The Src family tyrosine kinases src and yes have differential effects on inflammation-induced apoptosis in human pulmonary microvascular endothelial cells

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Nelin LD, White HA, Jin Y, Trittmann JK, Chen B, Liu Y. The Src family tyrosine kinases src and yes have differential effects on inflammation-induced apoptosis in human pulmonary microvascular endothelial cells. Am J Physiol Lung Cell Mol Physiol 310: L880–L888, 2016. — Endothelial cells are essential for normal lung function; they sense and respond to circulating factors and hemodynamic alterations. In inflammatory lung diseases such as acute respiratory distress syndrome, endothelial cell apoptosis is an inciting event in pathogenesis and a prominent pathological feature. Endothelial cell apoptosis is mediated by circulating inflammatory factors, which bind to receptors on the cell surface, activating signal transduction pathways, leading to caspase-3-mediated apoptosis. We hypothesized that yes and src have differential effects on caspase-3 activation in human pulmonary microvascular endothelial cells (hPMVEC) due to differential downstream signaling effects. To test this hypothesis, hPMVEC were treated with siRNA against src (siRNAsrc), siRNA against yes (siRNAyes), or their respective scramble controls. After recovery, the hPMVEC were treated with cytomix (LPS, IL-1β, TNF-α, and IFN-γ). Treatment with cytomix induced activation of the extracellular signal-regulated kinase (ERK) pathway and caspase-3-mediated apoptosis. Treatment with siRNAsrc blunted cytomix-induced ERK activation and enhanced cleaved caspase-3 levels, while treatment with siRNAyes enhanced cytomix-induced ERK activation and attenuated levels of cleaved caspase-3. Inhibition of the ERK pathway using U0126 enhanced cytomix-induced caspase-3 activity. Treatment of hPMVEC with cytomix induced Akt activation, which was inhibited by siRNAsrc. Inhibition of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway using LY294002 prevented cytomix-induced ERK activation and augmented cytomix-induced caspase-3 cleavage. Together, our data demonstrate that, in hPMVEC, yes activation blunts the ERK cascade in response to cytomix, resulting in greater apoptosis, while cytomix-induced src activation induces the phosphatidylinositol 3-kinase pathway, which leads to activation of Akt and ERK and attenuation of apoptosis.

caspase-3; extracellular signal-regulated kinase; cytokines

INFLAMMATION IS INVOLVED in the pathogenesis of almost all lung diseases. As one important example, inflammation underlies the acute lung injury (ALI) that leads to acute respiratory distress syndrome (ARDS) (34, 36). ALI/ARDS usually develops in hospitalized patients within 24–72 h of the onset of their illness and is secondary to inflammatory conditions such as sepsis, pneumonia, and trauma (34, 36). Treatments for ALI/ARDS are largely supportive, consisting of treatment of the underlying symptoms, as well as judicious mechanical ventilation and fluid management (27). There is a real need for the development of pharmacotherapies directed at ALI/ARDS (4), particularly pharmacotherapies that could be given within 24–72 h of onset to prevent or attenuate the development of full-blown ARDS (25). Another example of an inflammatory lung disease is bronchopulmonary dysplasia (BPD). Much like ALI/ARDS, BPD develops in preterm infants secondary to ALI caused by inflammatory conditions such as surfactant deficiency, pneumonia, and sepsis (11, 35).

Apoptosis is central to the pathogenesis of the lung injury leading to ALI/ARDS and BPD. In ALI/ARDS, endothelial cell apoptosis is well established as a key step in the formation of pulmonary edema and inflammatory cell infiltration, which are the hallmarks of the disease (10, 36). Hashimoto et al. (13) found significant mRNA and protein markers for apoptotic mediators in bronchoalveolar lavage fluid from patients with early ALI/ARDS. In an autopsy study of BPD patients, endothelial apoptosis was substantially greater in the lungs from patients with BPD than in age-matched controls (22). Animal models of BPD also showed that apoptosis is central to the pathogenesis of the disease (1, 8, 23).

