Rho and Rho Kinase Mediate Thrombin-Stimulated Vascular Smooth Muscle Cell DNA Synthesis and Migration

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Abstract—Aberrant regulation of smooth muscle cell proliferation and migration is associated with the pathophysiology of vascular disorders such as hypertension, atherosclerosis, restenosis, and graft rejection. To elucidate molecular mechanisms that regulate proliferation and migration of vascular smooth muscle cells, we determined whether signaling through the small G protein Rho is involved in thrombin- and phenylephrine-stimulated proliferation and migration of rat aortic smooth muscle cells (RASMCs). Thrombin and the thrombin peptide SFLLRNP stimulated DNA synthesis of RASMCs as measured by [3H]thymidine incorporation. Both ligands also increased cell migration as measured by the Boyden chamber method. Phenylephrine failed to induce either of these responses but increased inositol phosphate accumulation and mitogen-activated protein kinase activation in these cells, which indicated that the cells were responsive to α1-adrenergic stimulation. The C3 exoenzyme, which ADP-ribosylates and inactivates Rho, fully inhibited both thrombin-stimulated proliferation and migration but had no effect on inositol phosphate accumulation. In addition, Y-27632, an inhibitor of the Rho effector p160ROCK/Rho kinase, decreased thrombin-stimulated DNA synthesis and migration. To directly examine Rho activation, Rho-[^35S]GTPγS binding was measured. The addition of the thrombin peptide SFLLRNP, but not phenylephrine, to RASMC lysates resulted in a significant increase in Rho[^35S]GTPγS binding. Thrombin and SFLLRNP, but not phenylephrine, also increased membrane-associated Rho in intact RASMCs, consistent with selective activation of Rho by thrombin. These results indicate that thrombin activates Rho in RASMCs and establish Rho as a critical mediator of thrombin receptor effects on DNA synthesis and cell migration in these cells. (Circ Res. 1999;84:1186-1193.)

Key Words: Rho ■ thrombin ■ vascular smooth muscle ■ DNA synthesis ■ cell migration

The low-molecular-weight G protein Rho is a member of the Rho family of small GTPases that also includes Rac and Cdc42. These GTPases act as molecular switches to regulate cellular functions, the best characterized of which are changes in the actin cytoskeleton. An increasing body of evidence has revealed that some heterotrimeric G protein-coupled receptors (GPCRs) signal through small G proteins such as Rho. GPCR agonists such as lysophosphatidic acid (LPA), carbachol, and phenylephrine have been shown to increase levels of membrane-associated Rho or decrease cytosolic Rho, which is indicative of Rho activation. Studies from our laboratory have demonstrated a role for Rho in α1-adrenergic receptor (α1,AdrR)–induced hypertrophy and gene expression in cardiac myocytes in vivo and in thrombin-induced mitogenesis and actin cytoskeletal rearrangement in astrocytoma cells. In addition, Rho has been implicated in GPCR-induced cytoskeletal responses, and Rho kinase, blocks contractions in response to phenylephrine (PE) in intact rabbit aorta and GTPγS in permeabilized rabbit aorta. Although results from the above-mentioned studies provide evidence for Rho and Rho kinase involvement in the phenomenon by which VSM contractility can be increased by agonists independent of increases in intracellular Ca2+.

The observation that increases in contractility are elicited by GTP and GTP analogs in permeabilized preparations suggested the involvement of G proteins in this response. The C3 exoenzyme from Clostridium botulinum, which specifically ADP-ribosylates and inactivates Rho, inhibits α1,AdrR and guanine nucleotide–dependent contraction of permeabilized blood vessels. In addition, C3 blocks the associated increase in myosin light chain phosphorylation in permeabilized smooth muscle cells. The signaling cascade elicited by Rho that results in Ca2+-sensitization involves the Rho-dependent kinases p160ROCK and Rho kinase. Rho kinase has been shown to phosphorylate the myosin-binding subunit of myosin light chain phosphatase, thus inhibiting its function and allowing for increases in levels of phosphorylated myosin. Studies performed in permeabilized vessels reveal that the addition of the catalytic subunit of Rho kinase elicits contraction. In addition, Y-27632, an inhibitor of p160ROCK and Rho kinase, blocks contractions in response to phenylephrine (PE) in intact rabbit aorta and GTPγS in permeabilized rabbit aorta.
vascular contractility, the role of Rho and Rho kinase in other vascular responses such as mitogenesis and cell migration has not been investigated. Understanding the mechanisms of vascular smooth muscle cell (VSMC) proliferation and migration is critical to evaluation of the involvement of these processes in the pathophysiology of vascular disorders such as hypertension, atherosclerosis, restenosis, and graft rejection. The possibility that Rho is involved in vascular proliferation and migration is suggested by the involvement of Rho in the growth of nonvascular cells in response to heterotrimeric G protein receptor stimulation and in the migration of endothelial cells in response to mechanical strain or tyrosine kinase growth factors.

