A Role for Gab1/SHP2 in Thrombin Activation of PAK1
Gene Transfer of Kinase-Dead PAK1 Inhibits Injury-Induced Restenosis

Dong Wang, Biman C. Paria, Qiuhua Zhang, Manjula Karpurapu, Quanyi Li, William T. Gerthoffer, Yoshikazu Nakaoka, Gadiparthi N. Rao

Abstract—To understand the role of epidermal growth factor receptor (EGFR) transactivation in G protein–coupled receptor (GPCR) agonist–induced signaling events, we have studied the capacity of thrombin in the activation of Gab1-SHP2 in vascular smooth muscle cells (VSMCs). Thrombin activated both Gab1 and SHP2 in EGFR-dependent manner. Similarly, thrombin induced Rac1 and Cdc42 activation, and these responses were suppressed when either Gab1 or SHP2 stimulation is blocked. Thrombin also induced PAK1 activation in a time- and EGFR-Gab1-SHP2-Rac1/Cdc42-dependent manner. Inhibition of activation of EGFR, Gab1, SHP2, Rac1, Cdc42, or PAK1 by pharmacological or genetic approaches attenuated thrombin-induced VSMC stress fiber formation and motility. Thrombin activated RhoA in a time-dependent manner in VSMCs. LARG, a RhoA-specific GEF (guanine nucleotide exchange factor), was found to be associated with Gab1 and siRNA-mediated depletion of its levels suppressed RhoA, Rac1 and PAK1 activation. Dominant negative mutant-mediated interference of RhoA activation inhibited thrombin-induced Rac1 and PAK1 stimulation in VSMCs and their stress fiber formation and migration. Balloon injury induced PAK1 activity and interference with its activation led to attenuation of SMC migration from media to intima, resulting in reduced neointima formation and increased lumen size. Inhibition of thrombin signaling by recombinant hirudin also blocked balloon injury–induced EGFR tyrosine phosphorylation and PAK1 activity. These results show that thrombin-mediated PAK1 activation plays a crucial role in vascular wall remodeling and it could be a potential target for drug development against these vascular lesions. (Circ Res. 2009;104:1066-1075.)

Key Words: RhoGEF ■ GTPases ■ PAK1 ■ neointima

Thrombin elicits both mitogenic and motogenic actions in a variety of cell types, including vascular smooth muscle cells (VSMCs).1-3 Thrombin mediates its effects via G protein–coupled protease-activated receptors (PARs), specifically the high-affinity receptor PAR-1.3,4,6 In addition, thrombin-induced mitogenic and motogenic effects exhibit a requirement for transactivation of receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR)-1, and insulin-like growth factor I receptor in various cell types, including VSMCs.7-11 Furthermore, it was reported that transactivation of RTKs, particularly EGFR by G protein–coupled receptor (GPCR) agonists such as thrombin, leads to activation of signaling events downstream to receptor activation.12,13 In this aspect, studies from others as well as our laboratory showed that transactivation of EGFR by thrombin leads to stimulation of extracellular signal-regulated kinases (ERKs) and phosphatidylinositol-3 kinase (PI3K).2,13 However, it is less clear the extent to which RTK transactivation, eg, EGFR, by GPCR agonists such as thrombin, leads to activation of signaling events that are otherwise stimulated in response to a true ligand-induced RTK activation. One of the signaling events that is activated upon EGFR tyrosine phosphorylation is the recruitment of Gab1 (Grb2-associated binder 1) and its associated phosphatase SHP2 onto the receptor.14,15 Using tissue-specific knockout or knock-in mouse models, many studies have shown that Gab1-SHP2 plays a crucial role in a variety of cellular functions including cell proliferation and migration.15,16

Although a large number of studies have reported activation of EGFR by thrombin, it is less clear whether this GPCR agonist possess the capacity to activate Gab1/SHP2. Furthermore, the role of Gab1/SHP2 in the activation small GTPases such as Rac1/Cdc42 and their downstream target PAK1 is also not known. To test this, we have studied thrombin effects on Gab1-SHP2 activation and their involvement in Rac1/Cdc42-mediated PAK1 stimulation in VSMCs. We found that thrombin activates PAK1 via a signaling involving EGFR, Gab1/SHP2, LARG, RhoA and Rac1 as well as Cdc42 in

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VSMCs. Thrombin-induced PAK1 activation was also found to be crucial in mediating vascular wall remodeling in response to injury. In addition, phosphorylation of Thr423 but not Ser144 of PAK1 was observed to be correlated with its kinase activity in VSMCs in response to thrombin and in the artery in response to injury.

