Angiotensin II Type 1 Receptor Mechanoactivation Involves RGS5 (Regulator of G Protein Signaling 5) in Skeletal Muscle Arteries

Impaired Trafficking of RGS5 in Hypertension

Kwangseok Hong, Min Li, Zahra Nourian, Gerald A. Meininger, Michael A. Hill

Abstract—Studies suggest that arteriolar pressure–induced vasoconstriction can be initiated by GPCRs (G protein–coupled receptors), including the AT_1R (angiotensin II type 1 receptor). This raises the question, are such mechanisms regulated by negative feedback? The present studies examined whether RGS (regulators of G protein signaling) proteins in vascular smooth muscle cells are colocalized with the AT_1R when activated by mechanical stress or angiotensin II and whether this modulates AT_1R-mediated vasoconstriction. To determine whether activation of the AT_1R recruits RGS5, an in situ proximity ligation assay was performed in primary cultures of cremaster muscle arteriolar vascular smooth muscle cells treated with angiotensin II or hypotonic solution in the absence or presence of candesartan (an AT_1R blocker). Proximity ligation assay results revealed a concentration-dependent increase in trafficking/translocation of RGS5 toward the activated AT_1R, which was attenuated by candesartan. In intact arterioles, knockdown of RGS5 enhanced constriction to angiotensin II and augmented myogenic responses to increased intraluminal pressure. Myogenic constriction was attenuated to a higher degree by candesartan in RGS5 siRNA-transfected arterioles, consistent with RGS5 contributing to downregulation of AT_1R-mediated signaling. Further, translocation of RGS5 was impaired in vascular smooth muscle cells of spontaneously hypertensive rats. This is consistent with dysregulated (RGS5-mediated) AT_1R signaling that could contribute to excessive vasoconstriction in hypertension. In intact vessels, candesartan reduced myogenic vasoconstriction to a greater extent in spontaneously hypertensive rats compared with controls. Collectively, these findings suggest that AT_1R activation results in translocation of RGS5 toward the plasma membrane, limiting AT_1R-mediated vasoconstriction through its role in $G_{q/11}$ protein–dependent signaling. **(*Hypertension*. 2017;70:1264-1272. DOI: 10.1161/HYPERTENSIONAHA.117.09757.)**
• **Online Data Supplement**

Key Words: angiotensin II ■ candesartan ■ cell membrane ■ hypertension ■ rats

Small arteries display mechanosensitivity, constricting and dilating in response to increases or decreases in intravascular pressure (myogenic response), respectively.[1,2] Physiologically, the arteriolar myogenic response is an important autoregulatory mechanism regulating local tissue blood flow while protecting tissues from fluctuations in blood pressure.[1,2] Further, it is an important regulator of peripheral vascular resistance and mean arterial pressure.[3–5] The AT_1R (angiotensin II type 1 receptor), a member of the family of GPCRs (G protein–coupled receptors), has been proposed to act as a mechanosensor in a ligand-independent manner.[6,7] Thus, in small arteries, an increase in intraluminal pressure may directly activate the AT_1R in vascular smooth muscle cells (VSMCs) resulting in production of $G_{q/11}$ protein–mediated second messengers (ie, inositol trisphosphate and diacylglycerol) and vasoconstriction.[6,8,9] Given a role for the AT_1R in myogenic vasoconstriction, it is likely that AT_1R-mediated signaling mechanisms are tightly controlled to maintain appropriate myogenic responsiveness. Little attention, however, has been given to specific negative feedback mechanisms that would prevent excessive AT_1R-mediated myogenic vasoconstriction.

Activation of the GPCR exchanges guanosine diphosphate for guanosine triphosphate (GTP) on the G_α subunit and dissociation from the $G_{\beta\gamma}$ subunit. The dissociated subunits then activate or inhibit various downstream effectors.[10] Inactivation of GPCR signaling follows GTP conversion to guanosine diphosphate on the G_α subunit and reassociation with the $G_{\beta\gamma}$ subunit.[11] However, cofactors are necessary for the inactivation of GPCRs because the G_α subunit has low intrinsic GTPase activity. Of these cofactors, RGS (regulators of G protein signaling) proteins interact with the activated G_α subunit, enhancing the intrinsic GTPase activity of the G_α subunit to

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From the Department of Medical Pharmacology and Physiology (K.H., M.L., G.A.M., M.A.H.) and Dalton Cardiovascular Research Center (K.H., Z.N., G.A.M., M.A.H.), University of Missouri, Columbia; and Robert M. Berne Cardiovascular Research Center, University of Virginia, Charlottesville (K.H.).

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Correspondence to Michael A. Hill, Dalton Cardiovascular Research Center, University of Missouri-Columbia, 134 Research Park Dr, Columbia, MO 65211. E-mail Hillmi@missouri.edu

facilitate the GPCR receptor inactivation process. RGS proteins are referred to as GAP (GTPase accelerating proteins) and thus act as negative regulators reducing GPCR signaling.[12]

As RGS proteins act to brake G protein–mediated signaling, their dysfunction may lead to cardiovascular dysfunction by augmenting GPCR signaling. Consistent with this suggestion, it has been reported that RGS2 and RGS5 regulate cardiac hypertrophy,[13] blood pressure,[14,15] and vessel wall remodeling.[16] In particular, the loss of RGS5 causes angiotensin II (Ang II)–mediated hypertension as a result of augmented $G_{q/11}$-mediated signaling.[16] More recently, RGS5 deficiency in pregnant mice has been identified to cause hypertension and preeclampsia during the gestational period.[17] Along with these observations, it is conceivable that mechanoactivation of the AT_1R (in association with myogenic vasoconstriction)[6,11,18–20] interacts with the function of RGS proteins to prevent AT_1R-mediated excessive vasoconstriction and cardiovascular disorders. Thus, the present studies aimed to determine in arteriolar VSMCs whether RGS proteins colocalize with the AT_1R when activated by either Ang II or mechanical stimulation and thereby modulate AT_1R-mediated myogenic constriction.

