Regulator of G-Protein Signaling 5 Prevents Smooth Muscle Cell Proliferation and Attenuates Neointima Formation

Jan-Marcus Daniel,* André Prock,* Jochen Dutzmann,* Kristina Sonnenschein, Thomas Thum, Johann Bauersachs, Daniel G. Sedding

Objective—Regulator of G-protein signaling 5 (RGS5) is abundantly expressed in vascular smooth muscle cells (SMCs) and inhibits G-protein signaling by enhancing the guanosine triphosphate–hydrolyzing activity of Gα-subunits. In the present study, we investigated the effects of RGS5 on vascular SMC function in vitro and neointima formation after wire-induced injury in mice and determined the underlying mechanisms.

Approach and Results—We found a robust expression of RGS5 in native arteries of C57BL/6 mice and a highly significant downregulation within neointimal lesions 10 and 21 days after vascular injury as assessed by quantitative polymerase chain reaction, immunoblotting, and immunohistochemistry. In vitro, RGS5 was found significantly downregulated after mitogenic stimulation of human coronary artery SMCs. To restore RGS5 levels, SMCs were transduced with adenoviral vectors encoding wild-type RGS5 or a nondegradable mutant. RGS5-WT and, even more prominently, the C2A-RGS5 mutant prevented SMC proliferation and migration. Following overexpression of RGS5, FACS analysis of propidium iodide–stained cells indicated cell cycle arrest in G0/G1 phase. Mechanistically, inhibition of ERK1/2 phosphorylation and MAPK downstream signaling was shown to be responsible for the anti-proliferative effect of RGS5. Following wire-induced injury of the femoral artery in C57BL/6 mice, adenoviral-mediated overexpression of RGS5-WT or C2A-RGS5 significantly reduced SMC proliferation and neointima formation in vivo.

Conclusions—Downregulation of RGS5 is an important prerequisite for SMC proliferation in vitro and in vivo. Therefore, reconstitution of RGS5 levels represents a promising therapeutic option to prevent vascular remodeling processes. (Arterioscler Thromb Vasc Biol. 2016;36:00-00. DOI: 10.1161/ATVBAHA.115.305974.)

Key Words: neointima ■ proliferation ■ signal transduction ■ vascular remodeling ■ vascular smooth muscle

The proliferation of vascular smooth muscle cells (SMCs) represents a key mechanism for the progression of vascular proliferative diseases, such as atherosclerosis, post-angioplasty restenosis, or vein bypass graft failure.1 Excessive neointimal hyperplasia after percutaneous transluminal angioplasty induces restenosis, which often necessitates a secondary revascularization procedure.2 During neointima formation, SMCs proliferate in response to mitogenic stimuli and migrate toward the luminal side of the artery.3 Given the high clinical relevance of vascular proliferative diseases, new and selective treatment strategies can only be based on a detailed understanding of the signaling mechanisms involved in SMC activation.4,5

Regulator of G-protein signaling 5 (RGS5) is a member of the family of RGS molecules, which regulate the signal transduction of G-protein–coupled receptors (GPCRs) and G-proteins.4 In nonactivated cells, G-proteins consist of a guanosine diphosphate–bound G protein and its associated Gβγ heterodimer. Activators of GPCRs, such as angiotensin II, induce the exchange of guanosine diphosphate to guanosine triphosphate (GTP) on the Gα subunit, which induces dissociation and further G-protein downstream signaling.7 This signaling cascade is limited by the intrinsic GTP-hydrolyzing activity of the Gα subunit. RGS5 acts as a GTPase-activating protein of the Gαq and Gαi subunits and thus effectively suppresses the GPCR-induced downstream signaling of well-known vasoconstrictors and pro-mitogenic molecules, such as angiotensin II, endothelin-1, norepinephrine, or thrombin.8

Initially, RGS5 was found to be specifically expressed in the SMCs of the aorta and peripheral arteries and in pericytes.9,10 More recent studies in mice lacking RGS5 expression...
have shown that a loss of RGS5 leads to hypertension, vessel hypertrophy, and vascular stiffening and correlates with hyper-responsiveness to angiotensin II, endothelin-1, norepinephrine, and thrombin, suggesting that RGS5 plays a pivotal role in attenuating the effects of the aforementioned vasoconstrictors. Inasmuch as these vasoconstrictors also drive SMC growth, RGS5 may also function as an inhibitor of chronic trophic signals within the artery wall. Moreover, RGS5 expression was downregulated in SMCs of atherosclerotic plaques, and platelet-derived growth factor-BB as one of the most critical mediators of SMC proliferation was shown to acutely repress RGS5 expression in SMCs.

RGS5 expression is also tightly controlled on the post-transcriptional level via enhanced degradation through the ubiquitin-dependent N-end rule pathway. The Cys-2 residue at the N-terminus of RGS5 is essential for this degradation, and mutants of RGS5 in which this residue cannot reside at the N-terminus (C2A mutant) are stable and long-lived in vivo because of the lacking degradation. The activity of this pathway is controlled by oxygen levels, reactive oxygen species, nitric oxide, and inflammatory cytokines. Because these mediators are largely perturbed after vascular injury, the N-end rule pathway most likely affects the turnover and expression of RGS5 during vascular remodeling processes.

