Regulation of cardiovascular GPCR signalling

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S1P receptor signalling and RGS proteins; expression and function in vascular smooth muscle cells and transfected CHO cells

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ABSTRACT

Sphingosine-1-phosphate (S1P) signalling via G protein-coupled receptors is important for regulation of cell function and differentiation. Specific Regulators of G protein Signalling (RGS) proteins modulate the function of these receptors in many cell types including vascular smooth muscle cells (VSMCs). Therefore, we investigated the role of altered expression levels of RGS proteins in S1P receptor function in VSMCs and transfected CHO cells.

The mRNA expression of the S1P\(_1\) receptor, RGS4 and RGS16 were down-regulated in VSMCs during phenotypic modulation induced by culturing, whereas mRNA levels of RGS2, RGS3, S1P\(_2\) and S1P\(_3\) receptors were unchanged. Interestingly, the expression level of RGS5 was transiently up-regulated. Despite major alterations in RGS levels, S1P-induced calcium elevation in VSMCs was not altered. Co-transfection of RGS2, RGS3, RGS4, RGS5 and RGS16 into CHO-Flp-In cells stably expressing the S1P\(_1\) or S1P\(_3\) receptor did not modify S1P-induced inhibition of cAMP accumulation to a major extent. Similar results were obtained with SEW2871, a selective S1P\(_1\) receptor agonist. However, the inhibition of cAMP accumulation by the agonist FTY720-P via the S1P\(_1\) receptor was significantly decreased by co-transfection with RGS5.

These results indicate that mRNA of the S1P\(_1\) receptor, RGS4, RGS5 and RGS16 are differentially regulated during phenotypic modulation. However, major alterations in RGS protein expression have only limited effect on S1P receptor function.
INTRODUCTION

The sphingomyelin metabolite sphingosine-1-phosphate (S1P) is a ubiquitous modulator of cellular functions (Kluk and Hla, 2002; Pyne and Pyne, 2000). For example, in blood vessels S1P can affect maturation, differentiation, proliferation and migration of both endothelial and smooth muscle cells (Peters and Alewijnse, 2007). S1P acts on a specific set of G-protein-coupled receptors; the S1P receptors (formerly known as Edg receptors) of which five subtypes have been identified (Chun et al., 2002). Within blood vessels mainly the receptor subtypes S1P₁, S1P₂ and S1P₃ are expressed; while S1P₃ is the most abundantly expressed subtype in the endothelium, most studies report that in the vascular smooth muscle cells (VSMCs) the S1P₂ subtype is most abundant, followed by the S1P₃ receptor (Michel et al., 2007). While S1P₁ receptors mainly couple to Gᵢ, S1P₂ and S1P₃ receptors couple to multiple G proteins including Gₒ and G₉₁₁ (Windh et al., 1999). S1P-induced elevations of intracellular Ca²⁺ concentrations in cultured rat VSMCs occur largely if not exclusively via S1P₂ receptors as no effect was seen for FTY720-P, a S1P receptor agonist devoid of S1P₂ receptor affinity (M. Jongsma et al., manuscript in preparation).

G protein signalling is fine-tuned by Regulators of G protein Signalling (RGS) proteins. They form a family of highly diverse and multifunctional signalling proteins that share a conserved RGS domain. The RGS domain binds directly to activated Gᵢ subunits of heterotrimeric G proteins and modulates G protein signalling by functioning as a GTPase-activating protein. To date more than 30 RGS and RGS-like proteins are known, which can be divided into six subfamilies based on their amino acid sequence (Hollinger and Hepler, 2002). Most RGS proteins are GTPase activating proteins for Gᵢₒ and/or Gᵣ₁₄ proteins. However, the functional role of RGS proteins apparently depends not only on the G protein but also on the receptor (Abramow-Newerly et al., 2006; Hendriks-Balk et al., 2008), indicating the possible existence of a multimeric protein complex involving the G protein-coupled receptor, G protein, RGS protein, and maybe also other proteins. Similar to S1P receptors (see above) RGS proteins, particularly RGS2 and probably also RGS5, are implicated in the control of vascular function (Calo et al., 2004; Cho et al., 2008; Heximer et al., 2003). A possible role of some RGS proteins in the function of S1P receptor subtypes has been postulated by one group of investigators based upon experiments using extracellular signal-regulated kinase (ERK) activation as functional read-out (Cho et al., 2003a; Cho et al., 2003b).

The modulation from a contractile (differentiated) to a more synthetic (dedifferentiated) phenotype of VSMCs plays an integral role in the development and progression of various vascular diseases, e.g. atherosclerosis, neointima formation after angioplasty and possibly hypertension (Campbell and Campbell, 1985; Shanahan and Weissberg, 1998). In contrast to the VSMCs with a contractile phenotype, the VSMCs with a synthetic phenotype are characterized by increased proliferation and migration and under growth promoting conditions, e.g. in culture, VSMCs rapidly change phenotype from contractile to synthetic due to the presence
of extracellular matrix proteins and growth factors in serum (Hayashi et al., 2001; Sobue et al., 1999). Both S1P (Boguslawski et al., 2002; Kluk and Hla, 2001; Lockman et al., 2004) and RGS proteins (Adams et al., 2006; Berger et al., 2005; Geary et al., 2002; Li et al., 2004) have been implicated in VSMC proliferation and migration and thus might play a role in the phenotypic modulation of VSMCs. Therefore, we investigated the role of altered expression levels of RGS proteins in S1P receptor function in VSMCs and transfected CHO cells.

