Novel Role of Proline-Rich Nonreceptor Tyrosine Kinase 2 in Vascular Wall Remodeling After Balloon Injury

Ravisekhar Gadeppalli, Nikhlesh K. Singh, Venkatesh Kundumani-Sridharan, Mark R. Heckle, Gadiparthi N. Rao

Objective—To investigate the role of Pyk2, a proline-rich nonreceptor tyrosine kinase, in G protein–coupled receptor agonist, thrombin-induced human aortic smooth muscle cell growth and migration, and injury-induced vascular wall remodeling.

Methods and Results—Thrombin, a G protein–coupled receptor agonist, activated Pyk2 in a time-dependent manner and inhibition of its stimulation attenuated thrombin-induced human aortic smooth muscle cell migration and proliferation. Thrombin also activated Grb2-associated binder protein 1, p115 Rho guanine nucleotide exchange factor, Rac1, RhoA, and p21-activated kinase 1 ( Pak1) and interference with stimulation of these molecules attenuated thrombin-induced human aortic smooth muscle cell migration and proliferation. In addition, adenovirus-mediated expression of dominant negative Pyk2 inhibited thrombin-induced Grb2-associated binder protein 1, p115 Rho guanine nucleotide exchange factor, Rac1, RhoA and Pak1 stimulation. Balloon injury also caused activation of Pyk2, Grb2-associated binder protein 1, p115 Rho guanine nucleotide exchange factor, Rac1, RhoA, and Pak1 in the carotid artery of rat, and these responses were sensitive to inhibition by the dominant negative Pyk2. Furthermore, inhibition of Pyk2 activation resulted in reduced recruitment of smooth muscle cells onto the luminal surface and their proliferation in the intimal region leading to suppression of neointima formation.

Conclusion—Together, these results demonstrate for the first time that Pyk2 plays a crucial role in G protein–coupled receptor agonist thrombin-induced human aortic smooth muscle cell growth and migration, as well as balloon injury–induced neointima formation. (Arterioscler Thromb Vasc Biol. 2012;32:2652-2661.)

Key Words: restenosis ■ signal transduction ■ vascular biology

Thrombin is generated at the sites of vascular injury because of the exposure of platelets to subendothelial collagen and subsequent activation of the blood clotting process. Thrombin, in addition to its indispensable role in blood clotting, acts as a mitogen and chemotactic factor to a variety of cell types, including fibroblasts and vascular smooth muscle cells (VSMCs). Thrombin mediates its effects via protease-activated receptors 1–4 that are coupled to various G proteins. Many studies have also demonstrated that G protein–coupled receptor (GPCR) agonists, such as angiotensin II, lysophosphatidic acid, and thrombin, transactivate receptor tyrosine kinases in mediating their growth and chemotactic effects. Among the G proteins, Gs, Gi, Gq/11, and G12/13 are coupled to protease-activated receptors 1–4 and mediate thrombin’s effects depending on the cell type. Activation of these G proteins via coupling to various phospholipase Cs leads to generation of second messenger molecules, diacylglycerol and inositol 1,4,5-trisphosphate, which in turn results in increased intracellular calcium levels and protein kinase C activation. One of the signaling molecules whose activation process depends on calcium is Pyk2. Pyk2 is a proline-rich nonreceptor tyrosine kinase whose function has been linked to cell motility. It was also reported that Pyk2 bridges the GPCR agonist, angiotensin II–mediated calcium-protein kinase C signaling with mitogen-activated protein kinases in VSMCs. Pyk2 has also been shown to be involved in VSMC growth in response to platelet-derived growth factor-BB. However, nothing is known with regards to the role of Pyk2 in thrombin-induced human aortic smooth muscle cell (HASMC) growth and migration and injury-induced vascular wall remodeling. Therefore, in the present investigation we sought to address the role of Pyk2 in thrombin-induced HASMC growth and migration. Our findings reveal that thrombin activates Pyk2 in HASMCs and mediates their growth and migration. In addition, activation of Pyk2 is essential for thrombin-induced Grb2-associated binder protein 1 (Gab1)-p115 Rho guanine nucleotide exchange factor (RhoGEF)-Rac1-RhoA-p21-activated kinase 1 (Pak1) stimulation leading to HASMC growth and migration. Furthermore, balloon injury (BI) caused activation of Pyk2, Gab1, p115 RhoGEF, Rac1, RhoA, and Pak1. Blockade of Pyk2 attenuated BI-induced Gab1, p115 RhoGEF, Rac1, RhoA, and Pak1 activation resulting in reduced SMC migration/proliferation and neointima formation.

