Regulator of G protein signaling 2 is a functionally important negative regulator of angiotensin II-induced cardiac fibroblast responses

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Submitted 5 January 2011; accepted in final form 13 April 2011

Zhang P, Su J, King ME, Maldonado AE, Park C, Mende U. Regulator of G protein signaling 2 is a functionally important negative regulator of angiotensin II–induced cardiac fibroblast responses. Am J Physiol Heart Circ Physiol 301: H147–H156, 2011. First published April 15, 2011; doi:10.1152/ajpheart.00026.2011.—Cardiac fibroblasts play a key role in fibrosis development in response to stress and injury. Angiotensin II (ANG II), which accelerate termination of G protein signaling, are expressed in the myocardium. Among them, RGS2 has emerged as an important player in modulating Gq-mediated hypertrophic remodeling in cardiac myocytes. To date, no information is available on RGS in cardiac fibroblasts. We tested the hypothesis that RGS2 is an important regulator of ANG II–induced signaling and function in ventricular fibroblasts. Using an in vitro model of fibroblast activation, we have demonstrated expression of several RGS isoforms, among which only RGS2 was transiently upregulated after short-term ANG II stimulation. Similar results were obtained in fibroblasts isolated from rat hearts after in vivo ANG II infusion via minipumps for 1 day. In contrast, prolonged ANG II stimulation (3–14 days) markedly downregulated RGS2 in vivo. To delineate the functional effects of RGS expression changes, we used gain- and loss-of-function approaches. Adenovirally infected RGS2 had a negative regulatory effect on ANG II–induced phospholipase Cβ activity, cell proliferation, and total collagen production, whereas RNA interference of endogenous RGS2 had opposite effects, despite the presence of several other RGS. Together, these data suggest that RGS2 is a functionally important negative regulator of ANG II–induced cardiac fibroblast responses that may play a role in ANG II–induced fibrosis development.

Collagen; cell proliferation; G proteins; myofibroblasts; phospholipase Cβ

CARDIAC FIBROBLASTS are the most prevalent cell type in the myocardium (2, 38, 63) and play a central role in the maintenance of extracellular matrix (ECM) in the normal heart. Together with myocytes, they are key determinants of cardiac development, myocardial structure, cell signaling, and electromechanical function (3, 8, 48). In response to stress and in the injured and failing heart, fibroblasts phenotypically transform into “activated” myofibroblasts with characteristic changes in gene expression, morphology, and function (33, 42, 55). Myofibroblasts are the predominant cells responsible for collagen formation at sites of repair in the heart and fibrosis development (60), a major contributor to the cardiac remodeling response of the injured and diseased heart. Fibrosis can impair contractile function and electrical coupling of myocytes, which ultimately contribute to heart failure and predispose the heart to arrhythmias and sudden cardiac death, respectively (51).

A key profibrotic activator of cardiac fibroblasts is angiotensin II (ANG II), which is produced by circulating and local renin-angiotensin-aldosterone systems that are upregulated in response to mechanical stretch and other cardiac insults (5, 26, 51, 59). Adult rat cardiac fibroblasts express primarily AT1 receptors (12, 58); their stimulation activates Gq-mediated phospholipase Cβ (PLCβ) (13) and induces secretion of collagen and other ECM proteins (6, 58), transforming growth factor-β (TGF-β) and other growth factors (10, 18, 32), and cell proliferation (4).

Gq family members and other heterotrimeric GTP-binding proteins (G proteins) serve as central switch boards that transfer extracellular signals from G protein–coupled receptors across the plasma membrane to intracellular effectors and thereby determine signal transduction efficiency and specificity (39). They are tightly controlled by regulators of G protein signaling (RGS) (25, 46). The RGS family includes 20 canonical proteins that are grouped into four subfamilies based on primary sequence homology and the presence of additional domains (44, 67). RGS share a conserved RGS core domain that is both necessary and sufficient to confer GTPase activity by binding directly to active, GTP-bound Go subunits (54). This results in acceleration of Go GTPase activity and signal termination, as well as in some cases blocked Go-mediated signal generation via effector antagonism (1, 22). Each cell type expresses a unique RGS complement. RGS expression profiles are primarily based on Northern blots, in situ hybridizations, and PCR analyses, because antibodies that unequivocally recognize endogenous RGS isoforms at the protein level are generally not available. Several canonical RGS are expressed in the mammalian myocardium (36, 43), as well as in cultured/isolated cardiac myocytes (14, 28). To date, no information is available on RGS expression in cardiac fibroblasts.

