Activation of SRC Tyrosine Kinases in Response to ICAM-1 Ligation in Pulmonary Microvascular Endothelial Cells*

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The mechanisms underlying SRC activation in response to ICAM-1 cross-linking have been examined in rat brain microvascular ECs, where activation of SRC requires activation of phospholipase C (6). In these ECs and human umbilical vein ECs, activation of SRC in turn results in tyrosine phosphorylation of cortactin, an actin-binding protein that modulates the F-actin cytoskeleton as well as EC locomotion (14–16). Activation of SRC tyrosine kinases in response to ICAM-1 ligation likely has other downstream targets besides cortactin, and modulation of these downstream targets may play important roles in mediating EC responses induced by ICAM-1 ligation.

We have demonstrated recently (4, 17) that neutrophil adhesion to TNF-α-pretreated human pulmonary microvascular ECs induces cytoskeletal changes that require ICAM-1-initiated signaling pathways and can be mimicked by cross-linking ICAM-1 with antibodies. Signaling pathways initiated by ICAM-1 ligation with neutrophils or cross-linking antibodies include ICAM-1 redistribution on EC surface, activation of xanthine oxidase, production of reactive oxygen species (ROS), and activation of p38 MAPK (4, 17). Activation of p38 MAPK...
results in phosphorylation of heat shock protein 27, an actin-binding protein that may induce actin polymerization when phosphorylated, and p38 activation is required for the cytoskeletal rearrangement induced by ICAM-1 ligation, as well as for neutrophil migration toward EC borders (4, 17, 18). The signaling pathways induced by ROS production that lead to p38 MAPK activation are not understood.

This present study examined the role of SRC tyrosine kinases in ICAM-1-initiated signaling pathways in these human pulmonary microvascular ECs and focused on understanding the upstream signaling pathways leading to SRC activation, as well as identifying potential downstream targets of SRC tyrosine kinases in response to ICAM-1 ligation. Our studies demonstrate the following: 1) cross-linking ICAM-1 induces activation of SRC tyrosine kinases; 2) activation of SRC tyrosine kinases requires production of reactive oxygen species generated from xanthine oxidase and the SRC homology 2-containing protein-tyrosine phosphatase-2 (SHP-2); 3) activation of SRC tyrosine kinases is required for tyrosine phosphorylation of ezrin as well as activation of p38 MAPK.

EXPERIMENTAL PROCEDURES

Materials

Recombinant human TNF-α was obtained from R & D Systems Inc. (Minneapolis, MN). Murine anti-human ICAM-1 antibody (clone 6.5B5) and rabbit anti-murine IgG were obtained from Dako (Carpinteria, CA). Goat anti-murine IgG fragment of IgG was obtained from BioSource (Camarillo, CA). Rabbit anti-threonine-phosphorylated ezrin (Thr-558) antibody (clone 225, Zymed Laboratories Inc. South San Francisco, CA) and rabbit anti-human ezrin antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-human ezrin antibody and anti-SRC antibody-conjugated agarose beads were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Allopurinol, phenylarsine oxide (PAO), and BCA assay reagents were obtained from Sigma. Murine anti-phosphotyrosine antibody (clone PY20) was obtained from Zymed Laboratories Inc. (Camarillo, CA). PP2 and the phospho-SRC peptide (524–536 for human SRC) phosphorylated on Tyr-530 were obtained from Calbiochem.