Altered responsiveness to thrombin and catecholamines has been implicated in the pathophysiology of vascular diseases. These agonists act on heterotrimeric G protein–linked receptors to regulate vascular contraction and have been reported to affect vascular growth responses. Thrombin also appears to regulate vascular cell migration. The studies presented here used rat aortic smooth muscle cells (RASMCs) to determine whether thrombin or PE signals through the small G protein Rho and through Rho kinase to regulate vascular mitogenesis and cell migration.

Materials and Methods

Materials

The Rho kinase inhibitor Y-27632 was a gift from Yoshitomi Pharmaceutical Industries, Ltd (Iruma-shi, Saitama, Japan). The monoclonal RhoA antibody was obtained from Santa Cruz Biotechnology.

C3 Exoenzyme and Rho ADP Ribosylation

The method used for preparation of the glutathione S-transferase (GST) fusion protein has been previously described. Briefly, cDNA for the GST-C3 fusion protein (a gift from Dr J. Meinmth, University of Pennsylvania) was used to transform Escherichia coli strain JM 109 to produce the protein for purification. After 3 hours, the cells were lysed, and extracts were clarified and incubated with GSH-Sepharose. The C3 toxin protein was cleaved from GST by overnight incubation with thrombin. Thrombin was removed by incubation with p-aminobenzamidine-Sepharose. The supernatant was concentrated to 5 mg/mL protein with a Centricon-10 (Amicon Inc., Beverly, Mass.). Cells were suspended in 40 μg/mL C3 toxin for 12 to 16 hours. To measure the extent of in vivo ADP ribosylation of Rho after 12 to 16 hours of extracellular treatment with 40 μg/mL C3, cells were rinsed in PBS and lysed in 0.1 mol/L phosphate buffer, pH 7.9, that contained 1% Triton X-100. Cells were scraped, and the lysate was centrifuged at 4°C for 5 minutes at 12 000g. The supernatant was collected, and in vitro ribosylation was performed at 30°C for 30 minutes in a reaction buffer that contained 100 mM Tris-HCl, 5 mM MgCl₂, 30 μM GSH, 0.2 mM GDP, 5 μM L-DTT, 5 μM GDP, 15 μCi/mL [¹³⁵S]GTP, and 10 μg/mL C3. The reaction was stopped with 4× Laemmli buffer and the samples boiled. Proteins were separated by SDS-PAGE, and the radioactivity was quantified on an AMBIS radioanalytic scanner.

Cell Culture Conditions

RASMCs were obtained from Dr Wolfgang Dillmann’s laboratory, University of California, San Diego. These cells were isolated from thoracic aortas of 8- to 12-week-old male Sprague-Dawley rats as described.59 Cells were grown in high glucose DMEM supplemented with 10% FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL) and kept in a 37°C humidified incubator at 5% CO₂.