In the cardiovascular system, RGS2 has emerged as a key player in regulating G protein signaling in cardiac myocytes and vascular smooth muscle cells (56) and plays an important role in modulating Gq-mediated hypertrophy development (52, 66) and vascular tone (23, 53). Like other members of the B/R4 subfamily, RGS2 mainly consists of the RGS core domain with short NH2- and COOH-terminal extensions. In contrast, RGS2 regulates Gq proteins, whereas the others also target G½6 (21). It has been proposed that RGS2 arose from the B/R4 subfamily to have specialized activity as a potent and selective Goq GTPase-activating protein that modulates cardiovascular function (31). Importantly, RGS2 is highly regulated in its expres-
expression by ANG II. Moreover, we have identified several other RGS proteins from adult rat ventricles, 2) is dynamically regulated by ANG II both in vitro and in vivo, and 3) exerts an inhibitory effect on ANG II-induced PLCβ activity, collagen production, and cell proliferation. Moreover, we have identified several other RGS isoforms in cardiac fibroblasts that are not changed in their expression by ANG II.

MATERIALS AND METHODS

Isolation and culture of adult rat ventricular cardiac fibroblasts. All animal studies conformed to the Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training, and all experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital. Hearts were quickly excised from anesthetized male Sprague-Dawley rats (5 wk old), retrogradely perfused for 2 min in Krebs-Henseleit buffer, and then switched to enzyme buffer 1 containing 0.3 mg/ml collagenase II (Worthington, Lakewood, NJ), 0.3 mg/ml hyaluronidase (Sigma, St. Louis, MO), and 50 μM CaCl2. After 20 min, the ventricular tissue was isolated, minced, and further digested at 37°C for 18 min in enzyme buffer 1 supplemented with increased CaCl2 (500 μM), trypsin IX (0.6 mg/ml; Sigma), and deoxyribonuclease (0.6 mg/ml; Sigma). The cell suspension was then filtered through 10 ml of DMEM/F12 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (complete medium) and centrifuged for 2 min at 200 g. The supernatant was removed from the pellet, and centrifuged again for 5 min at 800 g. The resulting fibroblast pellet was resuspended in complete medium and plated for 2 h, after which the medium was changed to remove unattached or loosely attached cells, including vascular smooth muscle cells, endothelial cells, and a few remaining myocytes. Originally plated cells (P0) and the first two passages (P1 and P2, each obtained by trypsinization after 3 days in complete medium) were used in each experiment. Fibroblasts plated on coverslips were expressing adenovirus (Ad-GFP) served as controls. Fibroblasts were subjected to specific conditions described for each experiment. Demembrane/F12 supplemented with 10 μg/ml insulin, 5.5 μg/ml transferrin, and 5 ng/ml sodium selenite (ITS; Sigma) was used as serum-free medium when indicated.

Immunofluorescence staining. Fibroblasts plated on coverslips were maintained in complete medium for 72 h, fixed with 4% formaldehyde, and permeabilized with 0.1% Triton X-100 in PBS [15 min at room temperature (RT) each]. Nonspecific binding sites were blocked with Image-IT FX signal enhancer (Invitrogen; 30 min at RT), followed by incubation (1 h at RT) with antibodies against vimentin (Sigma; 1:100), α-smooth muscle actin (α-SMA; Sigma; 1:400), the embryonic form of smooth muscle myosin heavy chain (Abcam, Cambridge, MA; 1:3,000), smooth muscle myosin (Sigma; 1:250), or von Willebrand factor (vWF; Sigma; 1:250), as well as Alexa Fluor 594-conjugated secondary antibodies (Invitrogen; 1:200). Coverslips were mounted with ProLong gold antifade reagent containing 4,6-diamidino-2-phenylindole (DAPI; Invitrogen).

Western blot analysis. Fibroblasts were lysed for 30 min at 4°C in 1X lysis buffer (Cell Signaling Technology, Danvers, MA) containing a protease inhibitor cocktail (Roche, Indianapolis, IN). Equal amounts of protein per lane were separated on 10% SDS-polyacrylamide (Tris/glycine) gels (7% gels for procollagen detection) and transferred to nitrocellulose membranes. After transfer, the membranes were stained with Ponceau S, blocked in phosphate-buffered saline (PBS) containing 5% nonfat dry milk, and probed with antibodies against vimentin (Sigma; 1:500), α-SMA (Sigma; 1:1,000), procollagen type I (Santa Cruz Biotechnology, Santa Cruz, CA; 1:3,000), FLAG (M2; Sigma; 1:1,500), and β-actin (Santa Cruz Biotechnology; 1:200). After three washes in PBS with 0.1% Tween 20 and incubation with appropriate peroxidase-conjugated secondary antibodies, proteins of interest were visualized by chemiluminescence (Thermo Scientific, Rockford, IL). Quantitative densitometry was performed using the public domain ImageJ program (developed at the U.S. National Institutes of Health and available online at http://rsb.info.nih.gov/ij/).