**Materials and Methods**

**Methods**

Isolation of rat VSMCs, Western blot analysis, rat carotid artery balloon injury (BI), in vitro and in vivo VSMC migration, and immunohistochemistry were performed as described previously. All of the animal protocols were performed in accordance with the...
relevant guidelines and regulations approved by the Internal Animal Care & Use Committee of the University of Tennessee Health Science Center.

Statistics
Data analysis for statistical significance of variance was performed by Student’s t test.

Results
To understand the mechanisms by which thrombin induces VSMC migration, we have tested the role of Gab1 and SHP2. Thrombin at 0.5 U/mL induced tyrosine phosphorylation of

Figure 2. Thrombin-induced VSMC F-actin stress fiber formation and motility require activation of Gab1, SHP2, and EGFR. A, VSMCs that were transduced with Ad-green fluorescent protein (Ad-GFP) (control), Ad-dnGab1, or Ad-dnSHP2 with 40 mois were quiesced and treated with and without thrombin (0.5 U/mL) for 30 minutes, and F-actin stress fiber formation was measured by TRITC-conjugated phalloidin staining. B, Quiescent VSMCs that were treated with and without thrombin (0.5 U/mL) in the presence and absence of AG1478 (500 nmol/L) for 30 minutes were analyzed for F-actin stress fiber formation as described for A. C, Conditions were the same as in A except that after quiescence, cells were subjected to thrombin-induced (0.5 U/mL) migration using modified Boyden chamber method. D, Quiescent VSMCs were subjected to thrombin-induced (0.5 U/mL) migration in the presence and absence of 500 nmol/L AG1478 as described for C. The bar graphs represent mean±SD values of 3 independent experiments. *P<0.01 vs control or Ad-GFP; **P<0.01 vs thrombin or Ad-GFP+thrombin treatment alone.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.
Gab1, as measured by immunoblotting of anti-Gab1 immunoprecipitates of control and thrombin-treated VSMCs with anti-PY20 antibodies. Increases in tyrosine phosphorylation of Gab1 were observed at 10 minutes and peaked at 30 to 60 minutes (Figure 1A). Sequential probing of this membrane with anti-SHP2 antibodies showed a band with molecular mass of 72 kDa whose intensities were found to be higher in thrombin-treated VSMCs as compared to control, suggesting association of SHP2 with tyrosine-phosphorylated Gab1 in response to thrombin. In a converse experiment, thrombin induced tyrosine phosphorylation of SHP2 (Figure 1B). To identify the upstream mechanisms of Gab1 and SHP2 tyrosine phosphorylation, we examined the role of EGFR. Thrombin induced tyrosine phosphorylation of EGFR, as measured by immunoblotting of anti-EGFR immunoprecipitates of control and thrombin-treated VSMCs with anti-PY20 antibodies (Figure 1C). Maximum increases in tyrosine phosphorylation of EGFR occurred at 10 minutes, and these increases were sustained at least for 2 hours. Sequential probing of this membrane with anti-Gab1 and anti-SHP2