Rho-¹³⁵[S]GTPγS Binding

The Rho-¹³⁵[S]GTPγS binding assay was a modification of that developed in the Manning laboratory for measuring [¹³⁵S]GTPγS binding to α-subunits of heterotrimeric G proteins. RASMCs (10-cm plates) were grown to confluency in 20% FCS. Cells were washed with ice-cold PBS followed by incomplete lysis buffer that contained 50 mM Tris-HCl, 10 mM MgCl₂, 2 mM EDTA, and 100 mM NaCl. Cells were lysed and scraped in complete lysis buffer that in addition contained 0.3 μM GDP, 20 μM aprotinin, 10 μg/mL leupeptin, and 0.1 mM PMSF. The lysate was passed through a 27-gauge needle 15 times and spun at 500g for 5 minutes at 4°C to remove unbroken cells. The supernatant was collected and diluted to 20 μg of protein per 50 μL. The reaction was initiated by the addition of the cell lysate (20 μg of protein) to [¹³⁵S]GTPγS (final concentration, 300 mM/L) in the presence or absence of agonist. The reaction was terminated by the addition of 600 μL of ice-cold immunoprecipitation (IP) buffer that contained 50 mM Tris, 20 mM MgCl₂, 150 mM NaCl, 0.5% NP-40, 20 μg/mL aprotinin, 100 μg/mL GDP, and 100 μg/mL GTP. Samples were shaken at 4°C for 30 minutes before IP overnight with mononclonal RhoA antibody and protein A Sepharose. The IPs were washed 3 times with IP buffer and 1 final time with IP buffer that lacked NP-40. Samples were then boiled in 500 μL of 0.5% SDS for 1 minute before addition of scintillant and quantitation with a liquid scintillation counter.

Measurement of Rho Distribution

Plates (10 cm) of 75% confluent RASMCs were washed twice with HEPES-buffered DMEM that contained 0.1% BSA and changed to media with or without agonist for 3 minutes at 37°C. The reaction was stopped by washing the plates with ice-cold PBS followed by the addition of incomplete lysis buffer that contained 10 mM/L HEPES, 2 mM/L EDTA, and 1 mM/L MgCl₂. Plates were then placed on ice, and the cells were treated with 800 μL of complete lysis buffer (complete lysis buffer also contained 10 mM/L Na₃PO₄, 500 μM/L Na₂VO₄, 10 μg/mL leupeptin, 10 μg/mL antipain, 1 mM/L DTT, and 1 mM/L PMSF) for 15 minutes. Cells were then scraped, triturated, transferred to microfuge tubes, and centrifuged at 4°C for 5 minutes at 500g. The low-speed pellet was discarded, and the supernatant was centrifuged at 4°C for 30 minutes at 37,000g to generate membrane and cytosolic fractions. The membrane pellet was resuspended in lysis buffer and fractions were boiled in Laemmli buffer. Proteins were separated with the use of SDS-PAGE, transferred to Immobilon membranes, and blocked with 3% BSA at room temperature for 1 hour. Blots were then incubated in mouse anti-Rho A (1:1000) followed by horseradish peroxidase–labeled goat anti-mouse (1:2000) and subsequent enhanced chemiluminescence. Blots were exposed to film that was analyzed by densitometry.

³[H]Thymidine Incorporation

Cells were set on 12-well plates (22 mm) and allowed to grow in 20% FCS for 8 days under control conditions. Cells were serum-starved for 24 hours before the addition of agonists for 48 hours. [³H]Thymidine (1 μCi/mL) was added for the last 24 hours of agonist stimulation. Cells were washed twice with ice-cold PBS and fixed in methanol for 10 minutes. Cells were washed twice with 10% trichloroacetic acid, once with 0.5% trichloroacetic acid, and dissolved in 0.5 mL 1N NaOH for 30 minutes at room temperature followed by the addition of 0.5 mL 1N HCl. Radioactivity was quantitated using a liquid scintillation counter.

Cell Migration

Cell migration was performed using the Boyden chamber method as described. Briefly, the lower well of the chemotaxis chamber was filled with 100 μL of DMEM that contained the appropriate concentration of agonist or vehicle. An 8-μm polycarbonate filter...
was placed between the upper and lower chambers. Cells were trypsinized, counted with a hemacytometer, and resuspended in serum-free DMEM to a concentration of $5 \times 10^4$ cells per 200 $\mu$L. This volume was then added to the upper chamber. After incubation for 24 hours at 37°C in a 5% CO$_2$ incubator, filters were removed and cells on the top surface of the membrane (which had not migrated through the filter) were removed with a cotton swab. Membranes were next washed with PBS, fixed with methanol, stained with hematoxylin, and mounted on glass slides. Cells were counted with the use of a Zeiss-Axiophot microscope with a Zeiss Plan-Apochromat 63× oil immersion lens.