Reverse transcription-PCR. Total RNA (1 μg) extracted from fibroblasts was reverse transcribed and amplified using the SuperScript One-Step RT-PCR kit (Invitrogen) with RGS isoform-specific primers (Table 1). One-Step RT-PCR was performed at 50°C for 30 min and 94°C for 2 min and then at 94°C for 30 s, 48–56°C for 30 s, and 72°C for 1 min for a total of 30–40 cycles, followed by 72°C for 5 min. RT-PCR products were visualized on ethidium bromide-stained agarose gels. RNA from adult rat cortex and adult ventricular myocytes were used for control and comparison, respectively.

Real-time PCR. Reverse-transcribed (TaqMan reverse transcription reagents) RNA samples from fibroblasts were subjected to real-time PCR using FAM-labeled TaqMan probes for RGS2, RGS3, RGS5, GAPDH, and 18S and universal PCR master mix according to the manufacturer’s instructions (Applied Biosystems, Carlsbad, CA). Each sample was assayed in duplicate in two independent PCR reactions and normalized to 18S expression. Samples without enzyme in the reverse transcription reaction or template during PCR served as negative controls. PCR cycling was performed at 95°C for 10 min, followed by 95°C for 15 s and 60°C for 1 min for a total of 40 cycles using ABI Prism 7500. The cycle threshold (Ct) values corresponding to the PCR cycle number at which fluorescence emission in real time reaches a threshold above the baseline emission were determined using sequence detection system software (SDS version 1.4; Applied Biosystems). Serial dilutions of cDNA plasmids for rat RGS2 and RGS5 (0.3–3×105 copies) confirmed the linearity of the resulting Ct values.

Adenoviral gene transfer. Adenovirus encoding NH2-terminally FLAG-tagged RGS2 (Ad-RGS2) was generated previously (21). Control adenovirus (Ad-Ct) and a green fluorescent protein (GFP)-expressing adenovirus (Ad-GFP) served as controls. Fibroblasts were cultured in complete medium and infected the next day. To ensure comparable multiplicity of infection (MOI) between passages with different growth rates, a representative well was trypsinized and counted. Appropriate amounts of adenovirus in DMEM/F12 containing 0.5 ml of ITS were then added to each well immediately after medium aspiration. Complete medium was added (1.5 ml) after 2 h. RNA interference. The short interfering RNA (siRNA) for RGS2 was previously generated with AAGGAAAATATACACCGACTT as target sequence (66). GAPDH siRNA and negative control siRNA without significant homology to any known rat gene (Applied Biosystems) served as controls. After optimization of the type/amount of transfection reagent and siRNA, the following conditions were determined to achieve effective gene suppression with a minimum amount of siRNA. Fibroblasts were cultured in complete medium and subjected to RNA interference (RNAi) the next day using siPORT amine
transfection agent diluted in OPTI-MEM (Invitrogen). After 10 min of incubation at RT, 75 nM siRNA was added and incubated for another 10 min. The siRNA/siPORT amine transfection complexes were then dispensed onto the cells right after medium change with antibiotic-free DMEM/F12 containing 2% FBS (2 ml/well). Cellular siRNA uptake was visualized by fluorescent microscopy 2 days after transfection of Cy3-labeled siRNA (Applied Biosystems).

**Total collagen assay.** After 20 h in serum-free medium, fibroblasts were incubated in ANG II (1 μM) for 48 h, lysed as described above, and sonicated for 3 s on ice. After centrifugation at 3,000 g, the collagen-Sircol dye complex was precipitated, unbound dye was removed with the supernatant, and collagen-bound dye was subsequently released and quantitated via spectrophotometry at 540 nm. Normalization to protein content was expressed as the percentage of BrdU-positive nuclei to DAPI-positive (total) nuclei.