Methods

Experimental protocols were approved by the Animal Care and Use Committee of the University of Missouri. Male Sprague–Dawley rats (6–8 weeks of age; 150–250 g), Wistar–Kyoto (WKY; 16–19 weeks of age; 320–380 g), and spontaneously hypertensive rats (SHR; 16–19 weeks of age; 320–380 g) were used in the present studies. Arterioles were isolated from cremaster muscle and used for end point/quantitative PCR, diameter studies with or without siRNA transfection via reversible permeabilization, and Western blotting. VSMCs were isolated and used in in situ proximity ligation assay (PLA) experiments. Detailed methods are described in the online-only Data Supplement.

Statistical Analysis

Data are represented as mean±SEM. Statistical analyses were performed using IBM SPSS Statistics or Prism GraphPad. Differences between 2 treatments or groups were evaluated using Student *t* tests. One-way ANOVA was conducted to assess statistical differences with >3 groups/treatments. A Bonferroni correction was used for post hoc testing. *P* values of <0.05 were considered statistically significant.

Results

RGS Protein mRNA Expression in Rat Skeletal Muscle Arterioles

On the basis of published studies,[16,21–23] mRNA expression of candidate RGS proteins was first determined in cremaster arterioles by qualitative end point (Figure 1A) and quantitative real-time (Figure 1B) PCR. Rat heart and brain tissues served as positive controls. Consistent with earlier studies,[24] RGS2, 4, and 5 were the most dominantly expressed mRNAs in arterioles, with RGS5 clearly the most abundant species (≈23-fold higher compared with RGS2; Figure 1B). As a result of these data, subsequent studies focused on the role of RGS5 in agonist-dependent and agonist-independent activation of the AT_1R.

Ligand-Dependent and Ligand-Independent Activation of the AT_1R Leads to Association With RGS5

An in situ PLA was used to determine whether RGS5 translocates to the AT_1R on ligand or mechanical activation of the receptor. The PLA provides a quantitative assay for protein–protein interactions for 2 proteins within 40 nm of each other. PLA was performed after either treating cultured arteriolar myoctyes with Ang II (10^{-7} and 10^{-6} mol/L) or swelling-induced mechanical stimulation with hypo-osmotic solution (240–150 mOsm/L). These experiments were performed in the absence or presence of the specific AT_1R blocker, candesartan (10^{-5} mol/L), to demonstrate involvement of mechanical, agonist-independent, activation of the receptor and its interaction with RGS5. The concentration of candesartan was chosen on the basis of our previous studies showing its efficacy in blocking mechanoactivation of the AT_1R.[8]

Ang II–induced activation of the AT_1R caused concentration-dependent RGS5 trafficking toward the receptor (relative PLA signals: 22.3±2.6 [control] versus 32.8±3.4 [Ang II 10^{-7} mol/L] and 34.4±3.1 [Ang II 10^{-6} mol/L]; P=0.008; Figure 2). Similarly, hypotonic buffer–induced membrane stretch of myocytes led to an increased PLA signal (22.3±2.6 [control] versus 29.7±2.0 [200 mOsm/L] and 33.4±2.6 [150 mOsm/L]; P=0.004; Figure 2), indicating increased colocalization between the AT_1R and RGS5. Strength of the PLA signal was dependent on the level of hypotonicity. Blockade of the AT_1R with candesartan significantly (P<0.05) decreased the PLA signal in response to both Ang II (34.4±3.1; Ang II 10^{-6} mol/L without candesartan versus 20.3±3.3; Ang II 10^{-6} mol/L with candesartan) and hypotonic buffer (33.4±2.6; 150 mOsm/L without candesartan versus 14.2±1.1; 150 mOsm/L with candesartan; Figure 2; Figure S1 in the online-only Data Supplement).

As a control experiment for specificity of the in situ PLA, additional studies were performed using antibodies against RGS16 and the AT_1R (Figure 3A and 3B). In contrast to the RGS5 studies, Ang II or membrane stretch did not result in colocalization between AT_1R and RGS16.

Knockdown of RGS5 Augments AT_1R-Mediated Myogenic Reactivity and Ang II–Induced Vasoconstriction

To determine the impact of RGS5 on myogenic reactivity of rat skeletal muscle arterioles (98±6 and 158±7 μm, active and passive diameters at 70 mm Hg, respectively, n=12), pressure–diameter relationships were determined after reduction of endogenous RGS5 by siRNA transfection (Figure 4A). Effectiveness of siRNA was verified by a significant decrease in RGS5 mRNA expression in cremaster arterioles (67% decrease compared with sham siRNA treatment, Figure S2). Myogenic vasoconstriction was significantly (P<0.05) enhanced in arterioles treated with siRNA-directed RGS5 (72.4±2.1 μm at 70 mm Hg, Figure 4A, closed circles) compared with those transfected with sham siRNA (52.2±2.1 μm at 70 mm Hg, Figure 4A, closed squares). The constrictor response to a single concentration of Ang II (10^{-7} mol/L) was also enhanced (P=0.005) after siRNA treatment (15.2±1.2%; sham siRNA versus 42.0±5.0%; RGS5 siRNA; Figure 4B). Collectively, these data suggest that RGS5 acts as an important modulator for AT_1R-mediated signaling evoked by both mechanical stimuli and agonists.

