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Kalirin Promotes Neointimal Hyperplasia by Activating Rac in Smooth Muscle Cells

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Abstract

Objective—Kalirin is a multifunctional protein that contains two guanine nucleotide exchange factor (GEF) domains for the GTPases Rac1 and RhoA. Variants of *KALRN* have been associated with atherosclerosis in humans, but Kalirin's activity has been characterized almost exclusively in the CNS. We therefore tested the hypothesis that Kalirin functions as a RhoGEF in arterial smooth muscle cells (SMCs).

Methods and Results—Kalirin-9 protein is expressed abundantly in aorta and bone marrow, as well as in cultured SMCs, endothelial cells, and macrophages. Moreover, arterial Kalirin was up-regulated during early atherogenesis in apolipoprotein E-deficient mice. In cultured SMCs, signaling was affected similarly in three models of Kalirin loss-of-function: heterozygous *Kalrn* deletion, Kalirin RNAi, and treatment with the Kalirin RhoGEF-1 inhibitor 1-(3-nitrophenyl)-1H-pyrrole-2,5-dione. With reduced Kalirin function, SMCs showed normal RhoA activation but diminished Rac1 activation, assessed as reduced Rac-GTP levels, p21-activated kinase autophosphorylation, and SMC migration. $Kalrn^{-/+}$ SMCs proliferated 30% less rapidly than WT SMCs. Neointimal hyperplasia engendered by carotid endothelial denudation was ~60% less in $Kalrn^{-/+}$ and SMC-specific $Kalrn^{-/+}$ mice than in control mice.

Conclusions—Kalirin functions as a GEF for Rac1 in SMCs, and promotes SMC migration and proliferation both in vitro and in vivo.

Keywords

signal transduction; guanine nucleotide exchange factors; smooth muscle cells; neointimal hyperplasia

Human atherosclerosis has been associated with variants of the gene *KALRN* in several independent human cohorts,[1-3] yet the function of the proteins encoded by *KALRN*—

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Kalirin isoforms—has been studied almost exclusively in neurons and pituitary cells. The most abundantly expressed Kalirin isoform outside the CNS is Kalirin-9, for which mRNA is detectable in aorta, liver and skeletal muscle of adult mice.[4] A 270-kDa protein, Kalirin-9 is one of only two mammalian proteins that contain 2 GDP/GTP exchange factors (GEFs) for the Rho family GTPases, which are critical for cytoskeletal dynamics and consequently for cell motility and proliferation.[5] Phylogenetic conservation from *C. elegans* to human attests to the importance of Kalirin-9 in cell biology.[6] Kalirin-9 comprises an N-terminal phospholipid-binding Sec14p domain followed by 9 spectrin repeats, its "RhoGEF1" domain, a Src-homology 3 (SH3) domain, its "RhoGEF2" domain, and a second SH3 domain (Figure 1A).[6] Whereas the "RhoGEF1" domain of Kalirin activates Rac1 and RhoG, the "RhoGEF2" domain activates RhoA.[6] The spectrin repeats are known thus far to bind to the N-terminal region of inducible nitric oxide synthase (NOS2),[7] to peptidylglycine α-amidating monooxygenase, Huntingtin-associated protein-1, Disrupted-in-Schizophrenia 1, Arf6, sorting nexins 1 and 2, and αII-spectrin.[6,8] Kalirin has one mammalian ortholog, named Trio, with which Kalirin shares 65-85% amino acid identity in the RhoGEF domains.[6] Nevertheless, Kalirin and Trio are not interchangeable: *Trio*$^{-/-}$ and *Kalrn*$^{-/-}$ mouse embryos die during development on the 129/C57BL/6 mixed genetic background,[4,6] and *Kalrn*$^{-/-}$ mice show diminished size and multiple abnormalities on the C57BL/6 background.[4]

Kalirin's Rho-GEF activity could plausibly augment atherogenesis by enhancing vascular smooth muscle cell (SMC) proliferation and migration[9-11] and endothelial dysfunction,[12] among other mechanisms. Of the 60-70 Rho-GEFs encoded by mammalian genomes,[13] twenty-seven are expressed as mRNAs in SMCs,[14] but only a third of these have been shown to serve unique functions in SMCs.[14-19] By activating Rac1 in SMCs, Kalirin's RhoGEF1 activity could promote SMC migration and proliferation by activating the p21-activated (Ser/Thr) kinase PAK,[20-22] and/or NADPH oxidases.[23] By activating RhoA, Kalirin's RhoGEF2 activity could promote not only SMC migration and proliferation[24] but also endothelial dysfunction[12]—through the action of the Rho-associated coiled-coil containing protein (Ser/Thr) kinases. We sought to test the hypothesis that Kalirin-9 functions as a dual Rho-GEF in SMCs and thereby promotes SMC activation in vitro and in vivo. To do so, we used primary aortic SMCs from WT and congenic *Kalrn*$^{-/+}$ mice, as well as wire-mediated carotid endothelial denudation injury in WT and congenic *Kalrn*$^{-/+}$ mice.