**MATERIALS AND METHODS**

**Materials**

Male Wistar rats (240-280g) were purchased from Charles River (Maastricht, The Netherlands). Collagenase I (crude), elastase type I, gelatin, soybean trypsin inhibitor, forskolin, 3-isobutyl-1-methylxanthine, activated charcoal, HEPES, Tween-20, Triton-X100, EGTA, probenecid and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

CHO-Flp-In cells, pcDNA3.1HisA/B/C, pcDNA5/FRT/TO, pOG44, Lipofectamine™ 2000, Opti-MEM, Medium199, F-12 nutrient mixture (Ham), penicillin/streptomycin, foetal bovine serum (FBS), trypsin/EDTA, phosphate buffered saline (PBS), Hank’s balanced salt solution (HBSS), Trizol reagent, DNase I (Amplification grade), LDS sample buffer, reducing agent, antioxidant, NUPAGE® Novex 10% Bis-Tris gels, NUPAGE® MOPS buffer, transfer buffer, Magic Marker, See-Blue prestained marker, anti-HisG antibody (mouse monoclonal IgG2a) and Invitrolon PVDF membranes were obtained from Invitrogen (Breda, The Netherlands). Hygromycin B and geneticin (G418 sulphate) were from PAA (Coelbe, Germany). iScript™cDNA Synthesis kit, iQ™ SYBR® Green Supermix and Experion RNA StdSense Analysis Kit were purchased from Bio-Rad Laboratories BV (Veenendaal, The Netherlands). Primers were from Biolegio (Nijmegen, The Netherlands).

BCA protein determination kit and HALT protease inhibitor cocktail were obtained from Pierce (via Perbio Science, Etten-Leur, The Netherlands). RIPA buffer with EDTA was from Boston Bioproducts (Worcester, UK). BM chemiluminescence blotting substrate (POD) was obtained from Roche Diagnostics (Almere, The Netherlands). ECL Hyperfilms were from Amersham Biosciences (Diegem, Belgium).

Goat anti-mouse AlexaFluor® 488, pluronic acid and Fluo-4 AM were obtained from Molecular Probes (via Invitrogen, Breda, The Netherlands). Mouse anti-HA was from Zymed (via Invitrogen, Breda, The Netherlands). Mouse anti-smooth muscle-actin was purchased from Dako Cytomation (Heverlee, Belgium). UltraCruz with DAPI, goat anti-RGS4 and goat anti-mouse-HRP were from SantaCruz Biotechnology (via Tebu-bio, Heerhugowaard, The Netherlands). Normal donkey serum and donkey anti-goat-HRP were obtained from Jackson
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Immunoresearch (via Brunschwig chemie, Amsterdam, The Netherlands). Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Chemicon (Amsterdam, The Netherlands). The LANCE cAMP 384 kit and 384-well optiplates were from Perkin-Elmer (Zaventem, Belgium). S1P was obtained from Avanti Polar Lipids Inc (via Instruchemie, Delfzijl, The Netherlands). 2-Amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol mono(dihydrogen phosphate) ester (FTY720-P) was synthesized according to previously described methods (Albert et al., 2005). SEW2871 (5-(4-Phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)-1,2,4-oxadiazole) was obtained from Calbiochem (via VWR, Amsterdam, The Netherlands). The expression vectors pcDNA3.1-S1P₁, pcDNA3.1-S1P₃, pcDNA3.1-3xHA-RGS2, pcDNA3.1-3xHA-RGS4, pcDNA3.1-3xHA-RGS5, pcDNA3.1-3xHA-RGS16 and pcDNA3.1-RGS3 were purchased from UMR cDNA Resource Center (Rolla, MO, USA).

Isolation and culture of vascular smooth muscle cells

The experimental protocol was approved by the Animal Ethical Committee of the University of Amsterdam, The Netherlands. Male Wistar rats were killed by an overdose pentobarbital (100 mg/kg, i.p.) and their thoracic aortas were removed. The aortas were cleaned of fat and connective tissue under aseptic conditions and rinsed several times in Medium 199 containing penicillin (100 U/ml) and streptomycin (100 µg/ml). The cleaned vessels were incubated, shaking, at 30 °C in 5 ml digestion medium (Medium 199 containing 1 mg/ml collagenase I, 0.1 mg/ml elastase, 0.5 mg/ml soybean trypsin inhibitor, 1 mg/ml BSA and penicillin/streptomycin) for 30 min before the adventitial layer was removed as an everted tube. The remaining tissue was rinsed with Medium 199 containing antibiotics and further incubated in 5 ml digestion medium at 37 °C for 45 min. The partly digested tissue was then rinsed in Medium 199 containing antibiotics, cut into very small pieces and incubated for a further 2.5 h in 8 ml digestion medium at 37 °C. After digestion single cells were prepared by tituration and washed twice by resuspension in Medium 199 supplemented with 20% FBS and antibiotics. The isolated VSMCs were cultured in flasks coated with 0.1% gelatine in Medium 199 supplemented with 20% FBS and antibiotics for one week with changing the medium every 2-3 days. These VSMCs were defined as passage 0 (p0). After the first subculturing using trypsin/EDTA VSMCs were cultured in Medium 199 containing 10% FBS and antibiotics (with 0.1% gelatine coating). They were cultured until confluency with change of medium every 2-3 days before the cells were split 1:2. After subculturing the passage number of the cells was increased by one. VSMCs were cultured until passage 6. Cells were cultured at 37 °C in humidified air containing 5% CO₂.
Staining of VSMCs for \(\alpha\)-actin

The VSMCs were seeded in an 8-well chamber slide. At 60-70% confluence the cells were washed twice with PBS and fixated with 4% cold formaldehyde for 30 min. Thereafter the cells were washed with PBS with 0.1% Tween-20 three times and blocked with 3% BSA in PBS with 0.1% Tween-20 and 0.2% Triton-X100 for 30 min. The cells were then incubated for 1 h with mouse anti-\(\alpha\)-actin (1:100) in PBS with 0.1% Tween-20 and 0.2% Triton-X100. After washing the cells were incubated with goat anti-mouse-AlexaFluor® 488 (1:100) in PBS with 0.1% Tween-20 and 0.2% Triton-X100 for 1 h. After washing, the chamber slide was mounted with UltraCruz containing DAPI and fluorescence was determined using fluorescence microscopy.