In apolipoprotein E−/− mice lacking RGS5 expression, atherosclerotic plaque progression was increased, which correlated with a more pronounced endothelial dysfunction and enhanced vascular inflammation through NF-κB activation.

In contrast, non-systematic data are currently available regarding the effects of RGS5 on SMC proliferation in vitro or on the SMC proliferation during neointimal lesion formation in vivo. Moreover, initial observations in non-human primates have suggested that RGS5 is only expressed in aortic or peripheral artery SMCs but not in coronary artery SMCs as assessed by in situ hybridization. In contrast, we now provide evidence that RGS5 is also abundantly expressed and strongly regulated in human coronary artery SMCs. Our data indicate that RGS5 expression is controlled at multiple levels because mitogenic stimulation reduces RGS5 mRNA expression, and posttranslational RGS5 protein expression is tightly controlled by a high turnover rate and degradation via the N-end rule pathway and proteosomal degradation. We further demonstrate that reconstitution of RGS5 prevents the angiotensin II–induced activation of G-protein signaling, resulting in diminished phosphorylation of the downstream signaling molecules MEK1 and ERK1/2. Reconstitution of downregulated RGS5 levels prevents SMC proliferation after mitogenic stimulation in vitro and neointima formation in a mouse model of wire-induced vascular injury in vivo. These data conclusively show that the tightly and multilevel redundantly controlled downregulation of RGS5 represents an important prerequisite for the proliferative response of SMCs following vascular injury and that the reconstitution of RGS5 expression levels is sufficient to prevent negative vascular remodeling processes.

Materials and Methods

Materials and methods are available in the online-only Data Supplement.

Results

RGS5 mRNA Expression Levels in SMCs In Vitro and in Neointimal Lesions In Vivo

To evaluate RGS5 expression levels in an in vivo model of neointima formation, we performed wire-induced injury of murine femoral arteries and analyzed RGS5 expression levels at different time points thereafter. As indicated by co-staining for α-smooth muscle actin and Ki-67, the proliferating cells in the neointimal lesions at 10 and 21 days after injury almost exclusively represent proliferating SMCs (Figure 1A, upper 2 panels). RGS5 expression could be located in the medial SMCs of native, uninjured mouse femoral arteries, and compared with uninjured arteries, the endogenous RGS5 expression was downregulated 10 and 21 days after vascular injury as assessed by immunohistochemistry (Figure 1A). Quantitative polymerase chain reaction analysis of RGS5 mRNA expression in the neointimal lesions indicated a significant downregulation of RGS5 10 and 21 days after vascular injury (10 days: 0.37±0.134 vs CTRL, *P<0.05; 21 days: 0.46±0.186 vs CTRL, *P<0.05 n=4; Figure 1B). Concomitantly, RGS5 protein levels were significantly downregulated 21 days after vascular injury, as quantified by immunoblotting (Figure 1C; *P<0.05, n=3).

In vitro, we detected strong RGS5 mRNA and protein expression levels in human coronary artery SMCs. RGS5 mRNA levels were significantly downregulated at 12 and 24 hours after mitogenic stimulation with growth medium as assessed by quantitative polymerase chain reaction (Figure 1D; 12 hours: 0.69±0.012 vs serum-free growth medium, *P<0.05; 24 hours: 0.38±0.019 vs serum-free growth medium, *P<0.05, n=4). Significant downregulation of RGS5 protein expression levels was also observed at 12 and 24 hours after mitogenic stimulation with growth medium (Figure 1E; *P<0.05, n=3).

RGS5 Protein Expression Is Tightly Controlled by a High Turnover Rate and by Degradation via the N-End Rule Pathway

To reconstitute the downregulated RGS5 levels under mitogenic conditions to assess the impact of RGS5 on SMC function, we transduced SMCs with an adenoviral vector encoding the WT form of RGS5. We detected enhanced expression of the recombinant RGS5-WT at 48 hours post-transduction as assessed by immunoblotting using a specific antibody for the C-terminally added V5-tag (Figure 2A). However, the expression of RGS5-WT protein was rather weak and of short duration (Figure 2B; downregulation after 72 hours). In contrast, the expression levels of recombinant
RGS5-WT were robustly enhanced in the presence of the proteasome inhibitor MG132 (Figure 2A), indicating that RGS5 expression is tightly controlled by a rapid turnover and posttranslational proteasomal degradation. Therefore, to consistently assess the functional effects of RGS5 reconstitution, we cloned a mutant form of RGS5, in which the C2 residue cannot become N-terminal (C2A mutant) and thus is resistant to N-end rule-dependent proteasomal degradation (Figure I in the online-only Data Supplement).