The Src family of tyrosine kinases (STK) represents a group of closely related evolutionarily conserved nonreceptor protein tyrosine kinases defined by a common molecular structure (3, 17) with distinct structural motifs termed Src homology (SH) domains. The STK consist of an NH2-terminal sequence (the SH4 domain) that directs myristoylation, resulting in membrane localization, followed by a nonconserved unique domain, an SH3 domain that directs binding to polyproline-rich sequences, an SH2 domain that binds phosphotyrosine, a catalytic domain (the SH1 domain), which includes the positive regulator site tyrosine 416, and a short COOH-terminal tail that contains a negative regulator site, tyrosine 527 (3, 17). We previously showed in human pulmonary microvascular endothelial cells (hPMVEC) that activation of the STK is necessary for LPS-induced inducible nitric oxide (NO) synthase expression (5). The STK include at least nine members: src, fyn, yes, lyn, lck, hck, blk, fgr, and yrk; however, yrk has not been found in humans. We previously reported that hPMVEC express yes, src, and fyn (5). The signal transduction pathways that mediate apoptosis and caspase-3 activation in endothelial cells are not well understood. We previously showed in hPMVEC that the individual STK could be knocked down using specific siRNA and demonstrated that cytomix-induced urea production was differentially regulated by the various STK family members (5). Moreover, studies using hPMVEC have revealed differential effects of src, fyn, and yes on TNF-α-induced permeability (2). Therefore, we hypothesize that the STK src and yes will have opposing effects on cytokine-induced apoptosis in hPM-
VEC and that these opposing effects will be due to differential downstream signaling effects.

**METHODS**

*Human pulmonary microvascular endothelial cells. hPMVEC (Lonza, Allendale, NJ)* were grown in six-well plates according to the manufacturer’s recommendations using endothelial cell basal medium (EBM2, Lonza) supplemented with an EGM-2 bullet kit (Lonza). On the day of study, the hPMVEC were washed three times with 2 ml of Hanks’ balanced salt solution (HBSS). Then 1 ml of medium was placed on the hPMVEC (control), and the cells were cultured in an incubator for 24 h at 37°C in 5% CO₂-balance air. Included in the

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**Graphs and Figures:**

- **A:** src
  - Cleaved caspase 3
  - Cleaved PARP

- **B:** Cleaved caspase 3 (α-actin) (fold change from control)
  - Control
  - Scramble
  - src siRNA

- **C:** Caspase 3 Activity (fold change from control)
  - Control
  - Scramble
  - src siRNA

- **D:** yes
  - Cleaved caspase 3
  - Cleaved PARP

- **E:** Cleaved caspase 3 (α-actin) (fold change from control)
  - Control
  - Scramble
  - yes siRNA

- **F:** Caspase 3 Activity (fold change from control)
  - Control
  - Scramble
  - yes siRNA

- **G:** Cleaved caspase-3
  - Cytomix
  - Scramble
  - Yes siRNA
  - Src siRNA

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*Note: Diagrams and figures are not transcribed due to the nature of the task.*
medium used to culture the cytomix-treated hPMVEC were 1.5 µg/ml LPS, 3 ng/ml TNF-α, 2 ng/ml IL-1β, and 30 ng/ml INF-γ (all from Sigma Chemical, St. Louis, MO), as previously described (5). After 24 h, the medium was removed and frozen at −70°C. The hPMVEC were washed three times with 4 ml of HBSS and treated with lysis buffer for protein extraction. A transfection reagent provided by the manufacturer (Dharmacon) was used to transfect the hPMVEC with the siRNA of interest (Dharmacon) or a scramble siRNA as a control, as previously described (5, 33). We previously showed that the siRNAs against the STK (src, fyn, yes, and lyn) are specific for their targeted family member and do not alter expression of the other family members in the hPMVEC (see Fig. 4D in Ref. 5). After 48 h, the hPMVEC were washed three times, and medium containing cytomix or vehicle was added to the cell culture wells. After an additional 24 h of incubation, the medium was harvested, and the cells were lysed for protein extraction.