**Cell proliferation.** Fibroblasts were cultured on coverslips and starved in serum-free medium for 20 h. Bromodeoxyuridine (BrdU; 30 nM) was added immediately before addition of ANG II (1 μM). After 48 h, cells were fixed in 4% formaldehyde and incorporated BrdU was identified with a mouse anti-BrdU antibody (Dako, Carpinteria, CA; 1:100) and Alexa Fluor 594-conjugated secondary antibody (Invitrogen; 1:200). Coverslips were mounted as described above. Experiments were done in triplicate, and five images (with 300–400 positive (total) nuclei) were taken randomly for each coverslip. Fibroblast proliferation was expressed as the percentage of BrdU-positive nuclei to DAPI-positive (total) nuclei.

**Chronic ANG II infusion model.** Male Sprague-Dawley rats (5–6 wk old) were anesthetized with ketamine and medetomidine (75 and 1 mg/kg body wt). Osmotic minipumps (Alzet, Cupertino, CA; models 1003D, 2001, or 2002) were used and primed in sterile 0.9% saline at 37°C overnight to ensure immediate delivery of ANG II (555 ng·kg⁻¹·min⁻¹) or 0.9% saline after subcutaneous implantation. After surgery, the animals received regular chow with 0.4% KCl in drinking water. At the indicated time points (5 h to 2 wk), hearts were removed for isolation of ventricular fibroblasts and myocytes to investigate the RGS2 expression or processed for histology.

**Gomori trichrome stain.** Cross sections (5 μm) of formalin-fixed and paraffin-embedded hearts were deparaffinized in xylene, rehydrated through ethanol gradient solutions to PBS, and treated with Bouin’s solution (Sigma). They were then stained with Weigert’s iron hematoxylin (Electron Microscopy Sciences, Hatfield, PA) for 10 min, followed by trichrome stain for 20 min. After dehydration, slides were mounted with SHUR/Mount toluene-based mounting medium (Triangle Biomedical Sciences, Durham, NC).

**Statistical analysis.** Data from representative assays are shown and expressed as means ± SD for n determinations (unless indicated otherwise). Statistical differences were assessed using unpaired twotailed Student’s t-test or two-way ANOVA for comparison of individual means. A P value <0.05 was considered statistically significant.

**RESULTS**

**In vitro model of cardiac fibroblasts and myofibroblasts.** In response to stress, cardiac fibroblasts undergo three phenotypic changes: they convert into activated (i.e., contractile and hypersecretory) myofibroblasts, proliferate, and produce ECM components (such as collagen I and III) (42, 55). In this study, we used the first three passages (P0–P2) of ventricular fibroblasts isolated from 5-wk-old rats under experimental conditions that mimic these changes.

First, we determined the purity of the cell preparations: cells in all three passages expressed vimentin at comparable levels (Fig. 1, A and B) but were negative for smooth muscle myosin and vWF, indicating the absence of vascular smooth muscle cells and endothelial cells, respectively (Fig. 1A). Cardiac myocytes, which can be easily identified by their rod shape, were also not present.
Using surrogate markers for myofibroblasts (15, 33, 55), we delineated the timing and extent of phenotypic changes of fibroblast across the three passages: α-SMA and the embryonic form of smooth muscle myosin heavy chain were expressed at very low levels in P0 cells and markedly increased in P1 and P2 fibroblasts (Fig. 1, A and B). P1/P2 cells were also significantly larger and contained an increasing amount of α-SMA-positive stress fibers (Fig. 1A), suggesting progressive transformation of fibroblasts into activated myofibroblasts from P0 to P1/P2. Consistent with this notion, cell numbers (Fig. 1C) and procollagen type I expression (Fig. 1D) were increased in P1 and P2 compared with P0. Total IP formation normalized to cell numbers in each passage was measured as a reflection of PLCβ activity: both basal and ANG II-induced PLCβ activity showed a gradual increase from P0 to P2 (Fig. 1E). Thus the first three passages of freshly isolated adult ventricular fibroblasts recapitulate key phenotypic features of fibroblasts (P0) and myofibroblasts (P1/P2).

Transient RGS2 upregulation in response to short-term ANG II stimulation. Using RT-PCR, we found that RGS2 is expressed in cardiac fibroblasts (Fig. 2A). Its mRNA level was comparable to that of myocytes (isolated from the same heart) and brain and was not significantly changed between cell passages. In response to short-term stimulation with ANG II, RGS2 mRNA was transiently upregulated as shown by real-time PCR (Fig. 2B). Of note, TGF-β, which is enhanced in its expression by ANG II (29), also increased RGS2 expression (see Supplemental Fig. S1 for 6-h stimulation; supplemental material for this article is available online at the American Journal of Physiology-Heart and Circulatory Physiology website), and its effect was transient within 24 h (data not shown). Both ANG II and TGF-β effects were more pronounced in P0 than in P1 and P2.