Indeed, following overexpression, robustly enhanced and stable expression levels of this C2A-RGS5 mutant were detected compared with the recombinant RGS5-WT form (Figure 2B). Moreover, expression levels of the recombinant C2A-RGS5 mutant were similar to the expression levels of the recombinant RGS5-WT form in the presence of the proteasome inhibitor MG132, indicating that the C2A mutation effectively prevents the proteasomal degradation of RGS5 (Figure 2C).

Figure 1. Regulator of G-protein signaling 5 (RGS5) is downregulated in proliferating smooth muscle cells (SMCs) in vitro and during neointima formation in vivo. A, Representative cross sections of mouse femoral arteries at the given time points after injury are shown (H&E staining). Co-staining of α-smooth muscle actin (α-SMA) and Ki-67 identifies proliferating SMC (second panel). The 4 lower panels show RGS5 expression in SMC at the given time points after injury. B, Quantitative polymerase chain reaction (qPCR) was performed to evaluate RGS5 expression in vivo (*P<0.05, n=4). C, Dilated mouse femoral arteries were excised 21 d after injury, and RGS5 protein expression levels were quantified and compared with uninjured arteries by immunoblotting (*P<0.05, n=3). D, PCR and real-time qPCR using specific primers to detect RGS5 mRNA expression levels were performed in human coronary artery SMCs under basal conditions and at the indicated time points after mitogenic stimulation with growth medium (*P<0.05, n=4). E, Immunoblotting was performed using specific antibodies to evaluate RGS5 protein expression levels in human coronary artery SMCs under basal conditions and at the indicated time points after mitogenic stimulation with growth medium (*P<0.05, n=3).
RGS5 Prevents SMC Proliferation and Migration In Vitro

To evaluate the functional effects of RGS5 on the proliferation and migration of SMCs in vitro, human coronary artery SMCs were transduced with adenoviral vectors containing RGS5-WT (Ad-RGS5-WT), the nondegradable mutant (Ad-C2A-RGS5), or a control vector (Ad-empty). At 24 hours after transduction, we detected high and dose-dependent mRNA expression levels of RGS5-WT or C2A-RGS5 in SMCs as assessed by quantitative polymerase chain reaction (n=4; Figure IIA in the online-only Data Supplement).

RGS5-WT overexpression significantly reduced SMC proliferation as assessed by bromodeoxyuridine incorporation after stimulation with growth medium (***P<0.001, n=4). This effect was dose-dependent and significantly stronger after overexpression of the nondegradable mutant C2A-RGS5 (***P<0.001, n=4; Figure 2D). Furthermore, RGS5-WT overexpression reduced SMC migration significantly as assessed by a modified Boyden chamber assay (**P<0.005; ***P<0.001, n=4; Figure 2E). However, this effect was already maximal at the lowest viral transfection titers and was not further enhanced after overexpression of the nondegradable mutant C2A-RGS5 (Figure 2E).
Then, we performed successful knockdown of RGS5 in SMCs using specific siRNA (Figure II in the online-only Data Supplement). Following siRNA-mediated knockdown, SMC proliferation was significantly increased as assessed by bromodeoxyuridine incorporation (**P<0.001, n=3; Figure 2F). Following RGS5 knockdown, SMC proliferation also increased significantly already under basal conditions, indicating that RGS5 may also be involved in the control of SMC function under nonstimulated conditions (**P<0.001, n=3; Figure 2F).

In contrast, overexpression of RGS5 had no effect on apoptosis of SMC, as assessed by TUNEL-based ELISA (P=ns; n=4; Figure III in the online-only Data Supplement).

RGS5 Prevents Cell Cycle Entry/Progression in G0/G1 Phase

To further elucidate the mechanisms responsible for the anti-proliferative effect of RGS5, we determined cell cycle progression and the expression of cell cycle–regulating proteins following overexpression or knockdown of RGS5. To achieve strong and constant expression levels and to assure robust inhibition of proliferation, we used the nondegradable mutant C2A-RGS5. As determined by FACS analysis of propidium iodide–stained cells, C2A-RGS5 overexpression (MOI=120) prevented cell cycle entry/progression in G0/G1 phase, indicating that RGS5 interferes with early processes of cell cycle entry/progression (*P<0.05, n=4; Figure 3A and 3B). RGS5 overexpression resulted in decreased protein levels of cyclin D1, CDK1, and proliferating cell nuclear antigen, which are essential for progression through G0/G1 phase, as assessed by immunoblotting. An arrest in G0/G1 phase was also confirmed by the lack of hyper-phosphorylation of the retinoblastoma gene product (Figure 3C). The concerted prevention of the upregulation/activation of molecules controlling cell cycle entry suggests that RGS5 may not affect the expression or function of a single cell cycle regulatory protein but rather may interfere with upstream signaling mechanisms that orchestrate early cell cycle entry.