RNA isolation and cDNA synthesis

After enzymatic isolation the VSMCs were cultured into two 6 cm culture dishes coated with 0.1% gelatine. The cells were grown until passage 6. At every passage cells were lysed with 2 ml Trizol after washing twice with PBS.

Total RNA was isolated according to the manufacturer’s protocol with minor changes, using a second chloroform extraction to remove traces of phenol in the aqueous phase, a high salt solution (0.8 M sodium citrate, 1.2 M NaCl) together with isopropanol to precipitate RNA and a second wash of the RNA pellet with 75% ethanol. RNA purity was verified by gel electrophoresis (Experion, Bio-Rad Laboratories) and the RNA concentration was determined by spectrophotometry (Nanodrop, Isogen Life Science). To eliminate genomic DNA contamination 1 \(\mu\)g of total RNA was treated with 1.5 \(\mu\)l DNAse I, Amp Grade. cDNA was synthesized by reverse transcription using the iScript cDNA Synthesis kit according to the manufacturer’s protocol. A control for the presence of genomic DNA, in which no cDNA was synthesized, was made for each sample. The cDNA of 1 \(\mu\)g RNA was diluted 1:50 for use in real-time quantitative PCR.

Design of primers

Oligonucleotide primers were designed using the D-LUX designer software (Invitrogen), based on sequences from the GenBank database (Table 1 and 2). Each primer pair was tested for selectivity, sensitivity and PCR efficiency. The expression of elongation factor-1 (EF-1), p0 ribosomal protein and GAPDH seemed to be constant in all passages. Therefore, these genes were selected as endogenous controls to correct for potential variation in RNA input (for selection criteria see (Hendriks-Balk et al., 2007)).


**Table 1.** Oligonucleotide primers used for real-time quantitative polymerase chain reaction on rat VSMC samples. Non-capital letters indicate that these nucleotides are added to form a hairpin.

<table>
<thead>
<tr>
<th>Gene</th>
<th>accession</th>
<th>sequence</th>
<th>amplicon</th>
</tr>
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<tr>
<td>α-smooth muscle-actin</td>
<td>X06801</td>
<td>forward ACTGCTGACGCTGAGATCGT</td>
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<td></td>
<td></td>
<td>reverse GCCCATCAAGGCAGTTCTG</td>
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<td>SM22α</td>
<td>M83107</td>
<td>forward ATATGGCAGAGTGCAAGG</td>
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<td></td>
<td></td>
<td>reverse TGCGGAAGATTGTTCAAGGTC</td>
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<td>Calponin1</td>
<td>X71071</td>
<td>forward GCCAGGTGATGATCACAAGT</td>
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<tr>
<td></td>
<td></td>
<td>reverse gcagcaATTGTGGTGAGCTG</td>
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<td>RGS2</td>
<td>NM_053453</td>
<td>forward TCTGTTGCTGGCAAGACT</td>
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<td></td>
<td></td>
<td>reverse cactgaCTTGGAGCTCTCTGATG</td>
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<td>RGS3</td>
<td>NM_019340</td>
<td>forward GAGGCTTCCAGCAACCTT</td>
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<td></td>
<td></td>
<td>reverse cacaggTCATCCACTATCTTG</td>
<td></td>
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<td>RGS4</td>
<td>AF117211</td>
<td>forward cggtatCTGATGAAAAGGATTCATCCG</td>
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<td></td>
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<td>RGS5</td>
<td>AF241259</td>
<td>forward TGCCAGAGACGCAAAGCA</td>
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<td></td>
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<td>S1P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>NM_017301</td>
<td>forward TTCTGTTGCTGGCTGCT</td>
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<td></td>
<td></td>
<td>reverse gactctTGAAATTGAGGGAGATC</td>
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<td>S1P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>NM_017192</td>
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<td></td>
<td>reverse ATGTCATCCCAAACGAGGACC</td>
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<tr>
<td>S1P&lt;sub&gt;3&lt;/sub&gt;</td>
<td>AF184914</td>
<td>forward cggagCACTCTCAACAGCATTCTG</td>
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<td></td>
<td></td>
<td>reverse CTGGCGTCTGGACTTGAG</td>
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<tr>
<td>EF-1</td>
<td>NM_175838</td>
<td>forward GCAAG GCCCATG TGTTGAA</td>
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<td></td>
<td></td>
<td>reverse TGATGACCCACAGCAACTG</td>
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<tr>
<td>GAPDH</td>
<td>M17701</td>
<td>forward CTTCCGTGTTCCTTACC</td>
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<td></td>
<td></td>
<td>reverse ACCTGCTCTACGATGAG</td>
<td></td>
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<tr>
<td>p0 ribosomal protein</td>
<td>X15096</td>
<td>forward cacagaAGGGTCTCTGGCTTTGCTG</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse CGCAAATGCGAGATGGATCG</td>
<td></td>
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**Real-time quantitative PCR**

Relative quantification of mRNA was performed on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories) following the thermal protocol: 95°C for 3 min to denature, 40 cycles at 95°C for 10 seconds followed by 60°C for 45 seconds for annealing and extension. The final reaction mixture of 15 µl consisted of diluted cDNA, 1x iQ Sybr Green Supermix, 200 nM of the forward primer and 200 nM of the reverse primer. All the reactions were performed in 96-well plates, in duplicate. Controls for genomic DNA were included for each cDNA sample and also a negative control containing only both primers and the iQ Sybr Green Supermix. All data were corrected for RNA input using the expression of EF-1, p0 ribosomal protein and GAPDH simultaneously. The relative expression of each gene is expressed against the expression level at passage 6 (p6).
Chapter 4