We asked whether other RGS are expressed and regulated by ANG II. Figure 2Ca shows that members of all four canonical RGS subfamilies are expressed in cardiac fibroblasts. Depending on the isoform but unrelated to the subfamily, their expression was equal (RGS3), higher (RGS8, RGS10, RGS11, RGS17, RGS18, RGS20), or lower (RGS5 and RGS7) than in myocytes. Importantly, in contrast to RGS2, the other RGS were not altered in response to ANG II stimulation (Fig. 2Cb; see also Fig. 2B for RGS5).

Transient upregulation of RGS2 was also observed in cardiac fibroblasts that were freshly isolated from rats that had...
been subjected to subcutaneous ANG II infusion in vivo for less than 2 days (Fig. 2D). These findings demonstrate the presence of RGS2 mRNA in cardiac fibroblasts at a level comparable to that in myocytes and selective transient RGS2 upregulation in response to short-term ANG II stimulation both in vitro and in vivo.

Selective RGS2 downregulation in response to prolonged ANG II stimulation. The in vivo model provided an opportunity to also investigate the effect of prolonged ANG II stimulation on RGS2 mRNA expression levels. After the transient rise within a day of subcutaneous ANG II infusion (see above), RGS2 was markedly downregulated as early as 3 days after ANG II pump insertion and until at least 14 days (end of study) (Fig. 3A). We
also observed RGS2 downregulation in cardiac myocytes after prolonged ANG II stimulation in vivo (64 ± 12% of vehicle control after 3 days, \( P < 0.05; 67 ± 29\% \) of vehicle control after 14 days, \( P = 0.18; n = 3 \) each), which followed transient RGS2 upregulation (2.2 ± 0.5 fold, \( n = 6; P < 0.05 \)) after 18 h of ANG II stimulation.

We excluded the possibility that ANG II may be degraded over time by demonstrating that the efficacy in activating PLC\(\beta\) activity in cultured fibroblasts (P2 cells) was comparable between ANG II that was extracted from the minipumps 5 days after insertion and ANG II that was freshly prepared on the day of the assay [1,697 ± 141 vs. 1,635 ± 56 counts per minute per 6 wells (cpm/6 wells), \( n = 3 \) each, not significant; basal 305 ± 14 cpm/6 wells]. Proper drug release was also confirmed. Subsequently, for the 2-wk time point, pumps were exchanged twice after 5 days each.

In contrast to RGS2, the other RGS isoforms were not altered, as shown for the 7-day time point in Fig. 3B. As expected, ANG II infusion for 2 wk induced cardiac hypertrophy (Supplemental Fig. S2A), indicated by an increase in ventricular weight/body weight and expression of atrial natriuretic factor and by interstitial and perivascular fibrosis (Supplemental Fig. S2B). Matrix metalloproteinase (MMP)-2, MMP-9, and MMP-13 mRNA levels were unchanged (Supplemental Fig. S2C), but altered activity cannot be excluded.

**RGS2 overexpression inhibits ANG II-induced effects in cardiac fibroblasts.** To investigate the functional effects of increased RGS2 expression, we overexpressed RGS2 in P0–P2 cells via adenoviral gene transfer. We did not observe any apparent changes in cell morphology or viability after adenoviral gene transfer with different MOIs (MOIs 1, 3, and 10; data not shown). As expected, the infection efficiency in fibroblasts was dependent on the MOI, as shown with Ad-GFP for P1 cells in Supplemental Fig. S3A (similar results were obtained in P0 and P2). Accordingly, FLAG-tagged RGS2 protein expression could be titrated (Fig. 4A). The characteristic ANG II-induced rise in IP formation in cells infected with Ad-Ctr was dose-dependently inhibited on Ad-RGS2 infection at MOIs 1, 3, and 10 (shown for P1 cells in Supplemental Fig. S3B, top). At MOI 10, RGS2 inhibited IP formation by 65–75% in all three passages (Supplemental Fig. S3B, bottom).

