The aorta and heart differentially express RGS (regulators of G-protein signalling) proteins that selectively regulate sphingosine 1-phosphate, angiotensin II and endothelin-1 signalling

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Normal cardiovascular development and physiology depend in part upon signalling through G-protein-coupled receptors (GPCRs), such as the angiotensin II type 1 (AT₁) receptor, sphingosine 1-phosphate (S1P) receptors and endothelin-1 (ET-1) receptor. Since regulator of G-protein signalling (RGS) proteins function as GTPase-activating proteins for the G α subunit of heterotrimeric G-proteins, these proteins undoubtedly have functional roles in the cardiovascular system. In the present paper, we show that human aorta and heart differentially express RGS1, RGS2, RGS3S (short-form), RGS3L (long-form), PDZ-RGS3 (PDZ domain-containing) and RGS4. The aorta prominently expresses mRNAs for all these RGS proteins except PDZ-RGS3. Various stimuli that are critical for both cardiovascular development and function regulate dynamically the mRNA levels of several of these RGS proteins in primary human aortic

smooth muscle cells. Both RGS1 and RGS3 inhibit signalling through the S1P₁ (formerly known as EDG-1), S1P₂ (formerly known as EDG-5) and S1P₃ (formerly known as EDG-3) receptors, whereas RGS2 and RGS4 selectively attenuate S1P₂- and S1P₃-receptor signalling respectively. All of the tested RGS proteins inhibit AT₁-receptor signalling, whereas only RGS3 and, to a lesser extent, RGS4 inhibit ET_A-receptor signalling. The conspicuous expression of RGS proteins in the cardiovascular system and their selective effects on relevant GPCR-signalling pathways provide additional evidence that they have functional roles in cardiovascular development and physiology.

Key words: heterotrimeric G-protein signalling, regulators of G-protein signalling (RGS) protein, sphingosine 1-phosphate (S1P) receptors.

INTRODUCTION

The heterotrimeric G-protein consists of G α , G β and G γ subunits and functions as a signal transducer linking extracellular signals that activate G-protein-coupled receptors (GPCRs) to intracellular second messengers. GPCRs activated by ligand binding function as guanine nucleotide exchange factors for the G α subunit, exchanging GDP for GTP. This leads to dissociation of the trimeric proteins into GTP-bound G α and G $\beta\gamma$ dimers that then activate their own downstream effectors. The intrinsic GTPase activity of the G α subunit hydrolyses GTP to GDP, promoting reassociation of the trimeric G-protein, thereby terminating the GPCR-mediated signalling.

Cells modulate GPCR-mediated signalling by a variety of mechanisms, including the employment of members of a recently identified family of proteins called RGS (regulators of G-protein signalling). RGS proteins can act as GTPase-activating proteins (GAPs) for G α subunits by stabilizing the transition state of G α during GTP hydrolysis. They can also attenuate GPCR-mediated signalling by blocking the interaction between GTP-bound G α and its downstream effectors [1]. In addition, they can directly inhibit the activity of downstream effectors such as adenylate cyclase III in the olfactory system [2] and calcium channels activated by γ -aminobutyric acid ('GABA') receptors in chick

embryonic neuronal cells [3]. Functional roles for RGS proteins in the visual system, in olfaction and in the regulation of neurotransmitter and hormone signalling have been identified [2,4,5].

Previous studies have supported roles for RGS proteins in cardiovascular physiology. Mouse cardiomyocytes and rat aortic smooth muscle cells express mRNA transcripts for multiple RGS proteins [6,7]. Several RGS proteins impair signalling through GPCRs that are known to regulate cardiovascular function, such as the receptors for angiotensin II (AT-II), endothelin-1 (ET-1) and phenylephrine [8–11]. In addition, cardiac hypertrophy and heart failure can alter the expression levels of specific RGS proteins [12]. Finally, the transgenic expression of RGS4 in mice inhibits pressure-overload-induced cardiac hypertrophy, a normal compensatory mechanism [13].

A subfamily of GPCRs recently implicated in cardiovascular function is the endothelial differentiation gene receptor family, which serves as receptors for sphingosine 1-phosphate (S1P) [14]. S1P, a bioactive lipid, regulates diverse cellular processes including cell proliferation, differentiation, survival and migration. In vascular endothelial cells, S1P induces cell survival and morphogenesis via S1P₁ and S1P₃ receptors [15]. S1P can induce cardiac hypertrophy in rat neonatal myocytes via the S1P₁ receptor [16]. Verifying their essential roles in cardiovascular

Abbreviations used: AT-II, angiotensin II; AT₁ receptor, AT-II type 1 receptor; bFGF, basic fibroblast growth factor; ERK, extracellular-signal-regulated kinase; ET-1, endothelin-1; ET_A receptor, endothelin type A receptor; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GAP, GTPase-activating protein; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; HA, haemagglutinin; HAEC, human aortic endothelial cell; HASMC, human aortic smooth muscle cell; PDGF, platelet-derived growth factor; RGS, regulators of G-protein signalling; RGS3L, RGS3S and PDZ-RGS3, long form, short form and PDZ domain-containing form of RGS3 respectively; RT, reverse transcriptase; S1P, sphingosine 1-phosphate; S1P₁ (etc.), S1P receptor 1 (etc.); TGF- β 1, transforming growth factor β 1; vSMC, vascular smooth muscle cell.