Adenoviral Studies

The adenoviral vectors that encode LacZ, activated L63 RhoA, and activated V12 Ras were prepared as described previously. RASMCs were plated at $1 \times 10^5$ cells per well on 12-well plates and grown for 4 days before being serum-starved. The following morning, cells were infected with the adenovirus at titers ranging from 250 to 750 viral particles per cell. Twenty-four hours after infection, cells were washed with serum-free media. [$^3$H]Thymidine (1 $\mu$Ci/mL) was added $\approx 30$ hours after infection, and [$^3$H]thymidine incorporation was measured (as described above) $\approx 52$ hours after infection.

Statistics

Data were analyzed by ANOVA. Postanalysis was performed with the use of the Tukey test, with $P<0.05$. Probability values are given in the figure legends.

Results

To verify that the RASMCs used in our studies contained functional receptors for thrombin and PE, the ability of these agonists to stimulate phospholipase C (PLC) and activate MAP kinase was examined. Maximal concentrations of either agonist (0.5 U/mL thrombin and 100 $\mu$mol/L PE) produced similar increases in total [$^3$H]inositol phosphate formation, and both agonists elicited significant increases in MAP kinase activity (data not shown). The response to PE was blocked by 0.3 $\mu$mol/L prazosin hydrochloride but not by 2 $\mu$mol/L propranolol hydrochloride. These data confirm the existence of thrombin and $\alpha_1$AdRs coupled to the activation of PLC and MAP kinase in these cells as previously shown by other groups.

Experiments were next performed to determine whether these agonists stimulated DNA synthesis in RASMCs. When cells were grown to confluence and serum-starved for 1 day before the addition of agonist, thrombin (0.5 U/mL) consistently increased [$^3$H]thymidine incorporation (Figure 1A). Smaller responses were observed in nonconfluent cells or cells starved for 2 to 3 days. Unlike thrombin, PE (100 $\mu$mol/L) did not stimulate [$^3$H]thymidine incorporation (Figure 1A). A range of concentrations of PE (100 $\mu$mol/L to 100 $\mu$mol/L) was tested on [$^3$H]thymidine incorporation with similar negative results (data not shown). DNA synthesis was also assessed by immunocytochemical analysis with bromodeoxyuridine. Sixty-five percent of cells stimulated with thrombin (0.5 U/mL) were found to incorporate bromodeoxyuridine compared with 19% of control cells (data not shown). Migration of RASMCs in response to the two PLC-coupled agonists was also examined with the use of the Boyden chamber method (Figure 1B). Analogous to the findings for DNA synthesis, thrombin (0.5 U/mL) but not PE (100 $\mu$mol/L) was able to increase cell migration.

Similar experiments were also performed with the thrombin receptor–activating peptide SFLLRNP, which lacks the proteolytic activity of thrombin but mimics the effects of the tethered ligand exposed after cleavage of the thrombin receptor with thrombin. Although this peptide sequence is based on that of the human receptor and differs from the rat receptor by 1 amino acid, it was clearly equal to or more efficacious than thrombin at eliciting the responses of interest. Figure 1A and 1B show that the thrombin receptor peptide (100 $\mu$mol/L) stimulates both DNA synthesis and migration of RASMCs. These results confirm that the observed stimulation of DNA synthesis and cell migration is mediated through the cell surface thrombin receptor.

To determine whether the small G protein Rho is involved in thrombin-stimulated DNA synthesis and the migration of RASMCs, the C3 exoenzyme from C botulinum was used to ADP-ribosylate and inactivate Rho. C3 exoenzyme treatment (40 $\mu$g/mL C3 toxin for 12 to 16 hours) fully inhibited both thrombin-stimulated DNA synthesis and migration of
RASMCs (Figure 1A and 1B). This was associated with ribosylation of at least 50% of cellular Rho (data not shown). Thrombin-stimulated accumulation of inositol phosphates was not inhibited (data not shown), which indicated that C3 exoenzyme treatment does not nonspecifically block receptor function. This observation further indicates that C3 inhibits DNA synthesis and migration without blocking the generation of PLC-derived second messengers.

Rho kinase and the closely related p160ROCK are putative effectors of Rho that we considered possible candidates for mediating thrombin-stimulated VSMC mitogenesis and migration. Recently, the agent Y-27632 has been shown to specifically inhibit these Rho-dependent kinases.19 In RASMCs, treatment with 10 μmol/L Y-27632 prevented thrombin from producing significant increases in [3H]thymidine incorporation and cell migration (Figure 2).