RGS2 expression negatively regulated ANG II-induced PLC\(\beta\) activity, total collagen production, and cell proliferation in adult ventricular fibroblasts: the ANG II-induced rise in IP formation was reduced from 4.8-fold in Ad-RGS2-infected cells to 2.6-fold in control cells after 48 h at the indicated multiplicity of infection (MOI). A: basal and ANG II-induced phospholipase C\(\beta\) (PLC\(\beta\)) activity (0.3 \( \mu \)M ANG II, 0.5 h; top), collagen amount (1 \( \mu \)M, 48 h; middle), and bromodeoxyuridine (BrdU) incorporation (1 \( \mu \)M, 48 h; bottom). Proper drug release was also confirmed. Subsequently, for the 2-wk time point, pumps were exchanged twice after 5 days each.

RGS2 knockdown enhances ANG II-induced effects in cardiac fibroblasts.** We used RNAi to examine the functional consequences of downregulation of endogenous RGS2. Phase-contrast and fluorescent images of fibroblasts transfected with Cy3-labeled siRNA show uniform cellular siRNA uptake that was detectable in more than 95% of cells, with no apparent changes in cell viability or morphology (Supplemental Fig. S4A). Compared with cells transfected with negative control siRNA, we were able to achieve up to 80% reduction in GAPDH mRNA (Supplemental Fig. S4B) and up to 60% reduction in RGS2 mRNA (Fig. 5A) using gene-specific siRNAs. Importantly, mRNA expression of other isoforms such as RGS5 (Fig. 5A) and RGS3 (data not shown) was not changed in cells transfected with RGS2 siRNA.

Selective reduction in RGS2 expression markedly enhanced ANG II-induced PLC\(\beta\) activity, cell proliferation, and total collagen production: \( G_{\text{q}} \)-mediated IP formation over basal was increased from 4.5-fold in control cells to 11.4-fold in RGS2 siRNA-transfected cells (Fig. 5B, top). In addition, RGS2 RNAi increased ANG II-induced total collagen production

![Fig. 4. Functional effects of RGS2 overexpression.](http://ajpheart.physiology.org/)

**Fig. 4. Functional effects of RGS2 overexpression.** A: representative Western blot (probed with an anti-FLAG-antibody) containing lysates from P1 fibroblasts infected with adenovirus encoding FLAG-tagged RGS2 (Ad-RGS2) for 48 h at the indicated multiplicity of infection (MOI). B: basal and ANG II-induced phospholipase C\(\beta\) (PLC\(\beta\)) activity (0.3 \( \mu \)M ANG II, 0.5 h; top), collagen amount (1 \( \mu \)M, 48 h; middle), and bromodeoxyuridine (BrdU) incorporation (1 \( \mu \)M, 48 h; bottom) 72 h after infection with Ad-RGS2 or control adenovirus (Ad-Ctr) (\( n = 3 \) each). For ANG II induction, \( *P < 0.05 \), ANG II vs. basal. \#P < 0.05, Ad-RGS2 vs. Ad-Ctr.
DISCUSSION

ANG II is a well known profibrotic factor in many mammalian species, including humans (29). The majority of downstream effects are mediated via Gq-coupled AT1 receptors. RGS2 is an important regulator of Gq signaling in cardiac myocytes; however, no information is available for cardiac fibroblasts. We therefore investigated RGS2 expression, regulation, and function, using rat ventricular fibroblasts and myofibroblasts in the presence or absence of ANG II in vitro, as well as ANG II infusion in vivo, combined with molecular gain- and loss-of-function approaches. We have demonstrated that RGS2 expression is uniquely susceptible to ANG II-induced dynamic and biphasic regulation in vitro and in vivo and have shown that RGS2 is a functionally important negative regulator of ANG II-induced signaling and functional effects in cardiac fibroblasts despite the presence of several other RGS.

**Experimental models.** Primary isolates of fibroblasts from the heart and other organs are widely used to study their signaling properties and cellular functions under defined experimental conditions. The purity of the cell preparations for our in vitro model was confirmed by positive staining for vimentin and the absence of staining for endothelial cells and vascular smooth muscle cell markers. The first three passages represent different fibroblast phenotypes (Fig. 1): consistent with two other reports (16, 34), P0 cells that had a fibroblast-like appearance gradually converted into myofibroblast-like phenotype after plating at 200 cells/mm² and dual passage for 3 days each in complete medium, as evidenced by expression of smooth muscle cell markers (α-SMA and embryonic form of smooth muscle myosin heavy chain) and the presence of stress fibers (24). In addition, P1 and P2 cells increased in number more than P0 cells and had increased procollagen type I expression as well as basal and ANG II-stimulated PLC activity, which is consistent with a myofibroblast phenotype.

