Receptor for Activated Protein Kinase C1 Regulates Cell Proliferation by Modulating Calcium Signaling


Abstract—Receptor for activated protein kinase C1 (RACK1) is an intracellular scaffolding protein known to interact with the inositol-1,4,5-trisphosphate receptor and thereby enhance calcium release from the sarcoplasmic reticulum. Because calcium signaling may affect vascular smooth muscle cell proliferation, we investigated whether RACK1 regulates proliferation of rat preglomerular microvascular smooth muscle cells. Western blot analysis indicated that preglomerular microvascular smooth muscle cells robustly express RACK1 protein, and coimmunoprecipitation experiments demonstrated that RACK1 binds the inositol-1,4,5-trisphosphate receptor. RACK1 small interfering RNA (siRNA) decreased RACK1 mRNA and protein expression, significantly \((P=0.0225)\) reduced steady-state basal levels of intracellular calcium \((6712\pm156\) versus \(7408\pm248\), arbitrary fluorescence units in RACK1 siRNA-treated versus control cells, respectively) and significantly \((P<0.0001)\) decreased cell proliferation by \( \approx 50\%\). Xestospongin C and 2-aminoethoxydiethyl phosphorylborate (antagonists of inositol-1,4,5-trisphosphate receptors), cyclopiazonic acid (sarcoplasmic reticulum \( Ca^{2+}\)-ATPase inhibitor), and calmidazolium (calmodulin inhibitor) mimicked the effects of RACK1 siRNA on proliferation, and RACK1 siRNA had no additional effects on proliferation in the presence of these agents. RACK1 siRNA did not affect the expression of cyclin D1/2 or phosphorylation of retinoblastoma protein (progrowth cell cycle regulators), yet it caused compensatory decreases in the expression of p21Cip1/Waf1 and p27Kip1 (antigrowth cell cycle regulators). Like preglomerular microvascular smooth muscle cells, glomerular mesangial cells also expressed high levels of RACK1, and RACK1 siRNA inhibited their proliferation. In conclusion, RACK1 modulates proliferation of preglomerular microvascular smooth muscle cells and glomerular mesangial cells, likely via the inositol-1,4,5-trisphosphate receptor/calciunc/calmodulin pathway. RACK1 may represent a novel druggable target for treating renal diseases, such as glomerulosclerosis. (Hypertension. 2011;58:689-695.) ● Online Data Supplement

Key Words: receptor for activated protein kinase C1 ■ RACK1 ■ cell proliferation ■ microvascular smooth muscle cells ■ glomerular mesangial cells ■ calcium ■ calmodulin

Healthy kidneys require a structurally appropriate renal microcirculation, which, in turn, depends on the proper regulation of proliferation of renal microvascular cellular elements, including renal microvascular smooth muscle cells, such as preglomerular vascular smooth muscle cells (PGVSMCs). Dysregulation of PGVSMCs and their phenotypically similar counterparts in the glomerulus (ie, glomerular mesangial cells [GMCs]) contributes to nephropathies such as hypertensive and diabetic renal disease.\(^1\)\(^-\)\(^3\) Thus, the elucidation of novel, drugable molecular targets that regulate growth of PGVSMCs and GMCs is a logical first step in the development pathway for generating new pharmacological therapies to treat renal diseases that involve the renal microcirculation and glomeruli.

Receptor for activated protein kinase C1 (RACK1) is a 7-sided propeller protein with 7 WD40 repeats, and RACK1 participates in cell signaling by functioning as a scaffold protein.\(^4\)\(^-\)\(^5\) Although RACK1’s name reflects its role in localizing activated C kinases, more recent studies confirm that RACK1 serves as a highly diverse scaffold protein that binds a large array of proteins and regulates multiple signaling pathways by interacting with WD40, SH2, C2, PH, and NUF domains in binding partner proteins.\(^5\) Importantly, recent studies show that, in some cell types, RACK1 binds to the inositol-1,4,5-trisphosphate (\( IP_3 \)) receptor (\( IP_3\)R) and increases its binding affinity for \( IP_3 \) and thereby increases intracellular calcium levels by triggering release of calcium from the sarcoplasmic reticulum (SR) calcium-releasing system. RACK1 also binds to \( \text{BK}_{\text{Ca}} \) channels in rat pulmonary artery smooth muscle cells and in rat basilar artery smooth muscle cells, a process that is thought to impair \( \text{BK}_{\text{Ca}} \) channel function and increase localized compartments of intracellular calcium.\(^7\) Because increases in intracellular calcium stimulate proliferation of vascular smooth muscle cells,\(^8\)\(^-\)\(^13\) we hypo-
esize that RACK1 may regulate growth of PGVSMCs and GMCs. The main goal of this study was to evaluate this hypothesis.

