Regulation of RelA/p65 and Endothelial Cell Inflammation by Proline-Rich Tyrosine Kinase 2

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We investigated the role of proline-rich tyrosine kinase 2 (Pyk2) in the mechanism of NF- κ B activation and endothelial cell (EC) inflammation induced by thrombin, a procoagulant serine protease released in high amounts during sepsis and other inflammatory conditions. Stimulation of ECs with thrombin resulted in a time-dependent activation of Pyk2. RNA interference knockdown of Pyk2 attenuated thrombin-induced activity of NF- κ B and expression of its target genes, vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1. Pyk2 knockdown impaired thrombin-induced activation of I κ B kinase (IKK) and phosphorylation (Ser32 and Ser36) of I κ B α , but, surprisingly, failed to prevent I κ B α degradation. However, depletion of IKK α or IKK β was effective in inhibiting I κ B α phosphorylation/degradation, as expected. Intriguingly, Pyk2 knockdown impaired nuclear translocation and DNA binding of RelA/p65, despite the inability to prevent I κ B α degradation. In addition, Pyk2 knockdown was associated with inhibition of RelA/p65 phosphorylation at Ser536, which is important for transcriptional activity of RelA/p65. Depletion of IKK α or IKK β each impaired RelA/p65 phosphorylation. Taken together, these data identify Pyk2 as a critical regulator of EC inflammation by virtue of engaging IKK to promote the release and the transcriptional capacity of RelA/p65, and, additionally, by its ability to facilitate the nuclear translocation of the released RelA/p65. Thus, specific targeting of Pyk2 may be an effective anti-inflammatory strategy in vascular diseases associated with EC inflammation and intravascular coagulation.

Keywords: endothelial cells; signal transduction; transcription factors; adhesion molecules; inflammation

The NF- κ B controls myriad biological effects, including immune and inflammatory responses, cell fate decisions, such as proliferation, differentiation, tumorigenesis, and apoptosis, and is implicated in many disease states, such as atherosclerosis, acute lung injury, arthritis, inflammatory bowel diseases, and chronic obstructive pulmonary diseases (1–8). The mammalian NF- κ B family consists of five members: RelA (p65), RelB, c-Rel, p50, and p52 (1–3). Of these, RelA/p65, c-Rel, and RelB are transcriptionally active, due to the presence of both transactivation and DNA-binding domains, whereas p50 and p52 serve primarily as DNA-binding subunits, as they lack the transactivation domain (1, 2). NF- κ B, typically a heterodimer of p50 and RelA/p65 subunits, is sequestered in an inactive state in the

CLINICAL RELEVANCE

Endothelial cell (EC) inflammation associated with intravascular coagulation is an important component of many diseases, including acute lung injury and acute respiratory distress syndrome. However, the mechanisms by which the procoagulant thrombin promotes EC inflammation are not fully understood. This study identifies proline-rich tyrosine kinase 2 as a critical determinant of EC inflammation by virtue of promoting the release, increasing the transcriptional capacity, and facilitating the nuclear translocation of the released RelA/p65.

cytoplasm through its association with I κ B α that masks the nuclear localization signal of RelA/p65 (2). Activation of NF- κ B requires phosphorylation of I κ B α at Ser32 and Ser36 by a macromolecular I κ B kinase (IKK) complex containing the catalytic subunits, IKK α and IKK β , and the regulatory subunit, NF- κ B essential modulator (NEMO)/IKK γ (1, 2). Phosphorylation initiates rapid polyubiquitination of I κ B α by the E3 Skp1-cullin-F-box protein β -transducin repeat-containing protein (E3-SCF β -TrCP) ubiquitin ligase, targeting it for degradation by the 26 S proteasome (1, 2). The released NF- κ B undergoes nuclear translocation, binds to cognate κ B enhancer elements in the target genes, and activates their transcription (1–3, 9, 10). Another component of NF- κ B activation involves phosphorylation of RelA/p65 that serves to increase the transcriptional capacity of the bound NF- κ B. Studies have shown that phosphorylation at serine 276, 311, 529, or 536 increases the transactivation potential of RelA/p65 in a cell- and stimulus-specific manner (1–3, 11).

Among the key targets of NF- κ B in endothelial cells (ECs) are vascular cell adhesion molecule (VCAM)-1 and monocyte chemoattractant protein (MCP)-1/CC-chemokine ligand 2. VCAM-1 is an inducible adhesive protein that interacts with its counter receptor, very late antigen-4 (CD49 d/CD29) present on the circulating monocytes and lymphocytes (12). MCP-1 is a secreted protein that specifically attracts monocytes to the site of inflammation (13). The coordinate action of VCAM-1 and MCP-1 serves to promote the adhesion of monocytes to the vascular endothelium and, subsequently, their transendothelial migration (TEM) to the peripheral tissues, resulting in tissue injury (12–14). Activation of NF- κ B and, thereby, expression of VCAM-1 and MCP-1 on the vascular endothelium is induced by a variety of proinflammatory mediators, including the procoagulant thrombin, a serine protease activated during intravascular coagulation initiated by tissue injury or sepsis (15). We and others have shown that thrombin-induced NF- κ B-dependent gene expression is initiated by ligation of the protease-activated receptor-1 and activation of downstream signals (9, 16, 17). A key signal involves activation of the protein kinase C- δ , which regulates NF- κ B activation by inducing IKK-dependent release, and p38 mitogen-activated protein kinase (MAPK)-dependent phosphorylation of RelA/p65 (10, 17).