Materials and Methods
The construction of adenoviral vectors, cell culture, cell growth and migration, double immunofluorescence staining, histological staining,
immunoprecipitation, pull-down assays, transfections and transductions, Western blotting, common carotid artery BI, and in vivo smooth muscle cell migration were previously described. \(^1\) All of the experiments involving animals were performed in accordance with the relevant guidelines and regulations approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center, Memphis, TN. For a detailed description of the methods, please refer to the online-only Data Supplement.

**Results**

To understand the role of GPCR signaling events in vascular wall remodeling, we have studied the role of Pyk2 in thrombin-induced HASMC growth and migration. Thrombin stimulated Pyk2 tyrosine phosphorylation in a time-dependent manner (Figure 1A). Interference with Pyk2 activation by adenovirus-mediated expression of its dominant negative mutant attenuated thrombin-induced HASMC growth and migration (Figure 1B and 1C). To explore the signaling events of how Pyk2 mediates HASMC growth and migration, we studied the role of Gab1, a scaffolding adapter protein. \(^2\) Thrombin induced Gab1 tyrosine phosphorylation in a time-dependent manner (Figure 2A). Inhibition of Gab1 by its dominant negative mutant blocked thrombin-induced HASMC growth and migration (Figure 2B and 2C). In addition, inhibition of Pyk2 activation attenuated thrombin-induced Gab1 tyrosine phosphorylation (Figure 2D). To find whether Pyk2-Gab1 signaling leads to activation of any RhoGEFs, we tested the effect of thrombin on the stimulation of p115 RhoGEF, PDZ RhoGEF and leukemia-associated RhoGEF, the RhoA-specific GEFs. \(^2\) Thrombin stimulated the tyrosine phosphorylation of p115 RhoGEF and PDZ RhoGEF but not leukemia-associated RhoGEF in HASMCs (Figure 3A). Because p115 RhoGEF activation was found to be robust, we next focused on the role of this RhoGEF in thrombin-induced HASMC growth and migration, siRNA-mediated downregulation of p115 RhoGEF substantially inhibited thrombin-induced HASMC growth and migration (Figure 3B−3D). Furthermore, adenovirus-mediated expression of either dominant negative Pyk2 or dominant negative Gab1 inhibited thrombin-induced p115 RhoGEF tyrosine phosphorylation (Figure 3E and 3F). Because Gab1 is an adaptor molecule, we asked the question whether it directly interacts with p115 RhoGEF. In response to thrombin, p115 RhoGEF was found to be associated with Gab1 in a time-dependent manner (Figure 3G). In addition, inhibition of Gab1 activation attenuated thrombin-induced association of p115 RhoGEF with Gab1 (Figure 3H). RhoGEFs mediate activation of Rho GTPases by facilitating the exchange of GDP for GTP. \(^2\) To identify the Rho GTPases activated by p115 RhoGEF, first we studied the time course effect of thrombin on Rac1 and RhoA activation. Thrombin stimulated Rac1 and RhoA in a time-dependent manner (Figure 4A). Interestingly, dominant negative mutant–mediated blockade of either Rac1 or RhoA inhibited thrombin-induced HASMC growth and migration (Figure 4B and 4C). Downregulation of p115 RhoGEF by its siRNA prevented thrombin-induced Rac1 and RhoA activation (Figure 4D). Similarly, inhibition of either Gab1 or Pyk2 by adenovirus-mediated expression of their respective dominant negative mutants also attenuated both Rac1 and RhoA activation (Figure 4E and 4F). Previously, we reported that RhoA mediates Rac1 activation downstream to leukemia-associated RhoGEF in response to thrombin in rat aortic smooth muscle cells. \(^1\) So, we asked whether there