To further examine the role of RGS5 in AT_1R-mediated myogenic responsiveness, pressure–diameter relationships

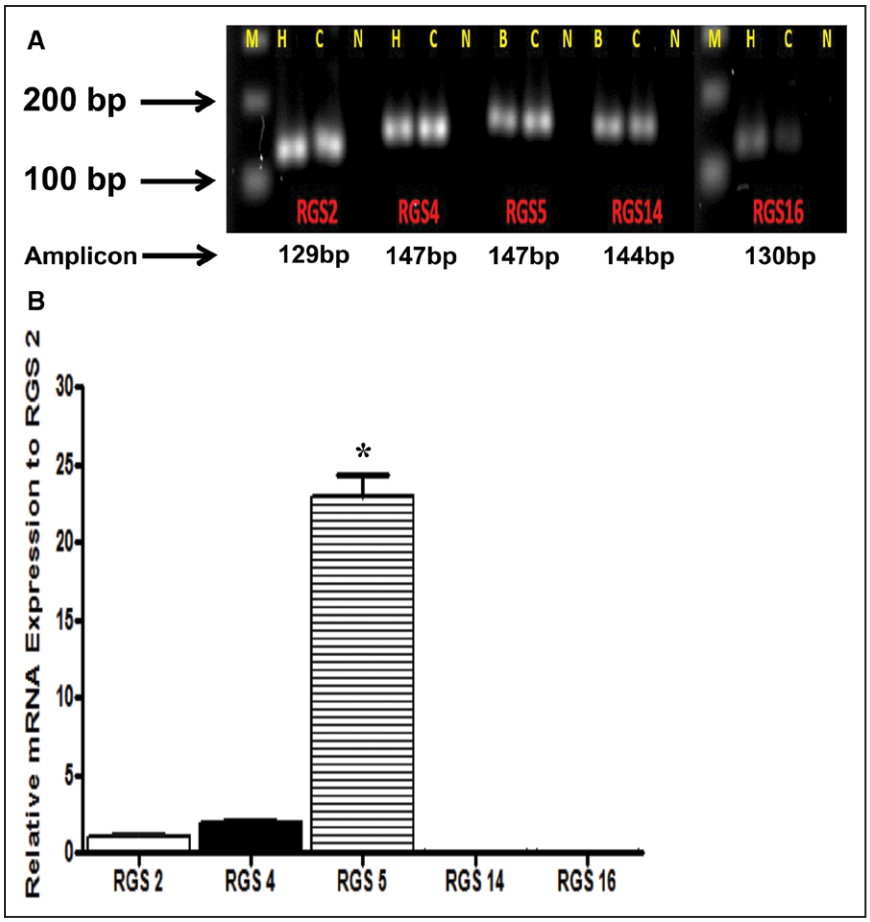

Figure 1. RGS (regulators of G protein signaling) mRNA gene expression profile in rat skeletal muscle arterioles. **A**, End point PCR products were amplified from cDNA isolated from rat heart, brain, and cremaster arterioles. The end point PCR images are representative of n=3 separate experiments. **B**, Real-time PCR assays were performed in cremaster muscle arterioles using β-actin as a housekeeping gene. Relative mRNA expression levels were normalized to the mRNA expression of RGS2 (n=6). **P*<0.05. Group data are presented as mean±SEM. B indicates brain (a positive control for RGS5 and 14); C, cremaster arterioles; H, heart (a positive control for RGS2, 4, 16); M, marker; and N, no template (negative control).

were determined in cremaster arterioles (transfected with RGS5 siRNA) in the absence or presence of candesartan (10^{-5} mol/L; Figure S3). Although candesartan caused a reduction of myogenic reactivity in arterioles transfected with both sham and RGS5 siRNA (Figure S3A through S3D), the magnitude of the decrease in myogenic responsiveness to candesartan (calculated as the delta reduction in diameter [Figure S3E]) was significantly greater in those treated with RGS5 siRNA. These data also indicate that mechanical activation of the AT_1R during myogenic vasoconstriction is modulated by RGS5.

Reduced Colocalization of RGS5 With the AT_1R in SHR

Additional studies were performed to examine the hypothesis that hypertension may be associated with diminished trafficking of RGS5 thereby contributing to dysregulation of AT_1R signaling. Similar to the above, in situ PLA was performed with myocytes isolated from cremaster arterioles of WKY and SHR rats. Consistent with the data from Sprague–Dawley rats, Ang II (10^{-7} mol/L) and hypotonic solution (150 mOsm/L) PLA signals showed trafficking of RGS5 to the AT_1R in vessels from WKY controls (Figure 5; Figure S4A). Similarly, the increased translocation of RGS5 was attenuated by candesartan in WKY arterioles (Figure 5; Figure S4A). These data also show that the average number of puncta per VSMC, indicative of colocalization between AT_1R and RGS5, was markedly lower in SHR compared with WKY (Ang II 10^{-7} mol/L: 76.6±5.4 [WKY] versus 45.4±3.3 [SHR]; $P<0.001$; Hypotonic 150 mOsm/L: 58.2±4.0 [WKY] versus 32.3±2.3 [SHR]; $P=0.000$; Figure 5; Figure S4A and S4B), suggesting that RGS-mediated regulation of AT_1R signaling pathways may be impaired in the SHR. In the presence of candesartan, no marked differences in trafficking of RGS5 were observed between WKY and SHR (Figure 5; Figure S4A and S4B), consistent with the differences between SHR and control cells being mediated by the AT_1R.

Effect of Genetic Hypertension on RGS5 Protein mRNA Expression in Cremaster Arterioles

To determine whether the differences in VSMC PLA signals could relate to levels of RGS5 expression, mRNA was evaluated in homogenates of cremaster arterioles from WKY and SHR. No significant difference in RGS5 mRNA expression was detected between the controls and hypertensive animals (Figure 6A).