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development, null mutants for the $S1P_1$ receptor or an $S1P_2$ -like receptor causes severe defects in blood vessel maturation or the heart respectively [17,18].

Knowledge of the expression patterns of the RGS proteins in the cardiovascular system and their roles in the cardiovascular GPCR-mediated-signalling pathways is crucial in assessing their importance in cardiovascular development and physiology. In the present study, we have examined the expression of RGS1, RGS2, RGS3 and RGS4 mRNA transcripts in aorta, various regions of heart, human aortic smooth muscle cells (HASMCs) and human aortic endothelial cells (HAECs). We also checked whether or not GPCR-coupled ligands and other stimuli that are known to have important roles in the cardiovascular system regulate the expression levels of these RGS proteins and/or their intracellular localization. Finally, we have tested their ability to attenuate $S1P$ -, $AT-II$ -, and $ET-1$ -induced extracellular-signal-regulated kinase (ERK) phosphorylation in cell lines and primary HASMCs.

EXPERIMENTAL

Cell culture, transfection, reagents and DNA constructs

Proliferating HASMCs and HAECs were purchased from Clonetics (Walkersville, MD, U.S.A.) and were grown as indicated in the manufacturer's protocol. HEK-293 and HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal calf serum. Transfections were performed using Superfect for HEK-293 and HeLa cells (Qiagen, Valencia, CA, U.S.A.). To transfect HASMCs, a newly developed transfection method by Amaxa Biosystems (Cologne, Germany) was used. Cells transfected with various RGS-green fluorescent protein (GFP) fusion proteins were evaluated for transfection efficiency using a Calibur FACS flow cytometer. The total amount of plasmid DNA for each transfection was always normalized with empty vector DNA. $S1P$ and human $AT-II$ were purchased from Sigma (St. Louis, MO, U.S.A.). Human platelet-derived growth factor (PDGF) AA-chain homodimer (PDGF-AA), human PDGF BB-chain homodimer (PDGF-BB), human basic fibroblast growth factor (bFGF), forskolin, $ET-1$ and PMA were purchased from Calbiochem (San Diego, CA, U.S.A.). Human transforming growth factor- $\beta 1$ (TGF- $\beta 1$) was obtained from R & D Systems, Inc. (Minneapolis, MN, U.S.A.). The use of human RGS1, 2, 3 or 4 cDNA clones was previously described in [2,19–21]. The constructs encoding RGS-GFP fusion proteins

were used previously and shown to be functional in mammalian cells [22].

Northern blot and reverse transcriptase (RT)-PCR

Various DNA fragments containing partial human RGS1, 2, 3 or 4 cDNA were generated by either restriction endonuclease digestion or by PCR amplification. The fragments were then labelled with [^{32}P]dCTP (Amersham, Piscataway, NJ, U.S.A.) and used as probes for hybridization with a human cardiovascular RNA blot (Clontech, Palo Alto, CA, U.S.A.). Hybridization was performed at 60 °C for 2 h using QuikHyb® (Stratagene, Cedar Creek, TX, U.S.A.), washed in $2 \times$ SSC (where $1 \times$ SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0)/0.1% (w/v) SDS twice for 15 min each at room temperature (22 °C), and then in $0.1 \times$ SSC/0.1% SDS at 50 °C for 30 min. For a loading control, a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe (Clontech) was used.

For RT-PCR, HAECs and HASMCs were harvested as recommended by Clonetics and lysed in RNA isolation buffer (RNeasy mini kit, Qiagen). Total RNA was extracted using the RNeasy mini kit as described in the manual for the Qiagen kit and then used to generate cDNAs with Advantage™ RT-for-PCR kit (Clontech). Human cDNAs of adult heart, foetal heart and aorta were purchased from Clontech. For PCR amplification, a specific set of primers was designed based on each human RGS protein sequence (Table 1). The resulting PCR products were cloned into pCR3.1 and their identity was confirmed by DNA sequencing.

ERK-signalling assay

To determine the effect of RGS proteins in $S1P$ -receptor-mediated signalling pathways, HEK-293 cells were transfected with a construct directing the expression of ERK-2-haemagglutinin (HA) in the presence of an RGS protein tagged with HA or FLAG. A construct directing the expression of $S1P_1$, $S1P_2$ or $S1P_3$ receptors was also transfected. The $S1P$ receptors were obtained from Dr T. Hla at the University of Connecticut (Farmington, CT, U.S.A.). For HASMCs, cells were transfected with a construct directing the expression of ERK-2-HA in the presence of one of the RGS-GFP fusion proteins. Cells were serum-starved overnight and then stimulated with $S1P$ (100 nM) for 5 min. After the stimulation, cells were washed twice in cold PBS and lysed in kinase lysis buffer [20 mM Hepes, 2 mM

Table 1 PCR primers

PCR primers were designed specifically for each human RGS protein subtype based on their reported sequences.