Methods

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Mice

All mice were congenic on the C57BL/6 background (≥10 generations back-crossed). The Kalirin spectrin repeat knockout mouse was created as described;[4] *Kalrn*$^{-/+}$ and littermate WT mice were created from matings of *Kalrn*$^{-/+}$ mice, which appeared phenotypically indistinguishable from WT mice. SMC-specific *Kalrn*$^{-/+}$ mice were created by mating *Kalrn*$^{flox/+}$ with SM22α-Cre mice (Jackson Laboratory stock #004746), to obtain SM22α-Cre^{+}/*Kalrn*$^{flox/+}$ (SMC-*Kalrn*$^{-/+}$) mice.

Cell Culture and Assays

Mouse aortic SMCs were isolated by enzymatic digestion of aortas and passaged as described.[9,25] SMC proliferation and migration studies as well as immunoblotting assays were performed as described.[9] We previously developed rabbit polyclonal antibodies against the spectrin domain of Kalirin[26] and Trio.[27] Determinations of RhoA-GTP and Rac1-GTP levels, respectively, were made with RhoA and Rac1 G-LISAs (Cytoskeleton, Inc.)

Mouse Surgeries

Wire-mediated endothelial denudation of the common carotid[9,28] was performed as described.

Results

Vascular Expression of Kalirin

To delineate potential loci of Kalirin activity in vascular biology, we first immunoblotted mouse tissues and cells to determine their Kalirin expression levels relative to those obtaining in mouse brain. As Figure 1B shows, primary SMCs, endothelial cells, and macrophages all express Kalirin-9 at levels comparable to those obtained from mouse brain, and similar findings were obtained by using extracts from mouse aorta or bone marrow. In contrast, aortas from *Kalrn*$^{-/-}$ mice demonstrated no immunoreactivity corresponding to Kalirin-9 (Figure 1C). To determine whether Kalirin expression changed in the context of atherogenesis, we compared thoracic aortas from WT and *Apoe*$^{-/-}$ mice at the age of 8 wk, before intimal macrophage infiltration occurs.[29] Kalirin-9 protein expression was 1.6±0.2-fold higher in aortas from *Apoe*$^{-/-}$ than from WT mice (p<0.05, Figure 1C). Thus, vascular cell expression of Kalirin-9 is up-regulated during the earliest stages of atherogenesis.

Kalirin Activates Rac1 in SMCs

To test whether Kalirin function affects SMC physiology, we first inhibited Kalirin's RhoGEF1 domain by treating primary SMCs with the cell-permeant compound NPPD (1-(3-nitrophenyl)-1*H*-pyrrole-2,5-dione), which selectively inhibits GDP/GTP exchange activity promoted by the highly homologous RhoGEF1 domains of Kalirin and its lone ortholog, Trio.[30] Kalirin's RhoGEF1 and RhoGEF2 domains activate Rac1 and RhoA, respectively (Figure 1A).[6] We therefore assessed SMC Rac1 and RhoA activity, by determining the activity of their respective effector kinases: p21-activated kinase (PAK1), which when activated by Rac1-GTP autophosphorylates on Thr-423;[31] and Rho-associated coiled-coil containing protein kinases (ROCK1, ROCK2), which when activated by RhoA-GTP phosphorylate the myosin phosphatase targeting subunit 1 (MYPT1) on Thr-853.[32] PAK1 autophosphorylation induced by platelet-derived growth factor (PDGF) was reduced by 40±20% (p < 0.05) in SMCs treated with NPPD (Figure 2A, B). In contrast, (ROCK-mediated) MYPT1 phosphorylation was not affected by NPPD (Figure 2A, B). Thus, the RhoGEF1 domain of Kalirin or Trio—known to activate Rac1, but not RhoA[6]—appears to mediate Rac1 activation downstream of the PDGF receptor in SMCs. To distinguish Kalirin from Trio in this NPPD-sensitive SMC Rac1 activation, we compared Kalirin and Trio expression in SMCs with that in brain tissue, in which Trio and Kalirin are expressed at comparable levels (qRT-PCR data not shown). Whereas SMC Trio protein levels were <5% of brain levels, SMC Kalirin protein levels were comparable to those in brain tissue (Figure 2C). Consequently, we inferred that most of the NPPD-inhibited, PDGF-induced Rac-GEF activity in SMCs is attributable to Kalirin, rather than to Trio.

To complement NPPD-mediated inhibition of Kalirin's RhoGEF1 domain, and to determine whether Kalirin's RhoGEF2 domain is also important in SMC physiology, we used Kalirin RNAi. Relative to SMCs transfected with control siRNA, SMCs transfected with Kalirin-targeting siRNA demonstrated 43±9% less Kalirin protein expression and 50±20% less PAK1 autophosphorylation in response to PDGF or serum (p < 0.05, Figure 2D-F). Nevertheless, Kalirin knock-down SMCs demonstrated MYPT1 phosphorylation (evidence of ROCK activity[32]) that was equivalent to control SMCs (Figure 2E, F). Thus, we obtained equivalent results in SMCs with Kalirin knock-down and chemical inhibition of Kalirin's RhoGEF1 domain, and Kalirin appears to function in SMCs as an important GEF for Rac1, but not for RhoA.

