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RGS2 is upregulated by and attenuates the hypertrophic effect of α_1 -adrenergic activation in cultured ventricular myocytes

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Abstract

Regulator of G protein signaling (RGS) proteins counter the effects of G protein-coupled receptors (GPCRs) by limiting the abilities of G proteins to propagate signals, although little is known concerning their role in cardiac pathophysiology. We investigated the potential role of RGS proteins on α_1 -adrenergic receptor signals associated with hypertrophy in primary cultures of neonatal rat cardiomyocytes. Levels of mRNA encoding RGS proteins 1–5 were examined, and the α_1 -adrenergic agonist phenylephrine (PE) significantly increased RGS2 gene expression but had little or no effect on the others. The greatest changes in RGS2 mRNA occurred within the first hour of agonist addition. We next investigated the effects of RGS2 overexpression produced by infecting cells with an adenovirus encoding RGS2-cDNA on cardiomyocyte responses to PE. As expected, PE increased cardiomyocyte size and also significantly upregulated α -skeletal actin and ANP expression, the markers of hypertrophy, as well as the Na-H exchanger 1 isoform. These effects were blocked in cells infected with the adenovirus expressing RGS2. We also examined hypertrophy-associated MAP kinase pathways, and RGS2 overexpression completely prevented the activation of ERK by PE. In contrast, the activation of both JNK and p38 unexpectedly were increased by RGS2, although the ability of PE to further activate the p38 pathway was reduced. These results indicate that RGS2 is an important negative-regulatory factor in cardiac hypertrophy produced by α_1 -adrenergic receptor stimulation through complex mechanisms involving the modulation of mitogen-activated protein kinase signaling pathways.

Keywords: Regulator of G protein signaling protein (RGS protein); RGS2; α_1 -Adrenergic receptor; Cardiomyocyte hypertrophy; Mitogen-activated protein kinase signalling (MAP kinase signalling); G protein

1. Introduction

G protein-coupled receptors (GPCRs) play a vital role in regulating cardiovascular function and adaptation to changes under both physiological and pathophysiological conditions. Notable among these are the Gq-coupled α_1 -adrenergic receptors whose prolonged activation likely contributes to hypertrophy under conditions of increased catecholamine release [1,2]. Thus, under pathological conditions α_1 -receptor signals may become chronically activated to initiate hypertrophic responses that eventually result in cardiac dysfunction [3,4]. The intracellular cell signaling changes that underlie cardiomyocyte hypertrophy have yet to be fully characterized, although a number of constituents have been identified [3,5].

In recent years, the regulator of G protein signaling (RGS) proteins have been found to attenuate receptor signals by promoting G protein deactivation via increased GTP hydrolysis and/or blocking effector activation [6]. Not including various

Abbreviations: α MHC, α -myosin heavy chain; β MHC, β -myosin heavy chain; ANP, atrial natriuretic peptide; ET-1, endothelin-1; GPCR, G proteincoupled receptor; NHE1, Na⁺/H⁺ exchanger isoform 1; PBS, phosphatebuffered saline; PCR, polymerase chain reaction; PE, phenylephrine; PKC, protein kinase C; PMA, phorbol myristate acetate; RGS, regulator of G protein signaling; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase.

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"RGS-like" proteins, twenty distinct mammalian genes for RGS proteins have been identified, some with splice variants, and these have been grouped into the four subfamilies A/RZ, B/R4, C/R7 and D/R12 [7,8]. RGS proteins belonging to the B/R4 subfamily are characterized by relatively short amino and carboxy termini flanking the conserved ~120 amino acid RGS domain [7], and they generally appear to be more abundant than members of other RGS subfamilies [9]. In the human heart, mRNA levels point to the B/R4 members RGS2, RGS3 and RGS5 as being the prevalent species [9,10], although at least a dozen additional types of RGS protein have been detected in mammalian cardiac myocytes and myocardial tissue [9,11].

The wide variety of RGS proteins present suggests that they are important in cardiomyocyte G protein signaling, however little information is available concerning their precise roles in regulating the myriad GPCR pathways that convey normal and pathogenic signals in these cells. A few studies have examined RGS protein regulation in cardiovascular disease, and isoforms identified as being upregulated in experimental models and human forms of hypertrophy and heart failure include RGS2 [12], RGS3 [12–14] and RGS4 [10,13,14]. There is some variability between studies as to which particular RGS proteins may be upregulated, and this may reflect differences related to species and/or etiology.

A possible function of RGS protein upregulation in heart failure may be to mitigate the GPCR signals that promote hypertrophy. Indeed, RGS2, RGS3 and RGS4 all are known to attenuate Gq-mediated signals by increasing the rate of Gq GTPase activity and by interfering with effector activation [15–19]. Thus they may exert an inhibitory effect against α_1 adrenergic mediated signals that contribute to heart failure. RGS2 is unique among the RGS proteins in that it interacts only weakly with G proteins of the Gi subfamily, whereas all other RGS proteins appear to have effects on Gi that are greater than or equal to their effects on Gq [6]. Interestingly, it has recently been found that RGS2 binds directly to the third intracellular loop of the α_{1a} adrenergic receptor to inhibit its activity [20], while a functional interaction between RGS2 and the α_{1b} adrenergic receptor subtype is mediated by the scaffolding protein spinophilin [21]. Accordingly, in the present study we determined the effects of the α_1 -adrenergic agonist phenylephrine (PE) on the expression levels of RGS protein B/R4 subfamily members RGS1-5. These results showed that only RGS2 was upregulated and we therefore investigated the effects of increased RGS2 on the responsiveness of cardiomyocytes to hypertrophy produced by α_1 -adrenergic receptor activation.

