells demonstrate a greater intensity of fluorescence. Representative data each from one of three experiments are shown.
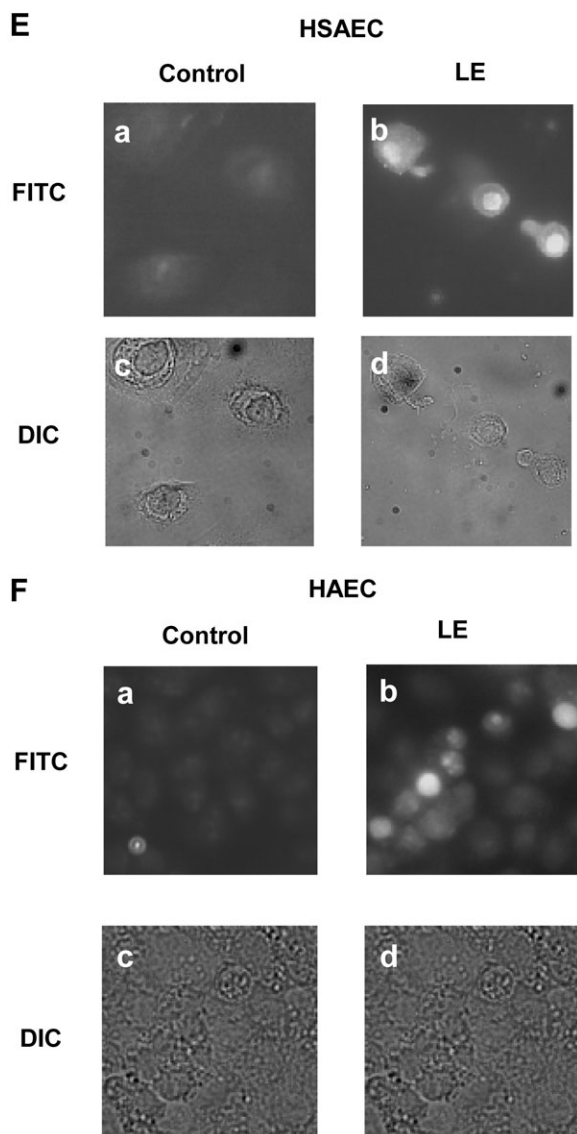
E

HSAEC

Control   **LE**

F

HAEC

Control   **LE**

Figure 1. Continued

Reagents

Human leukocyte elastase from human sputum was from EPC (Owensville, MO). PAR-1–activating peptide (PAR-1AP: Thr-Phe-Leu-Leu-Arg, TFLLR-NH$_2$) and PAR-1 control peptide (Arg-Leu-Leu-Phe-Thr, RLLFT-NH$_2$) were obtained from the Alberta Peptide Institute (Edmonton, AB, Canada). Thrombin from human plasma was from Sigma-Aldrich. SP600125, a selective JNK inhibitor, was obtained from A.G. Scientific, Inc. (San Diego, CA). The PAR-1 antagonist, SCH79797, was obtained from Tocris-Cookson Inc. (Ellisville, MO).

Antibodies

Primary antibodies included: anti–thrombin R (mouse monoclonal) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Akt (rabbit polyclonal); anti-phospho Ser-473 Akt (rabbit polyclonal); anti-SAPK/JNK (rabbit polyclonal); anti–phospho SAPK/JNK (rabbit polyclonal); anti–caspase 3 (rabbit polyclonal); anti–caspase 9 (rabbit polyclonal); anti–cytochrome *c* (rabbit polyclonal) (all from Cell Signaling Technology, Beverly, MA); anti-actin (murine monoclonal) (ICN, Aurora, OH); and anti-mitochondria COX IV (mouse monoclonal) (Abcam, Cambridge, MA).

Apoptosis Analysis

Human lung epithelial apoptosis was quantified 4 and 12 h after treatment with leukocyte elastase, PAR-1 AP, control peptide, and thrombin using the Cell Death Detection ELISA kit (Roche, Mannheim, Germany) that specifically detects the histone region (H1, H2A, H2B, H3, and H4) of mono- and oligonucleosomes that are released during apoptosis. Absorbance at 405 nm in a 96-well plate was measured using a microplate reader (THERMO max; Molecular Devices, Sunnyvale, CA). Apoptosis was measured in duplicate from 10^5 lung epithelial cells from each treatment group, and expressed as the absorbance ratio relative to control (28). Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) was performed on cells grown on coated glass slides (Lab-Tek, Naperville, IL) using an *in situ* Cell Death Detection Kit, Fluorescein (Roche) according to the manufacturer's instructions. Monolayers were mounted with fluorescent mounting media (DAKO, Carpinteria, CA) onto slides and visualized using a fluorescence microscope (LEICA DM-IRB2) controlled by Open Lab software (Improvision Inc., Lexington, MA).

## RNA Extraction and Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated from cultured lung epithelial cells using Trizol reagent (TRI Reagent; Sigma) following the manufacturer's protocol. Purity was checked by the A_{260}/A_{280} ratio. Total RNA was reverse transcribed using random hexamer priming and SuperScript II Rnase H$^-$ Reverse Transcriptase (RT) (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen, Carlsbad, CA). cDNA was prepared from 5 µg RNA in a 20-µl volume, adding 50 units SuperScript II RT, 2 µl 10× RT buffer, 40 units RNaseOut Recombinant RNase Inhibitor, 5 mM MgCl$_2$, 10 mM dithiothreitol, 0.5 mM dNTP mix, and 7.5 ng/µl random hexamers. A negative control reaction lacking RT was also performed for each RNA sample. The random hexamer primer was annealed for 10 min at 25°C. cDNA synthesis was performed for 50 min at 42°C, followed by 15 min at 70°C to terminate the reaction. One microliter of RNAse H was added to each tube and incubated for 20 min at 37°C, and cDNA was stored at −20°C until used. PCR was performed in 100 µl of reaction solution, containing 0.2 mM dNTPs, 1.5 mM MgCl$_2$, 2.5 U/100 µl of Taq polymerase (*Taq* DNA polymerase; Fermentas, Burlington, ON, Canada), 0.5 µM oligonucleotide primers, human PAR-1, Accession No. M62424, sense 5′-TGTGAACTGATCATGTTTATG-3′, antisense 5′-TTCGTAAGATAAGAGATATGT-3′, PCR product 708 bp; human GAPDH, Accession No. M33197, sense 5′-TCAACGACCACTTTGTCAAGCTCA-3′, antisense 5′-GCTGGTGGTCCAGGGGTCTTACT-3′, PCR product 120 bp and first-strand cDNA products. After an initial denaturation step (3 min at 94°C), PCR mixtures were amplified by 30 cycles (30 s at 94°C, 30 s at 55°C, 30 s at 72°C). The final extension period was of 7 min at 72°C. The PCR products were analyzed by electrophoresis using a 2% agarose gel with ethidium bromide.

Measurement of Intracellular Ca^{2+}

Human lung epithelial cells were cultured on 25 mm^2 microscope cover slips coated with human type VI collagen. Coverslips were placed in a sterile microscope chamber (Attofluor; Molecular Probes, Eugene, OR) and epithelial cells loaded with 3 µM Fura-2/AM (Molecular Probes) in DMEM/F12 medium at 37°C, 5% CO$_2$ for 25 min, and then washed three times with the same medium. Dynamic changes in intracellular calcium concentration ([Ca^{2+}]$_i$) in individual cells was measured using a Nikon inverted fluorescent microscope equipped with a CCD camera (Orca; Hamamatsu, Bridgewater, NJ) as previously described in detail (32). Images were acquired and analyzed using SimplePCI software (Compix, Cranberry Township, PA). Cellular fluorescence was monitored for at least 2 min to ensure stability before cells were challenged with agonists including leukocyte elastase, PAR-1AP, or PBS buffer control. Data were recorded for 15 min for each sample.

siRNA Design and Transfection

The different siRNAs were designed corresponding to the human PAR-1 gene sequence (Accession No. M62424). Sequences were chosen on the basis of guidelines from QIAGEN (Mississauga, ON, Canada). siRNA sequences were found using BLAST software from the National Center