To corroborate these data obtained with Kalirin RNAi, we used 5 independently isolated lines of SMCs from age- and gender-matched *Kalrn*$^{-/+}$ and littermate WT mice. *Kalrn*$^{-/+}$ SMCs expressed only 45±5% as much Kalirin-9 protein as WT SMCs, but equivalent levels of proteins critical for the receptor tyrosine kinase and (heterotrimeric) $G_{q/11}$ signal transduction pathways under investigation: PDGF receptor-β, endothelin receptor type A, Rac1, RhoA, PAK1, and MYPT1 (Figure 3A). When stimulated with serum, PDGF, or endothelin-1, *Kalrn*$^{-/+}$ SMCs demonstrated only ~60% of WT PAK1 activation, as assessed by PAK1 autophosphorylation (Figure 3B, C). In contrast, *Kalrn*$^{-/+}$ SMCs demonstrated WT levels of ROCK activation, as assessed by MYPT1 phosphorylation[32] (Figure 3B, C). Because ROCK isoforms can be activated not only by RhoA (the target of Kalirin's RhoGEF2 domain) but also by RhoB and RhoC,[33] we also assessed SMC RhoA-GTP levels. Consistent with MYPT1 phosphorylation data, *Kalrn*$^{-/+}$ and WT SMCs demonstrated equivalent, 2-3-fold over basal stimulation of RhoA GTP loading in response to serum (Figure 3D). In contrast, *Kalrn*$^{-/+}$ SMCs demonstrated 30% less Rac1 GTP loading than WT SMCs (Figure 3D). Thus, assessed at the level of the GTPase or the effector kinases, Kalirin appears to promote the activation of Rac1, but not RhoA in SMCs.

Kalirin Promotes SMC Migration and Proliferation

Because Rac1 and PAK1 signaling are critical for SMC migration,[20,21] we reasoned in light of Figure 3 that loss of Kalirin function would reduce SMC migration. To test this hypothesis, we studied SMC migration in modified Boyden chambers. Stimulus-independent SMC migration was not affected by loss of Kalirin function (Figure 4). However, whether engendered by chemical inhibition of Kalirin's RhoGEF1 domain (by NPPD), by Kalirin RNAi, or by Kalirin haploinsufficiency, loss of Kalirin function was associated with a ~35% reduction in PDGF- or serum-evoked SMC migration ($p < 0.05$, Figure 4). To determine whether loss of Kalirin function reduced SMC migration principally by reducing Rac1 activation, we treated SMCs with the cell-permeant Kalirin RhoGEF1 inhibitor ITX3[34] or the Rac1 inhibitor Z62954982, using conditions that have been shown to inhibit PDGF-induced GTP loading of Rac1 by ~50% without affecting GTP loading of the related Rho GTPases Cdc42 or RhoA.[35] Kalirin RhoGEF1 or Rac1 inhibition with ITX3 or Z62954982, respectively, reduced by 35-40% the migration of WT SMCs stimulated by PDGF (Figure 4D). This attenuation of SMC migration was comparable to that seen with the loss of Kalirin function observed in *Kalrn*$^{-/+}$ SMCs. Furthermore, the impaired migration of *Kalrn*$^{-/+}$ SMCs was not further reduced by ITX3 or Z62954982 (Figure 4D). In parallel experiments with WT SMCs, Z62954982 inhibited PAK1 autophosphorylation by 40±10%, but neither ITX3 nor Z62954982 inhibited ROCK activity, manifest as MYPT1 phosphorylation, or MEK1 activation, manifest as phosphorylation of ERK1/2 (Figure 4E and data not shown). As a further demonstration of inhibitor specificity, neither ITX3 nor Z62954982 reduced the SMC migration promoted by fetal bovine serum under the conditions prevailing in these experiments (Figure 4D). Taken together, these data suggest that Kalirin mediates Rac1 activation in SMCs downstream of the PDGF receptor, and thereby promotes SMC migration.

Whereas cellular migration can be promoted by Rac1 activity at the plasma membrane,[20] cellular proliferation appears to require Rac1 nuclear localization.[36] Thus, despite the observation that Kalirin promoted Rac1-dependent SMC migration, the effects of Kalirin on SMC proliferation remained uncertain. To test whether Kalirin activity promotes SMC proliferation, therefore, we quantitated WT and *Kalrn*$^{-/+}$ SMCs at several time points during exposure to serum. *Kalrn*$^{-/+}$ SMCs proliferated 50% less rapidly than WT SMCs in response to serum ($p < 0.03$, Figure 5). Thus, Kalirin appears to promote SMC proliferation.