Methods

Animals
Studies used male adult (16 weeks old) normotensive Wistar-Kyoto rats from Taconic Farms (Germantown, NY). The Institutional Animal Care and Use Committee approved all of the procedures. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85-23, revised 1996).

Culture of PGVSMCs and GMCs
PGVSMCs and GMCs were cultured by explant from freshly isolated rat renal microvessels and glomeruli as described in detail by us previously.14,15

Effects of RACK1 Small Interfering RNA on Cell Proliferation
Cells (third to fifth passage) were cultured in DMEM/F12 medium containing 10.0% ECS, 20 U/mL of penicillin, 20 μg/mL of streptomycin, and 0.05 μg/mL of amphotericin at 37°C with 5% CO₂. One day before transfection, cells were plated in 500 μL of DMEM/F12 medium containing 10.0% ECS (for [³H]thymidine incorporation studies) or 2.5% ECS (for cell number experiments) without antibiotics in a 24-well plate. On the day of transfection, 40 pmol of RACK1 small interfering RNA (siRNA) pool or nontargeting siRNA pool (Dharmacon, Lafayette, CO) and 1.5 pmol of RACK1 small interfering RNA (siRNA) pool or nontargeting siRNA pool were added to the transfection mixture. In some experiments, the transfection mixture included xestospongin C (5 μmol/L), 2-APB, cyclopiazonic acid, and calmidazolium were obtained from Sigma-Aldrich (St Louis, MO). The growth medium was removed from the cells and replaced with the transfection mixture at 37°C with 5% CO₂. For thymidine incorporation studies (DNA synthesis), at 68 hours, the medium was changed to DMEM/F12 medium containing 0.1% ECS (low serum) or 2.5% ECS (high serum) without antibiotics after 30 minutes, the sample was sonicated, incubated for 20 minutes at room temperature to allow transfection complexes to form. DMEM/F12 medium containing 0.1% ECS (low serum) or 2.5% ECS (high serum) without antibiotics were added to the complexes (transfection mixture). In some experiments, the transfection mixture included xestospongin C (5 μmol/L; antagonist of IP₃R), 2-aminoethoxydiphenylborate (2-APB; 100 μmol/L; alternative antagonist of IP₃R), cyclopiazonic acid (5 μmol/L; blocks calcium pump in the SR), or calmidazolium (1 μmol/L; inhibits calmodulin). Xestospongin C, 2-APB, cyclopiazonic acid, and calmidazolium were obtained from Sigma-Aldrich (St Louis, MO). The growth medium was removed from the cells and replaced with the transfection mixture, and the cells were incubated with the transfection mixture at 37°C with 5% CO₂. For thymidine incorporation studies (DNA synthesis), at 68 hours, the medium was changed to DMEM/F12 containing both 0.1% ECS and [³H]thymidine (1 μC/mL). Four hours later, the experiments were terminated by washing the cells twice with Dulbecco PBS and twice with ice-cold trichloroacetic acid (10%). The precipitate was solubilized in 500 μL of 0.3 N NaOH and 0.1% sodium dodecylsulfate after incubation at 50°C for 2 hours. Samples were mixed with 10 μL of scintillation fluid and counted in a liquid scintillation counter. For cell number experiments, at 72 hours, the transfection mixture was replaced with fresh DMEM/F12 medium containing either 0.1% or 2.5% ECS with or without xestospongin C, 2-APB, cyclopiazonic acid, or calmidazolium, and at 96 hours, cells were dislodged and counted on a Coulter counter.