RGS5 Inhibits Activation of MEK/ERK Signaling

Because the MEK/ERK signaling pathway is essential for cell cycle entry and is described to be regulated by the RGS5-protein family, we focused on the effects of RGS5 on the G-protein receptor–induced activation of this signaling pathway. The stable reconstitution of RGS5-WT and of the RGS-protein family, we focused on the effects of RGS5 on the G-protein receptor–induced activation of this signal-

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RGS5 Overexpression Prevents Proliferation of Neointimal SMCs Following Vascular Injury

To determine whether the in vitro results regarding the anti-proliferative effects of RGS5 reconstitution in SMCs are reproducible in vivo, we performed a wire-induced injury of the femoral artery in C57BL/6 mice. The administration of adenoviral vectors was performed extraluminally to the dilated artery in a thermosensitive and self-degrading Pluronic F-127 gel. Successful transduction and effective expression of recombinant RGS5 in medial cells were demonstrated by immunohistochemistry 4 days after injury (Figure 5A) and by Western blot analysis 7 days after injury (Figure 5B). In support of the in vitro observations, overexpression of RGS5-WT or C2A-RGS5 resulted in reduced ERK phosphorylation 7 days after injury (Figure 5B). The arteries were harvested 21 days after injury, and the proliferation of neointimal cells was significantly reduced after infection with Ad-RGS5-WT compared with vessels infected with the control vector alone, as assessed by the evaluation of the number of proliferating cell nuclear antigen–positive cells/all cells (10.4%±0.5% vs 7.07%±0.67%; *P<0.05, n=6). The reduction of the proliferation of neointimal cells was even more pronounced after application of the nondegradable mutant Ad-C2A-RGS5 (10.4%±0.5% vs 5.87%±0.41%; *P<0.05, n=6; Figure 5C and 5D).

RGS5 Overexpression Prevents Neointimal Lesion Formation and Luminal Stenosis

To evaluate the effect of RGS5 on neointimal lesion formation and luminal stenosis after vascular injury, mouse femoral arteries were excised 21 days after dilation, and morphometric analysis was performed. The administration of Ad-RGS5-WT resulted in a significant reduction in the neointima/media ratio, neointimal lesion size, medial size, and luminal stenosis but significantly increased the remaining lumen of injured vessels (**<P<0.0001, n=6; Figure 6A and 6B). Similar to its effects in vitro and on SMC proliferation in vivo, there was a trend towards an even more pronounced effect of the nondegradable mutant C2A-RGS5 on the reduction of neointimal lesion formation 21 days after vascular injury, which, however, just missed statistical significance (luminal stenosis P=0.052, n=6; Figure 6A and 6B).

C2A-RGS5 Overexpression Improves Re-Endothelialization

Following wire-induced injury, re-endothelialization of the dilated arteries was primarily complete 21 days after wire injury, and no differences were observed between the treatment groups (Figure 6C and 6D). Furthermore, early endothelial recovery was assessed by en face microscopy 5 days after electric injury of the carotid artery. At that time point, overexpression of Ad-RGS5-WT did not show a significant effect on re-endothelialization (P=ns, n=6). In contrast, endothelial recovery was slightly enhanced after application of the nondegradable mutant C2A-RGS5 (*P<0.05, n=6; Figure 6E and 6F).
Discussion

G-protein–coupled signaling pathways and ERK activation are known to be of paramount importance for the phenotypic plasticity of SMCs. Extracellular stimuli, such as hormones, neurotransmitters, and chemokines, exert their effects on SMCs by binding to GPCRs. RGS5 antagonizes the G-protein–induced signal transduction by its intrinsic GTP-hydrolyzing activity of the G\(\alpha\)(q) and G\(\alpha\)(i) subunits and thus modulates the subsequent signaling and cellular processes. However, the role of RGS5 in the response of SMC to acute vascular injury or during vascular remodeling remains insufficiently defined. In this study, we provide evidence that RGS5 is abundantly expressed in human coronary artery SMCs and that its expression is downregulated in both proliferating SMCs in vitro and in a model of neointima formation in vivo. The reconstitution of RGS5 expression levels prevents the activation of the MEK/ERK signaling cascade, which is essential for the induction of cell cycle entry. The reconstitution of RGS5 results in the downregulation of cyclin D1, proliferating cell nuclear antigen, and CDK1 and prevents the G0/G1/S phase transition of proliferating SMCs. RGS5 overexpression not only significantly reduces SMC proliferation in vitro but also is effective in attenuating SMC proliferation and neointima formation in vivo following wire-induced vascular injury.
RGS5 is known to be expressed in endothelial cells, SMCs, and pericytes of the vasculature. However, mice lacking RGS5 expression are viable and do not exhibit developmental defects or defects in vasculogenesis. In contrast, RGS5 deletion results in accelerated atherosclerotic lesion progression in apo-lipoprotein E−/− mice related to endothelial dysfunction because of higher rates of apoptotic endothelial cells and macrophages within the lesions. These data suggest a protective role of RGS5 in endothelial cells; however, the effect of RGS5 on SMC function during vascular remodeling remains to be defined.