Figure 3. Thrombin stimulates Rac1 and Cdc42 activation in Gab1/SHP2 and EGFR tyrosine kinase–dependent manner in VSMCs. A, An equal amount of protein from control and each time point of thrombin-treated (0.5 U/mL) VSMCs were subjected to pull-down assay using GST-PAK–conjugated Sepharose CL4B beads, and the resultant GST-PAK–bound proteins were analyzed by Western blotting for Rac1 (left) or Cdc42 (right) using their specific antibodies. The blots in the left and right images were reprobed with anti-Cdc42 and anti-Rac1 antibodies, respectively. The bottom Western blots show total levels of Rac1 and Cdc42. B, VSMCs that were transduced with Ad-GFP (control), Ad-dnGab1, or Ad-dnSHP2 with 40 mois and quiesced were treated with and without thrombin (0.5 U/mL) for 30 minutes, and cell extracts were prepared and analyzed for Rac1 and Cdc42 activation as described for A. C, Quiescent VSMCs were treated with and without thrombin (0.5 U/mL) in the presence and absence of AG1478 (500 nmol/L) for 30 minutes, and cell extracts were prepared and analyzed for Rac1 and Cdc42 activation as described for A. D and E, After transduction with 40 mois of Ad-GFP, Ad-dnRac1, or Ad-dnCdc42 and quiescence, VSMCs were subjected to thrombin-induced (0.5 U/mL) F-actin stress fiber formation or cell migration as described in the legend for Figure 2A and 2C. The bar graphs represent mean±SD values of 3 independent experiments. *P<0.01 vs control or Ad-GFP; **P<0.01 vs thrombin or Ad-GFP+thrombin treatment alone.
antibodies revealed their association with EGFR in tyrosine phosphorylation-dependent manner. To test whether EGFR tyrosine kinase activity is required for thrombin-induced Gab1 tyrosine phosphorylation and/or their association, quiescent VSMCs were treated with and without thrombin (0.5 U/mL) in the presence and absence of 500 nmol/L AG1478, a potent inhibitor of EGFR, cell extracts were prepared and analyzed for Gab1 tyrosine phosphorylation. AG1478 significantly blocked thrombin-induced Gab1 tyrosine phosphorylation (Figure 1D). Reprobing of this membrane with anti-SHP2 antibodies revealed association of SHP2 with Gab1 in EGFR tyrosine kinase activity–dependent manner. To find the functional significance of Gab1 and SHP2 activation, we further tested their role in thrombin-induced VSMC F-actin stress fiber formation and migration. Thrombin treatment caused extensive F-actin stress fiber formation, and these responses were completely blocked by adenovirus-mediated expression of dominant negative mutants of either Gab1 (dnGab1) or SHP2 (dnSHP2) (Figure 2A). Similarly, pretreatment with 500 nmol/L AG1478 inhibited thrombin-induced F-actin stress fiber formation (Figure 2B). Adenovirus-mediated expression of dnGab1 or dnSHP2 or pretreatment with AG1478 also reduced thrombin-induced VSMC migration, as measured by modified Boyden chamber method (Figure 2C and 2D).

The Rho family of GTPases plays a role in the regulation of F-actin stress fiber formation, which is essential for cell migration and proliferation. To identify the downstream effector molecules of EGFR-Gab1/SHP2 signaling, we next studied the role of Rac1 and Cdc42. Quiescent VSMCs were treated with and without 0.5 U/mL thrombin for various times, and cell extracts were prepared and analyzed by pull-down assay using glutathione S-transferase (GST)-PAK Sepharose-CL4B beads, followed by immunoblotting for Rac1 or Cdc42. Thrombin induced activation of both Rac1 and Cdc42 in a time-dependent manner, with maximum effect at 30 to 60 minutes (Figure 3A). Expression of either dnGab1 or dnSHP2 or pretreatment with AG1478 inhibited thrombin-induced Rac1 and Cdc42 activation by 80% (Figure 3B and 3C). Interference with activation of Rac1 or Cdc42 via adenovirus-mediated expression of their dominant negative mutants (dnRac1 and dnCdc42, respectively) attenuated thrombin-induced VSMC F-actin stress fiber formation and migration (Figure 3D and 3E). Many studies have demonstrated that Rac1/Cdc42 target PAK1 in the mediation of cell migration. Therefore, to determine whether this was the case for the role of Rac1/Cdc42, we next studied the time course effect of thrombin on activation of PAK1. Thrombin, while having no noticeable effect on Ser144 phosphorylation, induced Thr423 phosphorylation of PAK1 in a time-dependent manner, with maximum effect at 30 to 60 minutes (Figure 4A). To confirm the activation of PAK1 by thrombin, we also measured its activity by immunocomplex kinase assay. Consistent with its effect on Thr423 phosphorylation, thrombin induced PAK1 activity in a time-dependent manner, with a near maximum increase between 30 and 60 minutes (Figure 4B). To test the role of PAK1 in thrombin-induced VSMC migration, cells were transduced with dnPAK1 adenovirus at 40 multiplicities of infection (mois), quiesced, treated with and without thrombin (0.5 U/mL) for appropriate time periods, and tested for PAK1 activity, F-actin stress fiber formation, and VSMC migration. Adenovirus-mediated expression of dnPAK1 suppressed thrombin-induced PAK1 activity, F-actin stress fiber formation, and VSMC migration (Figure 4C through 4E). To determine the mechanisms by