Assessment of siRNA Knockdown of RACK1 mRNA
RNA was isolated (TRIzol Reagent, Life Technologies, Carlsbad, CA), and cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The RACK1 primers were as follows: forward, 5’-gtgcttcgctggctctc-3’; reverse, 5’-cggtggctgagggagaaagac-3’; 184 bp amplification product. β-Actin primers were as follows: forward, 5’-acacctccatccctc-3’; reverse, 5’-acacctccctagctgte-3’; 171 bp amplification product. Real-time PCR analysis was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in the AB 7300 Real-Time PCR System (Applied Biosystems). Threshold cycle (Ct) for target was subtracted from Ct for β-actin to calculate 2^(-ΔCt).

Assessment of siRNA Knockdown of RACK1 Protein
Protein was extracted (Mammalian Protein Extraction Reagent, Pierce Biotechnology Inc, Rockford, IL), measured (BCA assay; Pierce), and boiled (5 minutes in Laemmli buffer). SDS-PAGE was performed on polyacrylamide gels (8% to 16%) with 40 μg of protein per lane. Proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked in Tris-buffered saline containing 5% milk and probed with an anti-RACK1 mouse monoclonal primary antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were exposed to horseradish peroxidase–conjugated goat antimouse antibody (1:4000; Pierce) and visualized with the Bio-Rad VersaDoc Imaging System using luminal-based enhanced chemiluminescence substrate (Supersignal West Dura Extended Duration Substrate; Pierce).

Analysis of Cell Cycle Regulators
Cellular levels of cyclin D1/2, phosphorylated and hyperphosphorylated retinoblastoma protein, p21/Cip1/Waf1, and p27/Kip1 were measured by Western blotting, as described previously by us in detail.21

Comunoprecipitation of RACK1 With IP₃R and Calmodulin
Each batch of PGVSMCs was cultured in a 75-cm² flask and suspended in trypsin in a separate 15-mL tube. The sample was centrifuged at 4°C for 10 minutes (2000 rpm), and the pellet was resuspended in 10 mL of PBS and then again centrifuged for 10 minutes. Each pellet was resuspended in 1 mL of ice-cold radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology), and after 30 minutes, the sample was sonicated, incubated for another 30 minutes, centrifuged at 4°C for 10 minutes (10 000g), and the supernatant transferred to a 1.5-mL microcentrifuge tube. Pre-clearing B-Matrix-Rabbit solution (45 μL; Santa Cruz Biotechnology) was added to the sample, and the sample was incubated at 4°C for 10 minutes while rotating. Then each sample was centrifuged in a microcentrifuge at maximum speed for 30 seconds at 4°C, and the supernatant was transferred to a microfuge tube and placed on ice.

To prepare the precipitating complex, 45 μL of IP-Matrix (Exac-taCruz B, Santa Cruz Biotechnology), 500 μL of PBS, and 20 μL of IP₃R or calmodulin 11 rabbit polyclonal antibody (200 μg/mL; Santa Cruz Biotechnology) were incubated overnight at 4°C with rotation in a microcentrifuge tube. The precipitating complex was pelleted by microcentrifugation at maximum speed for 30 seconds at 4°C. The precipitate was resuspended in 500 μL of PBS and washed twice, and the precleared cell lysate was added to the pellet (IP-antibody + IP-matrix complex) and incubated at 4°C for 1 hour while rotating. The sample was pelleted by microcentrifugation at maximum speed for 30 seconds at 4°C and washed 3 times in PBS. The pellet was resuspended in sample buffer with reducing agent.

Sixteen micrograms of protein from each sample were placed in 16 μL of sample buffer, boiled for 10 minutes, incubated on ice for 5 minutes, flash centrifuged, and loaded onto an Invitrogen NuPAGE Novex Bis-Tris 12% gel. Gels were run in NuPAGE MOPS SDS running buffer at 120 V for 2 hours, and proteins were transferred onto a polyvinylidene fluoride membrane in NuPAGE transfer buffer at 0.21 A overnight. Polyvinylidene fluoride membranes were washed briefly with methanol and blocked in PBS (5% dry nonfat milk, 0.05% Tween-20) at room temperature for 2 hours. Blots were incubated with 1° RACK-1 mouse monoclonal antibody in PBS (5% dry nonfat milk, 0.05% Tween-20) at 1:350 ratio at 4°C overnight, washed 5 times in PBS, incubated with antimouse horseradish peroxidase–conjugated ExactaCruz detection reagent in PBS (5% dry nonfat milk, 0.05% Tween-20) at 1:8500 ratio for 1 hour at room temperature, washed 3 times in PBS, covered with SuperSignal West Femto reagent (Pierce), and imaged with a Fotodyne (Hartland, WI) gel imaging system for an 8-minute exposure.
High Serum
RACK1 siRNA negative control siRNA