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Proline-rich tyrosine kinase 2 (Pyk2), also known as calcium-dependent tyrosine kinase, cell-associated tyrosine kinase- β , and related adhesion focal tyrosine kinase, is a non-receptor tyrosine kinase and a member of the focal adhesion kinase family. It is expressed in various cell types, including neuronal cells and fibroblasts, but is abundant in cells of hematopoietic origin, especially B lymphocytes and monocytes/macrophages (18–20). Pyk2 is also present in different types of ECs, and has been implicated in various signaling pathways (21, 22). It is an important regulator of endothelial permeability and angiogenesis (23, 24), and has been shown to promote TEM of leukocytes via intercellular adhesion molecule-1 (ICAM-1)-dependent tyrosine phosphorylation of vascular endothelial cadherins and expression of IL-8 and MCP-1 (25–27). Thrombin has been shown to activate Pyk2 in ECs in a Ca^{2+} -dependent manner (21); however, the role of Pyk2 in thrombin-induced NF- κ B activation and proinflammatory gene expression remains unclear. In this study, we ascertained the role of Pyk2 in the mechanism of these responses. Our data show a pivotal role of Pyk2 in causing EC inflammation by its ability to activate IKK to promote the release and the transcriptional capacity of RelA/p65, and also by virtue of facilitating the nuclear translocation of RelA/p65.

MATERIALS AND METHODS

Reagents

Human thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Tyrphostin A9 was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). SB 203,580 was purchased from Enzo Life Sciences (Farmingdale, NY). Polyclonal antibodies to VCAM-1, RelA/p65, β -actin, I κ B α , Cu-Zn superoxide dismutase-1,

p38 MAPK and RNA polymerase II were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-(Ser180/Ser181)-IKK α /IKK β , phospho-(Ser32 and Ser36)-I κ B α , phospho-(Thr180/Tyr182)-p38 MAPK, phospho-(Ser536)-RelA/p65, phospho-(Tyr402)-Pyk2, IKK α , IKK β , Pyk2, and casein kinase (CK) II were obtained from Cell Signaling (Beverly, MA). All other materials were from VWR Scientific Products Corp. (Gaithersburg, MD).

EC Culture

Human pulmonary artery ECs (HPAECs) were purchased from Lonza (Walkersville, MD). HPAECs were cultured, as previously described (28), in gelatin-coated flasks using endothelial basal medium 2 with bullet kit additives (BioWhittaker, Walkersville, MD). HPAECs used in the experiments were between three and six passages.

Cell Lysis, Immunoprecipitation, and Immunoblotting

After treatment, cell lysates were prepared and analyzed by immunoblotting, as previously described (29). For immunoprecipitation, cell lysates were precleared with 10 μ l of protein A/G-agarose beads for 4 hours at 4°C. The precleared lysates were immunoprecipitated by incubating with 0.6–1.0 μ g of appropriate antibody and 10 μ l of the protein A/G-agarose beads at 4°C overnight with gentle shaking, as previously described (29). The immunoprecipitates were washed four times with the same volume of ice-cold phosphorylation lysis buffer and then extracted by boiling with 2 $\times$ SDS sample buffer for 5 minutes. The extracted proteins were then analyzed by immunoblotting (29).

ELISA

Total cell lysates were analyzed by ELISA for measurement of MCP-1 using the human cardiovascular disease panel Multiplex kit (Millipore, Bradford, MA), according to manufacturer's recommendations.