Our data now indicate that RGS5 is highly expressed in differentiated SMCs and that RGS5 expression is tightly controlled at multiple levels, including an altered mRNA expression and a rapid turnover and proteasomal degradation. Conclusively, previous studies reported that RGS5 can be regulated at the transcriptional level via platelet-derived growth factor.
factor–dependent signaling and at the post-translational level via enhanced N-end rule pathway–mediated proteosomal degradation. Interestingly, these signaling cascades have been shown to be crucially involved in vascular remodeling processes. Thus, the tightly and redundantly controlled regulation of RGS5 expression points toward a prominent role of RGS5 in SMC homeostasis and in SMC responses and adaptions to vascular injury.

In an initial study on non-human primates, Li et al described that RGS5 is downregulated in SMCs of atherosclerotic lesions compared with medial SMCs. However, the same study reported a lack of RGS5 expression in the coronary arteries of these primates as determined by in situ hybridization. In contrast, we found robust RGS5 mRNA and protein expression in human coronary artery SMCs and observed profound downregulation of RGS5 expression in proliferating SMCs in vitro and in vivo. These data indicate that RGS5 may also have a role in the development of coronary artery disease and restenosis after percutaneous coronary intervention.

Here, we provide evidence that RGS5 inhibits early cell cycle progression during G0/G1 phase before the cells begin to enter the cell cycle. Therefore, we hypothesize that RGS5 interferes with the upstream signaling that regulates G0/G1 phase entry/progression instead of regulating the specific

Figure 5. Regulator of G-protein signaling 5 (RGS5) overexpression inhibits vascular cell proliferation in vivo. A and B, Successful adenoviral transduction and expression of recombinant RGS5 in medial cells was assessed by immunohistochemistry using specific antibodies (V5, green staining) and by immunoblotting (V5). β-Tubulin served as a loading control. The densitometric analysis of the respective immunoblots is shown in the right panel. C, Representative cross sections of femoral arteries treated with the indicated vectors are stained for proliferating cell nuclear antigen (PCNA; brown) or hematoxylin (blue) 21 d after injury. D, The numbers of proliferating (PCNA-positive) cells within the neointima and media were determined by dividing the number of PCNA-positive cells per section by the total cell number of cells per section (*P<0.05, n=6). WT indicates wild-type.
expression of single, distinct cell cycle regulating molecules. Because we previously demonstrated that ERK activation is essential for the proper cell cycle entry and proliferation of vascular SMCs, we assessed the ability of RGS5 to interfere with this signaling pathway. Indeed, our data clearly show that RGS5 prevents phosphorylation of ERK after mitogenic stimulation and that after rescuing this inhibition by overexpressing a constitutive active MEK-mutant, the growth inhibitory effect of RGS5 is abolished. Thus, the inhibition of MEK/ERK activation identifies a key mechanism by which RGS5 regulates cell cycle entry and proliferation.

Because GPCRs regulate a wide variety of cellular functions in response to extracellular stimuli, other signaling pathways than the MEK/ERK signaling cascade may also be influenced by RGS5. A study on the effects of RGS5 on blood pressure homeostasis showed that Rho kinase signaling is responsible for the observed elevated blood pressure levels in RGS5-deficient mice. Importantly, these mice also showed characteristics of increased arterial stiffness and medial thickness because of SMC hypertrophy. RGS5 was also recently shown to repress the signaling of sonic hedgehog, a further important mediator of SMC differentiation and function. Moreover, platelet-derived growth factor–induced stimulation of SMC after knockout of the transcription factor CAMP response element modulator resulted in different expression levels of RGS5. Thus, in addition to preventing ERK-mediated proliferation and migration, RGS5 also controls other signaling mechanisms, which regulate different functions like differentiation, contraction, or hypertrophy, which may also contribute to the observed in vivo effects.

To more closely resemble the in vivo situation, we exposed the SMCs to growth medium, which contains a mix of FCS, as well as different growth factors. However, even after this redundant activation of multiple signaling cascades, the inhibition of MEK/ERK signaling by RGS5 was sufficient to prevent SMC proliferation. In conclusion with previous reports, these data indicate that (1) MEK/ERK signaling is a mandatory prerequisite for the cell cycle entry of SMC and that (2) interfering with this pathway, that is, by controlling RGS5 expression levels, is sufficient to prevent SMC proliferation even under redundant mitogenic stimulation of SMCs (Figure V in the online-only Data Supplement).

SMC migration is a further important component, which contributes to neointima formation. However, data regarding the effects of RGS5 overexpression/reconstitution were...
lacking. Here we demonstrate that overexpression of RGS5 significantly prevents the migration of human coronary artery SMCs. The prevention of cell migration by RGS5 may thus additionally contribute to reduced lesion formation in vivo. Mechanistically, it has been repeatedly demonstrated that ERK activation plays a crucial role in SMC migration. However, the observed antimitrotrophic effect of RGS5 may also be a rather unspecific and general response to cell cycle arrest because cell cycle inhibition not only prevents cellular proliferation but also modulates numerous cellular functions, such as differentiation, inflammation, and migration. Nevertheless, the specific role of RGS5 in the regulation of cell migration requires further investigation.