SMC Kalirin Promotes Neointimal Hyperplasia

SMC proliferation and migration are fundamental to the neointimal hyperplasia that develops in response to arterial endothelial denudation.[28] If Kalirin's positive effects on SMC migration and proliferation occurred not only in vitro but also in vivo, we reasoned that *Kalrn*$^{-/+}$ mice should develop less neointimal hyperplasia than WT mice. We therefore provoked carotid artery neointimal hyperplasia in congenic *Kalrn*$^{-/+}$ and WT mice by denuding the endothelium with a 0.36-mm wire—an approach that we have shown engenders neointimal hyperplasia comprising only SMCs from the arterial media.[28] Neointimal SMCs (Supplemental Figure I) demonstrated Kalirin up-regulation (Supplemental Figure II). Congruent with our SMC studies (Figures 3, 5), there was greater SMC PAK activation and proliferation in WT than in *Kalrn*$^{-/+}$ arteries (Supplemental Figures III, IV); nonetheless, arterial re-endothelialization, macrophage recruitment and collagen content were equivalent in WT and *Kalrn*$^{-/+}$ arteries (Supplemental Figures V, VI).

Kalrn$^{-/+}$ mice developed 60% less neointima than WT mice (Figure 6 and Supplemental Figure I). However, this result could have been produced by pleiotropic effects of Kalirin in endothelial cells or leukocytes.[37] We therefore sought to ascertain that SMC Kalirin activity, specifically, was promoting the in vivo SMC proliferation and migration of neointimal hyperplasia. To do so, we used mice with SMC-specific deletion of *Kalrn* (SMC-*Kalrn*$^{-/+}$, or SM22α-Cre^{+}/*Kalrn*$^{flox/+}$), and compared them with *Kalrn*$^{flox/+}$ control mice (which gave results equivalent to WT mice (data not shown)). Compared with WT or *Kalrn*$^{flox/+}$ control mice, SMC-*Kalrn*$^{-/+}$ mice expressed Kalirin-9 protein levels that were 50±5% less in endothelium-stripped aortas, but equivalent in whole brain extracts (Figure 6A). After carotid endothelial denudation, SMC-*Kalrn*$^{-/+}$ mice developed 65% less neointimal hyperplasia than in either control mouse cohort (Figure 6B, C). Prior to surgery, *Kalrn*$^{-/+}$, SMC-*Kalrn*$^{-/+}$ and WT mice had equivalent carotid dimensions (data not shown) and equivalent systolic blood pressures and heart rates: respectively, 121±7, 120±10, and 120±10 mm Hg; 700±40, 640±40, and 660±40 bpm (n=7/group). Thus, whether in SMCs alone or in combination with endothelial cells and leukocytes, Kalirin activity contributes to neointimal hyperplasia.

Discussion

This study establishes Kalirin, a human atherosclerosis candidate gene,[1-3] as a significant signaling intermediate that promotes SMC Rac1 activation, migration and proliferation downstream of the PDGF receptor-β and G protein-coupled receptors in vitro (Supplemental Figure VII). Consonant with this signaling activity in SMCs, Kalirin in vivo promotes neointimal hyperplasia induced by arterial endothelial denudation—whether Kalirin expression is manipulated globally, or just in SMCs. It is noteworthy that Kalirin's role in vascular biology manifests with loss-of-function models that allow ~50% of normal Kalirin activity. Consequently, the magnitude of Kalirin's total contribution to vascular pathophysiology may be underestimated by these models; nevertheless, incomplete loss-of-function models human disease and pharmacotherapeutic responses better than complete loss-of-function.[1-3]

Kalirin-promoted SMC migration and proliferation correlate with Kalirin's Rac1-GEF activity, triggered by the PDGF receptor-β: indeed, Kalirin deficiency impaired SMC migration only when Rac1 activity was intact (Figure 4). In neurons, Kalirin Rac-GEF activity is triggered by the receptor tyrosine kinase EphB2, which tyrosine-phosphorylates and recruits Kalirin to the plasma membrane.[38] Whether this method of Kalirin activation obtains downstream of the PDGF receptor-β tyrosine kinase remains to be established. However, SMCs express both EphB2 and its agonist ephrinB2, which appear to promote SMC migration in vivo;[39] thus, Kalirin could mediate SMC migration in vivo downstream

of multiple receptor tyrosine kinases. Furthermore, because EphB2 receptors on monocytes promote adhesion to arteries and because monocyte/macrophages express abundant Kalirin (Figure 1), it is conceivable that an EphB2-Kalirin signaling axis operates in monocytes to promote monocyte/macrophage infiltration of the (ephrinB2-expressing[39]) tunica media of injured arteries. Because monocyte/macrophages contribute significantly to neointimal hyperplasia,[40] Kalirin deficiency in monocytes could thus contribute to the reduction in neointimal hyperplasia we observed in *Kalrn*$^{-/+}$ as compared with WT mice (Figure 6).