RGS	GenBank® accession number	Primer sequences	Expected size (bp)
RGS1	NM002922	5'-AGGAGAATATTGAGTTCTGGCTGGC-3' 3'-TCACTTTAGGCTATTAGCCTGCAGG-5'	304
RGS2	NM002923	5'-GCCAGCAATATGGTCTTGCTGCA-3' 3'-TCATGTAGCATGAGGCTCTGTGGT-5'	371
PDZ-RGS3	AF463495	5'-AACCGCTTCAATGGGCTCTGCAAGGTGTGC-3' 3'-CCTCTTCTCATCTGCCTCCGCTCAACAT-5'	1200
RGS3L	U27655	5'-TTGGCTGTCAAGGCAAGCTGTACAATAGTGG-3' 3'-CGGAGCTCATGGTGTCTCAGTAAGGGCCT-5'	500
RGS3S	BF317203	5'-CTCCGAGGCATGTACCTCACTCGCAACGGG-3' 3'-CACTGAACCTAGTGCAGGAAGGCTTGA-5'	210
RGS4	NM005613	5'-AAATCACCATCTAACTAAGTCCCAAGGCC-3' 3'-CACACTGAGGGACGAGGAAGCACAGTCTG-5'	310
β -Actin	M10277	5'-ACCACCACGGCCGAGCGGGAATC-3' 3'-GCCGCCAGACAGCACTGTGTTGGC-5'	305

EGTA, 50 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1% (v/v) Triton X-100, 10% (v/v) glycerol, 100 mM NaCl, 10 mM NaF and protease inhibitor tablets (Boehringer Mannheim, Indianapolis, IN, U.S.A.). The resulting lysates were analysed by immunoblotting with anti-(phospho-ERK) monoclonal antibody (New England BioLabs Inc., Beverly, MA, U.S.A.), anti-HA (Covance, Richmond, CA, U.S.A.), anti-FLAG monoclonal antibody (Sigma) or anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

To determine the effect of RGS proteins in ET-1- or AT-II-mediated signalling pathways, HeLa cells were transfected with a construct directing the expression of ERK-1-HA (or ERK-2-HA) in the presence of one RGS protein tagged with HA or FLAG. Human endothelin type A (ET_A) receptor and human AT-II type 1 (AT_1) receptor were obtained from Dr N. Dulin at University of Chicago (Chicago, IL, U.S.A.) and Dr K. Hoe at Korean Research Institute of Bioscience and Biotechnology (Taejeon, Korea) respectively. The construct directing the expression of ET_A receptor or AT_1 receptor was also transfected. Cells were serum-starved overnight and then stimulated with ET-1 (100 nM) or AT-II (1 μM) for 5 min. After the stimulation, cells were treated and analysed by immunoblotting.

Confocal fluorescence microscopy

Images of HASMCs transfected with various RGS-GFP fusion proteins were collected on a Leica TCS-SP2 confocal microscope (Leica Microsystems, Exton, PA, U.S.A.) using a 40 $\times$ oil immersion objective (NA 1.32, confocal zoom 1). Fluorochromes were excited using an argon laser at 488 nm. Time-lapse recording of the confocal images was performed for 20 min with 15 s intervals between collections. Images were processed using the Leica TCS-SP2 software (version 2.770), Imaris 3.2 (Bitplane AG, Zurich, Switzerland) and Adobe Photoshop 5.5 (Adobe systems, San Jose, CA, U.S.A.).

RESULTS

RGS1, RGS2, RGS3 and RGS4 mRNAs are differentially expressed in human aorta and heart

To examine mRNA expression of various RGS proteins in aorta and heart, a Northern blot analysis of human RNA prepared from aorta and different portions of the heart was performed using RGS1, RGS2, RGS3 and RGS4 cDNA probes (Figure 1A). The human RGS3 probe detected three known splice variants of RGS3: RGS3S (short-form), RGS3L (long-form) and PDZ-RGS3 (PDZ-domain-containing) (Table 1) [23]. In addition, we observed a transcript of approx. 4.5 kb above the 4.2 kb PDZ-RGS3 transcript and a 1.3 kb mRNA present below the RGS3S transcript, suggesting the presence of two other splice variants of RGS3. The 4.5 kb RGS3 transcript probably arises from previously unrecognized exons upstream of the characterized RGS3 gene, and the 1.3 kb RGS3 transcript probably arises from the usage of an uncharacterized exon that splices to the original RGS3 exon 4 [23]. The RGS1, RGS2 and RGS4 cDNA probes identified single mRNA transcripts of approximately 1.8, 1.9 and 3.5 kb respectively.