To directly examine the ability of the GPCR agonists to activate Rho, Rho-[35S]GTPγS binding was measured in RASMC lysates. The thrombin peptide SFLLRNP produced significant increases in Rho-[35S]GTPγS binding, although PE did not (Figure 3). Increases in membrane-bound Rho are also associated with Rho activation. To further investigate the ability of these GPCR agonists to activate Rho in intact RASMCs, we measured changes in membrane-associated Rho. In unstimulated RASMCs, ~20% of Rho was membrane-bound and 80% was cytosolic (data not shown). When stimulated with 0.5 U/mL thrombin or 100 μmol/L thrombin peptide, an approximate doubling in membrane-associated Rho was seen (Figure 4A). Concomitant decreases in cytosolic Rho were not consistently observed (data not shown).

Figure 2. Y-27632 inhibits thrombin-stimulated RASMC DNA synthesis and migration. Confluent, quiescent RASMCs were treated with either vehicle or 10 μmol/L Y-27632 for 30 minutes before stimulation with 0.5 U/mL Thr and subsequent measurement of [3H]thymidine incorporation or cell migration. There was no significant difference in DNA synthesis or cell migration in the presence or absence of Y-27632. Data represent the mean±SE from 3 experiments for both DNA synthesis and cell migration. *P<0.001 versus thrombin in the absence of inhibitor.

Figure 3. Effect of agonist stimulation on Rho-[35S]GTPγS binding. Ten-centimeter plates of confluent RASMCs were lysed and stimulated with vehicle (Ctr), 10 μmol/L Thr-P, or 100 μmol/L PE for 1 minute in the presence of 300 nmol/L [35S]GTPγS and 10 mmol/L MgCl2. After immunoprecipitation with monoclonal anti-RhoA, the amount of [35S]GTPγS bound to Rho was measured by scintillation counting. Data represent the mean±SE from 3 experiments performed in triplicate. *P<0.05 versus control.
Evidence is rapidly accumulating that suggests a role for Rho in heterotrimeric GPCR-mediated signaling. For example, Rho has been shown to be involved in GPCR activation of phospholipase D, focal adhesion formation, and sensitization associated with the tonic component of VSM contraction. Only a few reports demonstrate increases in Rho-GTP binding on Rho activation, although a fair number of studies have shown increases in membrane-associated Rho or decreases in cytosolic Rho. In this report we examined both Rho-[35S]GTP-S binding and Rho distribution to determine whether GPCR agonists activate Rho in intact RASMCs. Our data provides the first demonstration that Rho is activated by GPCR stimulation in intact VSMCs, a phenomenon previously demonstrated by guanine nucleotide and agonist treatment in permeabilized vessels. This is also the first report of Rho activation by thrombin in smooth muscle.

Both thrombin and PE couple to the activation of PLC, which presumably results in increases in intracellular Ca²⁺ and activation of protein kinase C. Thus, the observation that thrombin activates Rho, although PE does not, indicates that PLC-generated second messengers are not sufficient to induce this response. Rho-dependent changes in cytoskeletal reorganization also appear to be independent of Ca²⁺ and protein kinase C in other systems. Early work from our laboratory has shown that although thrombin and carbachol both activate PLC in 1321N1 astrocytoma cells, only thrombin mediates Rho-dependent cytoskeletal rearrangement and DNA synthesis. Similarly in PC12 cells, two PLC-coupled receptor agonists, LPA and bradykinin, differ in their ability to induce cytoskeletal change. These data are consistent with our current observation that the PLC pathway appears insufficient to activate Rho.

We hypothesize that the ability of receptor agonists to activate Rho reflects selective receptor-coupling to a heterotrimeric G protein other than or in addition to Gα₁₂. Recent reports suggest that the thrombin receptor can also signal through the pertussis toxin-insensitive Gα₁₃ and Gα₁₃ proteins to mediate cellular responses. Thrombin receptor stimulation has shown to increase GTP labeling of Gα₁₂/1₃, and Gα₁₃ is required for thrombin-stimulated DNA synthesis and cell migration. The relationship between Gα₁₂ and Rho has not yet been elucidated, although overexpression of activated Gα₁₂ or activated Gα₁₃ was shown to increase Rho-GTP binding in COS-7 cells. Most importantly, Gα₁₃ has been shown to activate a guanine nucleotide exchange factor for Rho, which suggests a pathway for activation of Rho by this G protein. Studies in our laboratory are currently underway to determine if selective coupling to Gα₁₂/1₃ in addition to Gα₃/PLC is required for thrombin to activate Rho and the subsequent induction of DNA synthesis and migration of RASMCs.