Effect of Candesartan on Arteriolar Pressure–Induced Vasoconstriction Is Ameliorated in SHR

The observations of enhanced myogenic vasoconstriction and increased sensitivity to candesartan after knockdown of RGS5, and that reduced colocalization between RGS5 and AT_1R occurred in SHR, predicted an increased sensitivity to candesartan in SHR. To examine this, experiments were performed to determine whether mechanosensitive AT_1R–mediated myogenic vasoconstriction was more

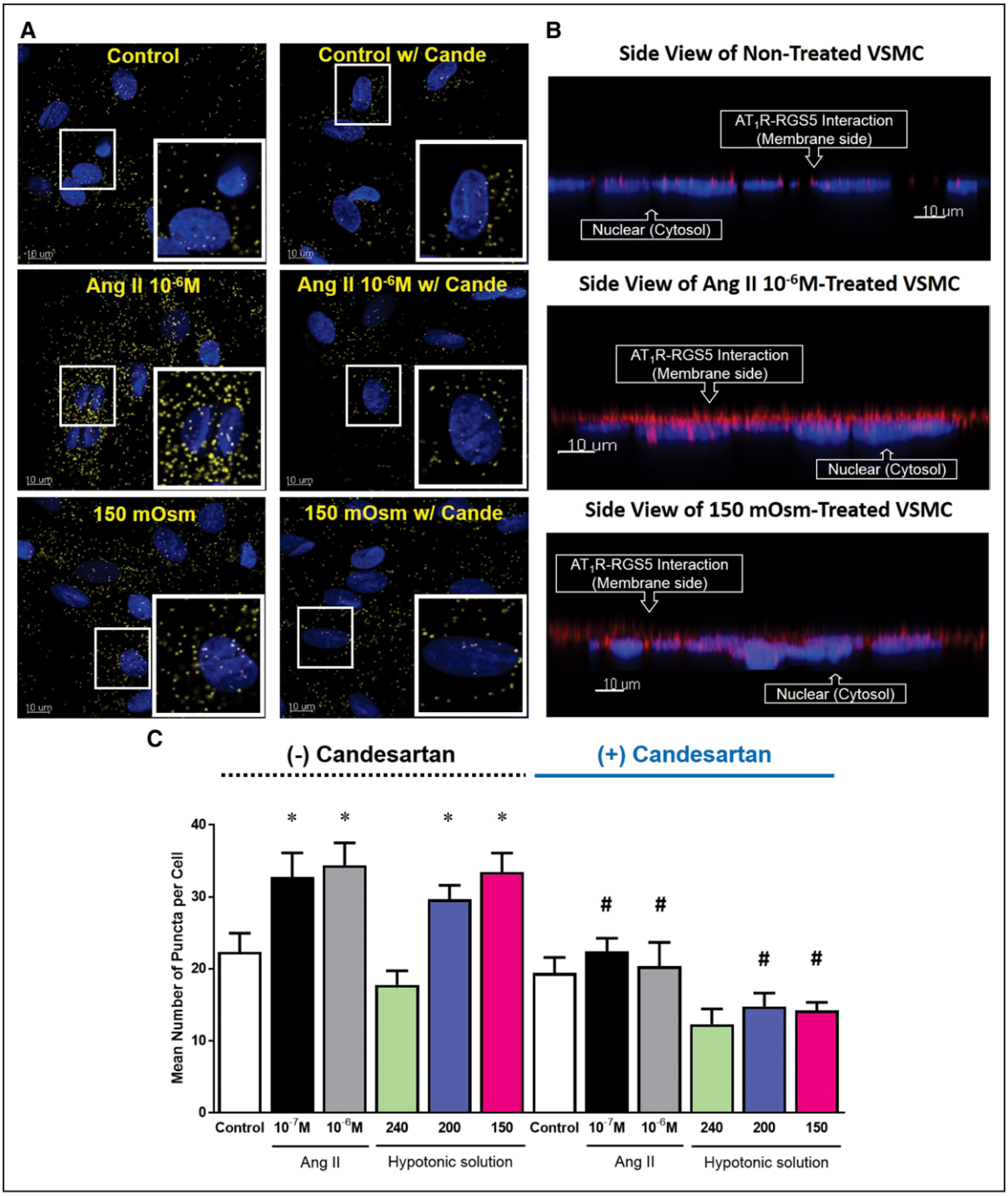

Figure 2. Angiotensin II (Ang II)–induced or membrane stretch–induced colocalization of RGS5 (regulators of G protein signaling) with the AT$_1$R (angiotensin II type 1 receptor). **A**, Representative confocal microscopy images collected from in situ proximity ligation assay (PLA). Arteriolar myocytes were treated with Ang II (10^{-6} mol/L) or hypo-osmotic solutions (150 mOsm/L) in the absence (**left**) or presence (**right**) of candesartan (10^{-5} mol/L). Insets (white boxes) represent magnified (×2) PLA images. **B**, Representative side view images of arteriolar myocytes with or without treatment of Ang II (10^{-6} mol/L) or hypotonic solution (150 mOsm/L) are exhibited. **C**, Quantification of puncta in 5 separate PLA experiments. *P<0.05 vs Control (–) Candesartan; #P<0.05 (–) vs (+) Candesartan. Group data are presented as mean±SEM. VSMC indicates vascular smooth muscle cell.

susceptible to candesartan in SHR. Pressure–diameter relationships were determined in the absence or presence of candesartan in arterioles from WKY and SHR (Figure 6B and 6C). Average active and passive arteriolar diameters at 70 mm Hg were 97±5 and 167±4 µm for WKY and 87±6 and 155±5 µm for SHR. As anticipated, the magnitude of the candesartan-induced decrease in myogenic vasoconstriction was markedly larger in SHR (Figure 6C), compared with WKY controls (Figure 6B).