**Figure 1.** Proline-rich nonreceptor tyrosine kinase 2 (Pyk2) mediates thrombin-induced human aortic smooth muscle cell (HASMC) growth and migration. **A,** An equal amount of protein from control and the indicated time periods of thrombin (0.5 U/mL) treated HASMCs was analyzed by Western blotting for phosphorylated Pyk2 (pPyk2) using its phosphospecific antibodies and the blot was normalized for the total Pyk2 levels. **B** and **C,** HASMCs that were transduced with adenovirus containing green fluorescent protein (Ad-GFP) or adenovirus containing dominant negative Pyk2 (Ad-dnPyk2) and quakecd were subjected to thrombin (0.5 U/mL) induced cell growth (B) or migration (C) assays. Cell growth was measured by [\(^3\)H]-thymidine incorporation, and MTT assay and cell migration was measured by modified Boyden chamber method. The bar graphs represent mean±SD of 3 independent experiments. *P<0.01 vs control or Ad-GFP; **P<0.01 vs thrombin or Ad-GFP+thrombin.
is any interaction between Rac1 and RhoA in HASMCs in response to thrombin. Inhibition of Rac1 blocked thrombin-induced RhoA activation (Figure 4G). However, blockade of RhoA stimulation had no effect on Rac1 activation (Figure 4H). Because both the Rho GTPases target Pak1, we next wanted to find whether thrombin activates Pak1 in HASMCs. Thrombin induced Pak1 activation in a time-dependent manner (Figure 5A). Furthermore, downregulation of Pak1 levels by its siRNA inhibited thrombin-induced HASMC growth and migration (Figure 5B−5D). In addition, adenovirus-mediated expression of either dominant negative Rac1 or dominant negative RhoA attenuated thrombin-induced Pak1 activation (Figure 5E and 5F). Downregulation of p115 RhoGEF levels by its siRNA or adenovirus-mediated expression of either dominant negative Gab1 or dominant negative Pyk2 also blocked thrombin-induced Pak1 activation (Figure 5G−5I). The observed effects with the use of adenoviral vectors for the delivery of dominant negative mutants of the indicated molecules into cells were not because of cell death as transduction with Ad-GFP had little (<5%) effect on HASMC viability (Figure I in the online-only Data Supplement).

To confirm the role of Pyk2 in thrombin-induced phosphorylation and activation of Gab1, p115 RhoGEF, Rac1, RhoA, and Pak1, we used the siRNA approach. Pyk2 depletion by its siRNA substantially inhibited thrombin-induced Gab1 and p115 RhoGEF tyrosine phosphorylation and Rac1, RhoA, and Pak1 activation (Figure II in the online-only Data Supplement). Consistent with these observations, siRNA-mediated downregulation of Pyk2 also attenuated thrombin-induced HASMC growth and migration (Figure III in the online-only Data Supplement). Furthermore, the blockade of Pyk2 activation by its dominant negative mutant had no effect on thrombin-induced protein kinase Cδ phosphorylation, suggesting that the role of Pyk2 on Gab1, p115 RhoGEF, Rac1, RhoA, and Pak1 stimulation by thrombin is selective (Figure IV in the online-only Data Supplement).