All of the RGS mRNA transcripts examined showed both a higher level of expression in aorta than that in heart and differential expression patterns in various regions of heart. Based on a G3PDH loading control, the adult-heart lane was underloaded and the right-ventricle lane slightly overloaded. The atria had higher levels of RGS1, RGS2 and PDZ-RGS3 transcripts than those in ventricles. RGS3L and RGS3S exhibited the opposite pattern of expression, being more highly expressed in

Figure 1 Expression of RGS1, RGS2, RGS3 and RGS4 mRNAs in human aorta and heart

(A) Northern blot containing polyadenylated RNAs isolated from human cardiovascular tissues hybridized with human RGS1, RGS2, RGS3 or RGS4 probes and also with G3PDH probe for loading control. FH, foetal heart; AH, adult heart; AO, aorta; AP, apex of heart; LA, left atrium; RA, right atrium; LV, left ventricle; and RV, right ventricle. PDZ-RGS3, RGS3L, RGS3S are indicated, together with two additional splice variants of RGS3 (one above PDZ-RGS3 and the other below RGS3S). (B) Semi-quantitative RT-PCR was performed with cDNAs generated from adult heart (AH), foetal heart (FH) and aorta (AO) to confirm further the presence of RGS3 isoforms (upper panel). The human primers described in Table 1 were used. G3PDH primers were also used as a control for cDNA synthesis. In addition, the presence of various RGS transcripts was examined in HAECs and HASMCs (lower panel). Lanes 1 and 3 are RT-PCR experiments performed with HAECs and HASMCs respectively. Lanes 2 and 4 represent 'no-RT' controls for HAECs and HASMCs respectively. Human β -actin primers were used as a control for cDNA synthesis. The results shown are representative of at least two experiments.

the ventricles. The left ventricle possessed the highest level of RGS4 mRNA as compared with other regions of heart. Considering that less RNA was loaded in the adult-heart lane, the expression levels of the RGS transcripts in the adult heart were similar to those in foetal heart, with the exception of RGS1. The level of RGS1 expression in foetal heart exceeded that in the adult heart by approx. 1.6-fold. In addition, semi-quantitative RT-PCR was performed to assess directly the expression of the RGS3 isoforms. Primers specifically designed for each RGS3 isoform (Table 1) using cDNAs generated from human aorta, and human adult and foetal heart confirmed the presence of all three RGS3 isoforms in accordance with the Northern result (Figure 1B).

The expression pattern of the RGS transcripts in cultured primary HASMCs and HAECs was then analysed by semi-quantitative RT-PCR (Figure 1B). The PDZ-RGS3 transcript was detected only in HAECs. RGS3S and RGS3L transcripts were present in both HAECs and HASMCs, but the expression appeared to be higher in HAECs. The expression levels of RGS4 mRNA in HAECs and HASMCs were similar. RGS2 exhibited a higher level of expression in HASMCs. In our multiple experiments performed with cDNAs that were generated from HASMCs and HAECs, the human RGS1 primers never produced an RGS1 band. Since RGS1 was readily detected in human aorta and heart RNAs by Northern blot analysis as well as by RT-

Figure 2 Dynamic regulation of RGS transcripts in HASMCs

HASMCs passed two to three times were serum-starved for 24 h before stimulation with various agonists. Serum-starved HASMCs remained unstimulated (C) or were treated with 1 μ M AT-II (lane 1), 20 ng/ml PDGF-AA (lane 2), 20 ng/ml PDGF-BB (lane 3), 100 nM S1P (lane 4), 10 ng/ml bFGF (lane 5), 1 μ g/ml TGF- β 1 (lane 6) or 100 μ M forskolin (lane 7) for 2 h, 4 h and 24 h. (A) Semi-quantitative RT-PCR was performed using the primers described in Table 1. Human β -actin was used as a control for cDNA synthesis. The results shown are representative of multiple experiments. The numbers shown below RT results represent a fold increase or decrease in the intensity of the DNA band compared with that of unstimulated HASMCs, normalized against the intensity of the corresponding β -actin band. Changes smaller than 2-fold are not shown. Densitometric analysis was performed using NIH Image software. (B) Induction of RGS2 mRNA upon stimulation with various agents. The relative intensities of DNA bands were quantified using NIH Image software as described above. Results are means $\pm$ S.E.M. of PCR results with varying numbers of amplification cycles between 23 and 26.

PCR (results not shown), the HASMCs and HAECs presumably down-regulate their expression of RGS1 during passage in culture. All of the primer sets produced bands of the appropriate sizes (Table 1). DNA sequencing revealed that the PCR products arose from the appropriate RGS mRNAs (results not shown).

Dynamic regulation of RGS mRNAs in HASMCs

We examined further whether or not the levels of RGS transcripts could be regulated by stimuli that are important for vascular function. HASMCs were serum-starved for 24 h, treated with AT-II, PDGF-AA, PDGF-BB, S1P, bFGF, TGF- β 1, or forskolin for 2 h, 4 h and 24 h, and semi-quantitative RT-PCR was performed with isolated RNAs (Figures 2A and 2B). To ensure the linearity of PCR amplification, multiple PCR reactions were performed with varying numbers of cycles from 20 to 29. The PCR amplification remained linear from cycle 23 to 26. Therefore, the relative intensities of DNA bands that resulted from these PCR amplification cycles were quantified using NIH Image software (version 1.62) and plotted (Figure 2B). Little change was observed in the mRNA levels of the RGS proteins by the 2 h time point (results not shown). Among the RGS proteins tested, RGS2 exhibited the most dynamic regulation by various agonists: forskolin induced a 5-fold increase, PDGF-BB 4-fold, PDGF-AA 3-fold and bFGF 2-fold at 24 h. Forskolin decreased RGS3S expression by the 2 h time point, which remained low by 24 h. TGF- β 1 moderately induced RGS3S gene expression by the 4 h time point. Since RGS1 and PDZ-RGS3 mRNAs were not