Despite containing a GEF domain specific for Rac1 and a GEF domain specific for RhoA,[6] Kalirin appears to function in SMCs as a GEF only for Rac1, and not for RhoA. This inference is based on comparisons among SMCs with Kalirin levels that were either 100% or ~50% of WT; consequently, it may be that 50% of normal Kalirin levels in SMCs suffice to drive normal activation of RhoA, but not Rac1. Alternatively, Kalirin's apparent Rho-family GTPase specificity in SMCs may result from distinct subcellular distributions and overlapping functions of the myriad Rac1- and RhoA-GEFs expressed along with Kalirin in SMCs.[14-19] Lastly, it is important to note that the overall GTPase activity reflects a balance between the activities of GEFs, which activate GTPases by promoting the exchange of GTP for GDP, and GAPs (GTPase-activating proteins), which de-activate GTPases by enhancing hydrolysis of GTP to GDP.[5] Thus, it may be that the functionality of GAPs for Rac1 exceeds that of GAPs for RhoA in SMCs; if so, we could ascertain differences in Kalirin-mediated activation of Rac1 more sensitively than we could for RhoA.

Kalirin may also contribute to neointimal hyperplasia through mechanisms distinct from its Rac1-GEF activity. For example, Kalirin's interaction with the N-terminal domain of NOS2 prevents NOS2 dimerization and thereby inhibits NOS2 activity.[7] Perhaps because NOS2-produced NO· reduces SMC proliferation and mitochondrial respiration,[41] *Nos2*$^{-/-}$ mice develop more medial thickening than WT mice subjected to carotid endothelial denudation.[42] Consequently, by inhibiting SMC NOS2, Kalirin could reduce NO·-mediated inhibition of SMC proliferation—and thereby contribute to neointimal hyperplasia. Whether such a mechanism obtains in SMCs remains to be established.

Achieved either genetically or by RNAi, just a 50% reduction in Kalirin activity suffices to reduce SMC proliferation and migration in vitro and in vivo. Consequently, Kalirin appears to be an appealing target for pharmacotherapy in SMC proliferative disorders like neointimal hyperplasia. Because interventions that diminish neointimal hyperplasia often diminish atherosclerosis, too,[28,43] the work presented here adds credence to human genetic data[1-3] implicating Kalirin in atherogenesis. Direct evidence to support Kalirin's role in atherosclerosis, of course, has yet to manifest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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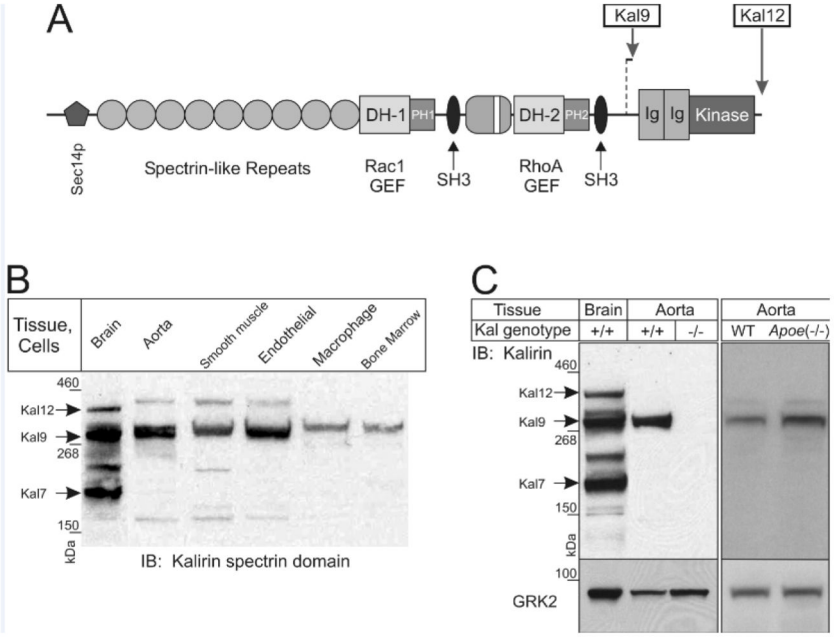

Figure 1.

Vascular and bone marrow-derived cells express Kalirin-9, which is up-regulated during atherogenesis. **A,** A scale drawing of the domain topography of Kalirins -9 and -12 (named for the size of their mRNA). Sec14p, phospholipid-binding; DH-1, Dbl (deleted in B-cell lymphoma) homology-1; PH, pleckstrin homology; SH3, Src homology 3; Ig, immunoglobulin; Kinase = Ser/Thr kinase. **B,** The indicated tissues and primary cell lines from WT mice were solubilized; 35 μg protein was subjected to SDS-PAGE and immunoblotted with anti-(Kalirin spectrin domain) IgG. Parallel blots probed with non-immune first IgG yielded no bands (not shown). Arrows indicate Kalirin isoforms. Shown is a single experiment, representative of 3 performed. **C,** Mice of the indicated genotype were sacrificed; 35 μg of tissue was immunoblotted for Kalirin as in panel B. The nitrocellulose was stripped and re-probed for G protein-coupled receptor kinase-2 (GRK2) as a loading control. Shown are immunoblots representative of 3 independent experiments.