To validate these signaling events in vivo, we used BI of rat carotid artery model. BI induced tyrosine phosphorylation of Pyk2, Gab1, and p115 RhoGEF, as well as caused Rac1, RhoA, and Pak1 activation (Figure 6A−6C). In addition, adenovirus-mediated transduction of dominant negative Pyk2 blocked BI-induced Pyk2, Gab1, and p115 RhoGEF phosphorylation, as well as Rac1, RhoA, and Pak1 activation (Figure 6A−6C). To understand the role of Pyk2 in neointima formation, its activation was blocked by its dominant negative mutant and tested its effects on BI-induced SMC migration and proliferation. Adenovirus-mediated expression of dominant negative Pyk2 substantially inhibited BI-induced SMC recruitment to luminal surface and their proliferation in intimal region leading to the suppression of neointima formation (Figure 6D−6F).
Figure 3. Proline-rich nonreceptor tyrosine kinase 2 (Pyk2) and Grb2-associated binder protein 1 (Gab1) mediate thrombin activation of rho guanine nucleotide exchange factor (RhoGEF). A, An equal amount of protein from control and each time period of thrombin (0.5 U/mL) treated human aortic smooth muscle cells (HASMCs) was immunoprecipitated with anti-PY20 or anti-p115 RhoGEF antibodies and the immunocomplexes were analyzed by Western blotting using p115 RhoGEF, PDZ RhoGEF, leukemia-associated RhoGEF (LARG), or PY20 antibodies. The blot on the right side was normalized for p115 RhoGEF levels. B, An equal amount of protein from cell extracts of HASMCs that were transfected with control or p115 RhoGEF small interfering RNA (siRNA) was analyzed by Western blotting for p115 RhoGEF and cyclin-dependent kinase 4 (CDK4) levels using their specific antibodies to show the effect of the indicated siRNA on its target and off-target molecules. C and D, HASMCs that were transfected with control or p115 RhoGEF siRNA and quiesced were subjected to thrombin (0.5 U/mL) induced cell growth (B) or migration (C) assays. E and F, An equal amount of protein from HASMCs that were transduced with adenovirus containing green fluorescent protein (Ad-GFP), adenovirus containing dominant negative Pyk2 (Ad-dnPyk2), or adenovirus containing dominant negative Gab1 (Ad-dnGab1), quiesced, and treated with and without thrombin (0.5 U/mL) for 30 minutes was immunoprecipitated with anti-p115 RhoGEF or anti-PY20 antibodies and the immunocomplexes were analyzed by Western blotting using anti-PY20 or anti-p115 RhoGEF antibodies. The blot in E was reprobed sequentially with anti-p115 RhoGEF and anti-Pyk2 antibodies for normalization of p115 RhoGEF levels and to show the overexpression of dnPyk2, respectively. The blot in F was reprobed with anti-Gab1 antibodies to show the overexpression of dnGab1.
RhoA, and Pak1 requires Pyk2 stimulation. Furthermore, the present observations clearly show that activation of Pyk2 is required for thrombin-induced HASMC growth and migration. Because both GPCR and receptor tyrosine kinase agonists–induced VSMC growth and migration appear to require Pyk2 activation, it might have a role in VSMC differentiation in response to cues that provoke vessel wall perturbation leading to vascular wall remodeling. Studies from our laboratory, as well as others, have previously reported that Gab1 plays a role in the regulation of VSMC growth/migration and cardiac hypertrophy. However, there appears to be no information on how Gab1 mediates VSMC migration and proliferation. In this regard, our studies show that Gab1 physically interacts with RhoGEFs, particularly p115 RhoGEF, and facilitates its Pyk2-mediated tyrosine phosphorylation in response to thrombin. A large body of literature suggests that p115 RhoGEF exhibits specificity for RhoA activation. However, our findings indicate that p115 RhoGEF mediates the activation of Rac1, which in turn, leads to RhoA stimulation in response to thrombin. It is interesting to note that in contrast to the antagonism reported between RhoA and Rac1 in the regulation of cell migration, the present observations reveal an interaction between these 2 Rho GTPases in thrombin-induced HASMC growth and migration. Although a similar interaction was observed between these 2 Rho GTPases in the modulation of rat aortic smooth muscle cell migration in response to thrombin, in these cells leukemia-associated RhoGEF–dependent RhoA stimulation is needed for Rac1 activation. Thus, these findings point out clear species differences between human and rat VSMCs in the interaction of Rac1 and RhoA and the mode of their activation by their upstream RhoGEFs in response to thrombin. The Rho GTPase effector Pak1 plays a vital role in cell migration. However, in the present study, we show that Pak1 in addition to its role in cell migration also plays a role in cell proliferation. Recently, it was demonstrated that Gab1 interacts with Pak4 in the regulation of Met receptor–mediated cell migration. It was also reported that Gab1 facilitates Ras-dependent mitogen-activated protein kinases activation in epidermal growth factor–induced epidermal cell proliferation. Based on all of these observations, as well as our previous and present findings, it may be suggested that Gab1 associates with many signaling molecules, including Rho and Ras-specific RhoGEFs, and mediates the activation of their respective downstream signaling events, such as MAPKs, in enhancing the agonist-induced cell migration and proliferation. In this context, we would like to point out that previously we reported a correlation between c-Jun N-terminal kinase 1 activation and VSMC growth in response to thrombin. In addition, many reports showed that small GTPases, Ras, Rac1, Cdc42, and RhoA mediate the activation of c-Jun N-terminal kinase 1 in response to various stimulants. In view of these observations, we may speculate that Pyk2-dependent Gab1-mediated p115 RhoGEF-Rac1/RhoA-Pak1 signaling activation may target c-Jun N-terminal kinase 1 stimulation in facilitating thrombin-induced HASMC growth and migration.