Methods

Cultivation of Human Pulmonary Microvascular ECs—Human pulmonary microvascular ECs were obtained from Clonetics (Walkersville, MD) and plated onto fibronectin-coated culture dishes, as described previously (17, 18). ECs were used between passage 7 and 15. ECs were allowed to grow to about 80% confluence. The cells were washed twice with PBS. Cell lysis was performed according to the manufacturer’s instructions. The supernatant was determined by BCA assay. Aliquots of supernatant were used for measuring SRC activity. Each sample was split into 2 aliquots of 40 μg of proteins. These 2 aliquots were pre-cleared with 20 μl of protein A/G plus beads for 30 min and incubated with or without 1 μg of rabbit anti-SHP-2 antibody for 2 h, followed by incubation with 20 μl of protein A/G plus beads for 1 h. The beads were washed twice with the cell lysis buffer and twice with the kinase assay buffer containing 50 mM Tris, pH 7.2, 1 mM EDTA, and 0.1% β-mercaptoethanol. The beads were incubated with 25 μl of phosphatase assay buffer containing 3 μg of the phospho-SRC peptide (524–536, phosphorylated on Tyr-530) for 30 min at 37 °C. To examine the tyrosine phosphorylation of the phospho-SRC peptide after the reaction, 5 μl of the samples was dot-blotted onto the nitrocellulose membrane, and phosphotyrosine was detected using an anti-phosphotyrosine antibody as described above. In addition, the quantity of immunoprecipitated SHP-2 was examined by immunoblot.

Activation of SRC upon ICAM-1 Ligation

Measurement of SRC Tyrosine Kinase Activity—The activity of SRC tyrosine kinase was evaluated by an in vitro kinase assay using Sam68–(331–443) as a substrate after immunoprecipitating SRC. Sam68–(331–443) is expressed in Escherichia coli as a 53-kDa fusion protein and is a very efficient substrate for SRC tyrosine kinases. After the indicated treatment, ECs were washed twice with PBS containing 2 mM Na3VO4. The cells were incubated on ice for 5 min in Triton X-100 lysis buffer with occasional agitation as described above. The cells were scraped off the dish, and the cell lysates were centrifuged at 14,000 rpm at 4 °C for 15 min. The supernatants were collected. The pellets were dissolved in equal volumes of SDS sample buffer and centrifuged again at 14,000 rpm at 4 °C for 15 min. The amount of tyrosine phosphorylated ezrin, threonine-phosphorylated ezrin, moesin, or ezrin in each fraction was detected by immunoblot.

Measurement of p38 MAPK Activity—The activity of p38 MAPK was measured using a p38 MAPK assay kit (New England Biolabs, Beverly, MA) as described previously (4). ECs were washed twice with ice-cold PBS. Cell lysis was performed according to the manufacturer’s protocols. Immunoprecipitated p38 MAPK was incubated using agarose beads conjugated with anti-phosphorylated p38 antibody. The activity of immunoprecipitated p38 MAPK was measured by an in vitro kinase assay using ATP-2 as a substrate according to the manufacturer’s protocols. Phosphorylation of ATP-2 was measured by immunoblot.
by using an antibody that recognizes phosphorylated ATF-2 at residue Thr-71 as described above. As a loading control, the amount of ezrin in each sample used for immunoprecipitation was examined by immunoblot as described above.

**Statistical Analysis**—Data were analyzed using the Student’s t test or one-way analysis of variance. A p value less than 0.05 was considered significant. The data are expressed as the mean value ± S.E. (n = 4). *, p < 0.05 when compared with controls.

**RESULTS**

ICAM-1 Cross-linking Induced Activation of SRC Tyrosine Kinases—To examine whether ICAM-1 cross-linking induced activation of SRC tyrosine kinases in TNF-α-pretreated human pulmonary microvascular ECs, SRC tyrosine kinases were immunoprecipitated by using agarose beads conjugated with anti-SRC tyrosine kinase antibody. The activity of immunoprecipitated SRC tyrosine kinases was evaluated by an *in vitro* kinase assay using Sam68-(331–443) as a substrate. SRC tyrosine phosphorylation of Sam68-(331–443) was detected by immunoblot using an anti-phosphotyrosine antibody as described under “Experimental Procedures.” A, representative immunoblot showing activation of SRC tyrosine kinases in response to ICAM-1 cross-linking. The amount of immunoprecipitated SRC was also determined for each sample. *Lanes* 1–5, SRC activity in ECs prior to (*lane* 1) or 0.25–15 min after ICAM-1 cross-linking (*lanes* 2–5). B, densitometric analysis of immunoblots. The activity of SRC tyrosine kinases was normalized by the amount of immunoprecipitated SRC. Data are presented as fold changes over the non-cross-linked controls and expressed as means ± S.E. (n = 4). *, p < 0.05 when compared with controls.