Figure 3 Selective inhibition by RGS proteins of S1P-induced ERK phosphorylation via the S1P₁, S1P₃ or S1P₂ receptor

(A) HEK-293 cells were transfected with a construct expressing S1P₁ (P₁), S1P₃ (P₃) or S1P₂ (P₂) receptor in the presence of ERK-2-HA. S1P-induced ERK activation in S1P-receptor-transfected cells was compared with that in vector (V)-transfected cells. In addition, HEK-293 cells were transfected with a construct directing expression of FLAG-tagged RGS1 (R1), HA-tagged RGS2 (R2), FLAG-tagged RGS3L (R3) or HA-tagged RGS4 (R4) in the absence of any exogenous S1P receptors. Similar experiments were performed with cells expressing the S1P₁ (B), S1P₃ (C), or S1P₂ (D) receptor in the presence of the RGS proteins. Cells were serum-starved, stimulated with S1P (100 nM) for 5 min, and harvested for immunoblotting with anti-phospho-ERK, anti-HA and anti-FLAG antibodies. The numbers shown above the RGS protein panels indicate the amount of transfected DNA (μ g) needed to normalize the expression level. All results shown are representative of at least two experiments. pERK-2-HA and pERK-1 represent phosphorylated, transfected ERK-2-HA and phosphorylated, endogenous ERK-1 respectively. The phosphorylated, endogenous ERK-1 is barely observed in panel (C) due to a shorter exposure because S1P via the S1P₃ receptor elicited a much stronger response.

detected in HASMCs, RT-PCR was not performed with these two genes. Human β -actin primers were used as RT-PCR controls.

Selective inhibition by RGS proteins of ERK phosphorylation induced by S1P, AT-II and ET-1

The differential expression pattern and dynamic regulation of the RGS proteins in the cardiovascular system prompted us to examine the role of these RGS proteins in GPCR-mediated

Figure 4 Attenuation of S1P-induced ERK phosphorylation by RGS proteins in HASMCs

HASMCs were transfected with a construct directing expression of RGS1–GFP, RGS2–GFP, RGS3L–GFP or RGS4–GFP in the presence of ERK-2–HA expression vector. A vector encoding GFP was also transfected to control cells to adjust the total amount of DNA. Cells were serum-starved, stimulated with S1P (100 nM) for 5 min, and harvested for immunoblotting with anti-(phospho-ERK), anti-HA and anti-GFP antibodies. Results shown are representative of at least two experiments. pERK-2–HA represents phosphorylated, transfected ERK-2–HA.

signalling pathways that are important for cardiovascular development and physiology. First, we examined whether or not RGS proteins modulated S1P-receptor signalling. ERK phosphorylation is a crucial step in many vascular functions and extensively used as a read-out to monitor the activation of GPCR-mediated signalling pathways. Therefore, we determined whether the expression of constructs that encode RGS proteins would inhibit S1P-induced ERK-2 phosphorylation in HEK-293 cells transfected with the S1P₁, S1P₂ or S1P₃ receptor (Figure 3). Since HEK-293 cells expressed endogenous S1P receptors, we compared the extent of S1P-induced ERK activation in S1P-receptor-transfected cells to that of vector-transfected cells (Figure 3A). Cells transfected with S1P receptors exhibited much larger S1P-stimulated ERK activation than did cells transfected with the vector control. S1P elicited a stronger phosphorylation of ERK-2 via the S1P₃ receptor than those observed in cells expressing either the S1P₁ or the S1P₂ receptor. The RGS proteins were also tested for their ability to inhibit endogenous S1P-receptor-mediated ERK activation (Figure 3A). RGS1 and RGS3 significantly, and RGS4 moderately, inhibited the endogenous S1P-receptor-mediated ERK activation. However, the inhibitory effect of RGS proteins on the endogenous S1P-receptor-mediated ERK activation in the S1P-receptor-transfected cells appeared minimal since the fold-increase in S1P-stimulated ERK activation via endogenous S1P receptors was small. In S1P-receptor-transfected cells (Figures 3B, 3C and 3D), RGS1 blocked ERK-2 phosphorylation induced via all three S1P receptor types. RGS4 potently attenuated S1P-induced ERK-2 phosphorylation mediated by the S1P₂ receptor; however, it showed little or no effect on ERK-2 phosphorylation induced via the S1P₁ or S1P₃ receptor. On the contrary, RGS2 significantly decreased ERK-2 phosphorylation induced via the S1P₃ receptor, whereas it did not inhibit S1P₁- and S1P₂-mediated ERK-2 phosphorylation. RGS3S, RGS3L and PDZ-RGS3 all contained an identical RGS domain and RGS3L was used for the S1P-signalling assays. RGS3L totally abolished ERK-2 phosphorylation induced by S1P via the S1P₃ receptor. It also significantly reduced ERK-2 phosphorylation mediated via the S1P₂ receptor and slightly blocked ERK-2 phosphorylation mediated via the S1P₁ receptor.