Figure 3. (Continued) G. An equal amount of protein from control and each time period of thrombin (0.5 U/mL) treated HASMCs was immunoprecipitated with anti-Gab1 or anti-p115 RhoGEF antibodies and the immunocomplexes were analyzed by Western blotting using anti-p115 RhoGEF or anti-Gab1 antibodies. The blots were normalized for Gab1 or p115 RhoGEF levels. H. All the conditions were the same as in F except that immunoprecipitation was performed with anti-Gab1 antibodies and the immunocomplexes were analyzed by Western blotting using anti-p115 RhoGEF antibodies. The blot was reprobed with anti-Gab1 antibodies to show the overexpression of dnGab1. The bar graphs represent mean±SD of 3 independent experiments. *P<0.01 vs control or Ad-GFP; **P<0.01 vs thrombin or Ad-GFP+thrombin. pp115 indicates phosphorylated p115; pPDZ, phosphorylated PDZ; pLARG, phosphorylated LARG.
Figure 4. Proline-rich nonreceptor tyrosine kinase 2 (Pyk2) and Grb2-associated binder protein 1 (Gab1) via p115 Rho guanine nucleotide exchange factor (RhoGEF) mediate thrombin-induced Rac1 and RhoA activation. A, An equal amount of protein from control and each time period of thrombin (0.5 U/mL) treated human aortic smooth muscle cell (HASMCs) was subjected to pull-down assay using GST-Pak or GST-Rhotekin conjugated Sepharose CL4B beads and the resultant GST-Pak and GST-Rhotekin–bound proteins were analyzed by Western blotting for total Rac1 and RhoA levels using their specific antibodies. B and C. After transduction with adenovirus containing green fluorescent protein (Ad-GFP), adenovirus containing dominant negative Rac1 (Ad-dnRac1), or adenovirus containing dominant negative RhoA (Ad-dnRhoA), and quiescence, HASMCs were subjected to thrombin (0.5 U/mL) induced cell growth (B) or migration (C) assays. D, An equal amount of protein from HASMCs that were transduced with control or p115 RhoGEF small interfering RNA (siRNA) and treated with and without thrombin (0.5 U/mL) for 30 minutes were analyzed for Rac1 and RhoA activation as described in A. An equal amount of protein from the same samples was analyzed by Western blotting for total Rac1 and RhoA levels using their specific antibodies. E and F, After transduction with adenovirus containing dominant negative Gab1 (Ad-dnGab1), or adenovirus containing dominant negative Pyk2 (Ad-dnPyk2), and quiescence, HASMCs were subjected to thrombin (0.5 U/mL) induced cell growth (E) or migration (F) assays. G and H, An equal amount of protein from HASMCs that were transduced with Ad-GFP, Ad-dnGab1, Ad-dnPyk2, Ad-dnRac1, or Ad-dnRhoA and treated with and without thrombin (0.5 U/mL) for 30 minutes were analyzed for Rac1 and RhoA activation as described in A. An equal amount of protein from the same samples was analyzed by Western blotting for Rac1, RhoA, Gab1, Pyk2, GFP, and β-tubulin levels to show the overexpression of their respective dominant negative mutants, vector control, or normalization. The bar graphs represent mean±SD of 3 independent experiments. *P<0.01 vs control or Ad-GFP; **P<0.01 vs thrombin or Ad-GFP+thrombin.
Figure 5. Proline-rich nonreceptor tyrosine kinase 2 (Pyk2), Grb2-associated binder protein 1 (Gab1), p115 rho guanine nucleotide exchange factor (RhoGEF), Rac1, and RhoA mediate thrombin-induced Pak1 activation. A, An equal amount of protein from control and each time period of thrombin (0.5 U/mL) treated human aortic smooth muscle cell (HASMCs) was analyzed by Western blotting for pPak1 levels using its specific antibodies and the blot was normalized for total Pak1 levels. B, An equal amount of protein from cell extracts of HASMCs that were transfected with control or Pak1 small interfering RNA (siRNA) was analyzed by Western blotting for Pak1 and β-tubulin levels using their specific antibodies to show the effect of the indicated siRNA on its target and off-target molecules. C and D, HASMCs that were transfected with control or Pak1 siRNA and quiesced were subjected to thrombin (0.5 U/mL) induced cell growth (C) or migration (D) assays. E–I, An equal amount of protein from HASMCs that were transduced with adenovirus containing green fluorescent protein (Ad-GFP), adenovirus containing dominant negative Rac (Ad-dnRac1), adenovirus containing dominant negative RhoA (Ad-dnRhoA), adenovirus containing dominant negative Gab1 (Ad-dnGab1), adenovirus containing dominant negative Pyk2 (Ad-dnPyk2), or transfected with control or p115 RhoGEF siRNA and treated with and without thrombin (0.5 U/mL) for 30 minutes were analyzed by Western blotting for pPak1 levels using its specific antibodies. The blots were probed with anti-Rac1, anti-RhoA, anti-p115 RhoGEF, anti-Gab1, anti-Pyk2, anti-GFP, anti-β-tubulin, or anti-cyclin-dependent kinase 4 (CDK4) antibodies to show the overexpression of their respective dominant negative mutants, the siRNA effect on its target molecule level, control vector or for normalization. The bar graphs represent mean±SD of 3 independent experiments. *P<0.01 vs control or Ad-GFP; **P<0.01 vs thrombin or Ad-GFP+thrombin. pPak1 indicates phosphorylated Pak1.
Figure 6. Blockade of proline-rich nonreceptor tyrosine kinase 2 (Pyk2) activation suppresses balloon injury (BI)-induced neointima formation. A–C, Common carotid arteries were dissected out at 12 hours after BI and transduction with adenovirus containing green fluorescent (Ad-GFP) or adenovirus containing dominant negative Pyk2 (Ad-dnPyk2) and tissue extracts were prepared. Tissue extracts containing an equal amount of protein were analyzed by Western blotting for pPyk2 levels using its specific antibodies (A), immunoprecipitated with anti-PY20 antibodies and the immunocomplexes were immunoblotted for Grb2-associated binder protein 1 (Gab1) or p115 rho guanine nucleotide exchange factor levels using their specific antibodies (B) or subjected to pull-down assay for Rac1 and RhoA activation (C). For testing Pak1 activation, tissue extracts were analyzed by Western blotting for pPak1 levels using its phosphospecific antibodies and blot was normalized for total Pak1 and β-tubulin levels (C). The blot in A was reprobed sequentially with anti-Pyk2 and anti-β-tubulin antibodies to show the overexpression of dnPyk2 and normalization, respectively. The bar graphs in A–C represent the mean±SD values of 3 experiments each involving 2- to 4-pooled arteries.