**Activation of SRC Was Inhibited by Allopurinol (a Xanthine**

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Fig. 1. ICAM-1 cross-linking induced time-dependent activation of SRC tyrosine kinases. TNF-α-pretreated ECs were incubated for 30 min with 10 μg/ml mouse anti-human ICAM-1 antibody and washed. A cross-linking secondary antibody was added for 0–15 min, and the activity of SRC was evaluated by an *in vitro* kinase assay using Sam68-(331–443) as a substrate after immunoprecipitating SRC. Tyrosine phosphorylation of Sam68-(331–443) was detected by immunoblot using an anti-phosphotyrosine antibody as described under “Experimental Procedures.” A, representative immunoblot showing activation of SRC tyrosine kinases in response to ICAM-1 cross-linking. The amount of immunoprecipitated SRC was also determined for each sample. *Lanes* 1–5, SRC activity in ECs prior to (*lane* 1) or 0.25–15 min after ICAM-1 cross-linking (*lanes* 2–5). B, densitometric analysis of immunoblots. The activity of SRC tyrosine kinases was normalized by the amount of immunoprecipitated SRC. Data are presented as fold changes over the non-cross-linked controls and expressed as means ± S.E. (n = 4). *, p < 0.05 when compared with controls.
Oxidase Inhibitor), Me₂SO (a Hydroxyl Radical Scavenger), or Deferoxamine (an Iron Chelator)—Production of xanthine oxidase-generated reactive oxygen species is implicated in the signaling events induced by ICAM-1 cross-linking in pulmonary microvascular ECs (4). To determine whether activation of SRC tyrosine kinases requires activation of xanthine oxidase, the effect of allopurinol, a xanthine oxidase inhibitor, on the activity of SRC tyrosine kinases was examined. Pretreatment of ECs with allopurinol did not alter the base-line activity of SRC tyrosine kinase. Following cross-linking, allopurinol significantly inhibited activation of SRC tyrosine kinases (Fig. 2A). These data suggest that xanthine oxidase-generated reactive oxygen species may be required for activation of SRC tyrosine kinases.

To examine further the role of reactive oxygen species, the effect of Me₂SO (a hydroxyl radical scavenger) or deferoxamine (an iron chelator) was examined. Both reagents inhibited activation of SRC tyrosine kinases induced by ICAM-1 cross-linking (Fig. 2B). Together, these data suggest that reactive oxygen species are required for activation of SRC tyrosine kinases.

Regulation of SRC Activity by SHP-2—Because phosphorylation and dephosphorylation at Tyr-419 and Tyr-530 represent an important mechanism for regulating SRC activity (10), the following experiments were performed to determine whether the activity of SRC in these ECs was regulated by tyrosine phosphatases. PAO, a tyrosine phosphatase inhibitor, was used to pretreat ECs. Pretreatment with PAO markedly reduced the base-line activity of SRC, suggesting that basal SRC activity depends upon the basal activity of tyrosine phosphatases (Fig. 3). Moreover, ICAM-1 cross-linking did not increase SRC activity in PAO-pretreated samples, suggesting that the induced SRC activity may also require tyrosine phosphatases (Fig. 3).