Figure 1. (A) Thrombin induces phosphorylation of proline-rich tyrosine kinase 2 (Pyk2) in endothelial cells (ECs). Confluent human pulmonary artery EC (HPAEC) monolayers were challenged with thrombin (5 U/ml) for the indicated time periods. Total cell lysates were subjected to immunoprecipitation (IP) with an antibody to Pyk2 or IgG, as described in MATERIALS AND METHODS. The immunoprecipitates were then immunoblotted with an antibody to phosphorylated (Tyr402) form of Pyk2 or an anti-Pyk2 antibody. The bar graph represents the level of Pyk2 phosphorylation induced by thrombin at different time points. The phosphorylated Pyk2 level normalized to the total Pyk2 is expressed relative to untreated control set at 1. Data are means ($\pm$ SE) ($n = 3$ for each condition); * $P < 0.05$ compared with thrombin-untreated control. (B) RNA interference knockdown of Pyk2. Cells were transfected with Pyk2 short interfering RNA (si-Pyk2) or with control siRNA (si-Con) for 24–36 hours, as described in MATERIALS AND METHODS. Total cell lysates were immunoblotted with an antibody to Pyk2 or focal adhesion kinase (FAK). Equal protein loading was monitored by immunoblotting the lysates with an antibody to Cu-Zn superoxide dismutase (SOD-1). The bar graph represents the level of Pyk2 in si-Pyk2 transfected cells. Pyk2 level normalized to SOD-1 level is expressed relative to si-Con set at 1. Data are means ($\pm$ SE) ($n = 5$ for each condition); *** $P < 0.001$ compared with cells transfected with si-Con. (C and D) Pyk2 mediates thrombin- and TNF- α -induced NF- κ B activity. HPAECs were transfected with si-Pyk2 or si-Con using DharmaFect1. Twelve hours later, cells were again transfected with NF- κ B luciferase (LUC) construct using diethylaminoethyl (DEAE)-Dextran, as described in MATERIALS AND METHODS. After 12 hours, cells were challenged with thrombin (5 U/ml) (C) or TNF- α (100 U/ml) (D) for 6 hours. Cell extracts were prepared and assayed for Firefly and Renilla luciferase activities. Data are means ($\pm$ SE) ($n = 4$ –6 for each condition). ** $P < 0.01$ compared with untreated controls; ### $P < 0.001$ compared with thrombin- or TNF- α -stimulated controls.

RNA Interference Knockdown

SMARTpool short interfering RNA duplexes targeting Pyk2, IKK α , IKK β , or CKII, and a nonspecific siRNA control were obtained from Dharmacon (Lafayette, CO). HPAECs were transfected with appropriate siRNAs using DharmaFect1 siRNA Transfection Reagent (Dharmacon), as previously described (28). Briefly, 50–100 nM siRNA was mixed with DharmaFect1 and added to cells that were approximately 50–60% confluent. After 24–36 hours, cell lysates were prepared and subjected to immunoblotting to assess the levels of Pyk2, CKII, IKK α , or IKK β .

Reporter Gene Transfection and Luciferase Assay

The plasmid, pNF- κ B-luciferase, containing five copies of consensus NF- κ B sequences linked to a minimal E1B promoter-luciferase gene, was from Stratagene (La Jolla, CA). The constructs, Gal4-p65 (286–551), Gal4-p65 (286–551, S536A), Gal4 Vector, and Gal4-luciferase (LUC), were kindly provided by M. W. Mayo (University of Virginia, Charlottesville, VA). Transfections were performed using the diethylaminoethyl (DEAE)-dextran method, essentially as previously described (29). A transfection efficiency of 16 ($\pm$ 3)% (mean [$\pm$ SD]; n = 3) was achieved with this protocol. The plasmid, pTKRLUC (Promega, Madison, WI), containing *Renilla* luciferase reporter gene driven by the constitutively active thymidine kinase promoter, was used to normalize the transfection efficiency. Cell extracts were assayed for Firefly and *Renilla* luciferase activities using the Promega Biotech Dual Luciferase Reporter Assay System. The data were expressed as a ratio of Firefly to *Renilla* luciferase activity.

Cytoplasmic and Nuclear Extract Preparation

After treatment, cytoplasmic and nuclear extracts were prepared essentially as described previously (28). The purity of nuclear and cytoplasmic extracts was confirmed by the presence or absence of RNA polymerase II in these extracts (data not shown). The concentration of the cytoplasmic and nuclear protein was determined using a Bio-Rad protein determination kit (Bio-Rad, Hercules, CA).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay was performed as previously described (29). Briefly, 10 μ g of nuclear extract was mixed with 1 μ g of poly (dI-dC) in a binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM

NaCl, 0.5 mM DTT, 10% glycerol [20 μ l final volume]) for 15 minutes at room temperature. The reaction mixture was incubated with [γ - 32 P]-labeled double-stranded oligonucleotides containing NF- κ B consensus sequence (5-GTTGAGGGGACT-TTCCCAGGC-3') (30) for 15 minutes at room temperature. The DNA-protein complexes were subjected to electrophoresis on a 5% native gel, dried on Whatman paper (Bio-Rad), and then exposed to radioactivity.

Statistical Analysis

Data are expressed as means ($\pm$ SE). Statistical significance was determined using one-way ANOVA followed by Tukey's *post hoc* test for multigroup comparisons using Prism 5.0 software (GraphPad Software, San Diego, CA). In some cases (Figures 1A and 1B), Student's *t* test was performed for comparisons between experimental groups. A *P* value less than 0.05 was considered significant.