Low Serum
RACK1 siRNA negative control siRNA

RACK1

β-actin

A

Expression of RACK1 Protein Normalized to β-Actin Protein

B

High Serum

C

Low Serum

Measurement of Intracellular Calcium
One day before transfection, 20,000 PGVSMCs were suspended in 100 μL of DMEM/F12 medium containing 2.5% FCS without antibiotics, and cells were placed in wells of a 96-well plate so that cells would be <30% confluent at the time of transfection. Eight picomoles of RACK1 siRNA pool or nontargeting siRNA pool (Dharmacon) and 0.3 μL of DharmaFECT 1 (Dharmacon) were diluted to 10 μL in Opti-MEM I medium and incubated for 5 minutes at room temperature. Then the diluted siRNA and DharmaFECT 1 were combined and incubated for 20 minutes at room temperature to allow transfection complexes to form. Eighty microliters of DMEM/F12 medium containing 2.5% FCS without antibiotics were added to the complexes (transfection mixture). The growth medium was removed from the cells and replaced with the transfection mixture, and the cells were incubated with the transfection mixture for 72 hours at 37°C with 5% CO2. At 72 hours, the transfection mixture was removed, and 100 μL of the dye loading solution (Fluo-4 NW Calcium Assay kit, Molecular Probes, Eugene, OR) were added to each well. Cells were incubated at 37°C for 30 minutes and then at room temperature for an additional 30 minutes. Fluorescence was measured using a Victor® plate reader (Perkin-Elmer, Waltham, MA) with instrument settings for excitation at 485 nm and emission at 535 nm.

Statistical Analysis
Variables were compared with either a Student t test or an independent-sampling 2-factor ANOVA. Statistical analysis was performed using the Number Cruncher Statistical System (Kaysville, UT), and all of the values in the text and figures refer to mean±SEM.

Results
Because the level of growth factors in the medium could alter the cellular expression of RACK1 binding partners, which could potentially alter the role of RACK1 in regulating growth, we conducted our initial studies in cells cultured under both high (2.5%) and low (0.1%) serum conditions. Accordingly, our first objective was to determine the feasibility of using RACK1 siRNA to reduce the expression of RACK1 under the cell culture conditions used in this study. Importantly, in PGVSMCs, RACK1 siRNA decreased RACK1 mRNA expression (as assessed by real-time PCR) by 70% (P=0.0014) and 87% (P=0.0001) in cells cultured under high and low serum conditions, respectively. Also, RACK1 siRNA reduced RACK1 protein expression by 67% (P=0.0003) and 45% (P=0.0345) in cells cultured under high (Figure 1A and 1B) and low (Figure 1A and 1C) serum conditions, respectively.

Having validated the ability of RACK1 siRNA to reduce the expression of RACK1, we next applied this approach to determine the role of RACK1 in modulating growth of PGVSMCs. In this regard, we first examined the effects of RACK1 siRNA on [3H]thymidine incorporation, because this index of DNA synthesis is a highly sensitive technique for assessing changes in cell growth. Importantly, in PGVSMCs cultured under low serum conditions (Figure 2A), RACK1 siRNA significantly reduced [3H]thymidine incorporation by 62% (P<0.0001).

This information provided confidence that cell counting experiments would be able to detect siRNA-induced changes
of the magnitude observed in the [3H]thymidine incorporation experiments, and from this point forward we used direct cell counting to assess cell proliferation, because this method is not subject to error introduced by aneuploidy. RACK1 siRNA significantly reduced cell counts in PGVSMCs by 55% (P<0.0001) and 65% (P<0.0001) when cells were cultured under low (Figure 2B) and high (Figure 2C) serum conditions, respectively. Because the results were similar when cells were cultured under high versus low serum conditions, in all of the subsequent proliferation studies we used high (2.5%) serum conditions to increase cell number and facilitate the detection of changes.