SHP-2, an SRC homology 2-containing protein-tyrosine phosphatase, has been implicated in the regulation of SRC tyrosine kinases (10, 20). These studies, along with our observation that tyrosine phosphatases modulate SRC activity in ECs, led us to examine the role of SHP-2. An antisense oligonucleotide was used to specifically inhibit the protein expression of SHP-2. Treatment with 10 nM SHP-2 antisense oligonucleotide, but not control oligonucleotide, completely inhibited the protein expression of SHP-2 (Fig. 4A). The effect of SHP-2 antisense appeared specific, because it did not inhibit the protein expression of SHP-1, a closely related tyrosine phosphatase that shares about 55% overall sequence identity with SHP-2 (21) (Fig. 4A). Inhibition of SHP-2
expression had no effect on the basal activity of SRC tyrosine
kinases but completely inhibited the induced SRC activity in re-
sponse to ICAM-1 cross-linking (Fig. 4B). Examination of ICAM-1
expression in response to TNF-α treatment by immunoblot indi-
cated that treatment with SHP-2 antisense had no effect on
ICAM-1 expression (data not shown). Taken together, these data
suggest that SHP-2 is required for the induced SRC activation in
response to ICAM-1 ligation and that tyrosine phosphatases other
than SHP-2 regulate the basal SRC activity.

The results of these studies, along with published work (22)
showing that SHP-2 is capable of selectively dephosphorylating
SRC at residue Tyr-530 when examined by using purified pro-
teins, led us to examine whether SHP-2 immunoprecipitated
from TNF-α-treated human pulmonary microvascular ECs can

![Fig. 3. Modulation of SRC activity by PAO, a tyrosine phosphatase inhibi-
tor. ECs were treated with 10 μg/ml anti-ICAM-1 along with control vehicle or
20 μg PAO for 30 min and washed. A cross-linking secondary antibody was
added for 0–6 min, and SRC activity was evaluated as described under “Experi-
mental Procedures.” Data are expressed as fold changes from the non-cross-linked
controls in vehicle-pretreated samples, and presented as mean ± S.E. (n = 4). *,
p < 0.05 when compared with the non-cross-linked controls; #, p < 0.05 when
compared with the vehicle-pretreated samples.](image)

![Fig. 4. Activation of SRC tyrosine kinases required SHP-2. ECs were
treated with 10 nM control or SHP-2 antisense oligonucleotides as described un-
der “Experimental Procedures.” A, the effect of SHP-2 antisense on the protein
expression of SHP-2 or SHP-1 in ECs as examined by immunoblot. B, the effect
of SHP-2 antisense on SRC activity before or after ICAM-1 cross-linking for 6 min.
Data are expressed as fold changes from the non-cross-linked controls and pre-
sented as means ± S.E. (n = 8). *, p < 0.05 when compared with the non-cross-
linked controls; #, p < 0.05 when compared with the control antisense-treated
samples.](image)
indeed dephosphorylate SRC at residue Tyr-530. Immunoprecipitated SHP-2 was incubated with purified SRC peptide phosphorylated at residue Tyr-530. When compared with the controls, where no SHP-2 was immunoprecipitated, incubation with immunoprecipitated SHP-2 resulted in a significant decrease in the phosphorylation levels of the phospho-peptide from 1 ± 0.03 to 0.50 ± 0.07 (p < 0.05, Fig. 5). These data indicate that immunoprecipitated SHP-2 is capable of dephosphorylating SRC at Tyr-530, suggesting that SHP-2 may regulate SRC activity by dephosphorylating SRC at this residue.

Activation of p38 MAPK Was Inhibited by PP2, a Specific Inhibitor of SRC Tyrosine Kinases—Cross-linking ICAM-1 in TNF-α-pretreated human pulmonary microvascular ECs induces activation of p38 MAPK that is required for the subsequent cytoskeletal rearrangement, as well as neutrophil migration toward EC borders (4). To determine whether activation of SRC tyrosine kinases is required for activation of p38 MAPK, the activity of p38 MAPK was compared in ECs pretreated with
vehicle or PP2, a specific inhibitor of SRC tyrosine kinases (23). In cells pretreated with the control vehicle, ICAM-1 cross-linking for 6 min induced a significant increase in p38 activity (Fig. 6). Pretreatment with PP2, however, completely inhibited p38 activation, suggesting that activation of SRC tyrosine kinases is required for p38 MAPK activation (Fig. 6).