A

PAR-1   708bp

GAPDH   120bp

B

C   LE 0.1 U/ml

D   PAR-1AP
100mM
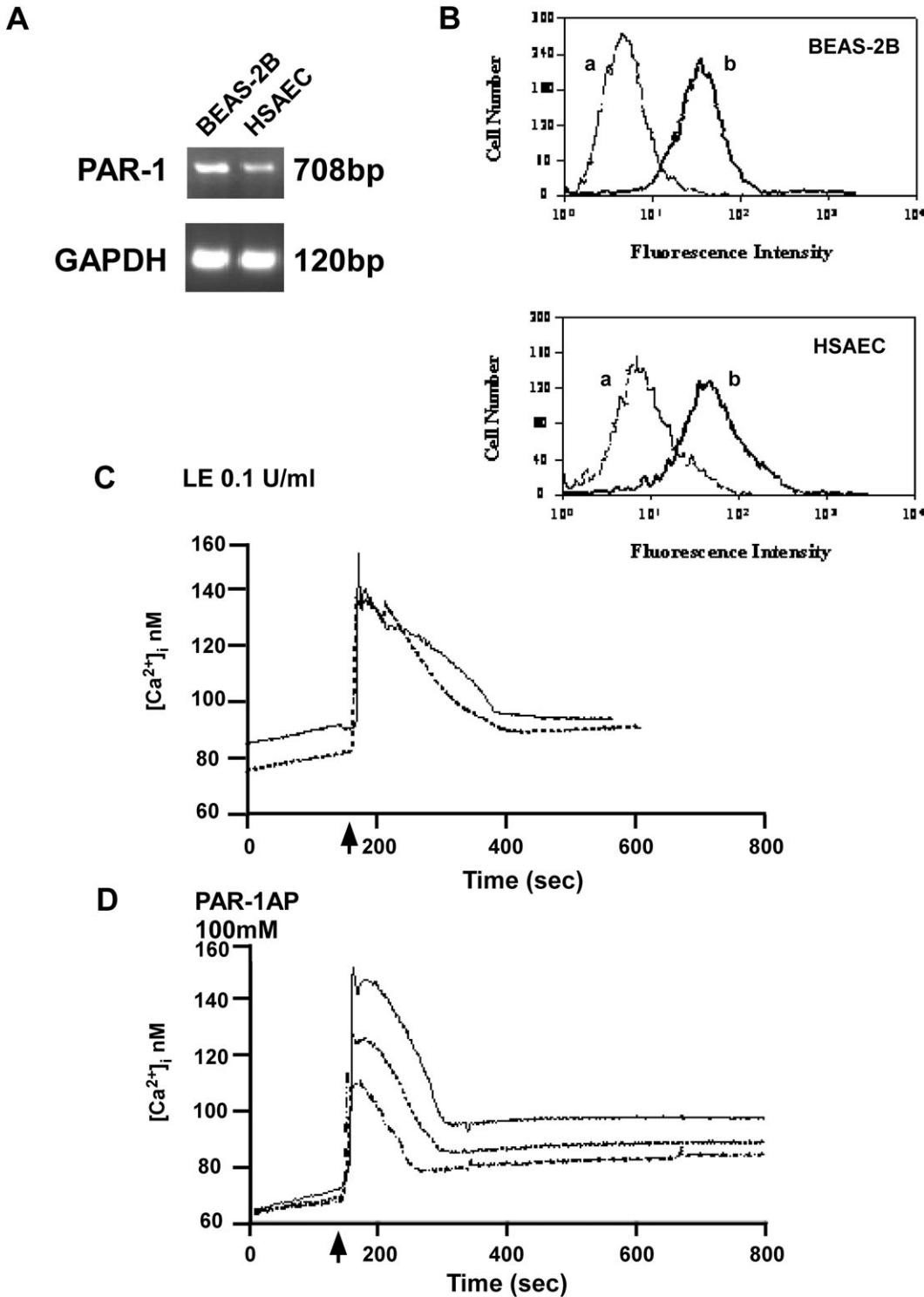
Figure 2. Expression of PAR-1 in lung epithelia as determined by RT-PCR and flow cytometry. (*A*) PAR-1 mRNA is detected in BEAS-2B and HSAE cells using RT-PCR. *Upper panel* represents PAR-1 and *lower panel* represents GAPDH expression, the latter used as a "housekeeping" gene. (*B*) Detection of PAR-1 cell surface receptor expression by FACS analysis. Cells were incubated with PAR-1 mouse monoclonal antibody FITC-conjugated (*b*) or with normal mouse IgG$_1$ FITC conjugated (*a*). Representative data each from one of four experiments are shown. (*C* and *D*) Functionality of PAR-1 as determined by [Ca^{2+}]$_i$ fluxes. Cells were challenged leukocyte elastase (LE) 0.1 U/ml or PAR-1AP 100 μM (*arrow*). Representative data each from one of three independent experiments are shown.

for Biotechnology Information (NCBI). A strategy using the "search for short nearly exact matches" mode against all human sequences deposited in the GenBank and RefSeq databases that also did not have significant homology (> 17 contiguous nucleotides of identity) to genes other than the targets (33) yielded appropriate sequences. PAR-1 siRNAs (listed in Table 1) were synthesized and annealed by QIAGEN. A quantity of 50 nM of control and PAR-1 siRNAs was transfected into BEAS-2B cells (30–50% confluence) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) on the basis of manufacturer's instructions. After 72 h incubation, cells were used for experiments as described below and harvested for flow cytometry and Western blot analysis.

Flow Cytometry

Adherent lung epithelial cells which were transfected with PAR-1 and control siRNA were detached from culture dishes with a cell scraper. Cells were sedimented by centrifugation, washed with PBS once, and resuspended in HBSS-BSA 2% (wt/vol) at a final concentration of 2 × 10^6 cells/ml. Cells were immunolabeled with either an FITC-conjugated anti–thrombin receptor-mouse monoclonal antibody or a control FITC-conjugated-mouse IgG$_1$ (Santa Cruz Biotechnology, Santa Cruz, CA), both at 1:500 dilution, for a 1 h at 4°C. Cells were then washed by FCM buffer (Santa Cruz Biotechnology, Santa Cruz, CA), and resuspended in

1% paraformaldehyde. Flow cytometry was performed on a FACScan using CELL-quest software (Becton Dickinson, Palo Alto, CA). Values are expressed as relative fluorescence index.

Cell Fractionation

Lung epithelial cells cultured in 60-mm plastic culture dishes were incubated with leukocyte elastase 0.1 U/ml and PAR-1AP 100 μM for 0, 2, 4, 12 h. They were washed once with PBS, scraped into PBS, pelleted at $1,000 \times g$ for 5 min, and resuspended in hypotonic buffer (10 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl [pH 7.5], 100 μM PMSF). Cells were allowed to swell on ice for 10 min and homogenized with a tight pestle using a 21-G needle (10 strokes) before lysis by additional of isotonic homogenizing buffer (2.5× MS buffer, 525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl [pH 7.5], and 2.5 mM EDTA [pH 7.5]). After mixing, cell fragments were sedimented at $1,300 \times g$. Supernatants were collected and sedimented at $17,000 \times g$ for 15 min. After centrifugation, pellets were resuspended in 1× MS buffer and used as the heavy membrane fraction containing mitochondria. The supernatants were further centrifuged at $100,000 \times g$ for 1 h, and resulting supernatants were used as the cytosol fraction.

SDS-PAGE and Immunoblotting

For SDS-PAGE of whole cell extracts, cells were collected with boiling lysis buffer (2% SDS, 10% glycerol, 65 mM Tris/HCl, pH 6.8, 50 mM dithiothreitol) supplemented with a protease inhibitor cocktail (Roche). Subcellular fractions were also collected with the same lysis buffer as described above. SDS-PAGE and immunoblotting were performed as described previously in detail (7). The data were analyzed by densitometry using NIH Image.

Mitochondrial Membrane Potential Assay

Lung epithelial cells were cultured on glass chamber slides (Lab-Tek, Naperville, IL) and incubated with PBS (as a negative control), 1 μM of valinomycin (as a positive control) (SIGMA, St. Louis, MO), leukocyte elastase, thrombin, and PAR-1AP for 30 min, 1 h, or 3 h. They were labeled with JC-1 using the DePsipher kit (Trevigen for R&D Systems Inc, Minneapolis, MN) according to the manufacturer's instructions. After labeling, cells were observed using fluorescence microscopy (LEICA DM-IRB), and Open lab (Improvision Inc., Lexington, MA) was used for data acquisition and analysis.

Statistical Analysis

Parametric data were compared by using *t* tests for mean values or ANOVA with correction for multiple comparisons (Fisher's PLSD test) when more than two variables were analyzed using STATview software.

RESULTS

Leukocyte Elastase Induces Apoptosis of Lung Epithelial Cells

We have recently reported that leukocyte elastase induces apoptosis of intestinal epithelial cells (7). To determine if there
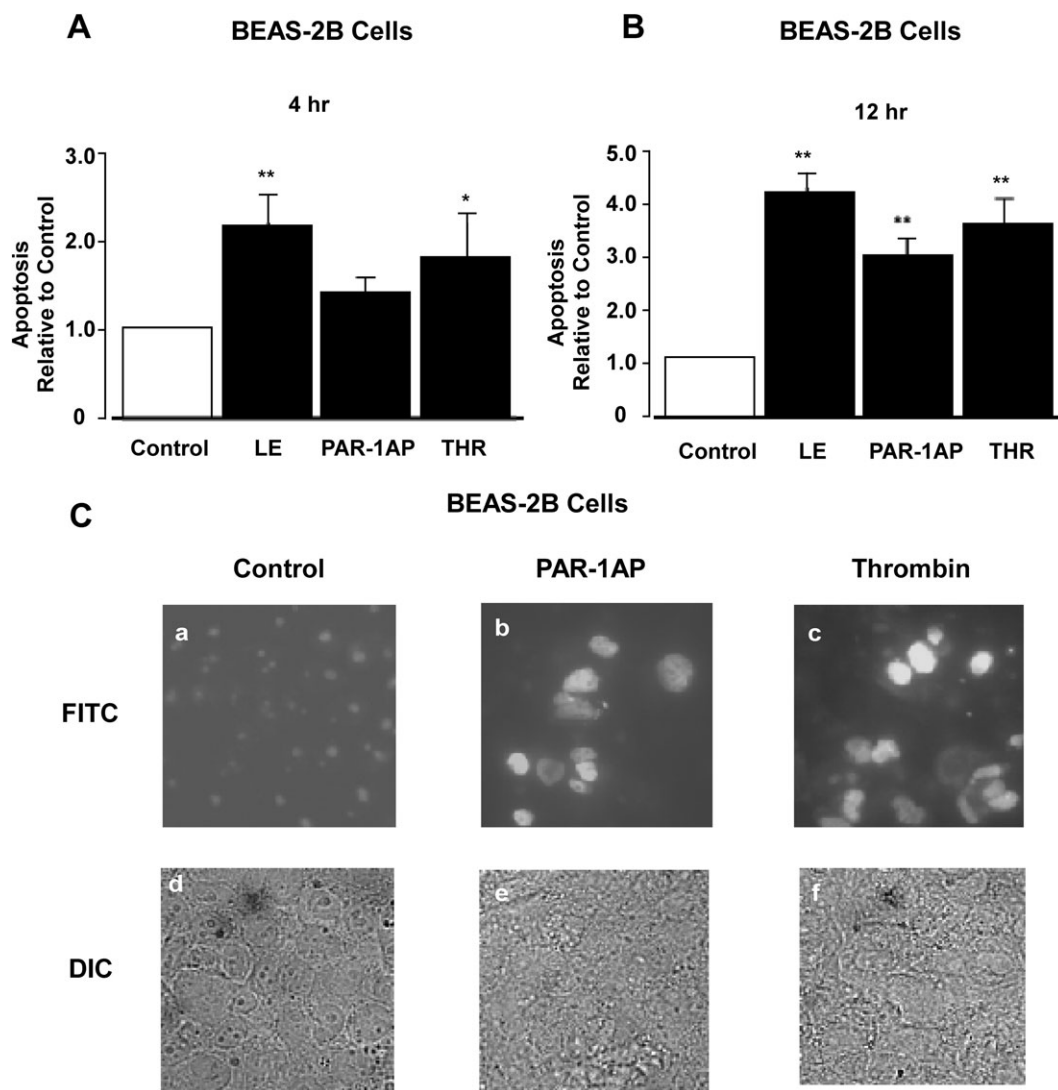
Figure 3. PAR-1 agonists induce lung epithelial apoptosis. Apoptosis was assessed using histone-associated DNA fragments (Cell Death Detection assay; *A, B, D, E, G, H*) at 4 and 12 h after the treatment with a control peptide (Control) 100 μM, leukocyte elastase (LE) 0.1 U/ml, PAR-1AP 100 μM, or Thrombin 1 U/ml. BEAS-2B cells were used in *A* and *B*, HSAE cells were used in *D* and *E*, and human alveolar epithelial (HAEC) cells were used in *G* and *H*. Values represent the mean ± SD; *$P <$ 0.05 and **$P <$ 0.01 compared with control. (*A*) $n = 4$; (*B*) $n = 7$; (*D, E, G, H*) $n = 3$. Apoptosis of lung epithelial cells, including BEAS-2B cells (*C*), HSAE cells (*F*), and HAEC (*I*) was determined using TUNEL labeling. Representative data from one of three experiments are shown.