Re-endothelialization of vascular lesions is important to prevent an acute thrombotic (re-)occlusion to attenuate the inflammatory response by preventing the continuous adhesion and invasion of circulating cells and to restore the vascular integrity and reactivity via the release of nitric oxide. Moreover, the combination of these effects is sufficient to significantly attenuate exaggerated SMC proliferation and neointima formation. Notably, we detected enhanced endothelial recovery following application of the nondegradable mutant C2A-RGS5. Importantly, previous studies reported a protective effect of RGS5 on endothelial function and endothelial cells survival, leading to increased atherosclerosis in RGS5−/− mice. In summary, these findings suggest that the specific role of RGS5 in the regulation of cell migration requires further investigation.

Taken together, we describe for the first time and provide cumulative evidence that RGS5 is an important negative regulator of the proliferative and migratory response of SMCs following acute vascular injury. Mechanistically, we demonstrate that the inhibition of MEK/ERK signaling and thus the control of early cell cycle entry is responsible for this effect. Because overexpression of RGS5 was able to prevent SMC proliferation and neointima formation without affecting re-endothelialization in a mouse model of vascular injury, RGS5 might represent a promising target for future therapeutic strategies to prevent vascular proliferative diseases.

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Disclosures

None.

References

The proliferation of vascular smooth muscle cells represents a key mechanism for the progression of vascular proliferative diseases. The regulator of G-protein signaling 5 (RGS5) is a negative regulator of G-protein–coupled receptor signaling. In this study, we describe for the first time that RGS5 is highly expressed in human coronary artery smooth muscle cell and is rapidly downregulated after mitogenic stimulation in vitro or after vascular injury in vivo. We provide cumulative evidence that RGS5 is a potent negative regulator of the proliferative and migratory response of smooth muscle cells in vitro and in vivo. Mechanistically, the inhibition of MEK/ERK-signaling and thus the control of early cell cycle entry is responsible for this effect. Because adenoviral overexpression of RGS5 is able to prevent smooth muscle cell proliferation and neointima formation without affecting re-endothelialization in a mouse model of vascular injury, RGS5 might represent a promising target for future therapeutic strategies to prevent vascular proliferative diseases.
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Materials and Methods

Cell culture and quantification of SMC proliferation, migration and apoptosis
Human coronary artery SMCs were purchased from Lonza (Cologne, Germany), and the cells were cultured in optimized growth medium (Lonza, SmGM™-2) supplemented with 5% FBS, epidermal growth factor, fibroblast growth factor-B, plateled-derived growth factor-BB and insulin. Cells between passages 3 and 6 were used for all experiments. Quantification of SMC proliferation was performed by a Bromodeoxyuridine (BrdU) assay after stimulation with SmGM as described previously according to the manufacturer’s instructions (Cell Proliferation ELISA, Roche, Mannheim, Germany). Migration assays were performed using 0.1% gelatin-coated Transwell 12-well tissue culture inserts with 8-µm pores (Corning Costar Corp, Koolhovenlaan, The Netherlands). Briefly, 5×10⁴ SMCs in 0.1 ml basal medium (Lonza, SmBM™) were added to the upper chamber, and SmGM was added to the lower chamber. The inserts were incubated for 6 h at 37 °C in an atmosphere containing 5% CO₂. Cells on the upper surface were removed with a cotton swab. Then, WST-1 dye (diluted 1:10) was added to each lower chamber and each insert, now containing only the migrated cells at the lower site, and was incubated for 4 h at 37 °C. The number of viable cells at the end of the treatment was determined by measuring the absorbance of the supernatant at a wavelength of 450 nm. For detection of apoptosis, SMCs were incubated in SmBM for 72 h at 37 °C in an atmosphere containing 5% CO₂ following transduction with the respective constructs. Apoptosis detection was performed by a TUNEL-assay (ELISA cell death detection assay, Roche) according to the manufacturer’s instructions.

Adenovirus production and infection procedure
RGS5-WT (wild-type), C2A-RGS5 mutant (a mutant that is resistant to degradation by the N-end rule pathway), and MEK1DD (S218D/S222D, DD) cDNA, encoding a constitutively active MEK1, was de novo synthesized, sequence-verified and cloned into pAd-CMV-Dest-V5 vectors by GeneArt (Life Technologies, Darmstadt, Germany). Recombinant RGS5-WT C2A-RGS5 and MEK1DD adenoviruses were generated as described previously. Recombinant adenoviral DNA that was isolated from kanamycin-resistant colonies was amplified in TOP10 cells (Invitrogen, Karlsruhe, Germany), purified by CsCl₂ density gradient centrifugation, linearized with PacI, and transfected into 293 cells using Lipofectamine (Life Technologies). After 7 to 10 d, the packaged virus was collected and used to infect fresh 293 cells. The amplified virus was isolated by freeze-thaw extraction, purified by CsCl₂ density gradient centrifugation, and subjected to titer determination by lysing the cells. Human coronary artery SMCs were infected with the indicated multiplicity of infection (MOI). For the infection procedure, SMCs were cultured in SmGM-2 medium (Lonza) for 24 h. Then, the cells were synchronized in serum-free medium for 24 h and subjected to the experiments as indicated.

siRNA transfection
SMCs were grown to 50% confluence and transfected using Lipofectamine® in Opti-MEM I® Reduced Serum Medium transfection reagent (Invitrogen) according to the manufacturers’ protocols. The siRNA sequences used were as follows: RGS5-1 sense, 5'-GGAGUUAUAUAAGUUA-3', and antisense, 5'-UUACUACUUGAUAAACUC-3'. Then, the cells were synchronized in serum-free medium for 24 h and subjected to the experiments as indicated.