**Tyrosine Phosphorylation of Ezrin Induced by ICAM-1 Cross-linking Was Inhibited by PP2**—ICAM-1 cross-linking also induced tyrosine phosphorylation of protein(s) that could be detected in total cell lysates by immunoblots using an anti-phosphotyrosine antibody (Fig. 7). Tyrosine phosphorylation was detected by 6 min of ICAM-1 cross-linking and persisted for at least 15 min (Fig. 7). The molecular masses of these tyrosine-phosphorylated protein(s) were about 80 kDa when separated under denatured and reducing conditions. This increase in tyrosine phosphorylation required the addition of cross-linking secondary antibody, because the addition of anti-ICAM-1 antibody alone did not induce any change in tyrosine phosphorylation (data not shown), consistent with our previous observations that a cross-linking secondary antibody is required to induce ICAM-1-initiated signaling events (17). Pretreatment of ECs with 20 μM PP2 completely inhibited tyrosine phosphorylation of the 80-kDa protein(s) induced by ICAM-1 cross-linking, suggesting that activation of SRC tyrosine kinases is required (Fig. 7).

The molecular weight of these tyrosine-phosphorylated protein(s) and the observations that ezrin can be phosphorylated at tyrosine residues in response to several stimuli (24) led us to examine the following: 1) whether tyrosine phosphorylation of ezrin occurred in response to ICAM-1 cross-linking; and 2) whether tyrosine phosphorylation of ezrin was also inhibited by PP2. Tyrosine phosphorylation of ezrin in both the Triton X-soluble and -insoluble fractions was examined using antibodies that recognize total ezrin, tyrosine-phosphorylated ezrin at residue Tyr-146, or tyrosine-phosphorylated ezrin at residue Tyr-354. After cross-linking ICAM-1 for 6 and 15 min, tyrosine-phosphorylated ezrin at residue Tyr-146 increased in both the Triton X-soluble and -insoluble fractions, and the amount of Tyr-146-phosphorylated ezrin recovered in the Triton X-insoluble fractions was at least half of that recovered in the Triton X-soluble fractions (Fig. 8, A and B). About 95% of total ezrin was recovered in the Triton X-soluble fraction prior to ICAM-1 cross-linking and did not change following cross-linking (Fig. 8, A and C). These data indicate that greater percentage of the
molecule in the Triton X-insoluble fraction was phosphorylated than in the Triton X-soluble fraction. No increase in tyrosine phosphorylation of ezrin at residue Tyr-354 was detected when examined using an antibody that recognized Tyr-354-phosphorylated ezrin (data not shown).

Pretreatment of ECs with 20 μM PP2 completely inhibited tyrosine phosphorylation of ezrin at 6 min, suggesting that activation of SRC tyrosine kinases is required (Fig. 9A). The arrow indicates the position of tyrosine-phosphorylated proteins in vehicle-pretreated samples. PP2 had no effect on the distribution of ezrin in the Triton X-soluble and -insoluble fractions (the percentage of ezrin recovered in the Triton X-soluble fraction before or 6 min after ICAM-1 cross-linking measured 92.8 and 93.2% in vehicle-pretreated samples and 95.5 and 94.6% in PP2-pretreated samples, respectively).

Because allopurinol inhibited the activation of SRC tyrosine kinases, the effect of allopurinol on tyrosine phosphorylation of ezrin was also examined. Pretreatment of ECs with 0.3 mg/ml allopurinol completely prevented tyrosine phosphorylation of ezrin at Tyr-146 in the Triton X-insoluble fraction and partially prevented this phosphorylation in the Triton X-soluble fraction (Fig. 9B). These studies further suggest that xanthine oxidase is upstream of SRC and SRC-dependent tyrosine phosphorylation of ezrin.