Because in some cell types RACK1 binds IP3Rs in the SR leading to increased IP3-induced release of SR calcium,6 we tested the hypothesis that RACK1 physically interacts with IP3Rs in PGVSMCs. In this regard, we performed coimmunoprecipitation experiments using an antibody to IP3Rs to immunoprecipitate proteins in PGVSMCs, followed by Western blotting and probing with a RACK1 antibody. As shown in Figure 3A, in PGVSMCs RACK1 strongly coimmunoprecipitated with IP3Rs. Moreover, using the Fluo-4 NW Calcium Assay Kit (Molecular Probes), we examined the effects of chronic RACK1 siRNA treatment on basal (steady-state) levels of intracellular calcium. These experiments revealed a significant (P=0.0225) reduction in intracellular calcium in RACK1-treated (n=25) versus control (n=26) PGVSMCs (6712±156 versus 7408±248, arbitrary fluorescence units in RACK1 siRNA versus control cells, respectively).

Because calmodulin mediates many of the signaling functions of intracellular calcium, we also investigated whether RACK1 scaffolds calmodulin. As shown in Figure 3B, coimmunoprecipitation experiments using an antibody to calmodulin to immunoprecipitate proteins in PGVSMCs, followed by Western blotting and probing with a RACK1 antibody, revealed that, in PGVSMCs, RACK1 strongly coimmunoprecipitated with calmodulin. Importantly, RACK1 was not detected in blots in which immunoprecipitation was performed with nonimmune serum (Figure 3C).

To further explore the hypothesis that RACK1 modulates proliferation of PGVSMCs via the IP3R/calcium/calmodulin pathway, we examined the effects of inhibition of IP3Rs with xestospongin C or 2-APB, depletion of SR calcium stores with cyclopiazonic, or inhibition of the calcium signaling transducer calmodulin with calmidazolium in control versus RACK1 siRNA-treated PGVSMCs. Xestospongin C, 2-APB, cyclopiazonic acid, and calmidazolium significantly (P<0.05) reduced cell proliferation (176 042±11 355, 118 229±7 556, 135 417±4 237 and 145 833±1 932 cells per well, respectively, versus 209 375±6 790 in control cells). Moreover, in the presence of these inhibitors, RACK1 siRNA had no additional effect on proliferation of PGVSMCs (Figure 4), suggesting a common mechanism of action of RACK1 siRNA and these inhibitors. In contrast to their effects on RACK1 siRNA, the inhibitors of calcium signaling did not affect the antiproliferative response to 2-chloroadenosine (data not shown), a metabolically stable analog of adenosine that inhibits vascular smooth muscle
cell proliferation via the adenosine A$_{2b}$ receptor by increasing the expression of p21$^{\text{Cip1}}$ (a negative growth regulator).\textsuperscript{22} These data suggest that the lack of growth inhibition by RACK1 siRNA in the presence of the inhibitors was not because of "nonspecific" interference with antiproliferative mechanisms.

Unlike 2-chloroadenosine (which increases the negative growth regulator p27$^{\text{Kip1}}$), RACK1 siRNA caused a compensatory decrease in the expression of the negative growth regulators p21$^{\text{Cip1/Wat1}}$ and p27$^{\text{Kip1}}$ (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). RACK1 siRNA did not affect the positive growth regulators cyclin D1/2 or hyperphosphorylated retinoblastoma protein (Figure S1).

GMCs, like PGVSMCs, are contractile cells that are similar to PGVSMCs and, like PGVSMCs, are involved in the pathophysiology of glomerulosclerosis. Therefore, we anticipated that RACK1 knockdown would have the same effects in GMCs as observed in PGVSMCs. As shown in Figure S2, RACK1 siRNA significantly decreased RACK1 mRNA expression in GMCs by 64% ($P<0.0001$) and decreased RACK1 protein expression in GMCs by 74% ($P=0.0036$). Consistent with our expectations, RACK1 siRNA reduced GMC proliferation by 45% ($P<0.0001$; Figure S2).