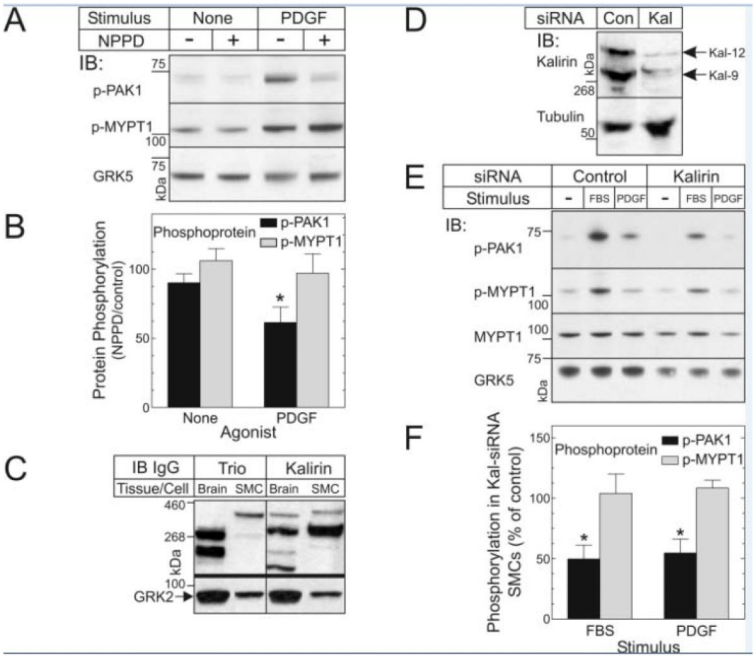

Figure 2.

SMC Kalirin activates Rac1, but not RhoA: inhibitor and RNAi data. **A**, Quiescent aortic SMCs were treated for 2.5 hr with 0.1% DMSO lacking (control) or containing the Kalirin RhoGEF1 domain inhibitor NPPD (100 μmol/L, [final]). Subsequently, SMCs were exposed to serum-free medium lacking (None) or containing 1 nmol/L PDGF-BB (10 min, 37 °C), solubilized, and immunoblotted for phospho-(p-)PAK1(p-Thr423), p-MYPT1(p-Thr853), and then for GRK5 (as a loading control). **B**, The indicated phosphoprotein band densities were normalized to cognate GRK5 bands on each blot; quotients were divided by those obtained from control-treated SMCs within each experiment to obtain "% of control," plotted as means±S.E. of 3 experiments. Compared with control: *, $p < 0.05$. **C**, Protein extracts (35 μg/lane) of whole mouse brain and SMCs were immunoblotted with IgG specific for Trio or Kalirin, and then re-probed for GRK2 (as a loading control). Results are from a single experiment representative of 3 performed. **D,** SMCs transfected with siRNA targeting no known protein (control, "Con") or Kalirin ("Kal") were immunoblotted for Kalirin and then tubulin; the mobility of Kalirin isoforms is indicated by arrows. Results shown are from a single experiment representative of 3 performed. **E,** SMCs from panel D were exposed to medium lacking or containing 10% FBS or 1 nmol/L PDGF-BB (10 min, 37 °C), and then processed for immunoblotting as in panel A. **F,** The indicated phosphoprotein band densities were normalized to cognate GRK5 bands on each blot; quotients from Kal-siRNA-transfected SMCs were divided by those obtained from control siRNA-transfected SMCs within each experiment to obtain "% of control," plotted as means ±S.E. of 3 experiments. Compared with control: *, $p < 0.05$.

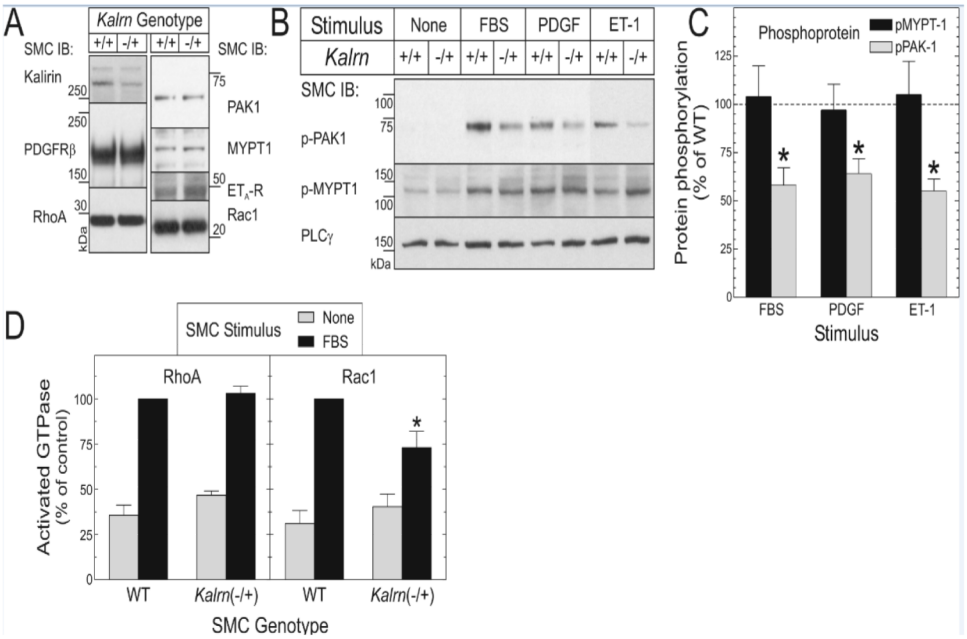

Figure 3.