Tyrosine Phosphorylation of Ezrin Induced by ICAM-1 Cross-linking Was Not Accompanied by Threonine Phosphorylation of Ezrin—The function of ezrin was also regulated by phospho- 
ylation of residue Thr-567 (reviewed in Ref. 25). Phosphorylation of this residue changed the conformation of ezrin and allowed ezrin to bind both F-actin and membrane receptors (25). An antibody that recognizes threonine-phosphorylated ezrin/radixin/moesin (ERM) was used to examine whether ICAM-1 cross-linking in TNF-α-pretreated ECs also induced...
Compared with untreated ECs, TNF-α/H9251 treatment for 24 h induced a 2.3-fold increase \((n = 7, p < 0.05)\) in threonine phosphorylation of ERM without altering the level of Tyr-146-phosphorylated ezrin or total ezrin (Fig. 10A). ICAM-1 cross-linking, however, did not induce a further increase in threonine phosphorylation of ERM (Fig. 10B). The distribution of threonine-phosphorylated ERM in Triton X-soluble and -insoluble fractions was also unaltered, and the majority was recovered in the Triton X-soluble fraction (Fig. 10).

**DISCUSSION**

This study evaluated the role of SRC tyrosine kinases in ICAM-1-induced signaling events in human pulmonary micro-

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**Fig. 8.** ICAM-1 cross-linking induced tyrosine phosphorylation of ezrin at Tyr-146 in both the Triton X-soluble and -insoluble fractions. ECs with or without ICAM-1 cross-linking were fractionated into Triton X-soluble and -insoluble fractions, and tyrosine phosphorylation of ezrin at residue Tyr-146 was determined by immunoblot using an anti-tyrosine-phosphorylated ezrin (pY146) antibody. The membrane was stripped and re-probed using an anti-ezrin antibody. A, representative immunoblot showing tyrosine phosphorylation of ezrin in response to ICAM-1 cross-linking. B, densitometric analysis of immunoblots showing tyrosine phosphorylation of ezrin. The data are expressed relative to the non-cross-linked controls in the Triton X-soluble fraction, which was defined as “1” in each experiment. Triton X-soluble fraction, open bars; Triton X-insoluble fraction, closed bars. C, densitometric analysis of immunoblots showing ezrin distribution. The data are expressed as percentage total ezrin recovered in the Triton X-soluble (open bars) and Triton X-insoluble fraction (closed bars). All the data are presented as means ± S.E. \((n = 3)\), \(^*\), \(p < 0.05\) when compared with non-cross-linked controls.
vascular ECs. The results show that ICAM-1 cross-linking induced an increase in the activity of SRC tyrosine kinases that required both the production of xanthine oxidase-generated reactive oxygen species and SHP-2, an SRC homology 2-containing protein-tyrosine phosphatase. Activation of SRC tyrosine kinases was required for both phosphorylation of ezrin at residue Tyr-146 and activation of p38 MAPK. This study demonstrated an essential role for SRC tyrosine kinases in ICAM-1-mediated signaling events in human pulmonary microvascular ECs.