**Figure 4.** Thrombin-induced VSMC migration requires PAK1 activation. A and B, An equal amount of protein from control and various time points of thrombin-treated (0.5 U/mL) VSMCs were analyzed either by Western blotting for PAK1 Ser144/Thr423 phosphorylation using their specific antibodies (A) or by immunocomplex kinase assay for PAK1 activity using MBP and [γ-32P]-ATP as substrates as described in Materials and Methods (B). C, VSMCs that were transduced with Ad-GFP or Ad-dnPAK1 at 40 mois and quiesced were treated with and without thrombin (0.5 U/mL) for 30 minutes, and cell extracts were prepared and analyzed for PAK1 activity as described for B, D and E. All the conditions were the same as for C except that after quiescence cells were subjected to thrombin-induced (0.5 U/mL) F-actin stress fiber formation (D) or migration (E) as described in the legend for Figure 2A and 2C, respectively. The bar graphs represent mean ± SD values of 3 independent experiments. *P<0.01 vs control or Ad-GFP; **P<0.001 vs Ad-GFP+thrombin treatment alone.
which thrombin activates PAK1, we next tested the role of EGFR-Gab1/SHP2-Rac1/Cdc42 signaling. Blockade of Gab1, SHP2, Rac1, or Cdc42 activation by adenovirus-mediated expression of their respective dominant negative mutants or suppression of EGFR activity by AG1478 inhibited both thrombin-induced PAK1 Thr423 phosphorylation and its activity (Figure 5A through 5F).

Guanine nucleotide exchange factors (GEFs) play an important role in agonist-induced activation of GTPases. To understand the mechanisms by which Gab1 mediates GTPase stimulation, we tested the role of GEFs. Coimmunoprecipitation assays revealed that LARG, a RhoA-specific GEF, forms a complex with Gab1 in a time-dependent manner in response to thrombin (Figure 6A). In addition, siRNA-mediated depletion of LARG inhibited thrombin-induced Rac1 and PAK1 activation (Figure 6B and 6C). Furthermore, thrombin stimulated RhoA in a time-dependent manner (Figure 6D). Inhibition of EGFR by AG1478 or siRNA-mediated depletion of either Gab1 or LARG levels substantially reduced thrombin-induced RhoA activation (Figure 6E through 6G). Adenovirus-mediated expression of dominant negative mutant of RhoA attenuated thrombin-induced Rac1 and PAK1 activation and stress fiber formation, resulting in reduced VSMC migration (Figure 6H through 6K).

To understand the role of PAK1 in vascular wall remodeling in vivo, we have examined its involvement in injury-induced SMC migration and neointima formation. First, mechanical injury of rat carotid artery induced both Ser144 and Thr423 phosphorylation of PAK1 as early as 6 hours after injury and peaked at 12 hours after injury (Figure 7A). However, the steady-state levels of PAK1 were decreased by 30% to 40% at these time periods after injury as compared to

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**Figure 5.** Gab1/SHP2 and Rac1/Cdc42 mediate thrombin-induced PAK1 activation in VSMCs. A, B, E, and F, VSMCs that were transduced with Ad-GFP, Ad-dnGab1, Ad-dnSHP2, Ad-dnRac1, or Ad-dnCdc42 at 40 mois and quiesced were treated with and without thrombin (0.5 U/mL) for 30 minutes, and cell extracts were prepared. Cell extracts containing an equal amount of protein from control and each treatment were analyzed by either Western blotting for PAK1 Thr423 phosphorylation using its specific antibodies or immunocomplex kinase assay for its activity using MBP and [$^32$P]-ATP as substrates. C and D, Quiescent VSMCs were treated with and without thrombin (0.5 U/mL) in the presence and absence of AG1478 (500 nmol/L) for 30 minutes, and cell extracts were prepared and analyzed for PAK1 Thr423 phosphorylation or kinase activity as described above for A and B, respectively. The bar graphs represent mean ± SD values of 3 independent experiments. *P<0.01 vs control or Ad-GFP; **P<0.01 vs thrombin or Ad-GFP + thrombin treatment alone.
its levels in uninjured arteries. Consistent with its phosphorylation, PAK1 activity was also increased in the arteries in response to injury (Figure 7B). To find whether activation of PAK1 occurs in SMC, double immunofluorescence staining was performed in the cryosections of injured and uninjured arteries for SMα-actin–and Thr423-phosphorylated PAK1. As shown in Figure 7C, double immunofluorescence staining for SMα-actin and PAK1 Thr423 phosphorylation revealed PAK1 activation in SMC in response to injury. Adenovirus-mediated expression of dnPAK1 in the arteries suppressed only Thr423 phosphorylation of PAK1 and its activity (Figure 8A and 8B). Dominant negative mutant-mediated inhibition of PAK1 activation also reduced injury-induced SMC migration from medial to luminal surface and thereby attenuated neointima formation by 60%, resulting in increased luminal size (Figure 8C and 8D). To find the link between thrombin, EGFR and PAK1 in BI-induced vascular wall remodeling, we tested the effect of recombinant...
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Figure 7. Balloon injury activates PAK1 in rat carotid artery. A and B, Carotid arteries were dissected out after the indicated time periods of BI and tissue extracts were prepared. The tissue extracts containing an equal amount of protein were analyzed by either Western blotting for PAK1 Ser144/Thr423 phosphorylation using their specific antibodies (A) or immunocomplex kinase assay for its activity using MBP and [γ-32P]-ATP as substrates (B). C, Twelve hours after BI, the injured and uninjured arteries were isolated and fixed, cryosections were made, and immunofluorescence was stained for SM-α-actin and pPAK1 (Thr423) colocalization using their specific antibodies.