Intracellular calcium measurements

VSMCs were plated in black, clear bottom 96 wells plates at 10,000 cells/well two days before the measurement. After serum starvation overnight, cells were loaded for 1 h with 4 μM Fluo-4 AM ester in HBSS containing 20 mM HEPES, 0.42 % v/v pluronic acid, 10 mg/ml BSA and 2.5 mM probenecid and incubated at 37 °C. After loading, cells were washed twice with HBSS containing 20 mM HEPES and 2.5 mM probenecid and incubated at 37 °C with HBSS containing 20 mM HEPES and 2.5 mM probenecid for 1 h. Fluorescence was measured at 37 °C using an excitation filter at 485 nm and emission filter at 520 nm on a NOVOstar plate reader (BMG Labtech, via Isogen, IJsselstein, The Netherlands). After measuring the basal level for 10 s, the ligand was added at 10 % v/v diluted in HBSS containing 20 mM HEPES and 2.5 mM probenecid. After 60 sec Triton X-100 was added (final concentration = 0.5 % v/v) to determine the maximal signal (F_{max}). After 80 sec EGTA was added (final concentration = 10 mM) to determine the minimal signal (F_{min}).

The increase in [Ca^{2+}]_{i} upon ligand stimulation was calculated as the difference between the basal [Ca^{2+}]_{i} level and the level after adding a ligand. The level of [Ca^{2+}]_{i} was calculated via the equation: [Ca^{2+}]_{i} = K_{d} * ((F_{min})/(F_{max}-F)). K_{d} is the dissociation constant of the binding of Fluo-4 to calcium (345 nM).

Creation of stable cell lines

CHO-Flp-In cells stably expressing the His-tagged human S1P_{1} (Jongsma et al., 2007) or S1P_{3} receptor were used. The cDNA of the S1P_{3} receptor obtained from UMR contained a point mutation (G^{623}A which results in R^{621}Q) that was removed by PCR using the following primers:

**Table 2.** Oligonucleotide primers used for real-time quantitative polymerase chain reaction on samples of the stable cell lines to detect mRNA expression of human RGS2, RGS3, RGS4, RGS5 and the S1P_{1} and S1P_{3} receptor. Non-capital letters indicate that these nucleotides are added to form a hairpin.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Sequence</th>
<th>Amplicon</th>
</tr>
</thead>
</table>
| S1P_{1} | NM_001400 | forward: cgtagtGCAGCTCGGTTCTGACTAcG  
 reverse: CTCTTTGCCCGCTGATATTC | 89 |
| S1P_{3} | NM_005226 | forward: cgctacAAACACAAACTCGGAcG  
 reverse: ACAGGCTACACAAATCACAc | 65 |
| RGS2 | NM_002923 | forward: gtagctTGGAAGACCGGTTTGAcTAc  
 reverse: CTCAGGAAGGCTTTGATGAAA | 107 |
| RGS3 | NM_144488 | forward: CAAGCCTTCTTCGCACGTGA  
 reverse: cacagtcTGATGCCATCTTGGAGCTG | 100 |
| RGS4 | NM_005613 | forward: cagccAGAGAAGTCAAGAAATGGGcTcG  
 reverse: TGTCCTGGAGACTTAGTcATG | 176 |
| RGS5 | NM_003617 | forward: TCATTCACGGAGGCCTCTAA  
 reverse: cacaagTGTCACCGAGCTCTCATTGcG | 79 |
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(forward: TGCCTGTCAGGGGGCGGGGGGCGGCG, reverse: CGGGCCCCCGCCCCCTGACCAG-GCA). The S1P\textsubscript{3} receptor coding DNA in the resulting plasmid as well as the stable cell line constructed with this plasmid has been confirmed by sequencing. The cell line stably expressing the human His-tagged S1P\textsubscript{3} receptor was constructed in a similar way as described previously (Jongsma et al., 2006).

Stable cell lines expressing either the S1P\textsubscript{1} or S1P\textsubscript{3} receptor were transfected with cDNA of RGS proteins using Lipofectamine 2000 according to the manufacturer’s protocol and stable cell lines were created by clonal selection using geneticin (500 µg/ml). All RGS proteins were HA-tagged except RGS3, which was either untagged (in cells with S1P\textsubscript{1} receptors) or His-tagged (cells with S1P\textsubscript{3} receptors). RGS expression was confirmed by real-time PCR and/or immunoblotting with antibodies against the HA- or the His-tag.

CHO-Flp-In cells stably expressing the His-tagged S1P\textsubscript{1} or S1P\textsubscript{3} receptor with RGS2, RGS3, RGS4, RGS5, RGS16 or empty vector were split 1:3 every 2 or 3 days in F-12 Nutrient Mixture (Ham) with L-glutamine, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 313 µg/ml hygromycin B, 350 µg/ml geneticin and 10% charcoal-stripped FBS. All cell lines were cultured at 37 °C in humidified air containing 5% CO\textsubscript{2}.