**Protein isolation.** Protein was isolated from the hPMVEC as previously described (5, 7, 33). Briefly, the cells were washed with HBSS, and lysis buffer (0.2 M NaOH and 0.2% SDS) was added. At 30 min before the hPMVEC were used, the following protease inhibitors were added to each milliliter of lysis buffer: 0.2 µl of aprotinin (10 mg/ml double-distilled H2O), 0.5 µl of leupeptin (10 mg/ml double-distilled H2O), 0.14 µl of pepstatin A (5 mg/ml methanol), and 5 µl of phenylmethylsulfonyl fluoride (34.8 mg/ml methanol). The cells were scraped and placed in sterile centrifuge tubes on ice. The supernatant was stored in 1-ml tubes at −70°C for Western blot analysis. Total protein concentration was determined by the Bradford method using a commercially available assay (Bio-Rad, Hercules, CA).

**Western blotting.** Cell lysates were assayed for cleaved caspase-3, fyn, src, yes, β-actin, phosphorylated and total amounts of extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38, as well as phosphorylated and total Akt, using Western blot analysis, as previously described (5, 16). Aliquots of cell lysate were diluted 1:1 with SDS sample buffer, heated to 80°C for 15 min, and then centrifuged at 10,000 × g at room temperature for 2 min. Aliquots of the supernatant were used for SDS-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes and blocked overnight in PBS with 0.1% Tween containing 5% nonfat dry milk and 3% albumin. The membranes were then incubated with the primary antibody of interest (cleaved caspase-3, 1:1,000 dilution; Cell Signaling Technology, Danvers, MA; all others, 1:1,000 dilution: Transduction Laboratories, Lexington, KY). The blots were washed with PBS with 0.1% Tween and 1% nonfat dry milk. The membranes were then incubated with the biotinylated IgG secondary antibody (1:5,000 dilution; Vector Laboratories, Burlingame, CA) for 1 h, washed, and incubated with streptavidin-horseradish peroxidase conjugate (1:1,500 dilution; Bio-Rad) for 30 min. The bands of interest were visualized using enhanced chemiluminescence (Amersham, Piscataway, NJ) and quantified using densitometry (Sigma Gel, Jandel Scientific, San Rafael, CA). To control for protein loading, the blots were stripped using a stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol). The blots for cleaved caspase-3, fyn, src, and yes were reprobed for β-actin (1:10,000 dilution; Abcam, Cambridge, MA), while the blots for the phosphorylated proteins were reprobed for the total amounts of the protein of interest [e.g., ERK for phosphorylated ERK (pERK) and Akt for phosphorylated Akt (pAkt)], as described above. The quantified densitometry data are shown as the protein of interest over the appropriate protein loading control normalized to the values for control such that control values are 1.

**Caspase-3 activity.** Caspase-3 activity was measured in cell lysates using a fluorescence-based assay (BioVision, Mountain View, CA).

**Proliferation assay.** Proliferation of the hPMVEC was determined in six-well plates, as previously described (33). Cultured hPMVEC, 5 × 104 cells, were plated into each well of six-well plates. The appropriate reagents were included in the medium, and the cells were incubated for 48 h. At the end of the experimental protocol, the cells were removed from the incubator and the plates were washed three times with HBSS. After the final wash, 1 ml of trypsin was added to each well, and the plates were incubated for 3 min; then the plates were incubated with 2 ml of trypsin-neutralizing solution. The cells from each well were placed in 15-ml conical tubes and centrifuged for 5 min at 1,220 g at 4°C. The supernatant was discarded, and the cells were resuspended in 1 ml of EGM. The cells were mixed 1:1 with Trypan blue and viable cells; in other words, cells that were not blue, were counted using a hemocytometer.