Discussion

The important findings of the present study are as follows. (1) Thrombin stimulated tyrosine phosphorylation of Gab1 and SHP2 in VSMCs. (2) Thrombin also induced tyrosine phosphorylation of EGFR with a time course similar to those of Gab1 and SHP2. (3) Both Gab1 and SHP2 were found to be associated with tyrosine-phosphorylated EGFR and inhibition of EGFR tyrosine kinase activity by AG1478 suppressed the actions of these agents on cell proliferation and migration.7–9,12 The present results reveal that stimulation of EGFR by thrombin is sufficient to activate the signaling events downstream to the receptor. In addition to SHP2, Gab1 has been shown to recruit PI3K, and via different interacting effector molecules, it targets the development of various organs. Specifically, it was demonstrated that the recruitment of PI3K by Gab1 is essential for EGFR-mediated embryonic eyelid closure and keratinocyte differentiation, whereas Gab1 association with SHP2 is required for Met receptor function in placental development and muscle progenitor cell migration to the limbs.15,16 Because thrombin activation of EGFR also caused the recruitment of Gab1/SHP2 onto the receptor, it is possible that this signaling complex participates in the regulation of VSMC motility. Evidence in support of this possibility comes by the finding that activation of Gab1/SHP2 is required for thrombin stimulation of Rac1/Cdc42, whose functions have been shown to be essential for F-actin stress fiber formation.18–21 The present findings also provide the first mechanistic evidence for the role of Gab1 in Rac1 activation. Specifically, Gab1 recruits LARG, a RhoA-specific GEF,28 which, in turn, via its influence on RhoA activation, leads to stimulation of Rac1. Although an antagonism was observed between RhoA and Rac1 in some cell types in response to many agonists,29 a potential role for RhoA in the activation of Rac1 has also been demonstrated in Swiss 3T3 fibroblasts.30 Based on these findings, it can be further speculated that Gab1 or SHP2 via recruiting and influencing either GEFs, GTPase-activating proteins (GAPs), or guanine nucleotide dissociation inhibitors may be facili-
tating Cdc42 activation by thrombin. Indeed, some reports showed that SHP2 via dephosphorylating p190-B RhoGAP mediates RhoA activation during myogenesis. A large body of data suggests that Rho GTPases via influencing the regulation of F-actin stress fiber formation play an important role in the mediation of cell motility. In fact, our finding that disruption of EGFR-dependent Gab1/SHP2-mediated RhoA, Rac1, or Cdc42 activation signaling aborts thrombin-induced VSMC F-actin stress fiber formation and migration suggests a role for this signaling axis in the regulation of GPCR agonist-induced cell motility.

Many reports showed that RhoA, Rac1, and Cdc42 play a role in the activation of PAK1. However, it is not known whether Gab1/SHP2 targets PAK1 in regulating either cell proliferation or migration in response to RTK or GPCR agonists. In this regard, the present study reveals that thrombin-induced Gab1/SHP2 leads to activation of PAK1. In addition, because blockade of EGFR or Gab1/SHP2 activation signaling aborts thrombin-induced VSMC F-actin stress fiber formation and migration suggests a role for this signaling axis in the regulation of GPCR agonist-induced cell motility.