D, Three days after BI and transduction with Ad-GFP or Ad-dnPyk2, injured common carotid arteries were dissected out, fixed, opened longitudinally, and stained with anti–smooth muscle cell α (SMCα)-actin antibodies. The SMCα-actin–positive cells were counted and SMC migration was expressed as the number of SMCα-actin–positive cells migrated onto a unit luminal surface area.

E, At 1 week post-BI and transduction with Ad-GFP or Ad-dnPyk2, injured common carotid arteries were dissected out, fixed, cryosections made, and stained with SMCα-actin and Ki67 using their specific antibodies. The SMCα-actin– and Ki67–positive cells were counted to measure neointimal SMC proliferation.

F, At 2 weeks post-BI and transduction with Ad-GFP or Ad-dnPyk2, injured common carotid arteries were isolated, fixed, cross sections made, stained with hematoxylin–eosin and the I/M ratios were calculated to measure neointima formation. The bar graphs in D–F represent the quantitative analysis of 6 animals. *P<0.05 vs uninjured; **P<0.05 vs Ad-GFP+BI.
well as their proliferation in the intimal region, resulting in decreased neointima formation. Based on these findings, we can conclude that Pyk2 plays an important role in the activation of RhoGEFs, such as p115 RhoGEF, which in turn mediates Rac1 and RhoA-dependent Pak1 activation. The connection between Pyk2 and p115 RhoGEF appears to be Gab1. The previous work from our as well as other laboratories reported that local expression or intravenous delivery of recombinant hirudin inhibits neointima formation.\(^{16,40}\) In addition, we have shown that recombinant hirudin attenuates BI-induced activation of RhoA, Rac1, and Pak1.\(^{18}\) Because thrombin activates Pyk2-Gab1-p115 RhoGEF-Rac1-RhoA-Pak1 in HASMCS and blockade of Pyk2 negates BI-induced Gab1, p115 RhoGEF, Rac1, RhoA, and Pak1, it is likely that thrombin-mediated GPCR signaling to Pyk2 activation plays a role in vascular wall remodeling. Whatever the cues that might be produced at the site of vascular injury, the present findings nonetheless suggest a prominent role for Pyk2 in vascular wall remodeling after injury.

**Acknowledgments**

This work was supported by National Institutes of Health grants HL103575 and HL069908 to G.N.R.

**Disclosures**

None.

**References**


Novel Role of Proline-Rich Nonreceptor Tyrosine Kinase 2 in Vascular Wall Remodeling After Balloon Injury

Ravisekhar Gadepalli, Nikhlesh K. Singh, Venkatesh Kundumani-Sridharan, Mark R. Heckle and Gadiparthi N. Rao

Arterioscler Thromb Vasc Biol. 2012;32:2652-2661; originally published online August 23, 2012;
doi: 10.1161/ATVBAHA.112.253112

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/32/11/2652

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2012/08/23/ATVBAHA.112.253112.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
SUPPLEMENT MATERIAL