RESULTS

Thrombin Activates Pyk2 to Induce NF- κ B Activity and Proinflammatory Gene Expression

To determine the involvement of Pyk2 in the mechanism of NF- κ B activation and proinflammatory gene expression, we first determined the time course of Pyk2 activation by thrombin in ECs. Because activation of Pyk2 is initiated through its phosphorylation at Tyr402 (31), we determined this response as an indication of Pyk2 activation. Thrombin induced Tyr402 phosphorylation of Pyk2 in a biphasic manner, with first peak phosphorylation occurring between 1 and 5 minutes and the second peak phosphorylation between 0.5 and 2 hours (Figure 1A and data not shown). We next examined whether Pyk2 is required for thrombin-induced NF- κ B activity. To assess this possibility, we evaluated the effect of RNA interference knockdown of Pyk2 on NF- κ B activity. Cells were transfected with siRNA targeting Pyk2 (si-Pyk2) or control siRNA, and Pyk2 expression was analyzed by immunoblotting. A marked depletion of Pyk2 was observed in si-Pyk2-transfected cells compared with the cells transfected with control siRNA (Figure 1B). Importantly, si-Pyk2 had no effect on the levels of focal adhesion kinase (Figure 1B), a structurally related family member with

Figure 2. (A and B) Effect of depletion or inhibition of Pyk2 on thrombin-induced vascular cell adhesion molecule (VCAM)-1 expression. HPAECs were transfected with si-Pyk2 or si-Con for 24–36 hours (A) or pretreated with tyrphostin A9 (5 μ M) for 1 hour (B). Cells were challenged with thrombin (5 U/ml) for 6 hours. Total cell lysates were immunoblotted with an antibody to VCAM-1. SOD-1 levels were used to monitor loading. The bar graph represents the effect of Pyk2 depletion on VCAM-1 protein expression normalized to SOD-1 level. Data are means ($\pm$ SE) (n = 3–5 for each condition). *** P < 0.001 compared with untreated controls; ## P < 0.01 compared with thrombin-stimulated controls. (C) Effect of Pyk2 depletion on monocyte chemoattractant protein (MCP)-1. HPAECs were transfected with si-Pyk2 or si-Con for 24–36 hours and then challenged with thrombin (5 U/ml) for 6 hours. Total cell lysates were used to determine MCP-1 levels by ELISA, as described in MATERIALS AND METHODS. Data are means ($\pm$ SE) (n = 5–11 for each condition). *** P < 0.001 compared with untreated controls; ### P < 0.001 compared with thrombin-stimulated controls.

IκBα normalized to RelA/p65 levels. Data are means ($\pm$ SE) ($n = 4$ for each condition). *** $P < 0.001$ compared with untreated controls; ## $P < 0.01$ compared with thrombin-stimulated controls.

approximately 48% amino acid identity (20), indicating the specificity of Pyk2 knockdown by this approach. Depletion of Pyk2 impaired thrombin-induced NF- κ B activity (Figure 1C). We also determined whether Pyk2 influences NF- κ B activity in response to TNF- α , a prototype of NF- κ B activator. Depletion of Pyk2 was effective in inhibiting TNF- α -induced NF- κ B activity as well (Figure 1D).

The above data led us to examine whether Pyk2 knockdown produces a similar effect on VCAM-1, a well defined NF- κ B target gene in ECs (3, 16, 17). Depletion of Pyk2 inhibited thrombin-induced VCAM-1 expression (Figure 2A), consistent with its effect on NF- κ B activity (Figure 1C). To determine whether kinase activity of Pyk2 is required, we assessed the effect of inhibiting Pyk2 on this response. Pretreatment of cells with tyrphostin A9, an inhibitor of Pyk2, abolished thrombin-induced VCAM-1 expression (Figure 2B). We also assessed the effect of depleting Pyk2 on MCP-1, another well defined NF- κ B target gene (32). Results showed that Pyk2 knockdown was

effective in suppressing the basal as well as thrombin-induced expression of MCP-1 (Figure 2C).

Pyk2 Controls Thrombin-Induced Activation of IKK and Phosphorylation of IκBα

Phosphorylation of IκBα at Ser32 and Ser36, and its subsequent degradation, is a requirement for the release of RelA/p65 for its translocation to the nucleus. Because phosphorylation of IκBα is mediated by IKK complex, we first evaluated the role of Pyk2 in IKK activation. Depletion of Pyk2 inhibited activation of IKK upon thrombin challenge, as determined by phosphorylation of IKKβ at Ser181 (Figure 3A). We next examined whether knockdown of Pyk2 affects phosphorylation and degradation of IκBα. Previously, our time course experiments established that thrombin-induced IκBα phosphorylation/degradation peaks at between 1 and 2 hours after stimulation (28, 33). We therefore used this time point in subsequent experiments to address the role of Pyk2 in the mechanism of NF- κ B activation. Depleting