Figure 8. Blockade of PAK1 activation suppresses BI-induced SMC migration from medial-to-intimal region and neointima formation in rat carotid arteries. A and B, Immediately after BI, Ad-GFP or Ad-dnPAK1 was transduced into injured arteries at 10^{10} pfu/mL. Carotid arteries were dissected out at 12 hours after BI, and tissue extracts were prepared and analyzed by either Western blotting for PAK1 Ser144/Thr423 phosphorylation using their specific antibodies (A) or immunocomplex kinase assay for its activity using MBP and [γ-^32P]-ATP as substrates (B). C, Three days after BI, injured and uninjured common carotid arteries were dissected out, fixed, opened longitudinally, and stained with SMα-actin antibodies, and the cells in the luminal region were counted. D, All of the conditions were the same as in C except that 2 weeks after BI, arteries were isolated and fixed, cross-sections were made and stained with hematoxylin/eosin, and morphometric analysis was performed and the I/M ratios were calculated. E, Before and soon after BI, r-hirudin was administered into animals. Injured and uninjured arteries were dissected out 16 hours after BI, and tissue extracts were prepared. The tissue extracts containing an equal amount of protein from each group were analyzed for EGFR tyrosine phosphorylation and PAK1 activation as described in the legends for Figures 1C, 4A, and 4B, respectively. The bar graphs in C and D represent means ± SD values of SMC migration and neointima formation/lumen size, respectively. *P<0.05 vs Ad-GFP BI alone (n=6).

Gab1 signaling to PAK1 activation via RhoA-dependent Rac1 stimulation. Furthermore, because no noticeable changes are observed in the Ser144 phosphorylation of PAK1 by thrombin, it is likely that EGFR-Gab1/SHP2-LARG-RhoA-Rac1/Cdc42 signaling does not affect the phosphorylation of this residue. It also appears that Ser144 phosphorylation is not required for PAK1 activity because there was no correlation between these 2 events in response to thrombin in VSMCs. It is noteworthy that adenovirus-mediated expression of kinase-dead PAK1, while enhancing Ser144 phosphorylation, reduced Thr423 phosphorylation and activity of endogenous PAK1, a finding that suggests a correlation between Thr423 phosphorylation and kinase activity. This result indicates that overexpression of kinase-dead PAK1 somehow sequesters endogenous PAK1 from being phosphorylated at Thr423 residue. It also suggests that Thr423 is present in the catalytic domain. With regard to PAK1 activation in the arteries in response to injury in vivo, its levels were decreased by approximately 30%, but its phosphorylation both at Ser144 and Thr423 and activity were increased very robustly. This finding suggests that although PAK1 levels were reduced by injury, its activity was increased by enhanced phosphorylation and this appears to be sufficient to activate its downstream signaling events necessary for SMC migration. Because downregulation of PAK1 activation significantly blocked injury-induced neointima formation, it is possible that PAK1 may also be involved in SMC multipli-
cation in response to injury. In fact, a large body of data suggests that PAK1 plays a role in the regulation of cell growth.33 Given the role of PAK1 in the regulation of both cell proliferation and migration, and downregulation of its activity inhibited injury-induced neointima formation, it is quite likely that PAK1 plays a crucial role in vascular wall remodeling. In addition, because downregulation of thrombin activity via r-hirudin inhibited injury-induced EGFR tyrosine phosphorylation and PAK1 activity, it is conceivable that endogenously produced thrombin activates both EGFR and PAK1 in vascular wall as well, contributing to neointima formation following angioplasty. The role of thrombin in injury-induced neointima formation has also been reported previously, but the underlying mechanisms were not explored.34,35 In this aspect, the present data provide mechanistic evidence for the role of thrombin in vascular wall remodeling.

Sources of Funding
This work was supported by NIH grant HL64165 (to G.N.R.)

Disclosures
None.

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Circ Res. 2009;104:1066-1075; originally published online April 9, 2009;
doi: 10.1161/CIRCRESAHA.109.196691

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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MATERIALS AND METHODS