**Immunoblotting**

Cells were scraped in PBS and lysed in RIPA buffer with protease inhibitor. Protein concentration was determined using the BCA kit according to the manufacturer’s protocol. Ten micrograms of protein was loaded onto a 10% NuPAGE® Bis–Tris Gel. Electrophoresis was carried out at 200 V for 55 min in NuPAGE® MOPS running buffer. Protein was transferred to Invitrolon™ PVDF blotting membranes at 30 V for 1 h. The membrane was blocked for 1 h at room temperature in 50 mg/ml non-fat dry milk in PBS with 0.1% Tween-20 followed by overnight incubation at 4°C with mouse anti-HA or mouse anti-HisG (1:1,000) or blocked in 5% normal donkey serum in PBS with 0.1% Tween-20 followed by overnight incubation at 4°C with goat anti-RGS4 (1:5,000) in 1% normal donkey serum in PBS with 0.1% Tween-20. After washing the membrane was incubated with goat anti-mouse-HRP IgG (1:5,000) or donkey anti-goat-HRP IgG (1:10,000) for 1 h at room temperature. Detection was done using BM Chemiluminescence Blotting Substrate and Hyperfilm ECL.

**cAMP measurement**

CHO-Flp-In cells were plated in 96 well plates at 20,000 cells/well two days before the measurement. After serum starvation overnight, cells were washed with stimulation buffer (HBSS, 0.05% BSA and 5 mM HEPES) and subsequently stimulated for 15 min with various concentrations of the ligands in stimulation buffer with 0.5 mM 3-isobutyl-1-methylxanthine and 3 µM forskolin at room temperature. After removal of the stimulation mixture the cells were lysed.
with 50 μl 0.5% Triton-X100 in stimulation buffer with 0.5 mM 3-isobutyl-1-methylxanthine and 10 μl of the lysate was added to the 384-well optiplate in triplicate. Detection of the cAMP formed during stimulation was performed with the LANCE cAMP 384 kit according to the manufacturer’s protocol. Measurements were carried out using a Victor plate reader (Wallac, Perkin-Elmer, Zaventem, Belgium) three hours (FTY720-P on cells with S1P\(_3\) receptors) or one day after adding detection buffer and antibody mixture.

**Data analysis**

QPCR data are analysed using the Gene expression Macro software (Bio-Rad Laboratories) based on algorithms of Vandesompele et al. (Vandesompele et al., 2002). The data are expressed as mean relative expression ± standard error of the mean (S.E.M.) and related to the expression level in VSMCs at p6. For statistical analysis relative expression was log-transformed and then one-sample t-tests were performed using GraphPad InStat (GraphPad Software, San Diego, CA, USA).

Densitometry of the bands on the western blots was done using ImageJ software (NIH, Bethesda, MD, USA). The data were normalized for GAPDH protein expression.

Data of the calcium and cAMP measurements were analysed using GraphPad Prism software. The data of the cAMP measurements were normalized to the top of each individual curve fit and expressed as mean ± S.E.M. Differences between cell lines were compared using one-way ANOVA with a Dunnett’s correction. A P<0.05 was considered as statistically significant.

**RESULTS**

**Expression of VSMC markers**

After enzymatic isolation of cells from the aorta we confirmed the presence of VSMC in our culture by staining for \(\alpha\)-smooth muscle-actin. More than 90% of the cells showed a positive staining for \(\alpha\)-smooth muscle-actin (Fig. 1A).

To investigate whether the VSMCs switched from a contractile to a more synthetic phenotype during culturing we determined the mRNA expression of two markers of the contractile phenotype, calponin1 and SM22\(\alpha\), and of \(\alpha\)-smooth muscle-actin in VSMCs at different passages. All three markers showed a significantly greater mRNA expression at p0 and p1 of culturing compared to p6 (Fig. 1B-D). The greatest difference in mRNA expression of the three markers was seen between p0 and p1.
Expression of S1P receptors and RGS proteins upon culturing of VSMCs

We investigated whether mRNA expression of the S1P₁, S1P₂ and S1P₃ receptor was changed upon VSMC culturing. The S1P₁ receptor showed a decline in mRNA expression upon culturing, whereas the S1P₂ and S1P₃ receptors were not affected by culturing (Fig. 2).

We also investigated the changes in mRNA expression of RGS2, RGS3, RGS4, RGS5 and RGS16 during VSMC culturing. RGS4 and RGS16 showed a greater mRNA expression at lower passage number, whereas RGS5 mRNA expression was transiently increased with a peak in p3 and a subsequent decline to levels similar to those observed in p6 (Fig. 3). RGS2 and RGS3 mRNA expression was not significantly regulated upon culturing.

Immunoblotting experiments to determine differences in RGS4 protein expression showed a decrease in protein expression at p2 and p6 compared to p0 (Fig. 4).
To investigate whether culturing of primary VSMCs influences S1P signalling we measured the S1P-induced elevation in \([\text{Ca}^{2+}]_i\) in VSMCs at p1, p2, p3 and p6. S1P caused a concentration-dependent increase in intracellular \([\text{Ca}^{2+}]_i\) with a similar potency in all VSMC passages (pEC\textsubscript{50} values: 7.0 ± 0.2, 7.2 ± 0.1, 7.0 ± 0.1 and 7.1 ± 0.1 for p1, p2, p3 and p6, respectively, n=5-14) (Fig. 5).

**Effects of VSMC culturing on S1P signalling**

To investigate whether culturing of primary VSMCs influences S1P signalling we measured the S1P-induced elevation in \([\text{Ca}^{2+}]_i\) in VSMCs at p1, p2, p3 and p6. S1P caused a concentration-dependent increase in intracellular \([\text{Ca}^{2+}]_i\) with a similar potency in all VSMC passages (pEC\textsubscript{50} values: 7.0 ± 0.2, 7.2 ± 0.1, 7.0 ± 0.1 and 7.1 ± 0.1 for p1, p2, p3 and p6, respectively, n=5-14) (Fig. 5).
We determined the effect of RGS protein expression on signalling of the S1P₁ and S1P₃ receptor by stably co-transfecting the His-tagged S1P receptor subtypes and specific RGS proteins in CHO-Flp-In cells. Addition of the N-terminal His-tag to the receptor did not influence S1P receptor signalling (Jongsma et al., 2007). Expression of the RGS proteins in these cells was
confirmed by real-time PCR (Ct values in co-transfection with S1P1 and S1P3 receptors were 29 and 32 for RGS2, 23 and 27 for RGS3, 24 and 24 for RGS4, 24 and 26 for RGS5, respectively; cells not transfected with RGS had Ct values >40, indicating the absence of human RGS proteins in these cells) and/or immunoblotting with antibodies against the HA- or the His-tag (Fig. 6A and B). No changes in mRNA or protein expression of S1P1 receptor and the S1P3 receptor were observed for the selected cell lines upon co-transfection with a specific RGS protein (data not shown).