Figure 3. Effect of Pyk2 depletion on thrombin-induced IKKβ phosphorylation, and IκBα phosphorylation and degradation. HPAECs were transfected with si-Pyk2 or si-Con for 24–36 hours and then challenged with thrombin (5 U/ml) for 1 hour. (A) Total cell lysates were immunoblotted with an anti-phospho IKKα (Ser180)/IKKβ (Ser181) antibody. Total IKKβ levels were used to monitor loading. The bar graph represents the effect of Pyk2 depletion on thrombin-induced phosphorylation of IKKα/β normalized to total IKKβ levels. Data are means ($\pm$ SE) ($n = 3$ for each condition). ** $P < 0.01$ compared with untreated controls; # $P < 0.05$ compared with thrombin-stimulated controls. (B) Lysates were immunoblotted with an anti-phospho IκBα (Ser32 and Ser36) antibody. SOD-1 levels were used to monitor loading. The bar graph represents the effect of Pyk2 depletion on thrombin-induced phosphorylation of IκBα normalized to SOD-1 levels. Data are means ($\pm$ SE) ($n = 3$ –4 for each condition). *** $P < 0.001$ compared with untreated controls; ## $P < 0.01$ compared with thrombin-stimulated controls. (C) Cytoplasmic extracts were immunoblotted with an antibody to IκBα. SOD-1 levels were used to monitor loading. The bar graph represents the effect of Pyk2 depletion on thrombin-induced degradation of IκBα normalized to SOD-1 level. Data are means ($\pm$ SE) ($n = 4$ for each condition). * $P < 0.05$ compared with untreated control. (D) Effect of IKK depletion on thrombin-induced IκBα phosphorylation and degradation. HPAECs were transfected with si-IKKα, si-IKKβ, or si-Con for 24–36 hours, and then challenged with thrombin (5 U/ml) for 1 hour. Total cell lysates were immunoblotted with an anti-phospho IκBα (Ser32 and Ser36) or an anti-IκBα antibody. Depletion of IKKα or IKKβ was determined by immunoblotting the lysates with an antibody to IKKα or IKKβ, respectively. RelA/p65 levels were used to monitor loading. The bar graph represents the effect of IKK depletion on thrombin-induced phosphorylation or degradation of IκBα.

Pyk2 was effective in inhibiting I κ B α phosphorylation, but, surprisingly, had no effect on I κ B α degradation (Figures 3B and 3C). However, depletion of IKK α or IKK β both reduced phosphorylation as well as degradation of I κ B α , as expected (Figure 3D). These results raise the possibility that Pyk2 knockdown exerts two effects on I κ B α degradation: one causes inhibition of IKK-dependent and the other promotes IKK-independent degradation of I κ B α , resulting in no net inhibition of thrombin-induced I κ B α degradation in Pyk2-depleted cells.

Pyk2 Controls Thrombin-Induced Nuclear Translocation and DNA Binding of RelA/p65 in the Presence of I κ B α Degradation

We next investigated the effect of depleting Pyk2 on nuclear translocation and DNA binding of RelA/p65. Analyses of nuclear extracts from control and thrombin-challenged cells by immunoblotting and electrophoretic mobility shift assay showed a marked inhibition in nuclear translocation and DNA binding of RelA/p65 in cells transfected with si-Pyk2 (Figures 4A and 4B). Together, these results show impaired nuclear translocation and DNA binding of RelA/p65 despite the degradation of I κ B α in cells depleted of Pyk2 (Figure 3C). Considered together, these results point to a role of Pyk2 in facilitating RelA/p65 translocation to the nucleus.

Figure 4. Effect of Pyk2 depletion on thrombin-induced nuclear translocation and DNA binding of RelA/p65. HPAECs were transfected with si-Pyk2 or si-Con for 24–36 hours and challenged with thrombin (5 U/ml) for 1 hour. (A) Nuclear extracts were immunoblotted with an antibody to RelA/p65. Actin levels were used to monitor loading. The bar graph represents the effect of Pyk2 depletion on thrombin-induced nuclear translocation of RelA/p65 normalized to actin. Data are means ($\pm$ SE) ($n = 3$ for each condition). *** $P < 0.001$ compared with untreated controls; ### $P < 0.001$ compared with thrombin-stimulated control. (B) Nuclear extracts were assayed for RelA/p65 DNA-binding activity by electrophoretic mobility shift assay, as described in MATERIALS AND METHODS. The bar graph represents the effect of Pyk2 depletion on thrombin-induced DNA binding of RelA/p65. Data are means ($\pm$ SE) ($n = 3$ –4 for each condition). *** $P < 0.001$ compared with untreated controls; ### $P < 0.001$ compared with thrombin-stimulated control.

Pyk2 Controls Transactivation Potential of NF- κ B through Ser536 Phosphorylation of RelA/p65

We also determined whether Pyk2 contributes to the transactivation potential of RelA/p65, in addition to promoting its nuclear translocation and DNA binding function. To this end, Pyk2-depleted cells, or cells pretreated with tyrphostin A9 were challenged with thrombin, and total cell lysates were analyzed for Ser536 phosphorylation of RelA/p65. Depletion or inhibition of Pyk2 each caused a significant inhibition in RelA/p65 phosphorylation induced by thrombin (Figures 5A and 5B). To definitively establish that Ser536 phosphorylation is critical for thrombin-induced transcriptional activity of RelA/p65, we used a construct Gal4-p65 that encodes a fusion protein containing DNA-binding domain of the yeast Gal4 transcription factor and transactivation domain (residues 286–551) of RelA/p65 (34). Cells were cotransfected with Gal4-p65 and Gal4-LUC, which contains four copies of Gal4 DNA-binding consensus sites from yeast *GAL4* gene promoter (34), and then challenged with thrombin. Expression of nuclear fusion protein Gal4-p65 caused a marked increase in constitutive transactivation of Gal4-LUC (Figure 5C), as expected. A further increase in transactivation by Gal4-p65 was noted upon stimulation with thrombin (Figure 5C). However, both basal and thrombin-induced transactivation potential of Gal4-p65 was lost upon mutation of Ser536 in transactivation domain of RelA/p65 (Figure 5C). These results indicate an essential role of Ser536 in controlling thrombin-induced transcriptional activity of RelA/p65.