Reagents: Aprotinin, dithiothreitol, FITC-conjugated anti-rabbit IgG, N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES), rHirudin, leupeptin, mouse monoclonal anti-SMα-actin antibodies, myelin basic protein, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, sodiumdeoxycholate, thrombin and TRITC-conjugated anti-mouse IgG were purchased from Sigma Chemical Company (St. Louis, MO). Anti-phospho-PAK1 (Thr423)/PAK2 (Thr402) antibodies (2601S), anti-phospho-PAK1 (Ser144)/PAK2 (Ser141) antibodies (2606S), anti-PAK1 antibodies (2602S), anti-phosphotyrosine mouse monoclonal antibodies (9411) were obtained from Cell Signaling Technology (Beverly, MA). Anti-Cdc42 antibodies (SC-87), anti-EGFR antibodies (SC-03), anti-Gab1 antibodies (SC-9049), anti-LARG antibodies (SC-25638), anti-phospho-PAK1 (Thr423) antibodies (SC-12925R), anti-PAK1 antibodies (SC-882) and anti-RhoA antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Rac1 antibodies (Cat. No. 05-389) were bought from Upstate Biotechnology (Lake Placid, New York). Anti-PTP1D/SHP-2 mouse monoclonal antibodies (Cat. No. 610621) was obtained from Transduction Laboratories (Lexington, KY). Growth factor-reduced Matrigel (Cat. No. 354250) was purchased from BD Biosciences (Bedford, MA). [γ^{32}P]-ATP (3000 Ci/mmol) was obtained from Amersham Biosciences (Piscataway, NJ). Hematoxylin (H-3404), biotinylated anti-mouse IgG (BA-9200), ABC kit (PK-6100) and DAB kit (SK-4100) were bought from Vector Laboratories (Burlingame, CA). Rat LARG siRNA (Cat No. ON-TARGETplus SMARTpool L-101376-01,
NM_001013246), rat Gab1 siRNA (Cat. No. ON-TARGETplus SMARTpool L-093469-00, XM_341667), siCONTROL nontargeting siRNA number 2 (Cat. No. D-0012-02-20) and DharmaFECT 2 transfection reagent (Cat. No. T-2002-03) were bought from Dharmacon RNAi Technologies (Chicago, IL).

**Cell culture:** Rat VSMCs were isolated and subcultured as described previously [1]. VSMCs were used between 6 and 12 passages.

**Construction of adeno viral vectors:** The Cdc42N17 and RhoAN19 were released from pCEV-Cdc42N17 and pCEV-RhoAN19, respectively, by digestion with BamHI and EcoRI (2) and subcloned into the same sites of pENTR3C to yield pENTR3C-Cdc42N17 and pENTR3C-RhoAN19, which were then subjected to recombination with pAdCMV/V5DEST to obtain pAd-Cdc42N17 and pAd-RhoAN19, respectively. SHP2CS (catalytic cysteine 459 was mutated to serine) was released from pCMV-SHP2CS by digestion with HindIII and XbaI and subcloned into the same sites of pBluescript II SK (+). The SHP2CS was then retrieved from pBluescript II SK(+) by digestion with KpnI and NotI and cloned into the same sites of pENTR3C to yield pENTR3C-SHP2CS. The pENTR3C-SHP2CS was recombinated with pAdCMV/V5DEST to yield pAd-SHP2CS. The construction of pAd-dnGab1, pAd-dnPAK1 and pAd-dnRac1 were described previously (3-5). The plasmids, pAd-GFP, pAd-dnCdc42N17, pAd-dnGab1, pAd-dnPAK1, pAd-Rac1N17, pAd-RhoAN19 and pAd-dnSHP2 were linearized by digestion with PacI and transfected into HEK293A cells. The resultant adenovirus was further amplified by infection of HEK293A cells and was purified by cesium chloride gradient ultracentrifugation (6).
Transfections and transductions: VSMCs were transfected with specific siRNA molecules at a final concentration of 100 nM using DharmaFECT 2 transfection reagent according to manufacturer's instructions. When adenoviral vectors were used to downregulate the function of a specific molecules, cells were transduced with carrying control GFP or appropriate target molecules at 40 moi overnight in complete medium. After transfections or transductions, cells were quiesced for 48 h and used as required.

Cell motility: Cell migration was measured using a modified Boyden chamber method (7). Wherever adenovirus was used, cells were first transduced with the respective adenovirus at a moi of 40 and growth-arrested before they were subjected to agonist-induced migration. Cell motility was presented as number of migrated cells/field.

Immunoprecipitation: After rinsing with cold phosphate-buffered saline (PBS), cells were lysed by freeze-thawing in 250 µl of lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml PMSF, 100 µg/ml aprotinin, 1 µg/ml leupeptin and 1 mM sodium orthovanadate) for 20 min on ice, whereas tissues were homogenized in the same buffer. The cell or tissue extracts were transferred into 1.5 ml Eppendorf tubes and cleared by centrifugation at 12,000 rpm for 20 min at 4°C. The cell or tissue extracts containing an equal amount of protein from control and each treatment were incubated with appropriate antibodies overnight at 4°C at which time protein A/B Sepharose CL4B beads were added and incubation continued for 2 hrs with gentle rocking. The beads were washed four times with lysis buffer and once with
PBS and the immunocomplexes were released by heating in Laemmeli sample buffer and analyzed by Western blotting for the indicated molecules using their specific antibodies.