Forskolin (3 μM) caused a cAMP accumulation of approximately 185 and 400 fmol/well in cells expressing S1P1 and S1P3 receptors, respectively, and similar values were found upon co-transfection with any of the RGS proteins (data not shown). S1P was somewhat more potent at S1P1 than at S1P3 receptors (Table 3). None of the co-transfected RGS proteins significantly altered the potency of S1P to inhibit the forskolin-induced cAMP formation via the S1P1 or the S1P3 receptor (Table 3 and 4). In addition, no significant alterations in S1P efficacy at both S1P receptors were found although RGSS5 caused a slight decrease in S1P efficacy on the S1P1 receptor mediated inhibition of cAMP signalling (Table 4). Similarly, no significant effects of the co-transfected RGS proteins were found on the potency and efficacy of SEW2871 to inhibit the forskolin-induced cAMP formation via the S1P1 receptor (Table 3 and 4).

The potency of FTY720-P to inhibit the forskolin-induced cAMP accumulation via the S1P1 or the S1P3 receptor was not significantly altered upon co-transfection with RGS proteins (Table 3). Also, co-transfection of RGS proteins did not significantly affect the efficacy of FTY720-P to inhibit S1P3 receptor-mediated inhibition of cAMP signalling (Table 4). However,
S1P receptor signalling and RGS proteins: expression and function in VSMCs and CHO cells

**Table 3.** Influence of co-transfection with RGS proteins on pEC$_{50}$ values of different S1P receptor agonists to inhibit forskolin-induced cAMP formation via His-tagged S1P$_1$ and S1P$_3$ receptor subtypes in CHO-Flp-In cells. Data are expressed as mean ± S.E.M. (n). Note that none of the observed differences was statistically significant when compared to mock-transfected cells.

<table>
<thead>
<tr>
<th></th>
<th>S1P</th>
<th>SEW2871</th>
<th>FTY720-P</th>
<th>S1P</th>
<th>FTY720-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>mock</td>
<td>9.2 ± 0.1 (11)</td>
<td>7.6 ± 0.1 (11)</td>
<td>8.8 ± 0.1 (9)</td>
<td>8.3 ± 0.1 (5)</td>
<td>7.7 ± 0.1 (4)</td>
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<tr>
<td>RGS2</td>
<td>9.4 ± 0.1 (4)</td>
<td>7.4 ± 0.3 (3)</td>
<td>8.7 ± 0.1 (9)</td>
<td>8.5 ± 0.1 (4)</td>
<td>7.5 ± 0.1 (4)</td>
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<tr>
<td>RGS3</td>
<td>9.0 ± 0.2 (3)</td>
<td>7.5 ± 0.2 (5)</td>
<td>8.9 ± 0.2 (4)</td>
<td>8.5 ± 0.2 (4)</td>
<td>7.6 ± 0.1 (4)</td>
</tr>
<tr>
<td>RGS4</td>
<td>9.5 ± 0.1 (4)</td>
<td>7.4 ± 0.1 (3)</td>
<td>9.0 ± 0.2 (7)</td>
<td>8.7 ± 0.1 (4)</td>
<td>7.7 ± 0.1 (4)</td>
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<tr>
<td>RGS5</td>
<td>9.2 ± 0.2 (7)</td>
<td>7.4 ± 0.1 (3)</td>
<td>8.6 ± 0.2 (8)</td>
<td>8.4 ± 0.1 (4)</td>
<td>7.7 ± 0.1 (4)</td>
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<tr>
<td>RGS16</td>
<td>9.2 ± 0.1 (4)</td>
<td>7.5 ± 0.1 (4)</td>
<td>8.9 ± 0.1 (6)</td>
<td>8.4 ± 0.1 (4)</td>
<td>7.5 ± 0.1 (4)</td>
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**Table 4.** Influence of co-transfection with RGS proteins on E$_{max}$ values (represented as % inhibition) of different S1P receptor agonists to inhibit forskolin-induced cAMP formation via His-tagged S1P$_1$ and S1P$_3$ receptor subtypes in CHO-Flp-In cells. Data are expressed as mean ± S.E.M. (n), *P<0.05 compared to mock-transfected cells.

<table>
<thead>
<tr>
<th></th>
<th>S1P</th>
<th>SEW2871</th>
<th>FTY720-P</th>
<th>S1P</th>
<th>FTY720-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>mock</td>
<td>66 ± 2 (11)</td>
<td>74 ± 3 (11)</td>
<td>75 ± 4 (9)</td>
<td>77 ± 3 (5)</td>
<td>89 ± 2 (4)</td>
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<tr>
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<td>75 ± 1 (3)</td>
<td>75 ± 2 (9)</td>
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<tr>
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<td>73 ± 3 (4)</td>
<td>77 ± 4 (4)</td>
<td>89 ± 3 (4)</td>
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<tr>
<td>RGS4</td>
<td>68 ± 5 (4)</td>
<td>69 ± 2 (3)</td>
<td>70 ± 3 (7)</td>
<td>83 ± 2 (4)</td>
<td>97 ± 1 (4)</td>
</tr>
<tr>
<td>RGS5</td>
<td>55 ± 4 (7)</td>
<td>69 ± 6 (3)</td>
<td>60 ± 3 (8)*</td>
<td>79 ± 2 (4)</td>
<td>94 ± 3 (4)</td>
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<tr>
<td>RGS16</td>
<td>63 ± 5 (4)</td>
<td>76 ± 5 (4)</td>
<td>66 ± 2 (6)</td>
<td>75 ± 1 (4)</td>
<td>95 ± 2 (4)</td>
</tr>
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the S1P$_1$ receptor-mediated inhibition of forskolin-induced cAMP formation showed a significant decrease in the efficacy of FTY720-P upon co-transfection with RGS5 whereas the other RGS proteins had no effect (Table 4).