D HSAEC 4 hr

E HSAEC 12 hr

F HSAEC

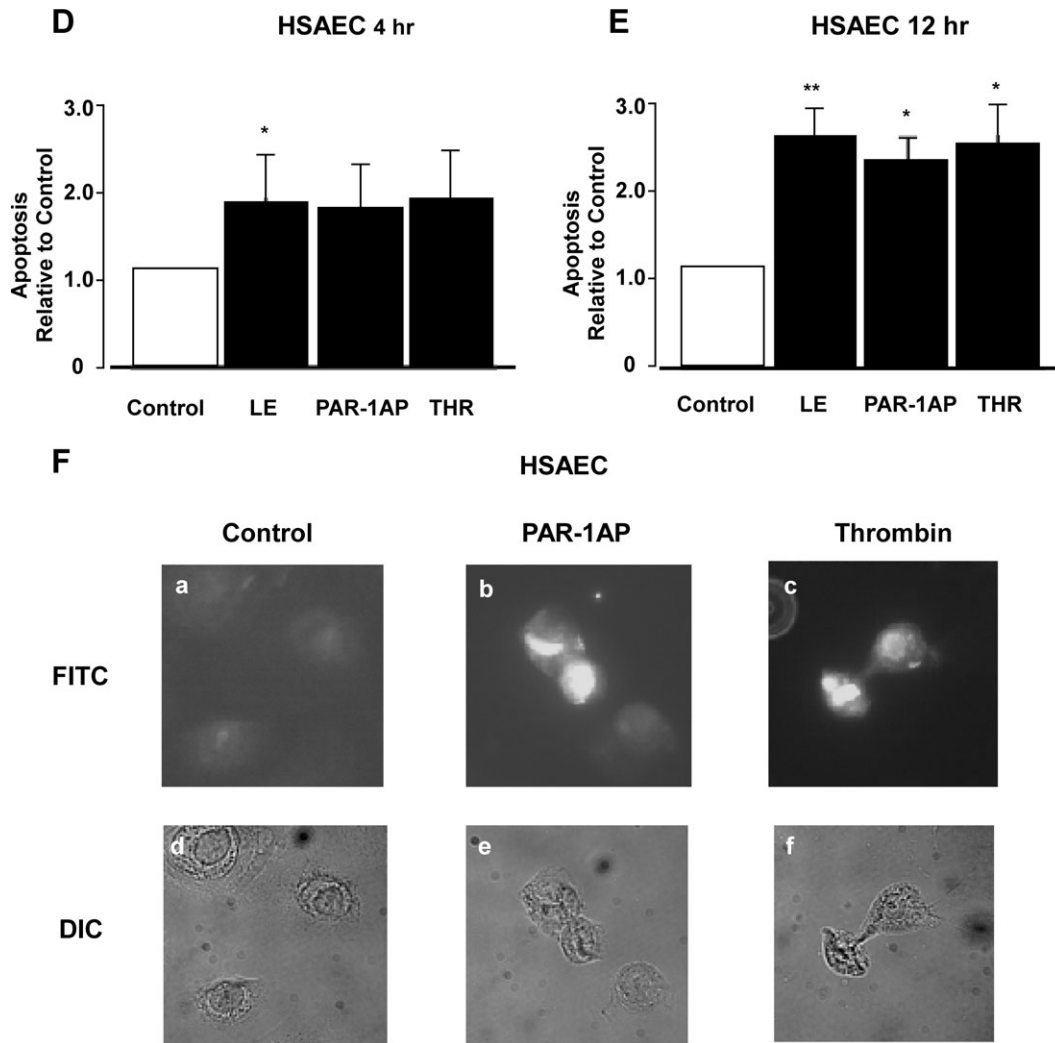Control   PAR-1AP   Thrombin

FITC

DIC

Figure 3. Continued

are analogous effects of elastase on lung epithelial cells, we quantified apoptosis in epithelial cells of both airway and alveolar origin in response to leukocyte elastase by monitoring the presence of histone associated mono- and oligonucleosomes. These studies revealed that exposure of the airway epithelial cell line BEAS-2B as well as primary cultures of human small airway epithelial (HSAE) and human alveolar type II cells to elastase-induced apoptosis in a dose-dependent manner that reached a maximum at 0.3 U/ml (Figures 1A–1C). Similar responses were noted in human A549 cells (data not shown). To confirm these observations by an independent method, we quantified lung epithelial cell apoptosis using TUNEL staining (Figures 1D–1F). These studies confirmed that treatment of both airway (BEAS-2B and HSAE) epithelial cells and alveolar type II epithelial cells with leukocyte elastase induced apoptosis. We conclude that leukocyte elastase induces apoptosis of lung epithelial cell lines and primary cultures of lung epithelial cells of both airway and alveolar origin in physiologic concentrations and in a dose-dependent manner.

Expression of Functional PAR-1 by Human Lung Epithelial Cells

To determine the mechanisms of leukocyte elastase–induced apoptosis, we focused on PAR-1, a membrane receptor known to be modulated by proteolytic cleavage. Using a combination of RT-PCR and Western analysis, we determined that BEAS-2B, HSAE

cells, and primary alveolar type II cells (not shown) express PAR-1 mRNA and protein (Figures 2A and 2B). To test the functionality of PAR-1, we measured intracellular calcium concentration using the calcium-sensitive fluorescent dye, FURA-2AM. The epithelial cells were challenged with either 0.1 U/ml leukocyte elastase or 100 μM PAR-1AP. As illustrated in Figures 2C and 2D, a reproducible response was observed in each case characterized by a rapid and transient rise of cytosolic Ca^{2+}, typical of receptor-mediated signaling events (24). These data indicate that exposure of cells to either leukocyte elastase or PAR-1AP induces rapid and transient intracellular calcium flux typical of receptor-mediated events; the similarity of these responses suggests that both may be mediated by PAR-1.