**Experimental protocols.** To examine the effect of src or yes knockdown on caspase-3 activity, we utilized Western blotting and activity assays for caspase-3. The hPMVEC were not transfected (control) or were transfected with a scramble siRNA or a siRNA against src (siRNAsrc) for 48 h. In parallel studies, the hPMVEC were not transfected or were transfected with a scramble siRNA or a siRNA against yes (siRNAyes) for 48 h. Then the cells were treated with cytomix for 24 h, and protein was harvested for Western blotting and activity assays for caspase-3.

To examine the effect of selective knockdown of src or yes on viable cell numbers, we used siRNA-induced selective knockdown of src or yes and Trypan blue exclusion to quantify viable cell numbers. The cells were not treated (controls) or were treated with a scramble siRNA, siRNAsrc, or siRNAyes. After 48 h, the cells were washed and trypsinized, and 5 × 104 cells were plated in each well of a six-well plate. The siRNA-transfected cells were treated with cytomix, while controls were left untreated; after 48 h, the number of viable cells was counted using Trypan blue exclusion.

To investigate the role of src and yes in cytomix-induced activation of the mitogen-activated protein kinases (MAPK), the hPMVEC were treated with siRNA src or a scramble siRNA; in parallel experiments, the hPMVEC were treated with siRNAyes or a scramble siRNA. After 48 h, the cells were washed and then treated with cytomix for an additional 24 h. Protein was harvested for Western blotting for total and phosphorylated ERK, p38, and JNK.

Next we looked at the effect of an ERK pathway inhibitor, U0126, on levels of cleaved caspase-3 in cytomix-treated hPMVEC. The cells were untreated (controls) or were treated with vehicle or 10 µM U0126.
U0126 and also given cytokimix. After 24 h, protein was harvested for Western blotting of phosphorylated and total ERK, JNK, and p38.

To determine if cytokimix-induced src and/or yes signaling activates the phosphatidylinositol 3-kinase (PI3K) pathway, we examined the activation of Akt following treatment with scramble siRNA, siRNA src, or siRNA yes. The cells were transfected with scramble RNA, siRNAsrc, or siRNAyes for 48 h or were not transfected (controls). The cells were then washed and treated with cytokimix for an additional 24 h, and the protein was subsequently harvested for Western blotting for phosphorylated and total Akt.

To determine if inhibition of PI3K activation would affect cytokimix-induced cleaved caspase-3 protein levels, we utilized the putative PI3K inhibitor LY294002. The cells were not treated (controls) or were treated with cytokimix together with vehicle or 10 μM LY294002; after 24 h, protein was harvested for Western blotting to detect cleaved caspase-3.

To determine if ERK plays a role in the inhibitory effect of PI3K on cytokimix-induced cleaved caspase-3 protein levels, the hPMVEC were not treated (controls) or were treated with cytokimix in the presence or absence of 10 μM LY294002. After 24 h, protein was harvested for Western blotting for phosphorylated and total ERK, JNK, and p38, respectively.

Statistical analysis. Values are means ± SE. One-way analysis of variance was used to compare the densitometry data between control and treated cells or cellular proliferation. Significant differences were identified using a Newman-Keuls post hoc test (SigmaStat, Jandel Scientific, Carlsbad, CA). Differences were considered significant when P < 0.05.

RESULTS

Knockdown of src augmented cytokimix-induced caspase-3 activation, while knockdown of yes attenuated cytokimix-induced caspase-3 activation. Transfection of the hPMVEC with a scramble siRNA and cytokimix had little effect on total src protein levels, while transfection with the siRNAsrc led to a dramatic src knockdown (Fig. 1A). Levels of cleaved caspase-3 protein were greater in the scramble siRNA-treated hPMVEC stimulated with cytokimix. Levels of cleaved caspase-3 protein were greater in the hPMVEC treated with siRNAsrc and cytokimix than in those treated with the scramble siRNA and cytokimix (Fig. 1B). This observation was confirmed with the caspase-3 activity assays. Caspase-3 activity was significantly greater in scramble siRNA-transfected hPMVEC following cytokimix treatment than in control cells, and cytokimix-induced caspase-3 activity was substantially greater in siRNAsrc-transfected hPMVEC than in controls or scramble-treated hPMVEC (Fig. 1C). Similar results were found when an siRNA against lyn was substituted for siRNAsrc (data not shown).