**DISCUSSION**

It has been well described that VSMCs undergo phenotypic modulation during cell culturing, i.e. switch from acontractile to a more synthetic phenotype which also is associated with processes involved in vascular remodelling such as migration and proliferation (Hayashi et al., 2001; Sobue et al., 1999). Well known biochemical markers of the contractile phenotype are calponin1 and SM22$\alpha$, whereas $\alpha$-smooth muscle-actin is a general marker of smooth muscle cells (Sobue et al., 1999; Worth et al., 2001). Prior to subculturing, more than 90% of our cells stained positive for $\alpha$-smooth muscle-actin (Fig. 1A), indicating that we have used a fairly pure VSMC culture isolated from rat thoracic aorta. In the present study the passage-dependent phenotypic modulation was confirmed by demonstrating a decline in the mRNA
expression of α-smooth muscle-actin, calponin1 and SM22α (Fig. 1B-D). While there was a continuous trend for lower expression with higher passage number, the greatest decrease for all three markers was seen between p0 and p1. Thus, our experimental conditions reproduce a passage-dependent phenotypic modulation from the contractile to the synthetic phenotype.

Apart from proteins directly related to the contractile phenotype, the expression of other proteins such as purinergic receptors (Erlinge et al., 1998), NADPH oxidase 1 (Arakawa et al., 2006) and potassium channels (Tharp et al., 2006), can also be regulated during phenotypic modulation in cultured smooth muscle cells. The present study extends this knowledge by investigating regulation of S1P receptors and RGS proteins upon culturing. This study demonstrates that the S1P receptor similar to the contractile markers calponin1 and SM22α is down-regulated with increasing passage number (Fig. 2), at least at the mRNA level. However, the S1P1 and S1P3 receptors did not exhibit passage-dependent regulation of mRNA expression (Fig. 2). Assessment of S1P receptor expression at the protein level by means of radioligand binding studies is technically difficult as available radioligands are very lipophilic, and validated selective antibodies are not commercially available making immunoblotting impossible.

Our data also reveal that several RGS proteins belong to the group of molecules that are regulated during phenotypic modulation (Fig. 3). Similar to the S1P receptor subtypes, this does not equally apply to all RGS proteins. Specifically, RGS2 and RGS3 exhibited only little regulation of mRNA expression, whereas RGS4, RGS5 and RGS16 mRNA expression changed with passage number. For RGS4 and RGS16 this involved a decline with increasing passage number, whereas RGS5 was transiently increased. A transient up-regulation of RGS5 mRNA in pericytes/SMC has also been shown during wound healing and active vessel remodelling (Berger et al., 2005).

While commercially available, selective antibodies are missing for several RGS proteins, some data indicate that at least for RGS2 (Li et al., 2005) and RGS16 (Patten et al., 2002) mRNA expression seems to be correlated with protein expression and/or functional receptor responses. We only could determine the protein expression of RGS4 during phenotypic modulation using immunoblotting. Similar to mRNA expression, the RGS4 protein expression changed during culturing. In p2 the RGS4 protein expression was reduced compared to levels observed in p0 (Fig. 4). However, unlike the mRNA expression, the RGS4 protein levels did not further decline in p6. Thus, the protein expression of RGS4 is, at least partly, down-regulated during phenotypic modulation although to a lesser extent than the mRNA expression.

The observed difference in RGS5 mRNA expression may contribute to changes in vascular tone since Rgs5-KO mice had persistent low blood pressure compared to control mice (Cho et al., 2008). However, the mean arterial pressure of these control mice was higher than normally reported whereas the mean arterial pressure of Rgs5-KO mice was comparable to those normally found in the wild type mouse strain (Cho et al., 2008; Mattson, 2001).
The observed up-regulation of RGS5 and down-regulation of RGS4 and RGS16 expression during phenotypic modulation of VSMCs might affect signalling of specific G protein-coupled receptors important in vascular function. Therefore, we used the phenotypic modulation to explore whether the changes in RGS4, RGS5 and RGS16 affect the signalling of certain S1P receptor subtypes. Agonist-induced elevation of intracellular Ca²⁺ concentration is a prototypical signalling response of several S1P receptor subtypes, but not for S1P₁ receptors (Kluk and Hla, 2002; Siehler and Manning, 2002). Although in some cell types S1P₁ and S1P₃ receptors can couple to Ca²⁺ elevation, the S1P-induced Ca²⁺ elevation in primary cultures of rat VSMCs occurs largely if not exclusively, via the S1P₂ subtype. In the present study S1P increased intracellular Ca²⁺ concentrations with almost super-imposable concentration-response curves in different passages (Fig. 5). These data show that major alterations in RGS4 mRNA and protein expression and RGS5 and RGS16 mRNA expression as observed in the present study are not linked to alterations in S1P₂ receptor-mediated Ca²⁺ signalling. While changes in mRNA expression have to be interpreted with caution, it is noteworthy that even 30, 12 and 40 fold changes in mRNA expression of RGS4, RGS5 and RGS16, respectively, or a two fold difference in protein expression of RGS4 were not associated with changes in S1P-induced Ca²⁺ elevation. Therefore, we propose that these three RGS proteins do not play a major role in regulating the function of S1P₂ receptors in rat aortic VSMCs and perhaps other cell types.