PAR-1 Agonists Induce Apoptosis of Lung Epithelial Cells

To provide more definitive evidence that apoptosis of lung epithelial cells induced by leukocyte elastase is mediated via PAR-1, BEAS-2B, HSAE and alveolar type II cells were treated with the prototypical PAR-1 activating proteinase, thrombin. Responses were compared with treatment with PAR-1 activating peptide (AP), a control inactive peptide of similar amino acid composition, and with leukocyte elastase. Figure 3 illustrates that thrombin, leukocyte elastase, and PAR-1AP but not the inactive peptide induced apoptosis of all three epithelial cell types. Apoptotic changes were detected after 4 h and reached a maximum at 12 h.
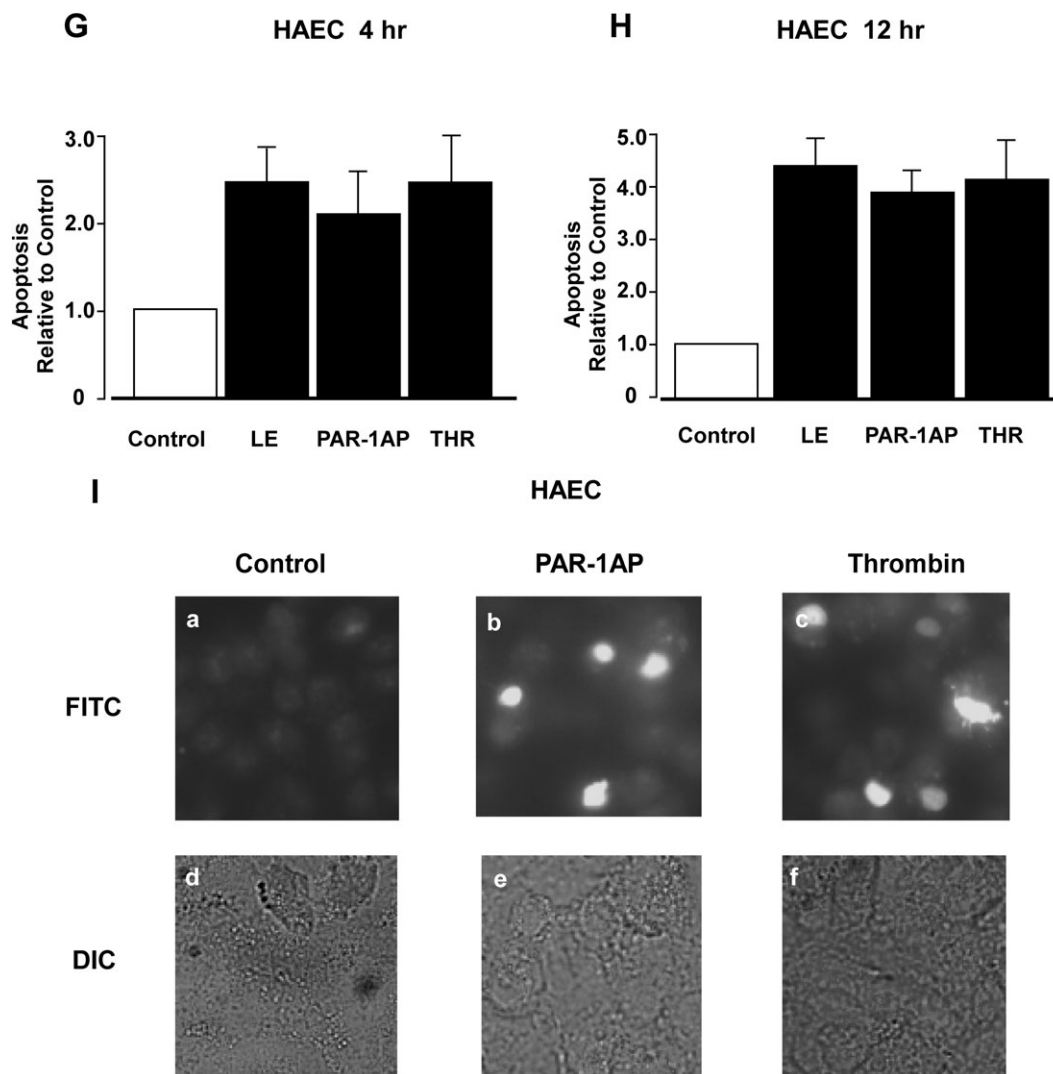
Figure 3. Continued

The magnitude of the response to PAR-1AP (3-fold) and to thrombin (3.6-fold) at 12 h was similar to the response observed with leukocyte elastase. Apoptosis of lung epithelial cells under these conditions was confirmed by TUNEL staining (Figures 3C, 3F, and 3I). Taken together, these observations indicate that direct activation of PAR-1 induces apoptosis of lung epithelial cells. Moreover, the responses are similar to those induced by leukocyte elastase suggesting that the effects of elastase may be mediated via PAR-1.

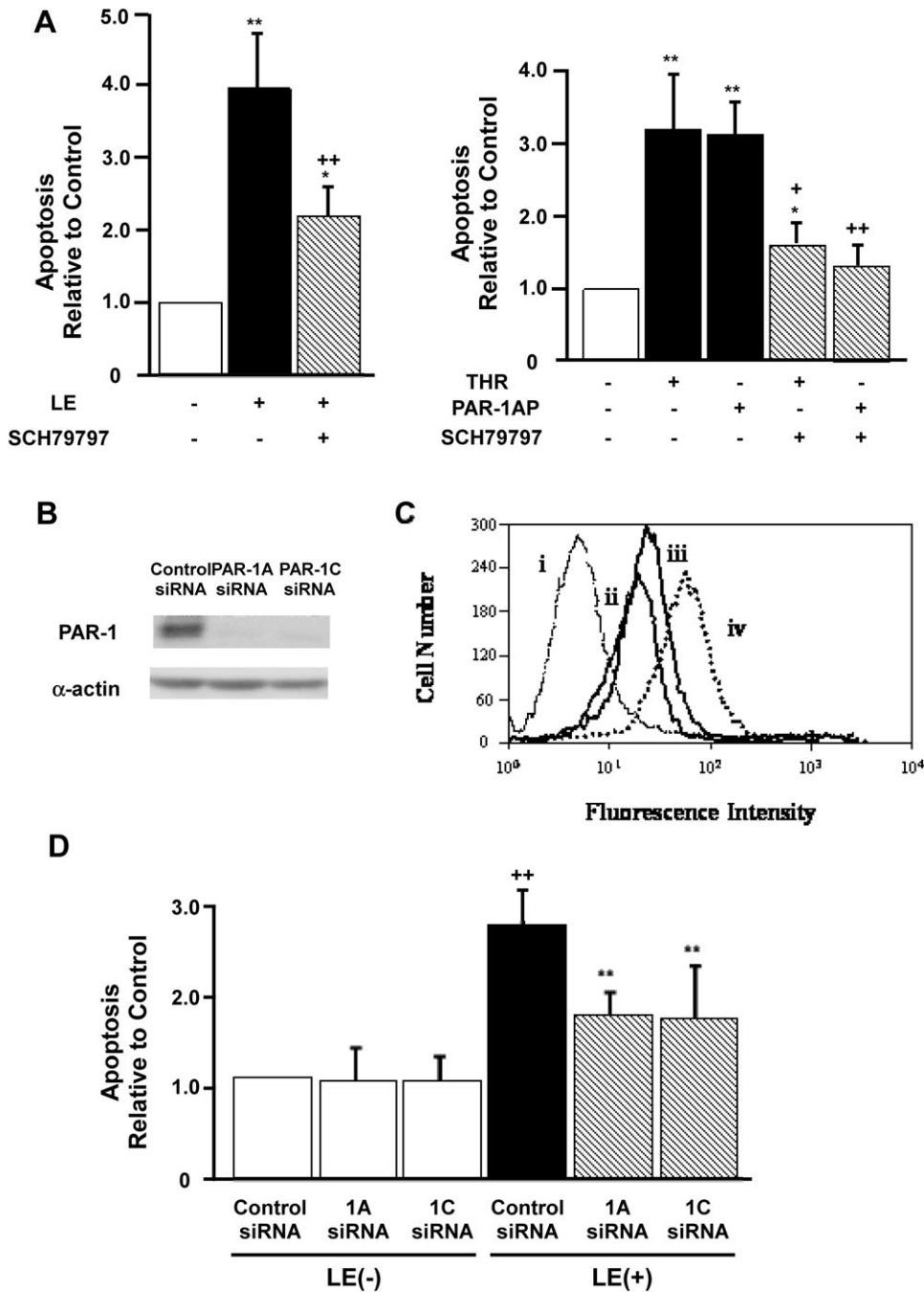### Leukocyte Elastase Induces Epithelial Apoptosis via PAR-1

To supply direct evidence for a role for PAR-1 in leukocyte elastase–induced apoptosis, two approaches were undertaken. First, we used a selective pharmacological inhibitor of PAR-1, SCH79797 (34). Treatment of cells with this inhibitor significantly diminished epithelial apoptosis induced by leukocyte elastase, thrombin, or PAR-1 AP (Figure 4A). Second, we employed gene-silencing with short interfering RNA directed at PAR-1. For these experiments, several sequences corresponding to distinct regions of PAR-1 mRNA were synthesized (Table 1) and introduced into BEAS-2B lung epithelial cells by transfection. Alterations in PAR-1 expression were assessed using Western analysis and flow cytometry with anti–PAR-1 antibodies (Figures 4B and 4C, respectively). These studies revealed that PAR-1

expression was significantly diminished ($>$ 70%) by two of the three siRNAs (PAR-1A and 1C). Additional studies demonstrated that unrelated cellular proteins including α-actin (Figure 4B) and intercellular adhesion molecule-1 (data not shown) were unaffected by this treatment indicating the specificity of gene silencing under these conditions.

Having established conditions where PAR-1 expression was selectively diminished by siRNA, we then assessed the apoptotic response of these cells to treatment with leukocyte elastase. Figure 4D illustrates that under conditions where PAR-1 expression was attenuated using siRNA, leukocyte elastase–induced apoptosis of lung epithelial cells was diminished. By contrast, leukocyte elastase–induced apoptosis was unaffected in lung epithelial cells transfected with control siRNA. It is noteworthy that treatment with siRNA by itself did not induce apoptosis under these conditions. We conclude that that leukocyte elastase–induced apoptosis is mediated at least in part by PAR-1.

Leukocyte Elastase and PAR-1–Activating Agents Increase Mitochondrial Permeability

To explore the mechanisms of apoptosis mediated by PAR-1, we focused initially on mitochondrial-dependent pathways and assessed alterations in mitochondrial membrane potential ($\Delta\Psi$) using the fluorescent probe, JC-1 (Figure 5A). Control lung

Figure 4. PAR-1 siRNA diminishes PAR-1 expression and inhibits leukocyte elastase (LE)-induced apoptosis in BEAS-2B cells. (*A*) The selective pharmacologic inhibitor of PAR-1, SCH79797, attenuates epithelial apoptosis by leukocyte elastase and thrombin. The data represent the mean $\pm$ SEM of $n = 3$ experiments. (*B*) *Upper panel* represents PAR-1 expression and the *lower panel* illustrates α-actin protein expression using with Western blot analysis. (*C*) PAR-1, cell surface receptor as detected by FACS analysis. Cells were incubated with a FITC-conjugated PAR-1 murine monoclonal antibody 72 h after transfection with control siRNA (iv), PAR-1A siRNA (ii), and PAR-1C siRNA (iii). Histogram (i) represents staining with FITC-conjugated isotype control murine IgG$_1$. Representative data each from one of five experiments are shown. (*D*) PAR-1 siRNA reduces leukocyte elastase–induced lung epithelial apoptosis. BEAS-2B cells were treated with leukocyte elastase 0.1 U/ml or buffer 12 h after transfection with siRNA. Values are mean $\pm$ SD; $++P < 0.01$ compared with the sample of control siRNA transfection and buffer only incubation, $**P < 0.01$ compared with the sample of control siRNA transfection and leukocyte elastase treatment ($n = 5$).