We previously found that selective knockdown of yes had little effect on urea and NO production in cytokimix-treated hPMVEC, while selective knockdown of lyn prevented cytokimix-induced urea and NO production in hPMVEC (5). As expected, siRNAyes effectively knocked down cytokimix-induced yes protein expression (Fig. 1D). Cytokimix-induced levels of cleaved caspase-3 were somewhat lower in the hPMVEC transfected with siRNAyes than in scramble-transfected cytokimix-stimulated hPMVEC (Fig. 1E). However, the levels of cytokimix-induced caspase-3 activity in cells transfected with siRNAyes were not significantly different from those in scramble-transfected hPMVEC (Fig. 1F).

To determine if transfection with the scramble siRNAs had an effect on cleaved caspase-3 expression, the hPMVEC were treated as described above with an additional group of scramble-transfected, vehicle-treated cells included. A representative Western blot, shown in Fig. 1G, demonstrates that transfection in the absence of cytokimix had no effect on cleaved caspase-3 levels in the hPMVEC. Furthermore, the representative yes and src Western blots (Fig. 1G) demonstrate that the siRNAs were specific for their targeted STK, as we showed previously (5).

Knockdown of src, but not yes, leads to decreased viable cell numbers. There were significantly fewer viable scramble siRNA-transfected cells treated with cytokimix than control cells in all experiments (Fig. 2). Selective knockdown of src resulted in substantially fewer viable cells than scramble siRNA transfection following cytokimix treatment (Fig. 2). In contrast, selective knockdown of yes resulted in similar numbers of viable cells, as seen in scramble-transfected cells following cytokimix treatment, such that, following cytokimix stimulation, there were significantly more viable siRNAyes- than siRNAsrc-transfected hPMVEC (Fig. 2).

Knockdown of src resulted in lower levels of cytokimix-induced phosphorylated ERK levels, while knockdown of yes augmented cytokimix-induced pERK levels, in the hPMVEC. Levels of pERK were lower in the hPMVEC treated with cytokimix and siRNAsrc than in cytokimix-stimulated scramble siRNA-transfected cells (Fig. 3A). siRNAsrc did not affect levels of phosphorylated JNK (pJNK) or phosphorylated p38 (pp38) (Fig. 3, C and D). Levels of pERK were greater in cytokimix-stimulated hPMVEC treated with siRNAyes than in scramble-treated cells (Fig. 3B). However, knockdown of yes had little effect on pJNK or pp38 levels in cytokimix-treated hPMVEC (Fig. 3, D and F).

Inhibition of ERK signaling augmented cytokimix-induced caspase-3 activity. pERK levels were greater in the hPMVEC treated with cytokimix alone than in controls (Fig. 4, A and B). pERK levels in the hPMVEC treated with cytokimix and U0126 were similar to those in the control cells (Fig. 4, A and B).
Cleaved caspase-3 protein levels were greater in the cells treated with cytomix than in control cells, and inhibition of ERK signaling with U0126 significantly augmented the cytomix-induced levels of cleaved caspase-3 (Fig. 4).

Knockdown of src, but not yes, resulted in lower levels of pAkt in cytomix-treated hPMVEC. Cytomix treatment resulted in greater levels of pAkt in scramble siRNA-treated cells (Fig. 5). siRNAsrc prevented the cytomix-induced increase in pAkt levels in the hPMVEC (Fig. 5A), while siRNAyes had no discernible effect on levels of pAkt protein in the hPMVEC (Fig. 5, C and D).