Discussion

Mechanical activation of VSMC AT$_1$R has been suggested to contribute to mechanisms underlying myogenic vasoconstriction.[6,8,25,26] The current experiments were aimed at extending

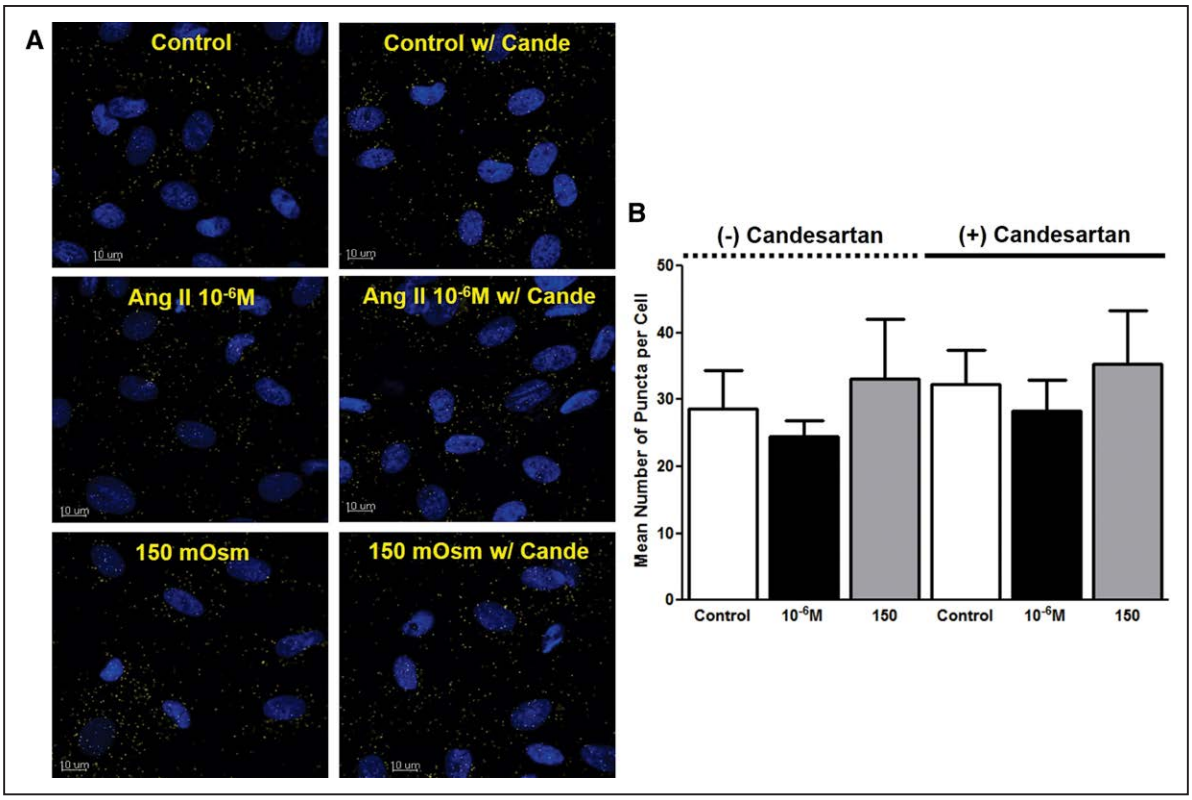

Figure 3. In situ proximity ligation assay (PLA) signals using antibodies for RGS16 (regulators of G protein signaling) and AT_1R (angiotensin II type 1 receptor) are unaffected by angiotensin II (Ang II) or hypo-osmotic solution. As an index of specificity of the PLA additional experiments were performed using the same AT_1R antibody together with an antibody to RGS16 (regulators of G protein signaling 16). **A**, Representative confocal microscopy images collected during in situ PLA. As in earlier experiments, arteriolar myocytes were treated with Ang II (10^{-6}) or hypo-osmotic solutions (150 mOsm/L) in the absence (**left**) or presence (**right**) of candesartan (10^{-5} mol/L). **B**, Group data showing puncta obtained in 4 separate PLA experiments. Data are presented as mean±SEM.

this to investigate how mechanosensitive GPCR–mediated myogenic responsiveness is regulated. Specifically, using isolated arteriolar VSMCs and cannulated arterioles, the present studies focused on RGS5-mediated regulation of the AT_1R during mechanical (ligand-independent) or Ang II (ligand-dependent) activation. Overall, our findings indicate that both ligand-dependent and ligand-independent activation of the AT_1R initiate trafficking of RGS5 toward the cell membrane (and presumably to the G_α subunit), consistent with limiting AT_1R-mediated vasoconstriction by terminating $G_{q/11}$ protein–dependent signaling. Further, trafficking of RGS5 is attenuated in arteriolar myocytes in a rat model of hypertension supporting a possible role for this impairment in enhanced AT_1R-mediated constriction.

Earlier studies examined the intracellular localization of RGS proteins in response to biological stimuli. For example, NO stimulates cGMP-dependent protein kinase I-α causing phosphorylation and membrane localization of RGS2, which subsequently decreases the downstream effects of G_q protein–mediated signaling.[15] Supporting this RGS2 is localized