Pyk2 Controls Ser536 Phosphorylation of RelA/p65 via IKK α / β

We previously demonstrated a role of p38 MAPK in regulating thrombin-induced Ser536 phosphorylation of RelA/p65 in ECs (35). We therefore asked whether Pyk2 is required for p38 MAPK activation. Depletion of Pyk2 failed to inhibit p38 MAPK activation, as determined by its phosphorylation at Thr180/Tyr182 (see Figure E1 in the online supplement). These data suggest the existence of an additional pathway for Ser536 phosphorylation of RelA/p65 that may be engaged by thrombin in a Pyk2-dependent manner. Given that Pyk2 depletion prevents activation of IKK (Figure 3A), and that IKK is implicated in Ser536 phosphorylation of RelA/p65 (36), we addressed the role of IKK in this response. We observed that depletion of IKK α and IKK β each inhibited RelA/p65 phosphorylation by thrombin (Figure 6). Unlike RelA/p65 phosphorylation, depletion of IKK α or IKK β showed no effect on p38 MAPK phosphorylation (Figure E2A). We also examined the other possibility, that p38 MAPK may regulate IKK activation, by monitoring the effect of p38 MAPK inhibition on I κ B α phosphorylation on Ser32 and Ser36. Inhibiting p38 MAPK failed to prevent I κ B α phosphorylation induced by thrombin (Figure E2B). These data are in line with our earlier studies showing that thrombin-induced I κ B α degradation and RelA/p65 nuclear DNA binding were resistant to p38 MAPK inhibition (10, 35). Taken together, these data are consistent with a model wherein thrombin engages Pyk2 as an additional mechanism of RelA/p65 phosphorylation that relies on IKK. To further verify this model, we evaluated the effect of Pyk2 knockdown and p38 MAPK inhibition on Ser536 phosphorylation of RelA/p65, and found that this approach resulted in additive inhibitory effect on thrombin-induced RelA/p65 phosphorylation (Figure E3).

DISCUSSION

The nonreceptor tyrosine kinase, Pyk2, is an important regulator of EC barrier function and angiogenesis (23, 24). Recently, it has

Figure 5. (A and B) Effect of depletion or inhibition of Pyk2 on thrombin-induced phosphorylation of RelA/p65. HPAECs were transfected with si-Pyk2 or si-Con for 24–36 hours (A), or pretreated with Tyrphostin A9 (5 μ M) for 1 hour (B). Cells were challenged with thrombin (5 U/ml) for 1 hour. Total cell lysates were immunoblotted with an anti-phospho RelA/p65 (Ser536) antibody. Total RelA/p65 was used to monitor loading. (A) The bar graph represents the effect of Pyk2 depletion on thrombin-induced RelA/p65 phosphorylation normalized to the total RelA/p65 level. Data are means ($\pm$ SE) ($n = 3$ –4 for each condition). * $P < 0.05$ compared with untreated controls; # $P < 0.05$ compared with thrombin-stimulated control. (B) Results are representative of two independent experiments. (C) Phosphorylation at Ser536 is required for thrombin-induced transcriptional activity of RelA/p65. HPAECs were cotransfected with Gal4-LUC in combination with Gal4 vector, Gal4-p65 (286–551), or Gal4-p65 (286–551, S536A). After 12 hours, cells were challenged with thrombin (5 U/ml) for 6 hours. Cell extracts were prepared and assayed for Firefly and *Renilla* luciferase activities. Data are means ($\pm$ SE) ($n = 6$ for each condition). $\psi\psi\psi P < 0.001$ compared with Gal-4 vector control; *** $P < 0.01$ compared with Gal4-p65 (286–551) untreated control; ### $P < 0.001$ compared with Gal4-p65 (286–551) thrombin-stimulated control.

also been implicated in proinflammatory gene expression and leukocyte TEM (26, 27); however, the mechanistic basis of these responses is only partially understood. Because NF- κ B is an essential regulator of genes involved in TEM of leukocytes, the present study was undertaken to address the role of Pyk2 in the mechanism of NF- κ B activation in ECs. Our data show that Pyk2 is an important determinant of thrombin-induced EC inflammation, and that it mediates this response by activating IKK to promote the release and the transcriptional capacity of RelA/p65, and additionally by facilitating the nuclear translocation of released RelA/p65. Importantly, Pyk2 also controls NF- κ B activation induced by TNF- α , suggesting that Pyk2 is a general regulator of NF- κ B signaling in the endothelium.