**Inhibition of PI3K augmented cytomix-induced cleaved caspase-3 expression.** While cytomix treatment resulted in an increase in cleaved caspase-3 protein levels in cells treated with vehicle relative to control cells, treatment with LY294002 resulted in a substantial increase in cleaved caspase-3 protein levels relative to cytomix-stimulated cells treated with vehicle (Fig. 6).

**Inhibition of PI3K activation prevented cytomix-induced ERK activation.** Levels of cytomix-induced pERK protein were significantly lower in LY294002- than vehicle-treated cells (Fig. 7A). Treatment of the hPMVEC with LY294002 had

Fig. 3. Of the MAPK, only cytomix-induced ERK activation was affected by src or yes knockdown. hPMVEC (n = 4 for each condition) were treated with siRNAsrc or scramble for 48 h, washed, and then treated with cytomix. After 24 h, protein was harvested for Western blotting of phosphorylated ERK (pERK), JNK (pJNK), or p38 (pp38); then the membranes were then stripped and reprobed for total amounts of ERK, JNK, or p38, respectively. Intensities of the bands were quantified by densitometry normalized to the respective total protein level. Experiment was repeated using siRNAyes and scramble, as described above. A: pERK levels were lower in siRNAsrc- than scramble-treated cells. B: pERK levels were augmented in siRNAyes- compared with scramble-treated cells. C and E: knockdown of src had little effect on cytomix-induced levels of pp38 or pJNK. D and F: knockdown of yes had little effect on cytomix-induced levels of pp38 or pJNK. *P < 0.05, cytomix vs. control. #P < 0.05, siRNA vs. scramble.
little effect on cytomix-induced levels of pp38 protein (Fig. 7B) or pJNK (Fig. 7C). These results suggest that PI3K inhibits apoptosis in cytomix-treated cells via the Akt and ERK pathways.

**DISCUSSION**

In the present study we demonstrate that the specific STK family members src and yes have differing effects on cytomix-induced apoptosis in hPMVEC. Inhibition of src using an siRNA resulted in a substantial augmentation of cytomix-induced cleaved caspase-3 protein expression and activity. In contrast, inhibition of yes with siRNAyes resulted in a small decrease in cleaved caspase-3 protein expression. The changes in cleaved caspase-3 expression paralleled viable cell numbers, such that siRNAsrc resulted in substantially fewer viable cells following cytomix treatment, while siRNAyes had little effect on the number of viable cells following cytomix treatment.

When exploring downstream signaling cascades activated by src and yes, we found that knockdown of src resulted in lower cytomix-induced pERK expression with little effect on pp38 or pJNK levels, while knockdown of yes augmented pERK expression with little effect on pp38 or pJNK levels. A small-molecule inhibitor of the ERK pathway resulted in augmentation of cytomix-induced cleaved caspase-3 protein expression in hPMVEC. Knockdown of src resulted in substantially lower levels of pAkt following cytomix treatment, while knockdown of yes had little effect on cytomix-induced pAkt levels in hPMVEC. Use of a small-molecule inhibitor of the PI3K/Akt pathway resulted in substantially increased levels of cleaved caspase-3 and decreased levels of pERK following cytomix treatment in hPMVEC, with no significant effect on cytomix-induced pp38 or pJNK levels. Together, our data demonstrate that src and yes differentially affect cytomix-induced apoptosis in hPMVEC, wherein src activates PI3K/Akt, which in turn activates ERK to prevent caspase-3 activation, while yes inhibits ERK activation, thereby augmenting caspase-3 activation (Fig. 8). Thus our findings support our hypothesis that src and yes have opposing effects on cytomix-induced caspase-3 activation, and this is due to the effects of src and yes on Akt/ERK signaling in hPMVEC. Further studies are needed to determine if these effects of src and yes on caspase-3 activation in hPMVEC correlate with disease pathogenesis or progression in vivo.