Myofibroblasts are typically not found in normal cardiac tissue but appear at sites of tissue repair (33). In contrast to other tissues, they persist in the myocardium long after the injury (61). The origin of myofibroblasts in the heart is still a matter of debate (64). Suspected sources include resident fibroblasts (62), endothelial cells (65), and bone marrow-derived hematopoietic precursor cells (17, 57). Our observations support the conventional view that resident fibroblasts in the heart can transform into myofibroblasts and suggest that circulating precursor cells may not be an absolute requirement. However, cell fate mapping studies are needed to determine the role of resident fibroblasts as source for activated myofibroblasts in vivo (64).

Subcutaneous ANG II infusion via osmotic minipumps was used to determine whether effects in vitro could also be observed in vivo. Moreover, it enabled us examine the effects of prolonged ANG II stimulation. This in vivo model has been widely used to test cardiac remodeling with ANG II delivery rates from 150 to 1,000 ng·kg⁻¹·min⁻¹ in rodents (4, 9). We chose 555·kg⁻¹·min⁻¹, plasma ANG II levels were shown to increase 6-fold compared with controls (11), which is in the same range (7-fold increase) as seen in heart failure patients (40). Consistent with other reports (27, 49, 50), ANG II infusion induced interstitial and perivascular fibrosis in the myocardium, as well as cardiac hypertrophy (Supplemental Fig. S2).
that RGS2 is transiently upregulated in fibroblasts and myofi-
broblasts in response to short-term ANG II stimulation (Fig. 2).
TGF-β had a similar effect in vitro (Supplemental Fig. S1).
Importantly, on prolonged ANG II infusion in vivo, we ob-
served marked RGS2 downregulation in cardiac fibroblasts
(Fig. 3). A similar biphasic regulation of RGS2 was also
observed in cardiomyocytes from the same hearts, consistent
with previous studies on RGS2 regulation in cardiac myocytes
in response to short-term and prolonged activation of the Gq
signaling pathway (21, 68). Ascending aortic constriction also
leads to marked RGS2 downregulation in ventricular tissues,
but cell type-specific RGS2 expression was not examined (66).

Further work is needed to determine the mechanisms for
the observed biphasic RGS2 regulation in cardiac fibroblasts on
ANG II stimulation. Our data suggest that TGF-β may be
involved. Reports in other cell types suggest that protein kinase
C (PKC)- and Ca²⁺-dependent mechanisms participate in
RGS2 upregulation after acute stimulation of the Gq signaling
pathway (30, 47). Much less is known about the mechanisms of
RGS2 downregulation. Prolonged activation of PKC can lead
to its downregulation, thereby potentially reducing a stimulus
for RGS2 expression. However, possible contributions of this
and/or other mechanisms to the downregulation of RGS2
expression have yet to be elucidated and were beyond the
scope of this study.

RGS2 function in cardiac fibroblasts. It is well known that
enhanced Gq-mediated signal transduction in fibroblasts leads
to an increase in cell proliferation and total collagen produc-
tion, both of which are profibrotic effects (5). Expression of
exogenous RGS2 was sufficient to inhibit ANG II-induced
PLCβ activity, fibroblast proliferation, and total collagen pro-
duction (Fig. 4). Conversely, knockdown of endogenous RGS2
markedly increased all three parameters (Fig. 5), indicating that
endogenous RGS2 exerts important inhibitory effects despite
the unperturbed presence of other RGS with potentially over-
lapping function (see below).

Given that RGS2 is a potent negative regulator of Gq in
many cell types, RGS2 upregulation in response to short-term
increase in Gq signaling is generally viewed as a negative
feedback mechanism (30). In this study, it was more pro-
nounced in nonactivated fibroblasts (P0) than in myofibroblasts
(P1/P2), suggesting less negative regulation of Gq signaling by
RGS2 in myofibroblasts under short-term ANG II stimulation.

The extent of RGS2 down-regulation after prolonged ANG
II infusion (42–58% reduction) was comparable to what we
achieved by RNAi in vitro (59% reduction). Functionally,
fibroblasts in vitro responded with enhanced profibrotic ANG
II effects on cell proliferation and total collagen production,
suggesting that RGS2 may play a role in exacerbating fibrosis
development in the stressed or injured heart with enhanced
ANG II stimulation (see also below). Further investigations are
needed to evaluate the role of RGS2 in regulating collagen
production and/or degradation. Importantly, RGS2 downregu-
lation in cardiac myocytes in response to enhanced Gq signal-
ing has been implicated to increase the hypertrophic response
in vitro (66) and in vivo (52). Thus the extent and timing of
RGS2 regulation appears to play a role in both aspects of the
cardiac remodeling response to stress.