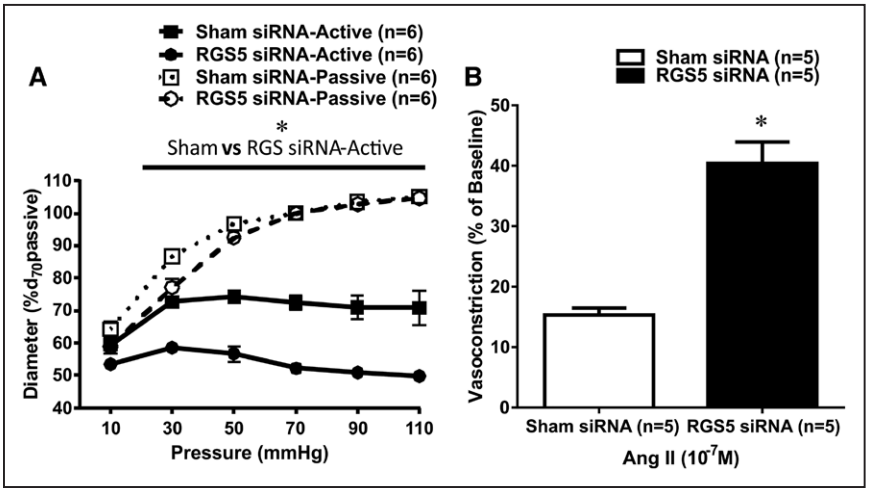

Figure 4. RGS5 (regulators of G protein signaling 5) downregulation enhances AT_1R (angiotensin II type 1 receptor)–mediated myogenic reactivity and vasoconstriction. **A**, Pressure–diameter relationships were assessed in cremaster arterioles transfected with either sham siRNA or siRNA-directed RGS5. Diameters were measured at each pressure and normalized to passive diameter at 70 mm Hg (%d_{70}passive). *P<0.05 sham siRNA active vs RGS5 siRNA active. **B**, Vasomotor response to angiotensin II (Ang II; 10^{-7} mol/L) was tested after transfection of sham siRNA or siRNA-targeted RGS5 (n=5). *P<0.05 sham siRNA vs RGS5 siRNA. Group data are presented as mean±SEM.

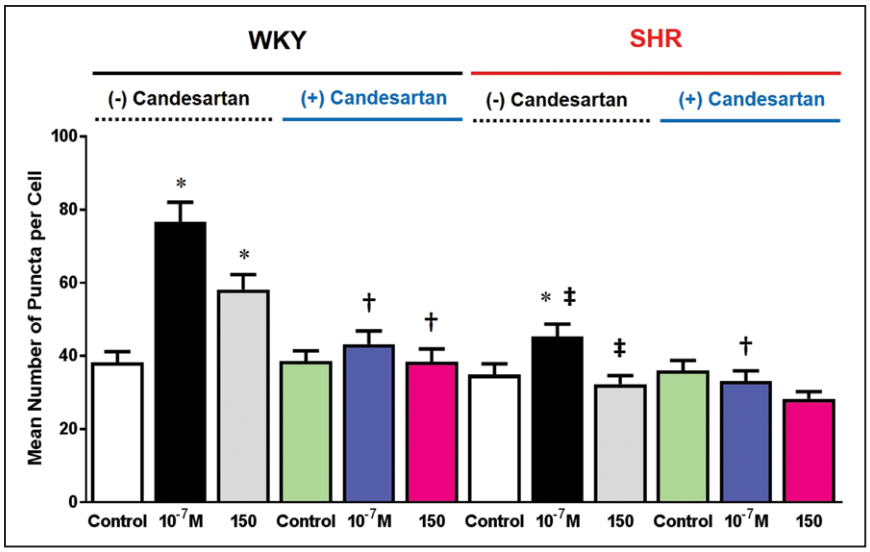

Figure 5. Ability of RGS5 (regulators of G protein signaling 5) to translocate toward activated AT_1R (angiotensin II type 1 receptor) seems lower in spontaneously hypertensive rats (SHR) than that in Wistar–Kyoto (WKY). Proximity ligation assay (PLA) was performed with cultured (passage 0) arteriolar myocytes isolated from WKY and SHR. The arteriolar myocytes were treated with Ang II (10^{-7} mol/L) or hypo-osmotic solution (150 mOsm/L) in the absence of candesartan (10^{-5} mol/L). Puncta obtained from 3 independent PLA experiments were quantified. **P*<0.05 vs each Control; †*P*<0.05 (–) vs (+) Candesartan; ‡*P*<0.05 WKY vs SHR. Group data are presented as mean±SEM.

between the plasma membrane and cytosol under resting conditions in a variety of cells.[15,27] In contrast, factors regulating cellular localization of RGS5 have received little attention, particularly in arteriolar VSMCs. To our knowledge, the present study is the first to demonstrate RGS5 movement toward the cell membrane in VSMCs after ligand-dependent/independent activation of the AT_1R. This is supported by the observation that Ang II– or hypotonic solution–mediated membrane stretch caused translocation/membrane localization of RGS5 and apparent interaction with the mechanosensitive AT_1R. Substantiating this, candesartan caused a decrease in the fluorescent PLA signal between RGS5 and the AT_1R.

Fluorescent puncta were apparent in nonstimulated arteriolar myocytes, perhaps indicative of some RGS5 interacting with the plasma membrane or other proteins closely associating with the AT_1R in the absence of stimuli. Further, RGS5 may be associated with the G_α subunit of the constitutively activated AT_1Rs as the receptor has been shown to exhibit a basal level of spontaneous activity without agonist-induced or mechanical stress–induced stimulation.[28] Interestingly, exaggerated constitutive activity of the AT_1R has been implicated in cardiovascular remodeling.[29] Criticism has, however, been leveled at the specificity of many commercially available AT_1R antibodies.[30,31] In rodents, AT_1R isoforms ($AT_{1a}R$ and $AT_{1b}R$) encode a 359 to 375 amino acid protein and have a predicted molecular weight of 42 kDa.[32] Consistent with this, a ≈42 kDa band was observed in cultured cremaster myocytes (Figure S5A, left). The AT_1R has also been shown to undergo post-translational glycosylation,[31] and the glycosylated AT_1R is predicted to have a higher molecular weight.[33] In the present study, rat kidney (used as a positive control) showed the ≈58 kDa glycosylated band, consistent with previous reports,[34] and this higher molecular weight band was also detected in the cultured cremaster myocytes (Figure S5A, left). As an index of specificity, Western blotting was performed after pretreating the antibody with a blocking peptide/antigen directed against the AT_1R (Figure S5A, right). In the presence of the peptide, the AT_1R bands were markedly diminished (Figure S5A, right), suggesting higher specificity of the antibody used in the present studies compared