The STK have been implicated in the pathogenesis of lung injury in animal models. In BALB/c mice (18, 30), it has been shown that LPS, administered intraperitoneally or intratracheally, leads to lung injury associated with STK activation and that when the STK are inhibited using small-molecule inhibitors, LPS-induced lung injury is attenuated. Oyaizu et al. (26) found that ischemia-reperfusion lung injury in Sprague-Dawley rats resulted in STK phosphorylation and that treatment of the mice with a small-molecule inhibitor of the STK attenuated lung injury and reduced the number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end label-positive cells in the lung. Zhao et al. (38) found that high-tidal-volume ventilation in Wistar rats resulted in lung injury and activation of STK and that the lung injury was significantly attenuated when STK activation was prevented by treatment with PP2, a STK inhibitor. Using a hyperoxia-and-high-tidal-volume model of ventilator-induced lung injury, Liu et al. (21) found that lung injury was attenuated in mice heterozygous for Src (Src+/-) compared with mice homozygous for Src (Src+/-). Han et al. (12) found greater susceptibility to LPS-induced lung injury in lymph-deficient than wild-type mice. These results highlight the importance of STK signaling in lung injury models and demonstrate that individual STK have a role in these lung injury models, although their role may depend on the model used to induce lung injury.

Our data support the concept that src is antiapoptotic, while yes is proapoptotic, in hPMVEC following cytomix exposure. This is consistent with findings in hepatocytes, where, in response to cell swelling, src is proproliferative, while yes is proapoptotic (28). On the other hand, in mesothelioma cell lines, knockdown of yes, but not src, using siRNA led to cell growth suppression due in part to enhanced apoptosis (29).
a neuroblastoma cell line, siRNA against either src or fyn resulted in decreased apoptosis following oxygen/glucose deprivation or amoylβ- peptide treatment (9). Similarly, in Hepa-1 cells (a mouse hepatoma cell line), overexpression of src or yes led to greater oxidant-induced apoptosis (24). Together, these findings support a differential role for the STK in apoptosis, although the effect of the individual STK family member likely depends on the specific cell type and/or the apoptotic stimulus. Indeed, Lewis-Tuffin et al. (19) recently reported that knockdown of the individual STK had highly variable effects in glioblastoma cell lines and xenograft models in mice, depending on the cell line. However, to the best of our knowledge, this is the first demonstration of a differential role of the STK in inflammation-induced apoptosis in hPMVEC.

We found that src, but not yes, was necessary for cytomix-induced Akt activation in hPMVEC. This finding is consistent with previous work showing that inhibition of STK signaling with PP2 inhibits phosphorylation of Akt (15). A specific role for src in Akt activation was demonstrated in mutant embryonic fibroblasts when knockout of src, fyn, and yes prevented estrogen-induced pAkt protein expression; if only yes and fyn were knocked out, there was an estrogen-induced increase in pAkt protein levels (14). We also found that Akt activity was necessary for attenuation of cytomix-induced apoptosis, given that inhibition of the PI3K/Akt pathway led to augmented cytomix-induced caspase-3 cleavage in hPMVEC. This finding is consistent with a report that inhibition of the PI3K/Akt pathway using LY294002 in human umbilical vein endothelial cells resulted in greater numbers of terminal deoxynucleotidyl transferase-mediated dUTP nick-end label-positive cells following TNF-α/cycloheximide treatment (37). Similarly, in bovine pulmonary artery endothelial cells, inhibition of Akt resulted in augmentation of LPS-induced caspase-3 activation (31). Thus, src mediates the Akt cascade in response to cytomix stimulation, and this src/Akt pathway is necessary to attenuate cytokine-induced apoptosis in hPMVEC.