Flow cytometric cell cycle analysis
Cells were harvested by trypsinization, fixed overnight in 75% methanol, washed and incubated for 1 h at 37 °C in PBS containing 100 µg/ml RNase A, 10 µg/ml propidium iodide (PI) and 3% FCS. After the cells were washed twice, they were resuspended in 500 µl PI-PBS and analyzed for DNA content (PI). The samples were analyzed using standard flow cytometry methods and a Coulter Epics XL-MCL flow cytometer (Beckman Coulter Inc., Brea, CA, USA). Duplet discrimination before cell cycle analysis was performed by blotting fluorescence width vs. fluorescence area. The data were computer-analyzed using MultiCycle Version 3.0 software (Phoenix Flow Systems, San Diego, CA, USA).
Preparation of cellular lysates and immunoblot analysis
Protein extraction and semi-quantitative protein analysis by immunoblotting were performed as described previously. Briefly, the cleared supernatants from lysates were run on polyacrylamide gels and then blotted onto nitrocellulose membranes using a commercial dry blotting system (Invitrogen). After blocking, the blots were incubated with primary antibodies for 24 h at 4 °C. Primary antibodies were diluted as follows: 1:3000 for anti-p-ERK1/2; 1:5000 for anti-ERK1/2; 1:200 for anti-PCNA; 1:1500 for anti-Cdc2; 1:1000 for anti-cyclin D1 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA); 1:1000 for anti-RGS5 (Abnova), 1:500 for anti-V5 (Life Technologies), 1:10000 for GAPDH (Abcam, Cambridge, UK), and 1:7000 for β-tubulin (Sigma-Aldrich, Munich, Germany). After labeling with horseradish peroxidase-labeled secondary antibody (1:2000 for 1 h, Cell Signaling, ), the proteins were visualized by enhanced chemiluminescence (ECL+, Amersham, Freiburg, Germany) according to the manufacturer’s instructions. Densitometric analysis of immunoblots was performed by determination of the area under the curve of the respective bands followed by normalization to the respective loading control using ImageJ software (at least n=3 for each experiment).

Quantitative reverse transcription–PCR analysis
Total RNA isolation from human coronary artery SMCs and cDNA synthesis were performed using commercial kits (RNeasy Mini and QuantiTect Rev. Transcription Kits, Qiagen, Hilden, Germany). Total RNA from mouse femoral arteries was extracted using a total RNA Miniprep Kit (Sigma-Aldrich). Real-time PCR was performed on a Stratagene MX300 Quantitative PCR System (Stratagene MxPro, La Jolla, CA, USA) using SYBR Green PCR Mix (PeqLab, Erlangen, Germany). The hydroxymethylbilane synthase (HMBS) gene served as an internal standard. The primers used were as follows: human RGS5: forward, 5′-GGTGGTTTGAAGATTAAATGTCATT-3′; reverse: 5′-GATGCTATATGCTAAAAAGAGAAA-3′; murine RGS5: forward, 5′-CGGAGAAGGCAAAGCAAT-3′; and reverse, 5′-CCAGGTTCTTCATGGGTAGTTTGCATT-3′; human HMBS: forward, 5′-GGCAATGCGGCTGCAA-3′; and reverse, 5′-GGGTACCCAGCACCACGCAATC-3′; murine HMBS: forward, 5′-AAGGGCTTTTCTGAGGCACC-3′; and reverse, 5′-AGTTGCCCCATCTTTCATCGT-3′.

Animals
All experiments were performed on adult male C57BL/6 mice purchased from Charles River (Sulzfeld, Germany). All procedures involving experimental animals were approved by the local government animal care committee (33.14-42502-04-14/1472) and complied with Directive 2010/63/EU of the European Parliament.