**PAK1 kinase assay:** After three to four washes in lysis buffer, the immunocomplex beads were rinsed once with kinase buffer (20 mM Hepes, pH 7.6, 20 mM MgCl₂, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol) and resuspended in 30 μl of the same buffer consisting of 5 μg MBP, 20 μM ATP, and 10 μCi of [γ-³²P]-ATP and incubated at 30°C for 30 min. At the end of incubation, the reaction was terminated by adding 30 μl of 4X Laemmeli sample buffer and boiling for 5 min. The reaction mix was then separated by 0.1% SDS-10% PAGE and the products were visualized by autoradiography.

**Pull-down assay:** An equal amount of protein from control and each treatment was incubated with GST-PAK1 (Cdc42 and Rac1 interactive binding domain) or GST-Rhotekin-conjugated Sepahrose CL4B beads 45 min at 4°C. The beads were collected by centrifugation, washed in lysis buffer, heated in Laemmeli sample buffer for 5 min and the released proteins were resolved on 0.1% SDS-12% PAGE and immunoblotted with anti-Rac1 antibodies, anti-Cdc42 antibodies or anti-RhoA antibodies. After incubation with Horseradish Peroxidase (HRP)-conjugated secondary antibodies, the antigen-antibody complexes were detected using a chemiluminescence reagent kit (Amersham Pharmacia Biotech).

**F-actin immunofluorescence staining:** VSMC were grown on glass coverslips coated with 10 μg/ml collagen (Roche Diagnostics Corporation, Indianapolis, IN). After appropriate treatments, cells were fixed in 3.7% formaldehyde in PBS for 20
min, permeabilized in 0.2% Triton X-100 for 5 min and blocked with 1% bovine serum albumin in PBS. Cells were then stained with 20 μM TRITC-labeled phalloidin (Biotium, Hayward, CA) for 30 min. Fluorescence was observed under Zeiss inverted microscope (Model: Axiovert 200 M).

**Rat carotid artery balloon injury:** All the animal protocols were performed in accordance with the relevant guidelines and regulations approved by the Internal Animal Care & Use Committee of the University of Tennessee Health Science Center, Memphis, TN. Balloon injury was performed essentially as described by us previously (8). Adenovirus ($10^{10}$ pfu/ml) in 150 μl of PBS was infused into the ligated segment of the common carotid artery for 30 min. In some experiments, r-Hirudin was administered intravenously into rats at a bolus dose of 75 units in a total volume of 0.5 ml of physiological saline, which is equivalent to 250 units/kg, via the right jugular vein immediately before balloon injury. After 8 hrs of surgery, another intravenous bolus dose of 75 units rHirudin was injected. In the control group, animals were administered only physiological saline via the same protocol. At different time points after balloon injury, the animals were sacrificed with an overdose of pentobarbital (200 mg/kg) and the carotid arteries were collected and processed for either protein isolation or morphometric analysis. For morphometric analysis, carotid arteries were fixed in formalin, dehydrated and embedded in OCT (Tissue Tek, Miles Inc., U.S.A.), sections (5 μm thick) made at equally spaced intervals in the middle of injured and uninjured common carotid artery segments and stained with Hematoxylin and Eosin. The intimal (I) and
medial (M) areas were measured using NIH ImageJ and the I/M ratios were calculated.

**Double immunofluorescence staining:** After blocking in normal goat serum, the cryosections (5 μm) were incubated first with anti-SMα-actin antibodies (1:500) followed by TRITC-conjugated secondary antibodies. After washing with PBS and blocking again in normal goat serum, these sections were incubated with anti-pPAK1 (1:100) antibodies followed by FITC-conjugated secondary antibodies. Fluorescence was observed under Zeiss inverted microscope (Model: Axiovert 200 M).

**In vivo SMC migration assay:** In vivo SMC migration was determined according to the method of Bendeck et al (9) as described previously (7).

**Western blot analysis:** Western blot analysis was performed as described previously (1).

**Statistics:** All the experiments were repeated three times with similar results. Data are presented as Means ± SD. The treatment effects were analyzed by Student's t test. p values < 0.05 were considered to be statistically significant. In the case of Western blotting and kinase assays, histochemistry and immunohistochemistry, one representative set of data is shown.

**REFERENCES**