SMC Kalirin activates Rac1, but not RhoA: data from *Kalrn*$^{-/+}$ and WT SMCs. **A,** Solubilized extracts (40 μg protein/lane) from WT (*Kalrn*$^{+/+}$) and *Kalrn*$^{-/+}$ SMCs were immunoblotted serially for the indicated proteins: PDGFRβ, PDGF receptor-β; ET_A-R, endothelin receptor type A. Shown are results from a single experiment representative of 5 performed. **B,** Quiescent SMCs from WT or *Kalrn*$^{-/+}$ mice were exposed for 10 min (37 °C) to serum-free medium lacking (None) or containing 10% FBS, 2 nmol/L PDGF-BB or 100 nmol/L endothelin-1 (ET-1), then solubilized and immunoblotted for the indicated (phospho)proteins. PLCγ (phospholipase C-γ) was used as a loading control. Shown are results from a single experiment, representative of 5 performed. **C,** The indicated phosphoprotein band densities were normalized to cognate PLCγ bands; quotients were divided by those of WT SMCs to obtain "% of WT," plotted as means±S.E. of 5 experiments with independently isolated WT and *Kalrn*$^{-/+}$ SMC lines. Compared with WT: *, $p < 0.05$. **D,** Quiescent WT and *Kalrn*$^{-/+}$ SMCs were stimulated as in panel B, lysed, and subjected to RhoA or Rac1 G-LISA (Cytoskeleton, Inc.). Absorbance values were normalized in each experiment to the value obtained for FBS-stimulated WT SMCs, to obtain "% of control." Shown are the means ± S.E. from ≥ 3 experiments performed in triplicate. Compared with WT: *, $p < 0.05$.

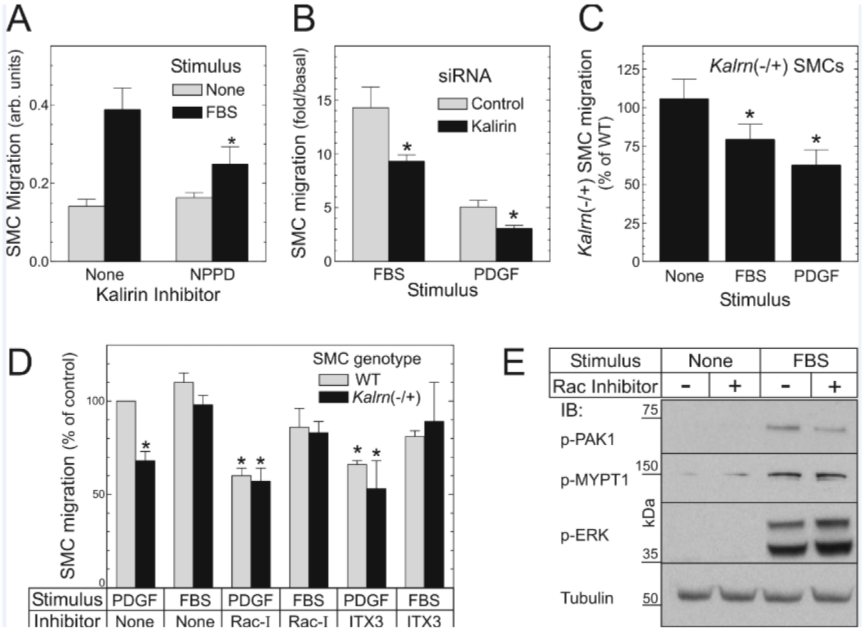

Figure 4.

Kalirin promotes SMC migration. Quiescent SMCs in modified Boyden chambers were exposed to vehicle ("basal"), 10% FBS, or 1 nmol/L PDGF-BB; migrated SMCs were quantitated colorimetrically (see Methods), and plotted as means ±SE of ≥3 experiments performed in triplicate: as arbitrary ("arb.") units (A), normalized to unstimulated SMC values (B), or normalized to cognate WT SMC values (C). Compared with control or WT: *, $p < 0.05$. **A,** WT SMCs were treated for 1 hr before migration assays with 0.1% DMSO lacking (None) or containing the Kalirin RhoGEF1 inhibitor NPPD (100 μmol/L). **B,** WT SMCs were transfected with the indicated siRNA, and subjected to migration assay 72 hr later. Values for "basal" migration in Kalirin knock-down SMCs were 95±8% of control SMC values. **C,** WT and *Kalrn*$^{-/+}$ SMCs were subjected to migration assays in parallel. **D,** WT and *Kalrn*$^{-/+}$ SMCs were exposed to 0.1% DMSO in serum-free medium lacking (None) or containing the Rac1 inhibitor (Rac-I) Z62954982 (10 μmol/L) or the Kalirin RhoGEF1 inhibitor ITX3 (100 μmol/L) for 4 hr prior to stimulation for migration assay. All migration values were normalized to those obtained for WT SMCs stimulated with 10% FBS, to obtain "% of control." Values for "basal" migration in *Kalrn*$^{-/+}$ SMCs were 100±10% of WT values. Compared with vehicle-treated WT SMCs: *, $p < 0.05$. **E,** Quiescent WT SMCs were pre-treated with vehicle or the Rac1 inhibitor as in panel D, challenged ±10% FBS (10 min, 37 °C), and then processed for IB as in Figure 3. Shown are results from a single experiment, representative of ≥3 performed.