We began our investigation by defining the time course of Pyk2 activation by thrombin. We noted a biphasic phosphorylation of Pyk2: an early and transient phase occurring between 1 and 5 minutes, followed by a late phase that begins at 30 minutes and is sustained for up to 2 hours after thrombin stimulation. The early phase of Pyk2 activation is in accord with a previous report (21) showing a similar time course of Pyk2 phosphorylation; however, the late phase was not detected in the earlier study, as Pyk2 phosphorylation was followed only up to 10 minutes after thrombin challenge. Thus, our data reveal a second phase (in addition to the early phase) of Pyk2 phosphorylation, and are consistent with two-stage mobilization of Ca^{2+} , a requirement for Pyk2 activation, induced by thrombin in ECs (37, 38).

Pyk2 activation was also observed in LPS-stimulated human umbilical vein ECs and human dermal microvascular ECs (26, 27), as well as in cyclic strain-exposed bovine aortic ECs (22). Thus, Pyk2 appears to be responsive to the proinflammatory environment in ECs from different vascular beds.

The activation of Pyk2 led us to assess its involvement in NF- κ B activation. To this end, we evaluated the effect of RNAi knockdown of Pyk2 on NF- κ B-dependent reporter activity. Depletion of Pyk2 abolished thrombin-induced NF- κ B activity, indicating the requirement of Pyk2 in the response. Our findings are consistent with those of earlier studies indicating the involvement of Pyk2 in G protein-coupled receptor (GPCR)-mediated signaling and NF- κ B activation (18, 39). We also examined if the impaired NF- κ B activity secondary to Pyk2 knockdown leads to reduced expression of VCAM-1 and MCP-1. A significant decrease in the expression of these genes was observed after thrombin stimulation of Pyk2-depleted cells. Notably, the role of Pyk2 in NF- κ B-dependent gene expression is not restricted to thrombin and the endothelium, but can also be induced by a variety of stimuli and in different cell types. Consistent with this, we found that depletion of Pyk2 was effective in inhibiting TNF- α -induced NF- κ B activity in ECs. Other studies showing a role of Pyk2 in LPS-induced expression of IL-8 and MCP-1 in ECs, carbacol-induced NF- κ B-dependent reporter activity in HeLa cells, and LPS- and peptidoglycan-induced IL-1 β expression in macrophages (26, 27, 39, 40) further underscore the importance of Pyk2 as a common mediator of NF- κ B signaling.

Figure 6. Effect of depletion of IKK α or IKK β on thrombin-induced RelA/p65 phosphorylation. HPAECs were transfected with si-IKK α , si-IKK β , or si-Con for 24–36 hours and then challenged with thrombin (5 U/ml) for 1 hour. Total cell lysates were immunoblotted with an anti-phospho RelA/p65 (Ser536) antibody. Total RelA/p65 levels were used to monitor loading. Depletion of IKK α or IKK β was determined by immunoblotting the lysates with an antibody to IKK α or IKK β , respectively. The bar graph represents the effect of depletion of IKK α or IKK β on thrombin-induced RelA/p65 phosphorylation normalized to total RelA/p65 level. Data are means ($\pm$ SE) ($n = 3$ –4 for each condition). *** $P < 0.001$ compared with untreated controls; ### $P < 0.01$ or # $P < 0.05$ compared with thrombin-treated control; # $P < 0.05$ compared with thrombin-treated control.

Our study yields the mechanistic basis of NF- κ B activation by Pyk2. We demonstrate that Pyk2 mediates NF- κ B activation by promoting the release, increasing the transcriptional capacity, and facilitating the nuclear translocation and DNA binding of RelA/p65. Nuclear translocation and DNA binding of RelA/p65 is contingent upon its release secondary to Ser32 and Ser36 phosphorylation, and, subsequently, degradation of I κ B α . Consistent with this, Pyk2 knockdown impaired the phosphorylation of IKK and I κ B α . Similarly, depletion of IKK α or IKK β also inhibited I κ B α phosphorylation and degradation. Intriguingly, however, Pyk2 depletion had no effect on I κ B α degradation by thrombin. This does not seem to be the result of a nonspecific effect of Pyk2-siRNA, as I κ B α level remained intact in unstimulated cells (Figure 3C). One possible explanation could be that a Ser32 and Ser36 phosphorylation-independent (i.e., IKK independent) mechanism of I κ B α degradation is activated by thrombin in the absence of Pyk2. Indeed, studies have shown that phosphorylation of I κ B α -PEST (proline–glutamic acid–serine–threonine) sequences by CKII-dependent mechanism also leads to its degradation (41, 42). It is likely that the inhibitory effect of Pyk2 depletion on IKK-dependent I κ B α degradation is masked by CKII-dependent degradation of I κ B α that may be activated by thrombin in the absence of Pyk2. Such a possibility is further supported by the requirement of Ca²⁺ for the latter mode of I κ B α degradation (41). Because Ca²⁺ is also required for Pyk2 activity, it is plausible that the depletion of Pyk2 increases the availability of Ca²⁺ to facilitate CKII-mediated

PEST sequence phosphorylation–dependent degradation of I κ B α by thrombin. In support of this model, we found that RNAi knockdown of CKII was effective in protecting I κ B α degradation only in cells depleted of Pyk2 (Figure E4). However, additional studies are required to unequivocally address this possibility.