**Discussion**

The research field regarding the role of RACK1 in regulating cell proliferation is in its infancy, and only a small number of publications in this potentially important area exists. Moreover, even within the small number of available reports, the findings are contradictory. This heterogeneity of results is not surprising, however, given that RACK1 functions as a scaffolding protein and that the number of binding partners interacting with RACK1 is remarkably large.\textsuperscript{3} Predictably, RACK1 should inhibit or promote cell growth depending on the precise expression levels of progrowth versus antigrowth RACK1 binding partners in the cell, which would, in turn, depend on the type of cell under consideration and on the culture conditions. For example, in human colon carcinoma cells in which Src tyrosine kinase activity is elevated, RACK1 inhibits cell proliferation by binding to and inhibiting Src activity, thus inhibiting progression of the cell cycle at the G$_1$ and mitotic checkpoints.\textsuperscript{23,24} In human pulmonary artery smooth muscle cells in which type II bone morphogenetic protein receptor is critical for inhibiting cell proliferation, RACK1 reduces cell growth by binding to the type II bone morphogenetic protein receptor and enabling Smad-mediated signaling.\textsuperscript{25} In NIH 3T3 cells that express high levels of type 1 insulin-like growth factor receptors (IGF-IRs), RACK1 binds to IGF-IRs and alters signaling such that overexpression of RACK1 reduces IGF-IR–induced cell growth yet increases IGF-IR–induced cell spreading and formation of stress fibers and focal adhesions.\textsuperscript{26}

In contrast to the aforementioned negative effects of RACK1 on cell proliferation, in other cell types, RACK1 promotes cell growth. For example, in human ovarian cancer cell lines, RACK1 siRNA decreases stimulated colony formation,\textsuperscript{27} and this effect of RACK1 is most likely attributed to RACK1–mediated scaffolding of STAT3 to the insulin receptor and IGF-IR. Interestingly, the African trypanosome Trypanosoma brucei expresses a RACK1 homologue called TRACK, and depletion of TRACK using RNA interference blocks the onset and progression of cytokinesis in this protozoan to the point that inducing TRACK RNA interference in infected...
mice eliminates parasites from peripheral blood within 3 days postinfection.28

The results of the present study support the conclusion that RACK1 is a positive modulator of proliferation of PGVSMCs. The evidence for this conclusion is that RACK1 is highly expressed in PGVSMCs, and reduction of RACK1 expression inhibits PGVSMC proliferation. The role of RACK1 as a positive modulator of proliferation in PGVSMCs is independent of whether the cells are grown under low or high serum conditions. This is important because the level of growth factors in the medium would be expected to alter the cellular expression of RACK1 binding partners, which could potentially profoundly alter the role of RACK1 in regulating growth. The fact that RACK1 siRNA inhibits PGVSMC growth similarly in cells cultured under both low and high serum conditions indicates that in PGVSMCs RACK1 is likely to positively modulate cell proliferation under a variety of physiological and pathophysiological settings.

We also cloned rat RACK1 and attempted to overexpress RACK1 in PGVSMCs using a plasmid-based vector. However, the basal level of expression of RACK1 was so high in PGVSMCs that we were unable to increase RACK1 expression (assessed by Western blotting) more than high basal levels. Nonetheless, we did observe a 40% increase in proliferation of PGVSMCs by transfecting cells with a RACK1 plasmid, and this response was blocked by xestospongin C and cyclopiazonic acid. Given the high basal expression of RACK1 in PGVSMCs, increasing RACK1 further by overexpression would not be expected to inform the physiological roles of endogenous RACK1 in PGVSMCs.

The mechanism by which RACK1 regulates cell growth most likely involves modulation of calcium signaling. The evidence for this conclusion is as follows: (1) increased cytosolic calcium levels stimulate proliferation of vascular smooth muscle cells8–13 and other contractile cells, for example, mesangial cells29,30; (2) studies by Patterson et al6 demonstrate that, in some cell types, RACK1 physiologically binds IP₃Rs and modulates calcium release by augmenting IP₃R binding affinity for IP₃; (3) in PGVSMCs, IP₃R and RACK1 also physically interact; (4) in PGVMSCs, RACK1 siRNA decreases basal (steady-state) levels of calcium; and (5) in PGVSMCs, inhibition of IP₃Rs (with xestospongin C or 2-APB) or depletion of SR calcium stores (with cyclopiazonic acid) or inhibition of calmodulin (with calmidazolium) decreases proliferation and blocks any further effects of RACK1 siRNA. These results are consistent with the hypothesis that RACK1 influences the proliferation of PGVSMCs via the IP₃R/calcium/calmodulin pathway.