METHODS

Reagents: Anti-SMCα-actin antibody, aprotinin, dithiothreitol, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate and sodium deoxycholate were obtained from Sigma Chemical Company (St. Louis, MO). Anti-Ki67 (ab15580), anti-pPak1 (Thr423, ab2477) and anti-Pyk2 (ab32571) antibodies were bought from Abcam Inc. (Cambridge, MA). Anti-Pak1 (2602S), anti-pPKCδ (Thr505, 9374S) and anti-pPyk2 (Tyr402, 3291S) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-CDK4 (SC-260), anti-Gab1 (SC-9049), anti-GFP (SC-9996), anti-LARG (SC-25638), anti-PKCδ (SC-937), anti-p115 RhoGEF (SC-20804), anti-PDZ-RhoGEF (SC-67024), anti-RhoA (SC-418) and anti-β-tubulin (SC-9104) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphotyrosine (Cat. No. 05-777) and anti-Rac1 (Cat. No. 05-389) antibodies were bought from Millipore Corporation (Temecula, CA). Growth factor-reduced Matrigel (Cat. No. 354250) was purchased from BD Biosciences (Bedford, MA). Lipofectamine 2000 transfection reagent (Cat. No. 11668-019) was obtained from Invitrogen Technologies (Carlsbad, CA). MTT cell proliferation assay kit (Cat. No. 30-1010K) was bought from ATCC (Manassas, VA). [3H]-Thymidine (s. a. 20 Ci/mmol) was procured from Perkin-Elmer (Waltham, MA). Human ARHGEF1 siRNA (Cat. No. ON-TARGETplus SMARTpool J-009421-05, NM_004706), human Pak1 siRNA (Cat. No. ON-TARGETplus SMARTpool J-003521-09,
NM_002576), human PTK2B siRNA (Cat. No. ON-TARGETplus SMARTpool L-003165-00-0010) and ON-TARGETplus SMARTpool nontargeting siRNA (Cat. No. D-001810-10-20) were bought from Dharmacon RNAi Technologies (Chicago, IL). The ECL Western blotting detection reagents (Cat. No. RPN2106V1) were obtained from GE Healthcare (Piscataway, NJ).

**Construction of adenoviral vectors:** Construction of pAd-dnGab1, pAd-dnPak1, pAd-dnPyk2, pAd-dnRac1, pAd-dnRhoA and pAd-GFP were described previously (1-4). The plasmids were linearized by digestion with PacI and transfected into HEK293A cells. The adenovirus was purified by cesium chloride gradient ultracentrifugation (5).

**Cell culture:** Human aortic smooth muscle cells were obtained from Cascade Biologics (Portland, OR) and subcultured in Medium 231 containing smooth muscle cell growth supplements and used between 6 and 12 passages.

**Transfections and transductions:** HASMCs were transfected with siRNA molecules at a final concentration of 100 nM using Lipofectamine 2000 transfection reagent according to manufacturer's instructions. When adenoviral vectors were used to block the function of a specific molecule, cells were transduced with adenovirus harboring either GFP or dominant negative mutant of the target molecule at 40 moi overnight in complete medium. After transfections or transductions, cells were growth-arrested for 48 hrs and used as required.

**Cell migration:** Cell migration was measured using a modified Boyden chamber method (1). Wherever adenovirus was used, cells were first transduced with the respective adenovirus at 40 moi and growth-arrested before they were subjected
to agonist-induced migration. Cell motility was presented as the number of migrated cells/field.

**DNA synthesis:** HASMC DNA synthesis was measured by $[^3]H$-thymidine incorporation as described previously (4).

**MTT assay:** HASMC growth was measured by MTT cell proliferation assay according to supplier’s instructions (ATCC, Manassas, VA).

**Immunoprecipitation:** After rinsing with cold phosphate-buffered saline (PBS), cells were lysed by freeze thawing in 250 µl of lysis buffer (PBS, 1% Nonidet P40, 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 the 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 additional 2 hrs with gentle rocking. The beads were collected by centrifugation at 4000 rpm per 1 min at 4°C and washed four times with lysis buffer and once with PBS. The immunocomplexes were released by heating the beads in 40 µl of Laemmeli sample buffer and analyzed by Western blotting for the indicated molecules using their specific antibodies.

**Pull-down assay:** An equal amount of protein from control and each treatment was incubated with GST-Pak1 or GST-Rhotekin-conjugated Sepharose 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 or anti-RhoA antibodies.

**Common carotid artery balloon injury:** All the experiments involving animals were performed in accordance with the relevant guidelines and regulations approved by the Institutional Animal Care & Use Committee of the University of Tennessee Health Science Center, Memphis, TN. Balloon injury was performed essentially as described previously (1). Adenovirus (10^{10} pfu/ml) in 150 µl of PBS was infused into the ligated segment of the common carotid artery for 30 min. At different time periods after balloon injury, the animals were euthanized with CO₂ inhalation followed by thoracotomy and the injured and uninjured carotid arteries were dissected and processed for either protein extraction or immunohistological staining.

**Double immunofluorescence staining:** The injured common carotid arteries were embedded in OCT compound (Tissue Tek, Sakura Finetek USA Inc., Torrance, CA) and sections (5 µm thick) were made at equally spaced intervals using Leica Kryostat (Model CM3050S, Leica, Wetzlar, Germany) in the middle of injured artery segments. After blocking in normal goat serum, the cryosections (5 µm) were incubated first with rabbit anti-Ki67 antibodies and mouse SMCα-actin antibodies followed by Alexa Flour 568-conjugated goat anti-rabbit and Alexa Flour 488-conjugated goat anti-mouse secondary antibodies. After washing with PBS and staining with Hoechst 33342, the sections were observed
under Zeiss Axio Observer Z1 Motarized inverted microscope (Model: AxioVision AX10).