Other RGS in cardiac fibroblasts. To our knowledge, this is
the first demonstration of canonical RGS expression in cardiac
fibroblasts. We report expression of members of each subfam-
ily in cardiac fibroblasts, which differed substantially from
cardiac myocytes. In addition, several isoforms appeared to be
altered in their expression during fibroblast-to-myofibroblast
transformation in vitro (see basal conditions in Fig. 2C). For
example, RGS11, RGS18, and RGS20 were decreased,
whereas RGS7 and RGS17 were increased. Much work needs
to be done to delineate the functional role of each RGS isoform
in cardiac fibroblasts and myofibroblasts both in vitro and in
vivo.

Although the A/RZ and C/R7 subfamily members are
expressed as selective for Goqα proteins, all B/R4 members accelerate
GTPase activity for Goqα proteins (46). In addition, RGS10
(D/R12) was shown to regulate Goq signaling (45). The fact that
selective RGS2 downregulation led to enhanced Goq-mediated
signaling and function (Fig. 5) suggests that the function of
RGS2 cannot be taken over by other Goq-regulating RGS
expressed in cardiac fibroblasts. Importantly, the other RGS
isoforms were not subject to regulation by ANG II (Figs. 2
and 3), highlighting a unique susceptibility of RGS2 to
regulation (21).

Conclusions and outlook. Together, the results from
the present study identify RGS2 as a novel negative regulator of
ANG II-induced signaling and function in adult cardiac fibro-
basts that is functionally important and highly regulated by
ANG II. RGS2 upregulation after short-term ANG II stimula-
tion might represent a brief period of negative feedback for
Goqα-mediated events, which is diminished in myofibroblasts.
RGS2 downregulation on prolonged ANG II stimulation might
facilitate the development of Goqα-mediated fibrosis.

Therapeutic benefits of reduction of ANG II production
and/or AT1 receptor blockade in heart failure are to a signifi-
cant extent derived from actions on cardiac fibroblasts and
fibrotic remodeling (7). Other Goq-coupled receptors [e.g., en-
dothelin receptors (37)] play a role in mediating fibrosis, as
well. It is therefore conceivable that RGS2 may emerge as a
therapeutic target in cardiac fibroblasts (in addition to myo-
cytes and vascular smooth muscle cells), because it mitigates
signaling at the G protein level.

Further investigations are required to delineate the physio-
ological and pathophysiological role of RGS2 in regulating
fibroblast behavior and fibrosis development in vivo. To that
end, studies in primary fibroblasts from normal and diseased
hearts, as well as transgenic and knockout approaches, parti-
cularly during cardiac remodeling and fibrosis in response to
stress, are needed. However, cardiac fibroblast-specific gene-
targeting experiments have been hampered by the lack of
identification of suitable promoter elements (41). In a mouse
model with global RGS2 deletion, pressure overload was
shown to be associated with increased fibrosis (52). However,
because RGS2 is ubiquitous with prominent expression in the
vasculature and nervous system in addition to the heart, RGS2
knockout mice have a multifaceted phenotype that includes
hypertension (23, 53) and an exaggerated hypertrophic re-
sponse to pressure overload or myocyte-specific transgenic
Goqα expression (52). An increase in autonomic tone has also
been suggested (20). Thus it is not possible to discern whether
an increased fibrotic response observed in this model is due to
the absence of RGS2 in cardiac fibroblasts or an indirect effect
resulting from RGS2 deletion in other cell types. Therefore,
animal models with RGS2 modifications restricted to fibro-

AJP-Heart Circ Physiol • VOL 301 • JULY 2011 • www.ajpheart.org
ACKNOWLEDGMENTS

We are grateful to Dr. Ian Dixon (St. Boniface General Hospital Research Centre, University of Manitoba, Winnipeg, Canada) for helpful discussions and advice in setting up the fibroblast cultures. We thank Leonard Chavez, Jr., for participation in osmotic minipump implantations and Lisa Rickey for the stain in Supplemental Fig. S2B.

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7. Brown RD, Ambler SK, Mitchell MD, Long CS. This work was supported by National Heart, Lung, and Blood Institute Grant HL-71274 and HL-80127 (to U. Mende) and American Heart Association Award 0740098N (to U. Mende).