Calmodulin binds calcium, and the calcium-calmodulin complex binds to and alters the activity of many downstream effector proteins. Thus, calmodulin is a critically important signal transduction component in calcium signaling. Our study is the first to show that RACK1 not only interacts with IP₃Rs in PGVSMCs but also binds to calmodulin. Although not investigated here, it is conceivable that RACK1 modulates the activity of calmodulin, for example, by positioning calmodulin near IP₃Rs for efficient calcium loading or near downstream effector molecules for efficient modulation of their activity. Thus, the regulation by RACK1 of the IP₃R/calcium/calmodulin pathway may involve multiple sites and mechanisms of action.

Recently we discovered that adenosine A₂B receptors inhibit growth of vascular smooth muscle cells by upregulating the expression of p27Kip1, a cell-signaling factor that inhibits the actions of cyclin-dependent kinases and, thus, restricts cell proliferation by impairing progression through the cell cycle.22

To test whether RACK1 siRNA triggers a similar mechanism, we examined in the current study the effects of RACK1 siRNA on the expression of not only p27Kip1 but also p21Cip1/Waf1, another inhibitor of cyclin-dependent kinases. Our results show that RACK1 siRNA downregulates, rather than upregulates, the expression of both p27Kip1 and p21Cip1/Waf1. Because downregulation of p27Kip1 and p21Cip1/Waf1 would be expected to increase, rather than decrease, cell proliferation, it appears that the change in expression of p27Kip1 and p21Cip1/Waf1 is a compensatory response to the inhibitory effects of RACK1 siRNA. We did not observe any effects of RACK1 siRNA on the expression of cyclin D1/2 or hyperphosphorylated retinoblastoma protein, which rules out an involvement of these key cell cycle regulators in the growth-modulatory effects of RACK1 in PGVSMCs.

The present study addresses the role of RACK1 as a regulator of microvascular smooth muscle cell proliferation. As with PGVSMCs, RACK1 also promotes the proliferation of GMCs. This is not surprising, because GMCs are phenotypically similar to PGVSMCs and can be viewed as the “contractile smooth muscle cell” of the glomerular vessels. In preliminary studies, we also examined the effects of RACK1 on proliferation of macrovascular smooth muscle cells including rat and human aortic and human coronary artery vascular smooth muscle cells. Importantly, in macrovascular smooth muscle cells, RACK1 siRNA did not inhibit cell proliferation yet did reduce RACK1 mRNA expression. These data suggest that the proliferative effects of RACK1 may be restricted to smooth muscle cells from resistance arteries.

Because RACK1 is involved in calcium signaling in microvascular smooth muscle cells and because calcium mediates smooth muscle cell contraction, it is conceivable that RACK1 also regulates microvascular tone and arterial blood pressure, and this hypothesis is under investigation. Interestingly, in preliminary studies, we detected RACK1 protein in the membrane, cytosolic, nuclear, and cytoskeletal fractions of PGVSMCs, and the expression levels were 2.5-fold higher in the membrane fraction of cells derived from spontaneously hypertensive rats versus normotensive Wistar-Kyoto rats. This finding is consistent with the fact that genetically hypertensive rats have increased renovascular tone, and PGVSMCs from spontaneously hypertensive rats proliferate more rapidly in culture.

Perspectives

Despite the growing awareness of the importance of RACK1 in cell biology,2,5 the roles of RACK1 in regulating the function, structure, and proliferation of renal microvascular smooth muscle cells are unknown. Indeed, as far as we know, this is the first report of the role of RACK1 in regulating the biology of microvascular smooth muscle cells from any source. Importantly, the present study indicates that RACK1 promotes proliferation of the preglomerular renal microvas-
cular smooth muscle cell and its phenotypically related cousin, the glomerular mesangial cell. Proliferation of renal preglomerular microvascular smooth muscle cells would be expected to increase resistance to blood flow and, therefore, could reduce renal blood flow and kidney function and increase systemic blood pressure. Moreover, proliferation of both preglomerular microvascular and glomerular mesangial cells is involved in the pathophysiology of hypertensive and diabetic renal diseases, as well as in other types of nephropathies. Therefore, RACK1 inhibitors may prove to be a new class of cardiorenal drugs.