Interestingly, Pyk2 knockdown was associated with impaired nuclear translocation and DNA binding of RelA/p65 in the face of I κ B α degradation. These results suggest that Pyk2 may also be involved in facilitating the translocation of released RelA/p65 to the nucleus. We recently found that dynamic changes in actin cytoskeleton induced by thrombin via RhoA/Rho-associated kinase (ROCK)/Cofilin pathway are necessary for nuclear translocation of RelA/p65 (43, 44). Given the involvement of Pyk2 and its downstream kinase c-Src in activating RhoA/ROCK pathway (19, 45, 46), it is possible that Pyk2 promotes nuclear translocation and, consequently, DNA binding of RelA/p65 by causing alterations in the actin cytoskeleton. Consistent with this possibility, inhibition of Pyk2 by tyrphostin A9 attenuates thrombin-induced Ser3 phosphorylation and, thereby, inactivation of cofilin1 (K.M.B., unpublished results), an actin-binding protein that occupies a central position in Rho–actin pathway mediating RelA/p65 nuclear translocation (43). However, in view of the reports that Pyk2 can also function downstream of RhoA/ROCK (47, 48), the other possibility that RhoA/ROCK engages Pyk2 to mediate the above responses cannot be excluded. Further studies are needed to establish how Pyk2 and RhoA/ROCK are linked in the mechanism of NF- κ B activation by thrombin.

An important event implicated in enhancing the transactivation potential of RelA/p65 includes its phosphorylation on serine residues, including Ser536 (10). We previously showed that thrombin induces Ser536 phosphorylation of RelA/p65, and that this response is mediated, in part, by p38 MAPK (34, 49). Because Pyk2 is required for p38 MAPK activation (50), and Pyk2 depletion reduces Ser536 phosphorylation of RelA/p65, we reasoned that Pyk2 may act upstream of p38 MAPK to mediate this response (RelA/p65 phosphorylation). However, our data that thrombin activates p38 MAPK in a Pyk2-independent manner argues against the involvement of Pyk2/p38 MAPK in RelA/p65 phosphorylation, and suggests the existence of an additional pathway that Pyk2 uses for this response. Indeed, we found that Pyk2 engages IKK (IKK α and IKK β) to promote RelA/p65 phosphorylation and, thereby, NF- κ B signaling induced by thrombin. Our findings that inhibition of p38 MAPK fails to prevent thrombin-induced I κ B α phosphorylation at Ser32 and Ser36 (Figure E2B), an IKK-mediated event required for I κ B α degradation and subsequently nuclear DNA binding of RelA/p65 (10, 35), also argues against the engagement of p38 MAPK upstream of IKK to mediate RelA/p65 phosphorylation. Thus, the inability of Pyk2 to mediate thrombin-induced p38 MAPK activation compared with an important role of Pyk2/p38 MAPK signaling in LPS-induced IL-8 and MCP-1 expression and vascular endothelial growth factor–induced EC migration (26, 27, 50) reveals a stimulus-specific activation of p38 MAPK by Pyk2 in ECs, and shows a fundamental signaling difference between thrombin and other receptors (GPCR versus LPS and vascular endothelial growth factor). It should also be stressed that the dominant inhibitory effect of Pyk2 depletion on thrombin-induced NF- κ B activation and EC inflammation may derive from the ability of Pyk2 to regulate RelA/p65 at multiple levels (release of RelA/p65 from I κ B α , nuclear translocation, and the transcriptional capacity of the released RelA/p65). Unlike Pyk2, p38 MAPK contributes to NF- κ B activity via RelA/p65 phosphorylation alone. Moreover, in a separate ongoing study, we have found that Ser536 phosphorylation is dependent

upon I κ B α degradation (K.M.B., unpublished results). Because p38 MAPK is not required for I κ B α degradation (10), its contribution to RelA/p65 phosphorylation and, thereby, EC inflammation may rely on Pyk2-dependent I κ B α degradation induced by thrombin.

In summary, this study identifies Pyk2 as a critical determinant of NF- κ B activation and EC inflammation. We show that Pyk2 engages IKK to liberate RelA/p65 from I κ B α and empower the liberated RelA/p65 with the transcriptional capacity. Additionally, Pyk2 is engaged to facilitate the nuclear accumulation and DNA binding of the released RelA/p65. Together, these events serve to promote EC inflammation. Thus, specific targeting of Pyk2 may be an effective anti-inflammatory strategy in vascular diseases associated with EC inflammation and intravascular coagulation.

Author disclosures are available with the text of this article at www.atsjournals.org.

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