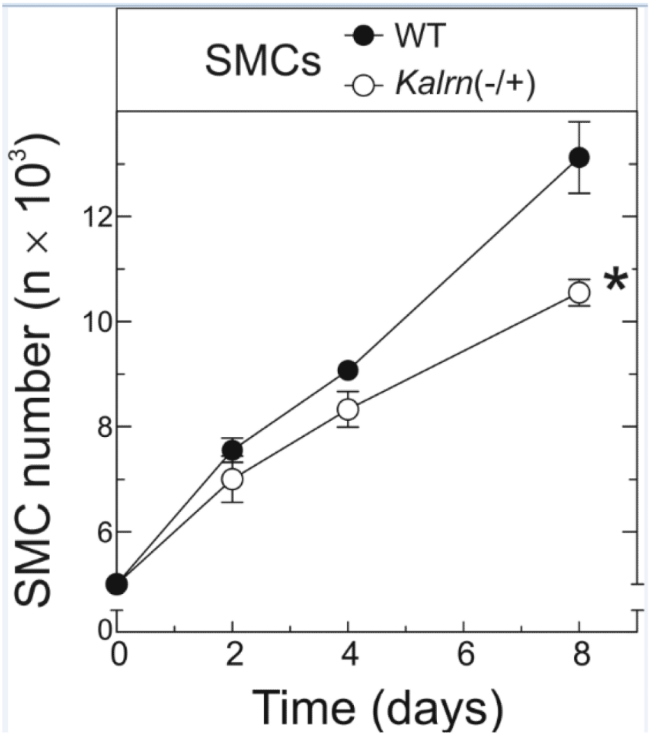

Figure 5.

Kalirin increases SMC proliferation. WT and *Kalrn*$^{-/+}$ SMCs were incubated for indicated times in 10% FBS/growth medium; SMC quantitation was performed as in Methods. Plotted are the means ± S.E. of SMC counts from 3 independent experiments with 3 independently isolated pairs of WT and *Kalrn*$^{-/+}$ SMC lines. Compared with the WT growth curve: *, $p <$ 0.001. The rate of SMC proliferation was obtained from linear regression (R^2 = 0.98-0.99).

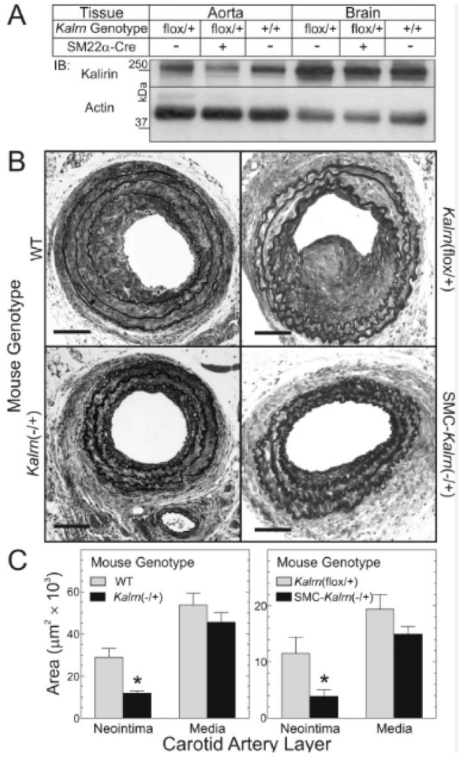

Figure 6.

SMC Kalirin promotes neointimal hyperplasia. **A,** Brains and aortas harvested from mice of the indicated genotype were solubilized and immunoblotted (40 μg protein/lane) serially for Kalirin and actin. Shown are results from a single experiment representative of 3 performed with independent mice. The ratio of Kalirin/actin was used for quantitation. **B,** Mice of the indicated genotype were subjected to carotid artery de-endothelialization and sacrificed 4 wk later. Perfusion-fixed carotids were stained with a modified connective tissue stain. Samples shown represent ≥5 of each genotype. Scale bars = 50 μm. **C,** Cross sectional areas for neointima and media are presented as means ± S.E. from ≥5 carotids of each genotype. Compared with WT: *, $p < 0.02$. Uninjured $Kalrn^{-/+}$ and WT carotid arteries demonstrated equivalent lumen and medial areas (not shown).