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Disclosures
None.

References
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RACK1 REGULATES CELL PROLIFERATION BY MODULATING CALCIUM SIGNALING

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Figure S1

A. Expression of p21 protein normalized to β-actin protein. No FCS vs FCS/Control vs FCS/RACK1 siRNA. Optical density ratio (% of No FCS) for p21: No FCS: 100, FCS/Control: 150, FCS/RACK1 siRNA: 70. p<0.05.

B. Expression of p27 protein normalized to β-actin protein. No FCS vs FCS/Control vs FCS/RACK1 siRNA. Optical density ratio (% of No FCS) for p27: No FCS: 100, FCS/Control: 90, FCS/RACK1 siRNA: 70. p<0.05.

C. Expression of Cyclin D1/2 protein normalized to β-actin protein. No FCS vs FCS/Control vs FCS/RACK1 siRNA. Optical density ratio (% of No FCS) for Cyclin D1/2: No FCS: 100, FCS/Control: 150, FCS/RACK1 siRNA: 100.

D. Expression of ppRb protein normalized to pRb protein. No FCS vs FCS/Control vs FCS/RACK1 siRNA. Optical density ratio (% of No FCS) for ppRb: No FCS: 100, FCS/Control: 150, FCS/RACK1 siRNA: 100.
**Figure S1.** Western blots in panels A-D illustrate effects of RACK1 siRNA (FCS/RACK1 siRNA) versus negative control siRNA (FCS/Control) on the expression in preglomerular vascular smooth muscle cells of p21<sup>Cip1/Waf1</sup> (p21; panel A), p27<sup>Kip1</sup> (p27; panel B), cyclin D1/2 (panel C) and hyperphosphorylated retinoblastoma protein (ppRb; panel D) in the presence of 2.5% fetal calf serum (FCS) in the medium. The protein expression of p21, p27 and cyclin D1/2 was normalized to β-actin protein expression by calculating the optical density ratio (optical density of p21, p27 or cyclin D1/2 band divided by optical density of β-actin band). The protein expression of ppRb was normalized to pRb protein expression by calculating the optical density ratio (optical density of ppRb band divided by optical density of pRb band). The optical density ratio for each protein was expressed as a % of the average optical density ratio for cells in the absence of FCS (No FCS). Values represent means ± SEM, and p-values (unpaired Student’s t-test) compare FCS/RACK1 siRNA to FCS/Control.
**Figure S2**

**A**

GMCs

- RACK1 siRNA
- Negative control siRNA

[Image of Western blot for RACK1 and β-actin]

**B**

GMCs

- Expression of RACK1 Protein Normalized to β-Actin Protein

- Optical Density (% of Control)

- Control vs. RACK1 siRNA

- p=0.0036

- (n=4)

- Cell Number (% of Control)

- Control vs. RACK1 siRNA

- (n=12)

- p<0.0001
Figure S2. Panel A shows Western blots demonstrating the effects of RACK1 siRNA versus negative control siRNA on RACK1 protein expression in glomerular mesangial cells (GMCs). For both negative control siRNA-treated cells (Control) and RACK1 siRNA-treated cells (RACK1 siRNA) the RACK1 protein expression was normalized to β-actin protein expression by calculating the optical density ratio (optical density of RACK1 band divided by optical density of β-actin band). In panel B, the optical density ratio for RACK1 siRNA cells was expressed as a % of the average optical density for the Control cells. Panel C depicts effects of RACK1 siRNA versus negative control siRNA (Control) on cell number in GMCs under high serum (2.5%) conditions. Cell number for RACK1 siRNA cells was expressed as a % of the average cell number for the Control cells. The cell count for control GMCs was 396,875. Values represent means ± SEM, and p-values are from unpaired Student’s t-test.