The RGS proteins were tested further for their ability in attenuating S1P-induced ERK activation in HASMCs known to contain multiple subtypes of S1P receptors [24]. HASMCs were transfected with constructs expressing ERK-2–HA and various RGS proteins, before being serum-starved, stimulated with S1P

Figure 5 RGS-protein-mediated inhibition of ET-1- or AT-II-induced ERK activation

HeLa cells were transfected with a construct directing expression of FLAG-tagged RGS1, HA-tagged RGS2, FLAG-tagged RGS3L or HA-tagged RGS4 in the presence of ERK-1–HA or ERK-2–HA expression vector. In addition, constructs expressing the ET_A receptor (A) or AT₁ receptor (B) were transfected. Cells were serum-starved, stimulated with ET-1 (100 nM) or AT-II (Ang II; 1 μM) for 5 min, and harvested for immunoblotting with anti-(phospho-ERK), anti-HA and anti-FLAG antibodies. The numbers above the RGS protein panels indicate the amount of transfected DNA (μg) needed to normalize the expression level. Results shown are representative of at least two experiments. pERK-1–HA and pERK-2–HA represent phosphorylated, transfected ERK-1–HA and ERK-2–HA respectively.

for 5 min and then harvested for the ERK signalling assay (Figure 4). To monitor transfection efficiencies and expression levels of RGS proteins in HASMCs, RGS–GFP fusion proteins were used. The new transfection method resulted in a transfection efficiency of 20–40 % as judged by GFP expression. RGS1 and RGS3L exhibited the most potent inhibition of S1P-induced phosphorylation of ERK in HASMCs. RGS4 also showed a significant decrease in S1P-induced ERK phosphorylation in HASMCs. RGS2 was least effective among the RGS proteins tested.

A similar set of experiments was performed analysing the effects of RGS1, RGS2, RGS3L and RGS4 on ET-1- and AT-II-induced ERK activation. Again, a considerable difference was noted in the efficacy of various RGS proteins to inhibit ERK phosphorylation induced by ET-1 (Figure 5A) or AT-II (Figure 5B). Transfection of a construct that expressed RGS3L potently inhibited ERK phosphorylation induced by ET-1, whereas RGS1 and RGS2 exhibited little effect. RGS4 showed a modest inhibition. Transfection of an expression construct for RGS1, RGS2 or RGS3L strongly attenuated AT-II-induced ERK-2 phosphorylation. RGS4 again exhibited a modest inhibition. Thus each of the S1P receptors, ET_A and AT₁ exhibited different sensitivities to these four RGS proteins (summarized in Table 2).

Intracellular localization of RGS proteins in HASMCs

To observe whether PDGF or S1P altered the intracellular localization of various RGS proteins in HASMCs, time-lapse recordings of live HASMCs transfected with RGS1–GFP, RGS2–GFP, RGS3–GFP or RGS4–GFP were performed using confocal fluorescence microscopy. HASMCs were transfected with various RGS–GFP fusion proteins, serum-starved, and

Table 2 Different sensitivities of various receptors towards RGS1, RGS2, RGS3L and RGS4

Relative degree of inhibition of ERK phosphorylation induced via S1P₁, ET_A, and AT₁ receptors by RGS proteins was scored based on the results shown in Figures 3 and 4. + + + +, potent; + + +, good; + +, modest; +, minimal; —, no inhibition.

	S1P ₁	S1P ₃	S1P ₂	ET _A	AT ₁
RGS1	+++	+++	++++	—	++++
RGS2	—	+++	—	—	+++
RGS3L	++	++++	+++	++++	++++
RGS4	+	—	+++	++	++

fusion protein expressed in human astrocytoma 1321N1 cells [25]. S1P did not elicit much change in intracellular distribution of any of the RGS-GFP fusion proteins. Treatment with PDGF caused membrane ruffling; however, none of the RGS-GFP fusion proteins significantly altered it (results not shown). The addition of PDGF did result in a significant accumulation of RGS2-GFP at the plasma membrane (Figure 6B). The initial recruitment of RGS2-GFP to the plasma membrane occurred approx. 1.5 min after the addition of the agonist and peaked around 10–12 min. In contrast, exposure to PDGF did not cause any significant translocation of the other RGS-GFP fusion proteins in HASMCs (results not shown).

DISCUSSION

There is a rapidly increasing body of knowledge showing that RGS proteins have significant functional roles in the cardiovascular system. In the present study, we systematically examined the expression patterns of four RGS proteins in the aorta and heart. Dynamic regulation of various RGS proteins by agonists that are critical for vascular development and function reinforces the notion that RGS proteins function in cardiovascular physiology. Importantly, the present study is the first to demonstrate the selective inhibition of RGS proteins in S1P-receptor-mediated signalling pathways and provides a comparison with their effects on the AT-II- and ET-1-receptor signalling pathways. We also show the utility of nucleofection for expressing constructs in HASMCs.