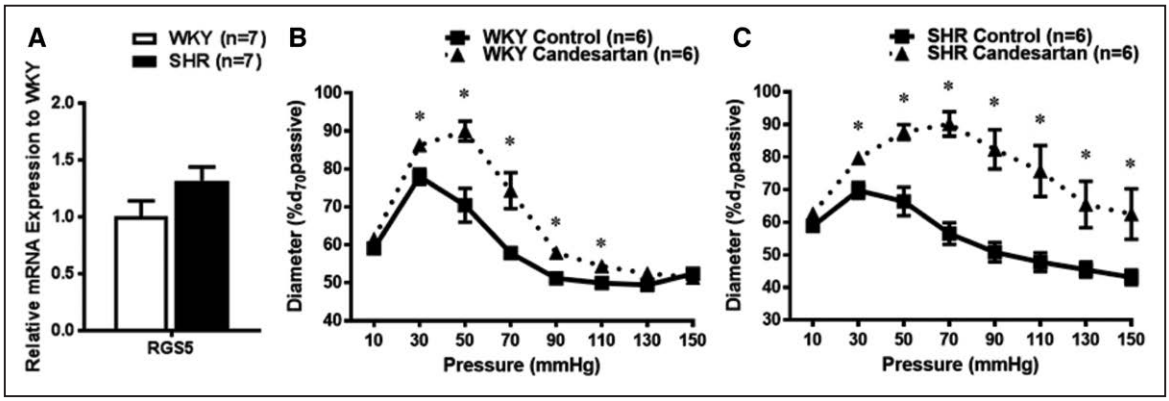

Figure 6. RGS5 (regulators of G protein signaling 5) mRNA expression is similar in skeletal muscle arterioles from Wistar–Kyoto (WKY) and spontaneously hypertensive rats (SHR) but AT_1R (angiotensin II type 1 receptor)–mediated vasoconstriction may be more sensitive to candesartan in SHR, compared with WKY. **A**, Quantitative PCR with SYBR green detection was performed to compare RGS5 mRNA expression in cremaster arterioles of WKY and SHR (n=7). Ribosomal protein S5 (*Rps5*) was used as the housekeeping gene. The comparative threshold (C_t) method ($2^{-\Delta\Delta Ct}$) was used to assess relative expression levels. Expression was normalized to corresponding WKY expression and presented as mean±SEM. **B** and **C**, Pressure–diameter relationships of arterioles of WKY and SHR were assessed in the absence or presence of candesartan (10^{-5} mol/L). Diameters were measured at each pressure and normalized to passive diameter at 70 mm Hg (%d_{70}passive). **P*<0.05 vs Control. Group data are presented as mean±SEM.

with commercially available antibodies discussed in previous studies.[30,31] Specificity of the RGS5 antibody was similarly evaluated (Figure S5B). According to the literature, RGS5 has a molecular weight of ≈26 to 28 kDa on gel electrophoresis.[35,36] In the present studies, although several additional bands were found, a distinct band was consistently observed at ≈28 kDa in rat heart with band intensity proportional to protein loading (Figure S5B, left). Further, pretreating the RGS5 antibody with the antigenic peptide entirely abolished the ≈28 kDa band (Figure S5B, right), suggesting that RGS5 antibody used in this study shows a degree of specificity. A further consideration is that nonspecific puncta would require close proximity of the 2 antibodies—conceivably this could occur stochastically or as a result of either one or both of the antibodies exhibiting nonspecific binding to similar sites. Regardless of this, any level of background did not prevent detection of a stimulus-induced increase in signal that was inhibited by candesartan.

Dysregulation of GPCR-mediated signal transduction is known to result in cardiovascular dysfunction.[37] In this context, it has been reported that systemic ablation of RGS2 causes hypertension as a result of exaggerated $G_{q/11}$-mediated vasoconstriction[14] and diminished NO-mediated vasodilation.[38] Further, decreased mRNA levels of RGS5 in VSMCs were detected in atherosclerotic lesions[39] and hypertension.[16] Given the findings that AT_1R mechanoactivation contributes to pressure-induced vasoconstriction[6] and RGS5 seems to colocalize with the G_α subunit of the AT_1R activated by agonists or mechanical stimuli, the present studies used siRNA to determine whether RGS5 functionally regulates myogenic vasoconstriction. Consistent with such a role, knockdown of RGS5 caused enhanced myogenic vasoconstriction. Our data are consistent with earlier studies showing increased myogenic tone or reactivity in interlobar arterioles of RGS2 protein–deleted mice[21] and after knockdown of RGS5 protein in mouse mesenteric arterioles.[22] However, whether mechanoactivation of the AT_1R is affected by knockdown of RGS5 has not been addressed, previously. Further supporting the interaction between the AT_1R and RGS5, candesartan significantly abrogated the enhanced myogenic vasoconstriction observed after downregulation of RGS5. On the basis of these observations, it is suggested that interaction between RGS5 and the AT_1R G_α subunit determines duration and intensity of AT_1R-mediated myogenic constriction.

Previous studies have shown that mRNA expression for RGS2 is reduced in saphenous arteries isolated from hypertensive rats.[40] In humans, gene polymorphisms of RGS2 and RGS5 proteins are associated with hypertension.[41,42] Reduction in RGS5 has been identified in bypass graft neointima, atherosclerotic arteries, and hypertension.[16,39,43] Further, RGS4 has been reported to inhibit endothelin-1–mediated signaling in human cardiomyocytes[44] and pressure-induced hypertension and cardiac hypertrophy.[45–47] On the basis of such observations and that we found a predominant expression of RGS5, we undertook studies to examine interactions between AT_1R and RGS5 in VSMCs from the SHR and age-matched WKY controls (16–19 weeks of age). In an initial study, we found RGS5 mRNA in cremaster arterioles to be similar for WKY and SHR rats. Although protein levels were not determined, this suggests that in this model RGS5 mRNA levels, alone, are not sufficient to account for hypertension.