ICAM-1 cross-linking in pulmonary microvascular ECs induced an increase in the activity of SRC tyrosine kinases, and this increase required tyrosine phosphatases. Pretreatment with PAO, an inhibitor of tyrosine phosphatases, resulted in a marked decrease in basal SRC activity, suggesting that the endogenous tyrosine phosphatases may represent one of the mechanisms that maintain the basal activity of SRC, probably by dephosphorylating Tyr-530 of SRC (10). Indeed, elevated SRC activity in several cancer cell lines correlates with enhanced tyrosine phosphatase activity in these cell lines (26, 27), whereas hyperphosphorylation of this residue occurs in cells deficient of tyrosine phosphatase-B, and reduced SRC activity is observed in cells deficient in protein-tyrosine phosphatase-α (28, 29). Interestingly, this study showed that specific inhibition of the protein expression of SHP-2 had no effect on the basal activity of SRC in these ECs, suggesting a role for phos-
phosphatases other than SHP-2. SHP-2 antisense as well as PAO also inhibited activation of SRC tyrosine kinases in response to ICAM-1 cross-linking. SHP-2 may be required for SRC activation through two mechanisms. 1) SHP-2 may directly dephosphorylate SRC at residue Tyr-530, resulting in SRC activation. In vitro, SHP-2 is indeed capable of selectively dephosphorylating SRC at residue Tyr-530 (22). This study using immunoprecipitated SHP-2 from ECs showed that SHP-2 can dephosphorylate SRC at residue Tyr-530, suggesting that this may indeed represent a mechanism through which SHP-2 can regulate the activity of SRC family tyrosine kinases in these ECs. 2) SHP-2 may regulate SRC activity through mechanisms that are independent of its phosphatase activity. Walter et al. (20) reported that overexpression of wild type or a catalytically inactive mutant of SHP-2 similarly enhanced SRC activity without significantly altering the phosphorylation status of SRC in a fibroblast cell line, and suggested that binding of SHP-2 to the SH-3 domain of SRC can be a mechanism for SRC activation.

How ICAM-1 ligation induces activation of SRC tyrosine kinases through SHP-2 remains to be determined. The cytoplasmic domain of ICAM-1 is relatively short with no apparent tyrosine kinase activity or SRC homology domains that can recruit tyrosine-phosphorylated proteins (2). This led to the hypothesis that ICAM-1 may transduce signaling pathways by recruiting other signaling molecules (2). Pluskota et al. (31) showed that ICAM-1 ligation by fibrinogen induces association of SHP-2 with ICAM-1 in ECs and that SHP-2 directly binds to the cytoplasmic domain of ICAM-1. In addition, association of SRC tyrosine kinases with ICAM-1 occurs following ICAM-1 ligation as determined by immunoprecipitation studies (7, 32). These studies led us to postulate that ICAM-1 ligation may bring SRC tyrosine kinases and SHP-2 into close proximity where SHP-2 may mediate SRC activation, possibly by dephosphorylating SRC tyrosine kinases at Tyr-530.

Activation of SRC was also inhibited by allopurinol (a xanthine oxidase inhibitor), Me$_2$SO (a hydroxyl radical scavenger), or deferoxamine (an iron chelator), suggesting that xanthine oxidase-generated ROS may be required for activation of SRC. Activation of SRC tyrosine kinases has been implicated in the signaling pathways induced by exogenously applied ROS (33–35). In human neutrophils, endogenous ROS are thought to play an important role in activating SRC tyrosine kinases (36). One of the identified downstream targets of ROS is tyrosine phosphatases. ROS cause oxidation of cysteine residues in the tyrosine phosphatase catalytic domain to form a disulfide bond, resulting in the inactivation of tyrosine phosphatases (37–41). Whether inactivation of tyrosine phosphatases is one of the underlying mechanisms whereby xanthine oxidase-dependent ROS production mediates activation of SRC tyrosine kinases remains to be determined. However, our studies using PAO, a global tyrosine phosphatase inhibitor, or SHP-2 antisense suggest that this is unlikely to be an important mechanism, because pretreatment with PAO or SHP-2 antisense prevented activation of SRC in response to ICAM-1 cross-linking.