epithelial cells loaded with JC-1 and viewed under a rhodamine filter exhibited a typical red punctuate distribution of JC-1, indicating accumulation of JC-1 aggregates in mitochondria (35). Valinomycin, used here as a positive control, disrupts the $\Delta\Psi$ and thus JC-1 translocates to cytoplasm reverting to its mono-meric form, indicated by more diffuse fluorescence when viewed under a fluorescein filter (Figure 5A, *panel g*). Similar effects were observed in cells treated with 0.1 U/ml leukocyte elastase (Figure 5A, *panel j*), 100 µM of PAR-1AP (Figure 5A, *panel h*), and 1 U/ml of thrombin treatment (Figure 5A, *panel i*). These

TABLE 1. SEQUENCES OF siRNA NUCLEOTIDES USED TO SILENCE PAR-1

siRNA Name	DNA Target Sequence	siRNA Sequence	Length
PAR-1A	AACATCATGGCCATCGTTGTG	CAUCAUGGCCAUCGUUGUGTT TTGUAGUACCGGUAGCAACAC	21
PAR-1C	AACGTCCTCCTGATTGCGCAT	CGUCCUCCUGAUUGCGCAUTT TTGCAGGAGGACUAACGCGUA	21

mitochondrial alterations were apparent as early as 30 min after treatment, persisted for at least 3 h, and were dose-dependent (data not shown). Taken together, these data suggest that PAR-1 activation, either by direct receptor activation (PAR-1AP) or by proteolytic cleavage (thrombin or leukocyte elastase), induces apoptosis via alterations in the mitochondrial membrane permeability of lung epithelial cells.

Given these changes in $\Delta\Psi$, we next examined the release of cytochrome *c* from the mitochondria to the cytosol as an independent assay of alterations mitochondrial membrane permeability. Subcellular fractions were prepared by cell lysis followed by sucrose density gradient ultracentrifugation with analysis of fractions by Western blotting. As illustrated in Figure 5B, after treatment with leukocyte elastase, the amount of mitochondrial cytochrome *c* was reduced, whereas cytosolic cytochrome *c* levels increased. Similar effects were noted after treatment of lung epithelial cells with PAR-1AP (Figure 5C). We conclude that PAR-1AP, thrombin, and leukocyte elastase induce release of cytochrome *c* to the cytosol, supporting the fluorescence studies indicating that these agents alter mitochondrial membrane permeability.

Leukocyte Elastase and PAR-1 Agonists Activate Caspase-9 and -3

We next sought to determine if downstream caspases were activated under these conditions. These experiments revealed that treatment with either leukocyte elastase or PAR-1AP induced cleavage of caspase-9 and -3 in a time-dependent manner, reaching a maximum at 12 h (Figures 6A and 6B).

Together, these data indicate that activation of PAR-1, either by specific activating peptide or by proteolytic cleavage, increases mitochondrial permeability with associated release of cytochrome *c* from the mitochondria to the cytosol, and activates caspase-9 and -3 leading to the biochemical and morphologic alterations of apoptosis. As there are multiple pathways that po-

tentially regulate apoptosis, we explored the role of two pathways involving Akt and JNK in mediating PAR-1–induced apoptosis.

Leukocyte Elastase Modulates Akt Phosphorylation through PAR-1

The serine/threonine kinase Akt is known to suppress apoptosis through phosphorylation and inhibition of caspase-9, and phosphorylation of BAD, one of the proapoptotic Bcl-2 members. To determine if PAR-1 activation by leukocyte elastase modulates apoptosis in lung epithelia via this pathway, we assessed the phosphorylation status of Akt in response to leukocyte elastase and to PAR-1AP. These experiments revealed that the phosphorylation of Akt was reduced by leukocyte elastase in a time-dependent manner, whereas total Akt was unchanged (Figure 7A). As noted above (Figure 6A), cleavage of caspase-9 in response to leukocyte elastase transpired with similar kinetics. By comparison, a similar reduction of phospho-Akt was observed after treatment of cells with PAR-1AP at early time points (1–4 h). However, the alteration in response to PAR-1AP was more evanescent, tending to return to baseline levels by 12 h (Figure 7B). These differences may be related to the kinetics of receptor activation by the peptide as compared with proteolytic cleavage by leukocyte elastase. Importantly, gene silencing with PAR-1 siRNA largely prevented the reduction of Akt phosphorylation in response to leukocyte elastase exposure for 12 h (Figure 7C). These data suggest leukocyte elastase and PAR-1AP may modulate epithelial apoptosis via effects on the phosphorylation status of Akt.

Leukocyte Elastase Modulates JNK through PAR-1

JNK is a serine/threonine kinase that is activated in response to diverse environmental stresses (36). The role of JNK in regulation of apoptosis is complex and depends on the cell type. JNK has been reported to promote or suppress apoptosis through diverse pathways, including mitochondrial, nuclear factor (NF)-κB–, or AP-1–dependent pathways (37). To determine if leukocyte elas-
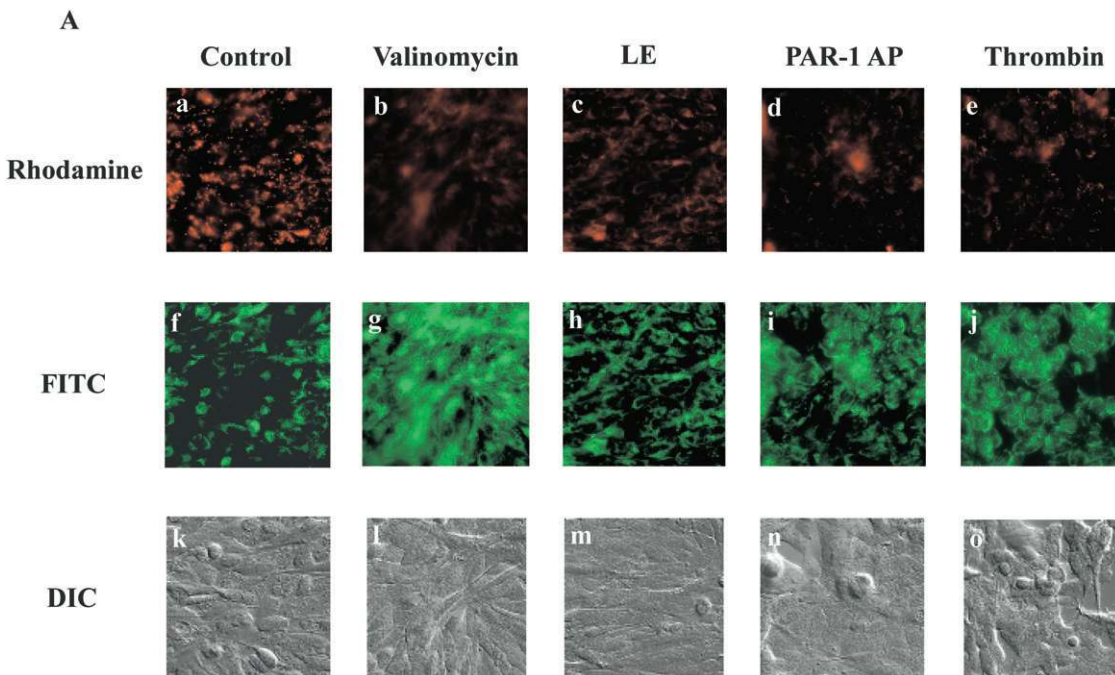
Figure 5. Leukocyte elastase (LE) and PAR-1 agonists reduce mitochondrial membrane potential ($\Delta\Psi$) in BEAS-2B cells. (*A*) *Upper panels* represent images acquired with a rhodamine filter, *middle panels* with an FITC filter, and *lower panels* show DIC images. Cells were treated with a control peptide 100 μM, valinomicin 1 μM, leukocyte elastase 0.1 U/ml, PAR-1AP 100 μM, and thrombin 1 U/ml for 1 h. Representative data each from one of five experiments are shown. (*B* and *C*) Cytochrome *c* release from mitochondria to cytosol in epithelial cells exposed to leukocyte elastase and PAR-1AP. Cells fractions were collected after 0, 4, 12 h of incubation. COX IV is used as a marker for the mitochondrial fraction and α-actin as a marker for the cytosolic fraction. The graph shows densitometry units normalized for COX IV and α-actin levels. Representative data each from one of four experiments are shown.
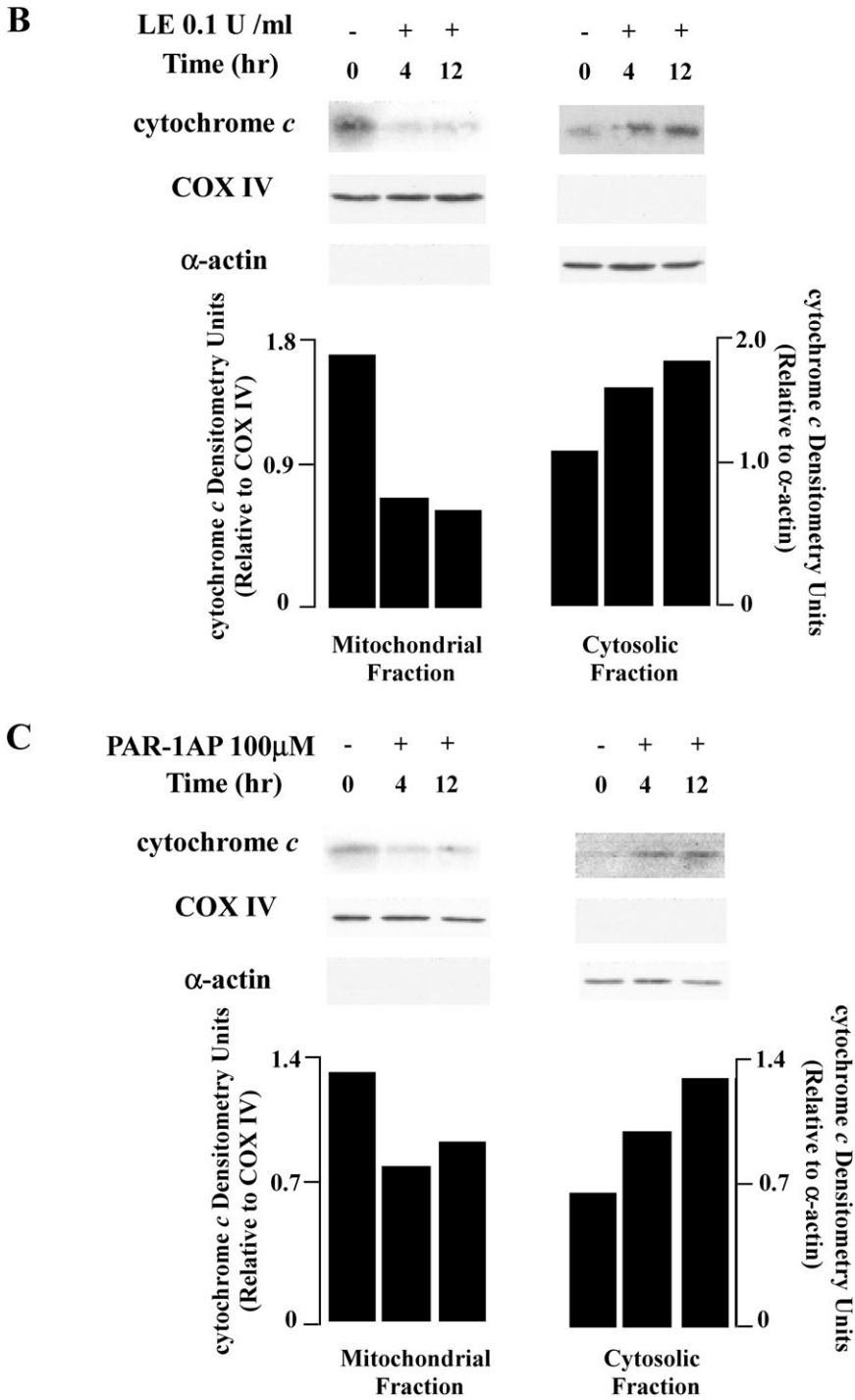
B