In subsequent studies, we, therefore, considered the possibility that trafficking of RGS5 toward the AT_1R G_α subunit in response to Ang II or membrane stretch may be impaired in the SHR, which could conceivably then contribute to hypertension by enhanced AT_1R signaling pathways. In support of this hypothesis, our data show impaired trafficking of RGS5 toward either the agonist or mechanically activated AT_1R in SHR compared with WKY (Figure 5; Figure S4A and S4B). Although it could be argued that this defect could relate to differences in AT_1R expression, we found no significant difference in mRNA expression levels for the $AT_{1a}R$ and $AT_{1b}R$ between WKY and SHR (Figure S6). On the basis of impaired translocation of RGS5 in the SHR, it was further anticipated that myogenic reactivity of resistance arterioles may be significantly enhanced in SHR. Consistent with previous studies,[3] arterioles from the SHR showed enhanced myogenic constriction at high pressures (>130 mm Hg; Figure S7). In the earlier study, myogenic tone was studied over a wider pressure range and showed that vessels from the SHR exhibited marked myogenic vasoconstriction at intraluminal pressures between 140 and 185 mm Hg, whereas myogenic responsiveness was lost in WKY arterioles.[3] Because the SHR group showed a significantly increased systolic blood pressure (185±4 mm Hg; Figure S8), excessive myogenic vasoconstriction may be suspected to have been present in vivo. Interestingly, candesartan caused a greater inhibition of pressure-induced vasoconstriction in arterioles of the SHR compared with those from the WKY, consistent with a heightened role for the AT_1R because of impaired trafficking of RGS5.

Study Limitations and Future Directions

Although our data show that both ligand-dependent and ligand-independent activation of the AT_1R result in trafficking of RGS5 toward the cell membrane and that RGS5 trafficking is attenuated in arteriolar myocytes in the SHR model of hypertension, several limitations should be considered. First, similarly to other studies,[16,17,22] we relied on mRNA measurements to reflect RGS protein levels and effectiveness of siRNA treatment. Future studies should include actual measurements at the protein level, although this will necessitate pooling of individual vessel segments. It should also be considered whether the results of these studies are applicable across vascular beds and between species. This may be particularly important when considering an actual mechanistic link between changes in AT_1R/RGS5 signaling and hypertension, as Ang II responsiveness varies between vascular beds with the renal vasculature being considered to be the most sensitive to its actions. Species differences may also contribute when considering the role of specific AT_1R subtypes. For example, rodents express 2 AT_1R subtypes, whereas humans have only 1 subtype,[48] and within rodents there are currently disparate findings as to the relative importance of the $AT_{1a}R$ and $AT_{1b}R$[8,19,25,49] (and current results). In addition, the current studies have not considered interactions between mechano- and agonist-induced activation of the AT_1R and how this may impact RGS5-mediated signaling. Under particular in vivo situations, prevailing levels of Ang II and intraluminal pressure

may coexist. Finally, an important direction will be to investigate which mechanosensitive GPCRs in VSMCs (contributing to myogenic responsiveness or vascular tone) are preferentially modulated by RGS5 (or other RGS proteins) to better understand GPCR signaling regulation.

Perspectives

RGS proteins are potent regulators of GPCRs. As GPCR-mediated signaling pathways are implicated in many cardiovascular disorders, considerable interest has developed in the role of RGS proteins. Genetic polymorphism, decreased mRNA/protein levels, and deficiency of RGS5 have been reported to cause increased vascular stiffness, preeclampsia, and hypertension.[16,17,22,50] The current study suggests that impaired translocation of RGS5, effectively removing a brake on AT_1R-dependent vasoconstriction, may be one factor associated with hypertension. In this scenario, increased duration of $G_{q/11}$-mediated signaling of the AT_1R would be expected to augment myogenic and Ang II–induced vasoconstriction. As the significance of RGS protein translocation has only recently received attention,[27] additional studies are required to fully comprehend mechanisms underlying RGS protein modulation of GPCR signaling in the cardiovascular system and how this may impact vascular disorders such as hypertension.

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Disclosures

None.

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Novelty and Significance

What Is New?

- The novel findings in this study suggest that both ligand-dependent and ligand-independent activation of the AT_1R (angiotensin II type 1 receptor) cause recruitment of RGS5 (regulators of G protein signaling 5) in arteriolar vascular smooth muscle cells, implying that downstream signaling pathways of the AT_1R and their physiological outcomes (eg, myogenic response, angiotensin II–induced vasoconstriction) may be tightly controlled by RGS5 terminating G protein–dependent signaling in resistance arterioles. Further, imparied trafficking of RGS5 is found in hypertension. This study supports the hypothesis that dysregulation of AT_1R signaling by abnormal activity of RGS proteins causes excessive myogenic and angiotensin II–mediated vasoconstriction that may contribute to the development or progression of hypertension.

What Is Relevant?

- As GPCRs (G protein–coupled receptors) are an important target for the treatment of hypertension, a complete understanding of the mechanisms for GPCR regulation is vital. Consistent with increased vascular resistance resulting from increased myogenic activation, exaggerated myogenic vasoconstriction has been found in a variety of vascular beds in hypertension. There is growing and convincing evidence that pressure-induced vasoconstriction is initiated by the mechanoactivation of GPCRs (eg, AT_1R) in resistance arteriolar myocytes, implicating that negative feedback regulatory mechanisms (ie, RGS proteins) for GPCR-related signaling are required to prevent excessive GPCR-dependent vasoconstriction. Thus, RGS proteins are considered as promising therapeutic targets for pharmacological drug discovery to prevent or treat GPCR-mediated cardiovascular disorders (ie, hypertension).

Summary

RGS5 is translocated from cytosol to the activated AT_1R (plasma membrane located) in arterial vascular smooth muscle cells and regulates endogenous ligand– and mechanical stress–mediated vasoconstriction induced by the AT_1R.