One substrate of SRC tyrosine kinases appears to be residue Tyr-146 of ezrin. Tyr-354 was not phosphorylated, although both residues are phosphorylated in response to epidermal growth factor in human epidermoid carcinoma A431 cells (24). A recent study by Autero et al. (42) demonstrated that in T
cells, ezrin is a substrate for Lck, a member of the SRC family and tyrosine kinases, and that Tyr-146 (not Tyr-354) is identified as the major phosphorylation site. Ezrin is a member of the ERM family proteins and shares a high degree of homology to the other two members of the family. It is a membrane cytoskeletal linker protein that has a binding site for F-actin at the C terminus and a binding site for membrane proteins such as CD43, CD44, ICAM-1, ICAM-2, and ICAM-3 at the N terminus (reviewed in Ref. 25). Functioning as a linkage between membrane proteins and the submembrane F-actin cytoskeleton, ezrin plays important roles in organizing membrane structures such as microvilli and in targeting membrane proteins such as ICAM-3 during lymphocyte migration (25, 43). Although not well understood, the activity of ERM proteins appears to be regulated by phosphorylation at a conserved threonine residue at the C terminus and/or binding to phosphatidylinositol 4,5-bisphosphate. The inactive ERM proteins have a phosphorylated threonine residue and/or binding to phosphatidylinositol 4,5-bisphosphate “opened” up the molecules, allowing them to function as a membrane-cytoskeletal linker (25). Our data indicate that phosphorylation of ERM proteins at this conserved threonine residue, but not tyrosine phosphorylation of ezrin at Tyr-146, increased in response to TNF-α treatment for 24 h. Cross-linking ICAM-1 in TNF-α-pretreated ECs, however, did not further increase threonine phosphorylation of ERM but rather induced phosphorylation of the threonine residue. Over 95% of the total ezrin was in the Triton X-soluble fraction, and this distribution was not affected by ICAM-1 cross-linking. These data suggest that tyrosine phosphorylation of ezrin does not occur preferentially in the Triton X-insoluble fraction. Tyrosine phosphorylation of ezrin occurs in response to growth factors or inhibition of tyrosine phosphatases, and it is implicated in altering subcellular localization of ezrin as well as transducing downstream signaling events (24, 44, 45). Mutation of tyrosine residues Tyr-145 and Tyr-353 to phenylalanine does not alter the subcellular localization of ezrin or the actin cytoskeleton in a pig kidney epithelial cell line, but does inhibit cell motility induced by hepatocyte growth factor (44).

Our data also demonstrate that activation of SRC tyrosine kinases was required for p38 MAPK activation, which occurs following phosphorylation at a tyrosine and a threonine residue. Upstream signaling mechanisms leading to activation of p38 MAPK are the least understood among the three MAPK members. Although many studies have provided evidence linking the activation of SRC tyrosine kinases to p38 MAPK (see Ref. 46 for example), there is no clear mechanism as to how SRC kinases may modulate the activity of p38 MAPK. In a study by Turkson et al. (47), activation of p38 in NIH 3T3 cells stably transformed by v-SRC requires Ras and Rac. Thus, it is likely that SRC kinases may regulate the activity of p38 through intermediate signaling pathways.

Activation of p38 upon ICAM-1 ligation has several downstream consequences in an inflammatory response, and the observations that activation of SRC tyrosine kinases is required for p38 activation suggest that this pathway is physiologically significant. First, our previous studies showed that activation of p38 leads to phosphorylation of heat shock protein 27 (hsp27) and is required for actin cytoskeletal changes in ECs induced by ICAM-1 ligation as well as neutrophil migration toward EC borders. This present study links SRC tyrosine kinases to activation of p38 MAPK and suggests that activation of SRC tyrosine kinases is an integral part of ICAM-1 signaling events that play important roles in modulating cytoskeletal changes in ECs, as well as neutrophil migration along ECs to reach the junctions. Second, activation of p38 MAPK is required for the transcription of several inflammatory genes such as interleukin-6 in astrocytes and ICAM-1 in human renal fibroblasts in response to ICAM-1 ligation (30, 48). Thus, activation of SRC tyrosine kinases upon ICAM-1 ligation in ECs may also modulate the transcription of inflammatory genes by regulating the activity of p38.

In summary, this study demonstrated that ICAM-1 cross-linking in human pulmonary microvascular ECs induced activation of SRC tyrosine kinases that required production of reactive oxygen species generated from xanthine oxidase. Activation of SRC was required for tyrosine phosphorylation of ezrin at residue Tyr-146 as well as activation of p38 MAPK.

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