Figure 5. Continued

C

tase–induced epithelial apoptosis involves JNK, we examined JNK phosphorylation using Western blot analysis. These studies revealed that leukocyte elastase treatment of lung epithelia induces rapid phosphorylation of JNK peaking at 30 min and returning to baseline by 12 h (Figure 8A). Similar responses were recorded after PAR-1AP treatment of epithelial cells (Figure 8B). To determine the relationship between JNK activation and apoptosis, we treated cells with a specific pharmacologic inhibitor of JNK, SP600125, and monitored apoptosis in response to leukocyte elastase and PAR-1AP. As illustrated in Figure 8D, leukocyte elastase–induced epithelial apoptosis was enhanced when JNK phosphorylation was blocked by SP600125 at a concentration of

10 μM (Figure 8C). We conclude that activation of JNK under these conditions provides an anti-apoptotic signal.

DISCUSSION

In this study, we provide evidence that leukocyte elastase induces apoptosis of human lung airway and alveolar epithelial cells through PAR-1. Our data indicate that the pro-apoptotic effects of leukocyte elastase are mediated via the intrinsic apoptotic pathway involving alterations in mitochondrial permeability (as manifest by decrease in $\Delta\Psi$), release of cytochrome c into the cytosol, and activation of caspase-9 and -3. In addition, exposure

A

B
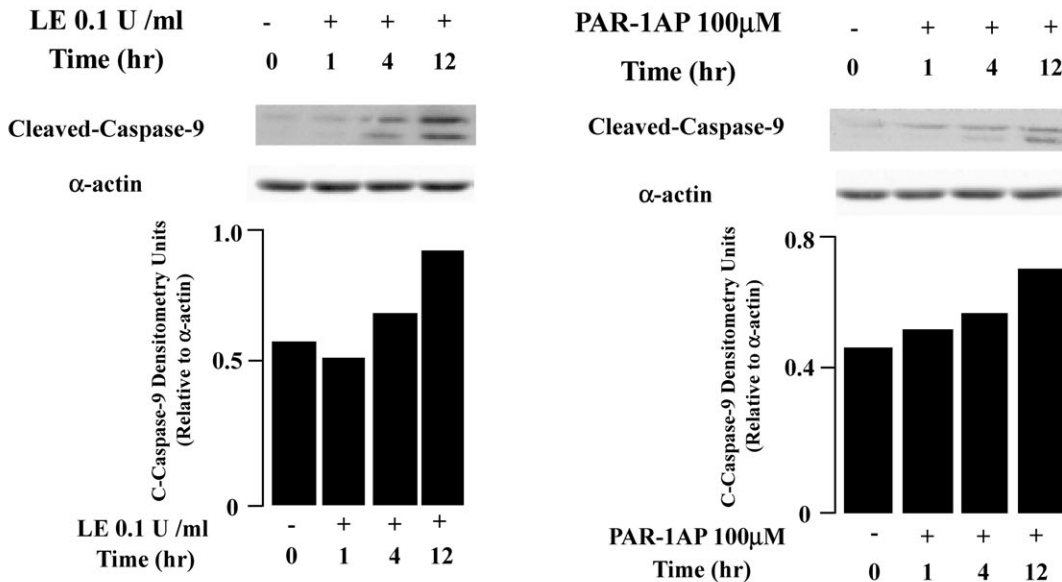
Figure 6. Caspase activation by leukocyte elastase (LE) and PAR-1AP exposure in lung epithelial cells. *A* illustrates cleavage of caspase-9, and *B* illustrates cleavage of caspase-3 in BEAS2-B epithelial cells by Western blot analysis. The graph represents quantification by densitometry normalized for levels of α-actin. Representative data each from one of three experiments are shown.
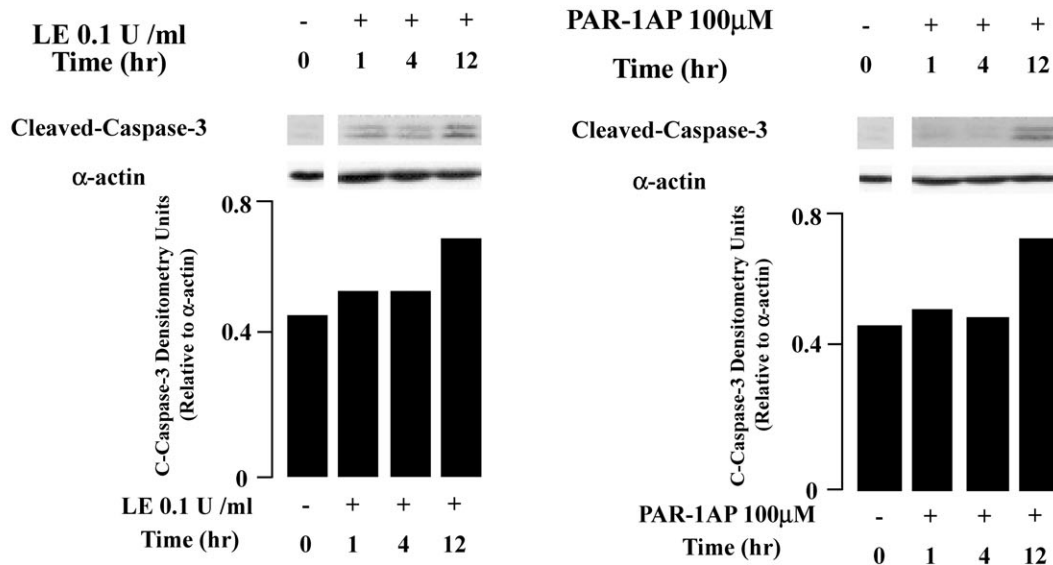
of epithelial cells to leukocyte elastase, in a PAR-1–dependent manner, results in inhibition of Akt and activation of JNK, a member of the MAP kinase family. Both of these effects modulate apoptosis. These observations have important implications for our understanding of the pathogenesis of acute and chronic lung injury including emphysema where inappropriate apoptosis induced by serum or leukocyte-derived proteinases, may contribute to lung epithelial injury and dysfunction.

In the current study, leukocyte elastase, thrombin, and PAR-1–activating peptide all induced apoptosis of human lung epithelial cells with a similar (but not identical) pattern. The similarity in responses to leukocyte elastase and to "authentic" PAR-1 activating agents supports the notion that the effects of

elastase are mediated via PAR-1. Consistent with this notion, epithelial apoptosis induced by leukocyte elastase was largely prevented by the selective PAR-1 antagonist, SCH79797 (Figure 4A). Additional and very strong evidence for a role of PAR-1 in elastase-induced epithelial apoptosis is provided by the studies using RNA interference (Figure 4), where PAR-1 siRNA diminished elastase-induced apoptosis by more than 50%. Although both PAR-1–activating peptide and thrombin activate PAR-1 directly (26, 27), the ability of leukocyte elastase to activate PAR-1 has not been previously reported. It is known that leukocyte serine proteinases including elastase, cathepsin G, and proteinase 3 can cleave and inactivate ("disarm") PAR-1 on human endothelial cells (38). However, in the case of PAR-2, proteolytic

cleavage of the receptor by leukocyte elastase can either activate or inactivate the receptor, depending on the site of cleavage (39, 40). By analogy, it is possible that proteolytic cleavage of PAR-1 might also activate or inactivate the receptor depending on the site of cleavage. Our data obtained with the use of the nonpeptide PAR-1 antagonist, SCH 79797, demonstrate clearly that the apoptotic action of leukocyte elastase is due to PAR-1 activation (41). Factors that could influence the site and extent of cleavage of the receptor include the extent of glycosylation of the receptor, the ratio of enzyme to receptor, and the presence of endogenous antiproteinases such as SLPI. These factors might differ between endothelial and epithelial cells and provide an explanation for differential responses between endothelial and epithelial cells. It is also possible that the effects of leukocyte elastase on PAR-1 are indirect and mediated for example, by release of surface bound proteinases such as matrix metalloproteinases (MMPs) that could then cleave and activate PAR-1. Further, the effects of leukocyte elastase might be mediated in part through other epithelial plasma membrane receptors or by release of surface-bound molecules such as growth factors that could in turn bind to their cognate receptors and initiate pro-apoptotic signaling pathways. Thus, although our data clearly indicate a role for PAR-1 in transducing the pro-apoptotic effects of leukocyte elastase, whether these are direct or indirect effects of the enzyme on the receptor remains to be clarified.