We found that ERK activation was necessary to attenuate cytokine-induced apoptosis in hPMVEC. When ERK activity was inhibited using U0126, cytokine-induced cleaved caspase-3 levels were considerably increased. Furthermore, the effect of siRNAsrc on enhancement of caspase-3 activation was associated with lower levels of pERK protein, while the effect of siRNAyes in decreasing cleaved caspase-3 expression following cytomix was associated with greater levels of pERK.
Finally, the effect of Akt inhibition on enhancement of cytomix-induced cleaved caspase-3 levels was associated with decreased pERK protein levels. These results demonstrate a central role for ERK in attenuation of cytomix-induced apoptosis in hPMVEC (Fig. 8). This is consistent with studies in endothelial cells. Taraseviciene-Stewart et al. (32) found that simvastatin suppressed pERK expression and induced caspase-3 activation in rat pulmonary microvascular endothelial cells. Chen et al. (6) reported that treatment of human umbilical vein endothelial cells with urotensin II resulted in ERK phosphorylation and protection of cells from doxorubicin-induced caspase-3 activation and apoptosis and that when ERK phosphorylation was prevented with U0126, urotensin II had no effect on doxorubicin-induced caspase-3 activation and apoptosis. Recently, using rat pulmonary microvascular endothelial cells, Li et al. (20) reported that inhibition of ERK augmented LPS-induced apoptosis, while inhibition of JNK protected cells from LPS-induced apoptosis, consistent with the notion that ERK is involved in attenuation of inflammation-induced apoptosis. However, their findings also suggest that the other MAPK may have a proapoptotic role in pulmonary endothelial cells. Our present findings that knockdown of src or yes resulted in no differences in p38 or JNK activation following cytomix treatment suggest that src and yes are not involved in p38 or JNK activation in hPMVEC.

In summary, we found opposing effects of two STK family members on caspase-3 activation in hPMVEC following cytomix treatment: src was antiapoptotic, while yes was proapoptotic (Fig. 8). The antiapoptotic effects of src were through downstream activation of Akt, which in turn led to activation of ERK and, subsequently, attenuated caspase-3 activation. Proapoptotic effects of yes were through blunting of ERK activation, which dampened the inhibitory effect of ERK on caspase-3, resulting in augmentation of caspase-3 activation. Agonists of src and/or Akt and antagonists of yes may be potential therapeutic targets in preventing inflammatory lung diseases.

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

L.D.N., B.C., and Y.L. developed the concept and designed the research; L.D.N., H.A.W., and Y.L. analyzed the data; L.D.N., Y.J., J.K.T., and Y.L. prepared the figures.

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**Fig. 7.** Inhibition of Akt activation prevented cytokine-induced pERK expression. hPMVEC were not treated (controls) or were treated with cytomix in the presence or absence of 10 μM LY294002 (n = 7 for each condition). After 24 h, protein was harvested for Western blotting for pERK, pJNK, or pp38, and blots were stripped and reprobed for total ERK, JNK, or p38, respectively. Treatment with LY294002 prevented only cytomix-induced pERK protein expression (A), with no significant effect on pJNK (B) or pp38 (C) expression. *P < 0.05, cytomix vs. control. #P < 0.05, LY294002 vs. vehicle.

**Fig. 8.** Proposed model for opposing effects of src and yes on caspase-3 activation. Antiapoptotic effects of src were through downstream activation of Akt, which in turn led to activation of ERK and, subsequently, attenuated caspase-3 activation. Proapoptotic effects of yes were through blunting of ERK activation, which dampened the inhibitory effect of ERK on caspase-3, resulting in augmentation of caspase-3 activation. Agonists of src and/or Akt and antagonists of yes may be potential therapeutic targets in preventing inflammatory lung diseases.
interpreted the results of the experiments: L.D.N. and Y.J. prepared the figures; L.D.N., H.A.W., J.K.T., and B.C. drafted the manuscript; L.D.N., H.A.W., J.K.T., B.C., and Y.L. edited and revised the manuscript; L.D.N., H.A.W., J.K.T., B.C., and Y.L. approved the final version of the manuscript; Y.J. performed the experiments.

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