**Histological staining:** For morphometric analysis, the injured common carotid arteries were fixed in formalin, dehydrated and embedded in OCT compound. The sections were made as described above 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.

**In vivo smooth muscle cell migration:** In vivo smooth muscle cell migration was determined according to the method of Bendeck et al (6) as described previously (1).

**Western blotting:** An equal amount of protein from control and treatment samples was separated by SDS-PAGE and immunoblotted for the indicated molecule using its specific antibodies. The antigen-antibody complexes were detected by enhanced chemiluminescence Western blotting detection reagents as described previously (4).

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

**REFERENCES**


**ONLINE FIGURE LEGENDS**

**Online Figure I**: Transduction with adenoviral vector(s) had little effect on HASMC viability. HASMCs were transduced with and without Ad-GFP at 40 moi for overnight in Medium 231 with smooth muscle growth supplements. After transduction, cells were allowed to recover in complete Medium 231 for 12 hrs, at which time they were quiesced for 24 hrs in smooth muscle growth supplement-
free Medium 231 and the cell viability was assessed by Trypan Blue Exclusion assay. The values are mean ± SD of three experiments.

**Online Figure II.** Depletion of Pyk2 levels block thrombin-induced Gab1 and p115 RhoGEF tyrosine phosphorylation as well as Rac1, RhoA and Pak1 activation. A & B. HASMCs that were transfected with control or Pyk2 siRNA and quiesced were treated with and without thrombin (0.5 U/ml) for 30 min and cell extracts were prepared. Cell extracts containing an equal amount of protein from control and each treatment were immunoprecipitated with anti-Gab1 or anti-p115 RhoGEF antibodies and the immunocomplexes were analyzed by Western blotting using PY20 antibodies. The blots were reprobed for Gab1 or p115 RhoGEF levels for normalization. An equal amount of protein from the same samples was also analyzed for Pyk2 and β-tubulin levels to show the effect of Pyk2 siRNA on the levels of its target and off-target molecules. C. All the conditions were the same as in panels A/B except that an equal amount of protein from control and each treatment was subjected to pull-down assay using GST-Pak or GST-Rhotekin conjugated Sepharose CL4B beads and the resultant GST-Pak and GST-Rhotekin-bound proteins were analyzed by Western blotting for Rac1 or RhoA levels using their specific antibodies. An equal amount of protein from the same samples was also analyzed by Western blotting for Rac1, RhoA, Pyk2 and β-tubulin levels using their specific antibodies to show the effect of Pyk2 siRNA on the levels of its target and off-target molecules. D. All the conditions were the same as in panels A/B except that an equal amount of protein from control and each treatment was analyzed by Western blotting for
pPak1 levels using its phosphospecific antibodies. The blot was reprobed sequentially with anti-Pak1, anti-Pyk2 or anti-β-tubulin antibodies for normalization or to show the effect of Pyk2 siRNA on the levels of its target and off-target molecules.

**Online Figure III.** Depletion of Pyk2 levels block thrombin-induced HASMC growth and migration. A & B. HASMCs that were transfected with control or Pyk2 siRNA and quiesced were subjected to thrombin (0.5 U/ml)-induced cell growth (A) or migration (B) assays. * p < 0.01 vs control siRNA; ** p < 0.01 vs control siRNA + thrombin.

**Online Figure IV.** Blockade of Pyk2 activation does not affect thrombin-induced phosphorylation of PKCδ. HASMCs that were transduced with Ad-GFP or Ad-dnPyk2 and quiesced were treated with and without thrombin (0.5 U/ml) for 30 min and cell extracts were prepared. Cell extracts containing an equal amount of protein from control and each treatment were analyzed by Western blotting for pPKCδ using its specific antibodies. The blot was reprobed sequentially with anti-PKCδ, anti-GFP and anti-Pyk2 antibodies for normalization and to show the over expression of control vector or dnPyk2.
Online Figure III

A

**[H]-Thymidine incorporation (10^3 cpm/dish)**

- Control siRNA + + - - -
- Pyk2 siRNA - - + + +

**MTT assay (absorbance at 570 nm)**

- Control siRNA + + - - -
- Pyk2 siRNA - - + + +

B

**Migration (cells/field)**

- Control siRNA + + - - -
- Pyk2 siRNA - - + + +

Online Figure IV

<table>
<thead>
<tr>
<th>Ad-GFP</th>
<th>Ad-dnPyk2</th>
<th>Thrombin</th>
<th>PKCδ</th>
<th>pPKCδ</th>
<th>GFP</th>
<th>Pyk2</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>