The involvement of PAR-1 in regulation of apoptosis has been recently recognized in other cell types including neurons, endothelium, intestinal epithelium, lung fibroblasts, and melanoma cells, suggesting a more generalized mechanism (28, 42–45). Under these circumstances, PAR-1 has been linked to both pro- and anti-apoptotic effects depending on the cell type and the experimental conditions (46). For example, activation of PAR-1 by thrombin can either induce or inhibit apoptosis in cultured neurons and astrocytes (47, 48). In gastrointestinal epithelium, activation of PAR-1 increases apoptosis and intestinal permeability in a caspase-3–dependent fashion (28). In the current study, we demonstrate that leukocyte elastase induces apoptosis in human lung epithelial cells of either airway (BEAS-2B and SAEC) or alveolar (primary alveolar type II cells) origin. The generalizability of these responses across a broad range of lung epithelial cell types supports their physiologic importance.

In the current studies, we noted some differences in the effects of PAR-1–activating peptide and leukocyte elastase on the kinetics of apoptosis (Figure 3). This might be explained by both rapid activation and termination of signals from PAR-1 by the activating peptide compared with more gradual but protracted activation of the receptor by leukocyte elastase. Further, leukocyte elastase may have additional effects including the ability to activate multiple receptors, the ability to generate cleaved peptides with biological activity, and the ability to alter cellular

processes independently of cell surface receptor modifications as discussed above.

In the present study, the concentration of leukocyte elastase was chosen on the basis of estimates of the amount of elastase released by neutrophils *in vitro* (7). In this regard, it is important
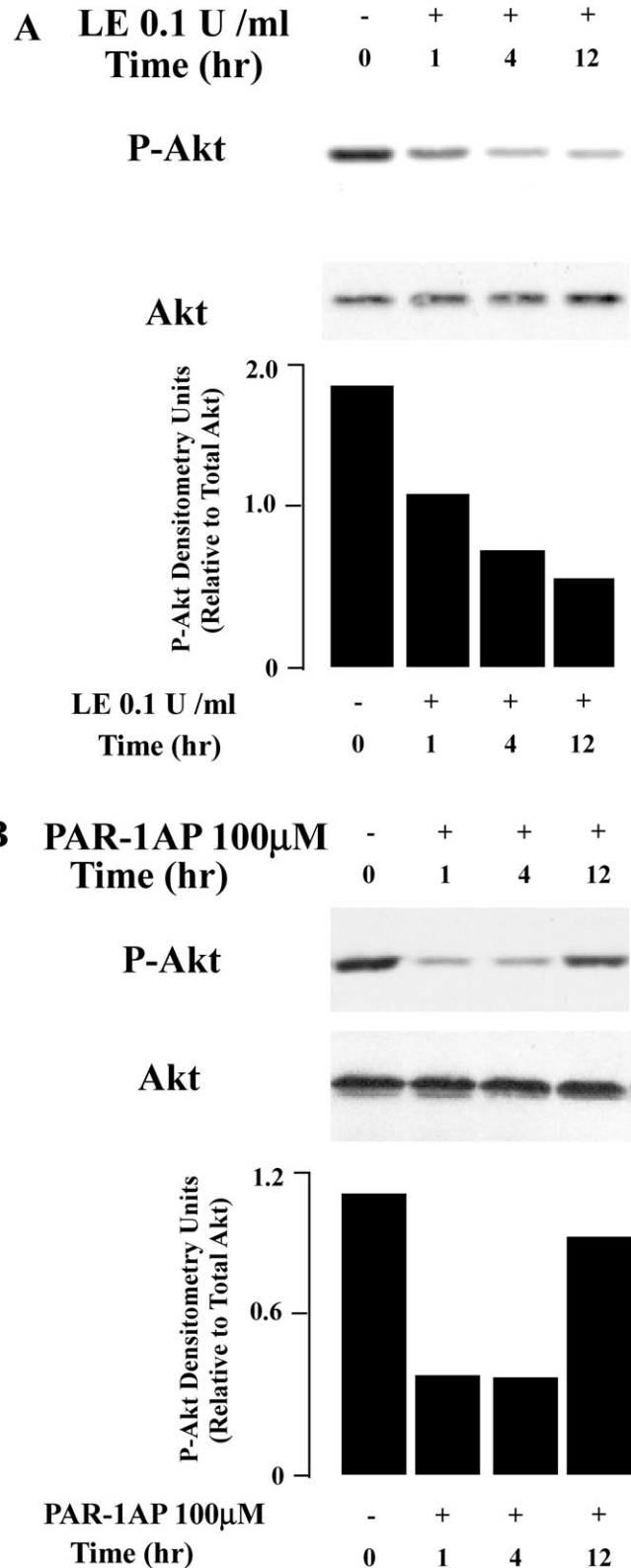

Figure 7. Akt phosphorylation is reduced by exposure to leukocyte elastase (LE) and PAR-1AP. Western analysis of total protein from BEAS-2B cells collected at 0, 1, 4, and 12 h after exposure to leukocyte elastase (*A*) or PAR-1AP (*B*). The graph represents quantification by densitometry normalized for levels of total Akt. Representative data from one of five independent experiments are shown. (*C*) Western analysis of total protein from BEAS-2B cells collected after PAR-1A siRNA or control siRNA transfection followed by incubation with leukocyte elastase or buffer control. Phospho-Akt and total Akt were assessed at 0 and 12 h. The accompanying graph represents quantification by densitometry normalized for levels of total Akt. Representative data from one of three independent experiments are shown.
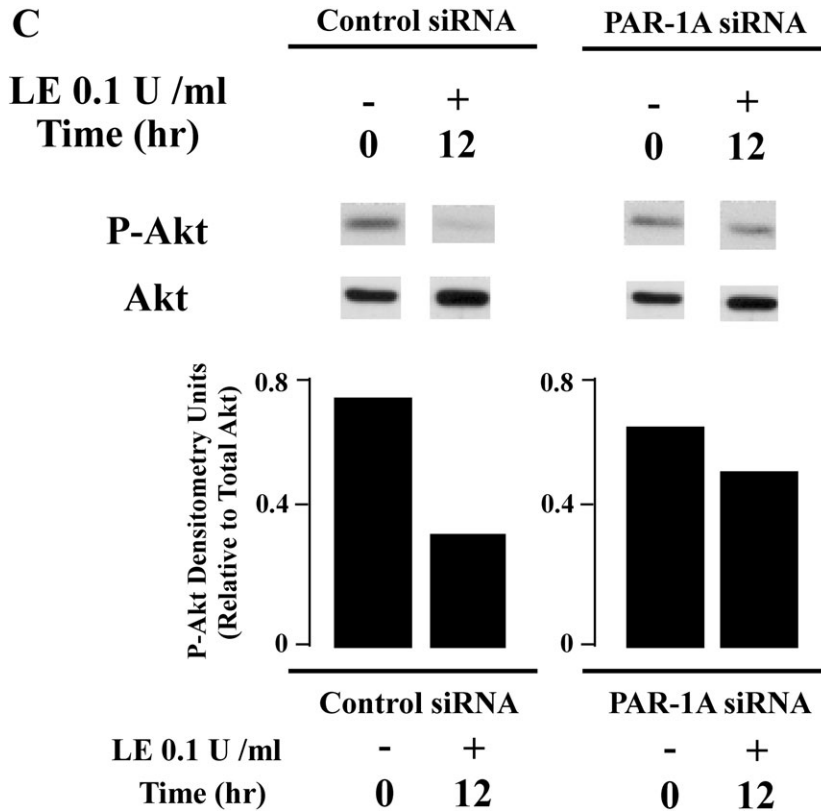
Figure 7. Continued

to appreciate that when leukocytes such as neutrophils are in close proximity to lung parenchymal cells, proteinases can be released into a "protected space" that forms between the epithelial cell and the neutrophil (2, 49). Large molecular weight anti-proteinases do not have access to this space, promoting the unopposed action of proteinases. Moreover, activated neutrophils release reactive oxygen species, which can inactivate anti-proteinases. Thus, it is likely that adjacent epithelial or endothelial cells are exposed to even higher local concentrations of proteinases.

Although it has been previously described that leukocyte-derived proteinases can lead to epithelial apoptosis (8, 50), the mechanisms remain incompletely understood. Research has focused on mechanisms involving modulation of plasma membrane receptors of the death domain family (i.e., the extrinsic pathway). Expression of Fas, a prototypical member of the death-receptor family, is increased in the alveolar epithelium of patients with ARDS and importantly, soluble Fas ligand (FasL) levels are elevated in the BAL fluid of patients with ARDS (51, 52). More recently, Nakamura and coworkers reported that proinflammatory cytokines sensitize the lung epithelium to Fas-induced cell death (53). As leukocyte elastase enhances production of proinflammatory cytokines such as IL-8 and IL-1β, it is possible that this effect may also lead to apoptosis. Elastase exposure has also been shown to increase expression of FasL in liver cells directly (54, 55). By analogy, a similar mechanism may be applicable to ALI/ARDS. Although our results indicate a role for the intrinsic apoptotic pathway, this does not exclude a potential role for the Fas–FasL pathway.