As the above findings are mostly S1P₂-mediated and hence do not allow conclusions about the role of RGS expression in the signalling of the S1P₁ and S1P₃ receptors, we switched to a different model system, i.e. CHO-Flp-In cells stably co-transfected with His-tagged S1P receptor subtypes and specific RGS proteins.

As S1P₁ receptors typically do not couple to Gq (Kluk and Hla, 2002; Siehler and Manning, 2002), our functional tests in the CHO-Flp-In cells were based upon inhibition of forskolin-induced cAMP accumulation, as both S1P₁ and S1P₃ receptors couple to Gi (Siehler and Manning, 2002). Treatment with pertussis toxin completely abolished the inhibition of forskolin-induced cAMP accumulation via the S1P₁ receptor as well as the S1P₃ receptor (data not shown). None of the co-transfected RGS protein significantly altered the potency of S1P to inhibit the forskolin-induced cAMP formation via the S1P₁, or the S1P₃ receptor (Table 3). To increase the robustness of this finding, we have additionally tested the specific S1P₁ agonist SEW2871 and FTY720-P, an agonist for all S1P receptors except S1P₂. None of the co-transfected RGS proteins affected the potencies of either SEW2871 or FTY720-P (Fig. 6, Table 3). However, FTY720-P acting on S1P, receptors had a significantly lower efficacy in RGS5 co-transfected cells, and a non-significant trend for a lowered efficacy was also seen for S1P acting on S1P receptors in the RGS5 co-transfected cells (Table 4). These data suggest that RGS5 may modulate S1P₁ receptor function, although the effect may not be sufficiently large to be consistently detectable with each agonist. Alternatively, the agonist-dependent effect of RGS5 on S1P₁ receptor function might be explained by agonist-dependent recruitment of different signalosomes. Interestingly, it has been shown by other researchers that
S1P and FTY720-P differentially affect regulation of the S1P₁ receptor (Oo et al., 2007). To draw definitive conclusions on the agonist-dependency of the effect of RGS5 protein expression further studies are required.

To the best of our knowledge, only one group of investigators has also explored the role of RGS protein expression on S1P receptor function (Cho et al., 2003a; Cho et al., 2003b). These investigators have used double or triple co-transfection of S1P receptors, RGS proteins (sometimes used as fusion proteins with green fluorescent protein) and HA-tagged ERK into CHO, NIH3T3, HEK293 or human aortic VSMCs. While no quantitative data were shown, the authors proposed, based upon representative experiments with a single agonist concentration, that signalling of the S1P₁, S1P₂ and S1P₃ receptor was attenuated by some RGS proteins, with each subtype exhibiting a distinct pattern of inhibition by RGS proteins (Cho et al., 2003a; Cho et al., 2003b). More recently, these investigators showed increased S1P-induced ERK phosphorylation in VSMCs of RGS5 deficient-mice (Cho et al., 2008) further confirming their previous studies. With the possible exception of the combination of S1P₁ receptors and RGS5, none of the S1P receptor subtype/RGS protein interactions proposed by these investigators was confirmed in the present study. While it cannot be excluded that these apparently contradictory findings are related to differences in the cell types being used, a more obvious candidate to explain this discrepancy in findings is a difference in signalling response. While Cho et al. (Cho et al., 2003a; Cho et al., 2003b) have used activation of ERK (by over-expression of HA-tagged ERK), we have used endogenous signalling responses such as elevation of intracellular Ca²⁺ and inhibition of cAMP accumulation.

This situation resembles findings with other receptors, e.g. RGS2 attenuates the angiotensin II AT₁ receptor-mediated inhibition of cAMP formation but not the ERK activity in VSMCs although it inhibits angiotensin II-induced ERK activity in HeLa cells (reviewed in (Hendriks-Balk et al., 2008).

In conclusion, our data show that the S1P₁ receptor, RGS4 and RGS16 mRNA expression is down-regulated during phenotypic modulation of VSMCs whereas mRNA levels of RGS2, RGS3, S1P₂ and S1P₃ receptors were unchanged. Interestingly, RGS5 mRNA expression is transiently up-regulated. The decline in RGS4 mRNA correlated with a reduction in RGS4 protein expression although at a lower extent.

In addition, our data show that major regulation of endogenous RGS4, RGS5 and RGS16 expression does not change S1P₂-mediated Ca²⁺ elevations by S1P in VSMCs and that co-transfection with RGS2, RGS3, RGS4 and RGS16 in CHO cells does not change S1P₁ and S1P₃-mediated inhibition of cAMP formation by S1P or other agonists at these receptor subtypes. Co-transfection with RGS5 resulted in a decreased efficacy of FTY720-P acting on the S1P₁ receptor, whereas the efficacies of S1P and SEW2871 were slightly or not reduced. Given the plethora of cellular S1P responses (Ishii et al., 2004; Kluk and Hla, 2002; Pyne and Pyne, 2000) and of RGS proteins which potentially can interact with S1P receptor subtypes (Cho et al.,
2003a; Cho et al., 2003b) our study obviously cannot claim to exclude an effect of any possible combination of S1P receptor subtype, cellular response and RGS protein.

Nevertheless, our data demonstrate that expression of various RGS proteins is regulated differentially during phenotypic modulation. Although this major regulation was not associated with altered S1P receptor function in our study, changes in expression of RGS proteins still might be important in VSMC function as more than 70 different G protein-coupled receptors are expressed in the vascular system, including many ‘orphan’ receptors (Hakak et al., 2003), and several other signalling pathways also play a role in vascular function.
REFERENCES