Differential expression undoubtedly partially determines the *in vivo* specificities of RGS proteins. Previous studies have shown region-specific expression patterns of RGS protein mRNAs in brain, in primary olfactory neurons and in the vomeronasal system, the latter implying a potential role for RGS proteins in spatially organized sensory coding [26,27]. At least five RGS proteins (RGS1 to RGS5) are abundantly expressed in aorta (the present study and [28]). A microarray study identified RGS5 as the most differentially expressed gene between artery and vein media [28]. In addition, rat aortic smooth muscle cells express multiple RGS mRNA transcripts [7], as did HASMCs and HAECs in the present study. Not only does the aorta express multiple RGS proteins, but so do rat heart and cardiomyocytes [6,7]. Our Northern blot and RT-PCR results revealed widespread, but differential, expression of RGS1, RGS2 and RGS3 isoforms, and RGS4 in the various regions of human heart. The highest expression levels of RGS1 and RGS2 occurred in the atria, whereas RGS3S and RGS3L predominated in the ventricles. The left ventricle had the highest level of RGS4. A recent study that examined the expression of RGS protein mRNAs in human myocardium also noted higher levels of RGS2 in the atria as compared with the left ventricle [29].

The striking expression of multiple RGS proteins in the aorta is consistent with the known importance of GPCR-mediated G-protein signalling in aortic development and function. Heterotrimeric G-protein signalling impacts both the control of aortic vascular tone and smooth-muscle proliferation. GPCR-mediated G α_q activation functions to relax vascular tone and to inhibit smooth-muscle growth [30,31]. In contrast, GPCR-mediated G α_q activation results in increasing vascular tone and augments smooth muscle growth [32,33]. RGS proteins such as RGS1, RGS3, RGS4 or RGS5 can by virtue of their GAP activities towards G α_q and G α_i reduce GPCR-mediated G α_q and G α_i activation, thus limiting GPCR-induced increases in vascular tone and signals for smooth-muscle proliferation. Reduced expression or functional inactivation of these RGS proteins may lead to excessive signalling through G α_q - and G α_i -linked GPCRs,

Figure 6 Intracellular localization of RGS proteins in HASMCs

(A) Confocal fluorescence images of live HASMCs expressing RGS1-GFP, RGS3-GFP or RGS4-GFP. Images were collected on a Leica TCS-SP2 confocal microscope using a 40 $\times$ oil immersion. The RGS1-GFP, RGS3-GFP and RGS4-GFP images shown were collected before the addition of any agonist. (B) Time-lapsed images of live RGS2-GFP-positive HASMCs stimulated with PDGF. HASMCs expressing RGS2-GFP were serum-starved and subsequently stimulated with 0.5 ml PDGF (at 40 ng/ml) added to the lower right corner of cell chambers. Confocal images were collected every 15 s for 20 min on a Leica TCS-SP2 confocal microscope using a 40 $\times$ oil immersion. The particular RGS2-GFP images shown were collected before the addition of the agonist, and 1.5, 12 and 20 min after. The arrowheads point to the area of RGS2-GFP recruitment at the plasma membrane.

were then stimulated with PDGF or S1P for 20 min during which time they were recorded. Before exposure to PDGF or S1P, we noted an even expression of RGS1-GFP and RGS4-GFP throughout the cell, including the nucleus, whereas RGS3-GFP was localized predominantly in the cytoplasm (Figure 6A). RGS2-GFP was prominently localized in the nucleus with some cytoplasm expression (Figure 6B). A previous study has shown similar localization of endogenous RGS2 as well as a RGS2-GFP

causing the opposite effect. RGS2 may have a dichotomous effect, limiting G_{α_q} signalling and G_{α_s} -mediated activation of adenylate cyclase III, a prominent isoform in the aorta. Among the RGS proteins that were detected in the aorta, PDZ-RGS3 was the only one uniquely expressed in HAECs. Since PDZ-RGS3 directly interacts with B-ephrins via its PDZ domain and because of the established role of ephrin signalling in sprouting angiogenesis and blood-vessel remodelling during vascular development [34,35], PDZ-RGS3 may function to link B-ephrin reverse signalling to GPCR signalling in vascular endothelial cells. In parallel with the suggested role in neuronal cell migration [34], PDZ-RGS3 may render endothelial cells insensitive to S1P or other G-protein-coupled ligands, when B-ephrin reverse signalling is activated. This may prevent endothelial cells from being recruited away by G-protein-coupled chemoattractants or selectively attenuate certain GPCR-mediated-signalling pathways during angiogenesis or vascular development.

Normal vascular-smooth-muscle-cell (vSMC) migration and proliferation depends upon intact signalling through the PDGF and S1P₁ receptors [36]. Lamellipodia extensions and cell motility induced by PDGF, but not cell proliferation, apparently requires intact S1P₁-receptor signalling [37]. Both PDGF-AA and PDGF-BB, but not S1P, significantly increased RGS2 mRNA expression in HASMCs, without affecting RGS3 and RGS4. However, since RGS2 did not affect S1P₁ signalling, its induction by PDGF probably affects other GPCR-linked signalling pathways. In HEK-293 and airway smooth muscle cells, to efficiently promote mitogenic signalling, the PDGF β receptor and S1P receptor formed complexes where RGS proteins could fine-tune the cross-communication between PDGF- and GPCR-mediated signalling pathways [38,39]. bFGF and TGF- β 1 also play crucial roles in vSMC differentiation and maturation [40]. Induction of RGS2 by bFGF and that of RGS3S by TGF- β -1 suggest cross-communication between GPCR-mediated and other signalling pathways, as previously reported with other RGS proteins [1,34]. Forskolin is a direct activator of adenylate cyclases and leads to an increase in intracellular cAMP levels. It potently increases RGS2 mRNA expression in HASMCs, as has been reported in human astrocytoma 1321N1 cells [25]. Since RGS2 strongly inhibits the activation of adenylate cyclases V and VI, two of the prominent adenylate cyclase isoforms in the heart [41], its induction by forskolin can provide a negative feedback inhibition.