Diverse and interconnected signaling molecules are known to be involved in apoptosis including NF-κB, mitogen-activated protein (MAP) kinases, phosphatidylinositide 3-kinase (PI3K),

and tyrosine kinases (44, 46, 56, 57). In the current study, we focused on two additional signaling molecules modulated by leukocyte elastase that lie downstream of PAR-1. The first of these is Akt, a serine/threonine kinase that is known to suppress apoptosis. Akt inactivates the pro-apoptotic protein BAD and caspase-9 by phosphorylation (58). We recently reported that treatment of GI epithelial cells with a pharmacologic inhibitor of Akt, amplifies leukocyte elastase–induced apoptosis (7) and we have observed similar effects in lung epithelial cells (data not shown). Thus under the conditions of our model system, Akt exerts primarily an anti-apoptotic influence and this is prevented by leukocyte elastase in a PAR-1–dependent manner.

In the current study, we provide evidence that elastase-induced activation of JNK, a member of the MAP kinase family, modulates apoptosis. The role of JNK in apoptosis is complex and both pro- and anti-apoptotic effects have been reported (59–63). Evidence for pro-apoptotic effects of JNK includes the observation that mouse embryonic fibroblasts deficient in both JNK1 and JNK2 are resistant to apoptosis (59). In addition, JNK stimulates BAX translocation to the mitochondria in ischemia-induced neuronal apoptosis (64) and phosphorylates Bcl-2 inter-acting mediators of cell death (Bim$_{EL}$) in apoptosis induced via the p75 neurotrophin receptor (p75^{NTR}) (65). Conversely, JNK activation may promote cell survival. For example, JNK1$^{-/-}$ and JNK2$^{-/-}$ embryos exhibit markedly increased apoptosis in the forebrain (66, 67). In addition, downregulation of JNK using antisense oligonucleotides results in increased apoptosis in human breast and colorectal carcinoma cells (68). In our current study, we observed that JNK phosphorylation rapidly (30–60 min) increased after exposure of epithelial cells to leukocyte elastase and PAR-1–activating peptide. Importantly, inhibition of JNK amplified leukocyte elastase–induced apoptosis indicating that
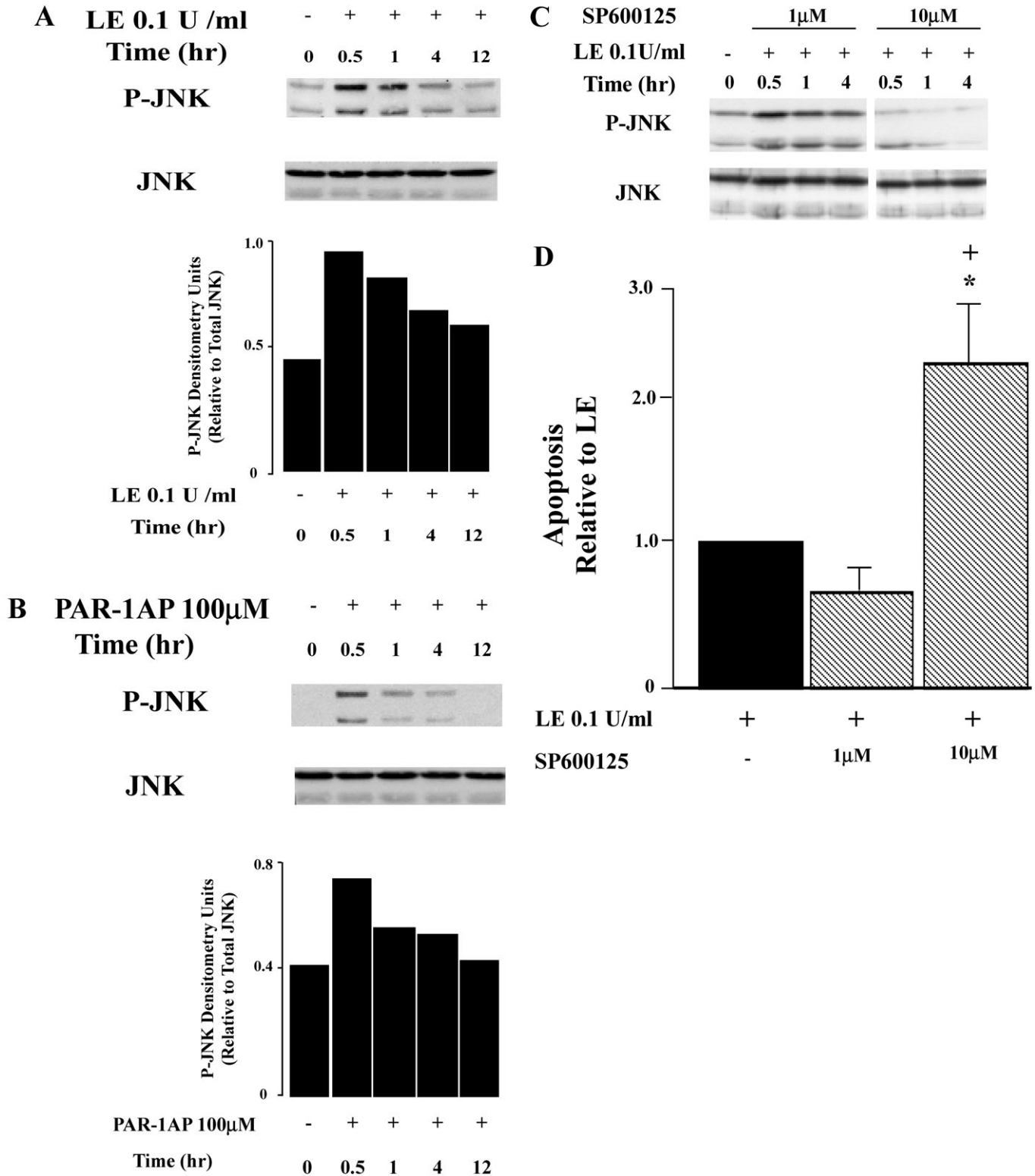
Figure 8. JNK phosphorylation is induced by exposure to leukocyte elastase (LE) and PAR-1AP. BEAS-2B cells were treated with leukocyte elastase (A) and PAR-1AP (B), and total protein was collected at 0, 0.5, 1, 4, and 12 h. The graph represents quantification by densitometry normalized for levels of total JNK. Representative data from one of four independent experiments are shown. (C) Cells were treated with SP600125, a specific JNK inhibitor 1 h before exposure to leukocyte elastase and PAR-1AP. Western blot analysis was used to assess levels of phospho-JNK and total JNK. (D) Treatment of cells with a JNK inhibitor enhances leukocyte elastase–induced epithelial apoptosis. Cells were pretreated with SP600125 1 μM and 10 μM and exposed to leukocyte elastase for an additional 12 h. The graph represents apoptosis relative to leukocyte elastase treatment samples. Values are mean ± SD; *$P < 0.05$ compared with the samples without SP600125; + $P < 0.05$ compared with the samples with SP600125 1 μM; $n = 3$.

under these circumstances, JNK exerts a net anti-apoptotic effect. These data do not preclude the possibility that JNK exerts both pro- and anti-apoptotic effects with the anti-apoptotic effect predominating under the conditions of the experiments. There is evidence that MAP kinases including JNK lie downstream of PAR-1 (69), but to our knowledge, this is the first report linking PAR-1 modulation of JNK to apoptosis.

A substantial body of experimental and clinical data support the role of apoptosis in the pathogenesis of inflammatory tissue injury in critical illness including acute lung injury and ARDS, myocardial infarction, and renal and gastrointestinal tract failure dysfunction in sepsis (52, 70–72). Although apoptosis is vital in many physiologically important processes, either inappropriate activation or inhibition of apoptosis can lead to disease, either because cells have prolonged survival (ongoing inflammation) or because they die prematurely and produce structural and functional changes in normal tissue. Similarly, in the context of ALI and ARDS, apoptosis may be either beneficial or harmful. For example, apoptosis of neutrophils may reduce inflammatory injury by induction of an anti-inflammatory phenotype in activated alveolar macrophages (72) and epithelial apoptosis is necessary for remodeling of tissue during repair. Conversely, during the acute phase, excessive epithelial apoptosis may aggravate lung injury by compromise of the alveolo–capillary membrane. Indeed, in both animal and human studies, increasing evidence suggests that apoptosis of distal lung epithelial cells appears to be pivotal in the pathogenesis of ALI (8, 10, 50). With lung epithelial loss, the permeability of alveolar capillary barrier is increased, and activated neutrophils as well as plasma proteins (thrombin) move from the bloodstream into the alveolar space. In this milieu, there is potential for alveolar epithelial cells to be exposed to PAR-1–activating proteases that may induce apoptosis and thus exacerbate lung injury. Therapeutic intervention aimed at modulating pro-apoptotic signals from PAR-1 might be considered under these circumstances. Clearly the balance between pro-apoptotic and anti-apoptotic factors in ALI is crucial and any therapeutic strategies directed at these processes must bear this in mind.

In summary, we have provided strong evidence that leukocyte elastase, acting via PAR-1, induces apoptosis of human lung epithelial cells by a mechanism involving increased mitochondrial permeability, release of cytocrome *c* to the cytosol, and activation of caspase-9 and -3 leading to apoptosis. In addition, PAR-1 activation by leukocyte elastase leads to inhibition of Akt and activation of JNK, responses that are pro-apoptotic. Together, these observations provide a potential mechanistic explanation for apoptosis of lung epithelial induced by exposure to leukocyte-derived proteinases, circumstances that are relevant to inflammatory lung injury such as occurs in ALI and ARDS (8, 11) as well as in chronic obstructive lung disease (11, 73).

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