The balance in favor of activation of Rho by a given GPCR is probably mediated by a complex network of signals that result from activation of a variety of G proteins and/or protein kinases. Cell-specific signaling to Rho may occur not only as a result of differential coupling of receptors to G proteins but also as a result of differential availability and/or coupling of G proteins to guanine nucleotide exchange factors, GDP dissociation inhibitors, and GTPase activating proteins. Thus, although α₁-AdR receptors have been shown to mediate Ca²⁺ sensitization, a Rho-dependent process, in VSM, vessel-specific differences in G protein coupling such as those described above could account for the lack of observed PE activation of Rho in RASMCs. In addition, previous studies on α₁-AdR regulation of Rho and Ca²⁺ sensitization were performed using permeabilized preparations. Exogenous guanine nucleotides added back to the permeabilized vessels may promiscuously activate G proteins and allow for activation of receptor-mediated pathways (ie, α₁-AdR coupling to Gα₁₃) not effectively engaged by receptor activation in the intact system.
A role for Rho and Rho-mediated myosin phosphorylation in the Ca\(^{2+}\)-sensitization component of VSM contraction has been extensively investigated and supported by studies from several laboratories including the Somlyo and Kaibuchi laboratories.\(^3\),\(^13\),\(^17\),\(^18\),\(^54\) In contrast, the role of Rho in DNA synthesis and the migration of VSMCs has not been investigated. Our finding that C3 fully blocks thrombin stimulation of these responses in RASMC provides the first evidence that Rho is involved in VSMC proliferation and migration. Our data also suggest that although Rho is necessary for thrombin-stimulated DNA synthesis, activation of this small G protein by itself is not sufficient to induce DNA synthesis in RASMCs. Instead, Rho appears to potentiate the effects of Ras, which is in agreement with results from other laboratories that demonstrate concerted effects of Rho activation on Ras-stimulated cell cycle progression.\(^3\)^\(^5\)

Although other investigators have demonstrated Rho involvement in cellular growth\(^4\),\(^9\),\(^10\) and migration responses,\(^20\),\(^21\) the downstream effectors of these responses have not been identified. GTP-bound Rho has been shown to bind to and activate Rho kinase\(^54\),\(^56\),\(^57\) as well as other putative Rho effectors.\(^54\),\(^58\)–\(^61\) Recent studies from our laboratory have demonstrated that cardiomyocyte hypertrophy and myofibrillar assembly are blocked by inhibitory mutants of Rho kinase, which suggests a role for this Rho effector in cellular growth responses.\(^5\) A recent report suggests that the binding of Rho to Rho kinase correlates with its ability to produce cell transformation.\(^62\) Our current results further support this idea and suggest that Rho kinase is involved in thrombin stimulation of both VSMC proliferation and migration.

In summary, results from these studies indicate that agonist stimulation of the G protein–coupled thrombin receptor activates the small G protein Rho in lysed and intact RASMCs. We further demonstrate that Rho is required for both thrombin-stimulated vascular proliferation and migration. With the use of the inhibitor Y-27632, we suggest that Rho kinase is required for thrombin-stimulated VSMC DNA synthesis and migration. Interestingly, VSMC proliferation,\(^63\),\(^64\) migration,\(^65\) and Ca\(^{2+}\)-sensitization\(^66\) are all responses that have been reported to be enhanced in experimental hypertension. A recent report demonstrated that acute administration of the Rho kinase inhibitor Y-27632 reduced blood pressure in three different experimental forms of hypertension.\(^19\) This acute reduction in blood pressure is presumably the result of decreases in Ca\(^{2+}\)-sensitization. Current studies provide evidence for a role for Rho and Rho kinase in two additional processes, vascular cell proliferation and migration, both of which have been implicated in the pathophysiology of atherosclerosis, restenosis, and graft rejection.\(^67\)–\(^70\) Therefore, a central role for disturbances in Rho and Rho kinase function in these vascular disorders warrants additional investigation.

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