Primary cells such as vSMCs, vascular endothelial cells and cardiomyocytes express multiple subtypes of S1P receptors. For example, vSMC prominently express S1P₂, with lesser amounts of S1P₁ and S1P₃ [24]. Because of the difficulty in discerning the effect of an individual RGS protein on a specific subtype receptor in these primary cells, we, as have others, used HEK-293 cells as a model cell line to study signalling events that occur downstream of the S1P receptors [37,42,43]. That information helped to interpret results obtained from examining S1P-induced ERK activation in HASMCs. RGS1 and RGS3, which inhibited signalling through each of the three S1P receptors in HEK-293 cells, also potently inhibited S1P-induced ERK phosphorylation in HASMCs. RGS4, which strongly inhibited ERK activation through the S1P₂ receptor, but not the S1P₁ receptor in HEK-293 cells, also significantly reduced S1P-induced ERK phosphorylation in HASMCs. RGS2, which only inhibited S1P₃-triggered ERK phosphorylation, only had a modest effect when introduced into HASMCs. We also examined whether these RGS proteins affected AT₁- and ET_A-mediated ERK phosphorylation. RGS1 and RGS2 blocked AT₁-receptor signalling, but not ET_A-receptor signalling, that led to ERK phosphorylation. RGS3 potently inhibited signalling through both receptors, whereas RGS4 modestly inhibited signalling.

RGS1, RGS3 and RGS4 all exhibit good GAP activities towards G_{α_i} and G_{α_q} , whereas RGS2 functions better as a G_{α_q} GAP than as a G_{α_i} GAP [1]. The S1P₁ receptor couples specifically to G_{α_i} , but is able to trigger Rac activation in a G_{α_i} -independent manner [14]. The S1P₃ receptor couples efficiently to G_{α_q} and also to $G_{\alpha_{12}}$, and the S1P₂ receptor couples to G_{α_i} , G_{α_q} and $G_{\alpha_{12}}$. The ET_A receptor couples predominantly to G_{α_q} , as does the AT₁ receptor [44,45]. Thus the GAP-activity profile of a specific RGS protein does not allow an accurate prediction of the sensitivity of a specific GPCR signalling pathway to the RGS protein. These results support other studies that have shown RGS proteins to exhibit receptor selectivity. The inhibition of G_{α_q} -mediated Ca²⁺ signalling by different RGS proteins varies dramatically depending on the triggering GPCR [46] and synthetic ribozymes designed to target either RGS3 or RGS5 differentially augmented signalling through the muscarinic M₃ and AT₁ receptors [47]. Because some cells express multiple S1P receptor isotypes, which seemingly mediate opposite signalling events such as S1P₁-stimulated versus S1P₂-inhibited activation of Rac and cell migration, RGS proteins may function as subtype-selective receptor antagonists. The net outcome of activation induced by S1P may depend on the network of RGS proteins in a particular cellular context. Furthermore, the cellular milieu may alter either the expression or intracellular localization of an RGS protein. For example, PDGF-induced induction of RGS2 mRNA and its recruitment of RGS2 to the plasma membrane may render cells insensitive to S1P₃-mediated signalling, but allow cellular processes mediated by S1P₁.

Gene-targeting studies of members of the S1P receptor family in mice and zebrafish have revealed their importance for cardiovascular development. Mice that lack the S1P₁ receptor and zebrafish that lack the S1P₂-like receptor exhibit severe defects in the development of blood vessels including the aorta and those in the heart respectively [17,18]. However, S1P₃ knock-out mice show no obvious defects although S1P₃-mediated signalling is clearly impaired [48]. Based on the importance of S1P₁ receptor in aorta development, the sensitivity of S1P₁-receptor signalling to RGS1 and the high level expression of RGS1 in adult aorta, it will be of interest to determine when RGS1 expression occurs developmentally.

In summary, the present study strongly supports the concept that RGS proteins participate in cardiovascular development and function by fine-tuning different GPCR-coupled signalling pathways. Furthermore, they may mediate cross-communication between GPCR-coupled signalling pathways and other signalling pathways such as those triggered by PDGF, FGF and TGF- β 1. Additional studies of RGS proteins using genetically modified animals in which components of GPCR-signalling pathways that are important for cardiovascular development and/or function have been disrupted and the examination of RGS proteins in disease alterations of the heart and blood vessels should provide a wealth of information about the overall importance of these proteins in the cardiovascular system.

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