Vascular injury models and gene transfer
Male C57BL/6 mice were administered general anesthesia using 100 mg ketamine hydrochloride/kg body weight (Anesketin, Albrecht, Aulendorf, Germany) and 16 mg xylazine hydrochloride/kg body weight (Rompun® 2%, Bayer, Leverkusen, Germany). The femoral artery was dilated using a straight spring wire (0.38 mm in diameter; Cook, Bloomington, IN, USA) inserted ~10 mm toward the iliac artery as described previously. The spring wire was inserted into the profunda femoris artery, and the blood flow was reconstituted after dilation by ligation of the profunda femoris branch. The electric de-endothelialization of the left common carotid artery was performed as described previously. In brief, the artery was injured with a bipolar microregulator (ICC50, ERBE-Elektromedizin GmbH, Tuebingen, Germany) distal to the bifurcation by applying an electric current of 2 W for 2 seconds over a total length of 4 mm. Immediately after dilation or electric injury, the artery was covered with 50 µl of a 25% thermosensitive Pluronic F-127 gel (Sigma-Aldrich) containing 5×10⁶ plaque forming units of Ad-RG55-WT, the non-degradable Ad-C2A-RGS5 mutant, or an empty control vector. This procedure was performed as described previously. Post-interventional analgesic therapy was performed by administering 0.1 mg buprenorphine/kg body weight for 3 days. Following wire-induced injury, the vessels were excised at the given time points. The
arteries used for morphometric analyses and Immunohistochemistry were fixed in 4% paraformaldehyde (PFA) and embedded in Tissue Tek OCT embedding medium (Sakura Finetek Europe B. V., Zoeterwoude, The Netherlands). The arteries used for qPCR were perfused with phosphate-buffered saline (PBS). All arteries were snap-frozen and stored at -80 °C until sectioning. Following electric injury, vessels were analyzed 5 days after injury. Re-endothelialization was determined by staining of the denuded area with 50µL of a 5% Evans blue dye via injection into the left ventricle. Pictures of the en face prepared injured carotid arteries were analyzed by Metamorph Imaging 7.0 software (Molecular Devices, Downingtown, PA, USA) and the ratio of re-endothelialized area (which is defined as area not stained with Evans blue / total injured surface area) was determined.

**Morphometric analysis**
The femoral artery was excised from the inguinal ligament to the branching of the profunda femoris artery and was cut into 6-µm serial sections, and 6 cross sections taken at regular intervals throughout the artery were stained with hematoxylin and eosin. For the morphometric analyses, MetaMorph Imaging 7.0 software (Molecular Devices, Sunnyvale, CA, USA) was used to measure the external elastic lamina, internal elastic lamina, and lumen circumference to calculate the luminal-medial- and neointimal area and the neointima/media ratio and the lumen loss 21 d after dilation.

**Immunohistochemistry**
After the slides were fixed and rehydrated, they were pre-incubated with 10% normal goat serum (Zymed Laboratories Inc., San Francisco, CA, USA), followed by incubation with the primary antibody directed against α-SMA (Sigma-Aldrich), CD31 (BD Pharmingen, Franklin Lakes, NJ, USA), Ki-67 (Abcam), RGS5 (Abnova), or V5 (Life Technologies). The subsequent incubations were performed with Alexa 488- or Cy3-coupled secondary antibodies (Molecular Probes, Eugene, OR, USA), and then the slides were counterstained with nuclear 4,6-diamidino-2-phenylindole (DAPI; Linaris, Wertheim, Germany). Monoclonal antibodies to α-SMA were labeled directly with Cy3. Negative controls were conducted by substituting primary antibody with an appropriate species- and isotype-matched control antibody (Santa Cruz Biotechnology). Analysis was performed on 6 cross sections of each artery taken at regular intervals (n=6). Microscopy was performed using a DMRB fluorescent microscope (Leica, Wetzlar, Germany). Staining for proliferating cell nuclear antigen (PCNA) was performed using a PCNA staining kit (Invitrogen) according to the manufacturer’s instructions.

**Statistical analysis**
The data are presented as the mean±SEM. The data were compared using analysis of variance (ANOVA), with Fisher’s corrected t-test as the post hoc analysis: Depending on the number of comparisons, ANOVA was followed by pair-wise multi-comparison using the Holm-Sidak method or the ANOVA on ranks test (comparison of 2 groups). *p<0.05 was considered statistically significant in all comparisons.
References


Supplemental Figure I: (A) Sequence of WT-RGS5 and the non-degradable C2A-RGS5 mutant which is resistant to degradation by the N-end rule pathway. (B) Schematic overview of RGS5-WT and C2A-RGS5 and its respective amino acid sequences as well as their predicted N-termini.
Supplemental figure II: (A) RGS5 mRNA expression levels were determined by qPCR after transfection of human coronary artery smooth muscle cells with the respective adenoviral constructs at the indicated concentrations (**P<0.01 ***P<0.001, n=4). (B) RGS5 mRNA expression levels were determined by qPCR after knock down of RGS5 using specific siRNA at the indicated concentrations (*P<0.05, n=4).
Supplemental figure III: Following adenoviral transduction using the non-degradable C2A-RGS5 mutant (MOIs as indicated), apoptosis of SMCs was assessed by TUNEL-based ELISA assay. SMC apoptosis is expressed as the mean OD405±SEM (P=n.s., n=4).
Supplemental figure IV: Following transduction of SMCs with the indicated adenoviral constructs (MOI=120), cells were stimulated with growth medium, and BrdU incorporation was determined after 24 h by anti-BrdU ELISA (****p<0.0001, n=4).
Supplemental figure V: This sketch provides a simplified overview on the molecular mechanisms by which RGS5 regulates cell cycle entry and proliferation of smooth muscle cells. Continuous lines show direct molecular interactions, broken lines indicate an indirect signaling impact.