Table 1

Primers used in RT	_PCR reactions to	o detect RGS	mRNA
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2. Materials and methods

2.1. Cell isolation and primary culture of neonatal rat ventricular myocytes

Ventricular myocytes were isolated from 2-day-old Sprague-Dawley rats and primary cardiomyocyte cultures were established and maintained as described previously [22,23].

2.2. Recombinant adenovirus

The cDNA encoding RGS2 was purchased from the Guthrie Institute, Danville, PA, USA. A replication defective adenovirus encoding RGS2 was generated by sub-cloning the RGS2 cDNA into the multi-cloning site of an adenovirus shuttle vector and rescued into viral particles in HEK 293 cells by methods previously described [24]. For all experiments, myocytes were infected with adenovirus encoding either RGS2 (AdRGS2) or GFP AdGFP (control) for 24 h [25] prior to treatment. Adenoviruses were propagated, harvested, titered, and plaque purified as previously reported [24].

2.3. Real-time PCR analysis of RGS mRNA levels in cardiomyocytes

Cultured cardiomyocytes were serum-starved for 24 h prior to treatment with PE (10 μ M) (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) for a further 24 h. Additional experiments were done in which the cells were treated instead with phorbol myristate acetate (PMA, 1 μ M) (Sigma-Aldrich) for an identical period. The cells were then lysed in 1 ml of Trizol reagent (Invitrogen Canada Inc., Burlington, ON, Canada) for total RNA isolation. Total RNA was then used for first-strand cDNA synthesis using SuperScriptTM II RNase H minus Reverse Transcriptase (Invitrogen Canada Inc) and was then used as a template in the PCR reactions. Real-time PCR was performed using the primers indicated in Table 1. The PCR cycles used were as follows: 94–95 °C for 20 sec, 57–60 °C for 30 sec and 72 °C for 30 sec for 35 cycles in a real-time PCR analysis (DNA Mr Research Engine Opticon 2 System; Bio-Rad Laboratories, Toronto, ON, Canada). The cDNA levels were quantified using a standard graph of the respective genes [22,26] and normalized to 18S rRNA measured in parallel. All the quantification values were within the range of standards used.

2.4. Immunofluorescence detection

To examine changes in endogenous RGS2 protein levels, primary cultures of neonatal rat ventricular myocytes that had been serum-starved overnight were treated with 10 μ M PE for 3 h, and subsequently fixed with periodate–lysine–paraformaldehyde fixative for 30 min [27]. Fixed cells were permeabilized with 0.3% Triton X-100 in PBS for 10 min and then blocked with 5% BSA for 20 min at room temperature to minimize nonspecific binding of antibodies. Cells were incubated overnight at 4 °C with a polyclonal goat antibody to RGS2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:250, and subsequently incubated at room temperature for 1 h with an anti-goat antibody conjugated to Texas Red-X (Molecular Probes, Eugene, OR, USA) diluted 1:1000. Following 3 washes with PBS, coverslips were mounted on slides with Immumount (Fisher Scientific, Nepean, ON, Canada) for visualization by confocal microscopy. Slides were examined using a Zeiss LSM 410 confocal microscope with a 566 nM laser and a rhodamine-Texas Red 630 nM filter for excitation and emission, respectively.

Protein	Forward primer	Reverse primer
RGS1	5'-GCCAACCAGATGGGTCAAAATCTC-3'	5'-ATGTGGGAGTTGGTGTTTTGATCTTC-3'
RGS2	5'-AGCAAATATGGGCTTGCTGCAT-3'	5'-GCCTCTTGGATATTTTGGGCAATCA-3'
RGS3	5'-TTTCAAGAAGGTCAAATCGCAGTCCA-3'	5'-GTCAAAGCAGCCTCGGGTGAT-3'
RGS4	5'-GACTTCTGGATCAGCTGTGA-3'	5'-CAGTCTGCAGAACTCTTGGC-3'
RGS5	5'-AAGTCCCCCATCAAAATGGCAGAGA-3'	5-CTGGGCCAGGTCAAAGCTGTG-3'

To analyze the effects of RGS2 on agonist-induced changes in cell morphology, myocytes that had been serum-starved overnight were infected with an adenovirus encoding RGS2-cDNA or a control construct lacking the RGS2 coding sequence. After 24 h of infection, cells were treated with 10 μ M PE for 24 h and then fixed and incubated with a murine antibody directed toward sarcomeric myosin heavy chain (1:5 dilution MF20 hybridoma) overnight at 4 °C. Using 10 μ g/ml rhodamine-conjugated sheep F(ab)'₂ anti-mouse IgG (Boehringer Mannheim GmbH, Mannheim, Germany) followed by Hoechst 33258 dye for nuclear morphology, and cells were assessed for surface area as described previously using Mocha software (Jandel, San Rafael, CA, USA) [22].

2.5. Cell infection and transfection, and reporter gene assays

After overnight incubation in Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (DF) 1:1, 17 mM HEPES, 3 mM NaHCO₃, 2 mM L-glutamine, 50 μ g/ml gentamycin, and 10% fetal bovine serum (FBS), cells were transferred to serum-free medium [28]. Myocyte cultures were infected with an adenovirus encoding RGS2 at multiplicity of infection (MOI) of 20 for 4 hr under serum-free conditions as previously reported [24]. This MOI achieves exogenous gene delivery to \geq 95% of neonatal ventricular cells under these conditions [24]. Following adenoviral infection, myocytes were transfected using Effectene (Qiagen Inc., Mississauga, ON,

Canada) immediately after removal of viral stocks with DMEM containing 2.5% calf serum, 5.0 μ g of luciferase reporter plasmid comprising of either the α -skeletal actin (α -S.actin), α - or β -myosin heavy chain (α MHC, or β MHC) plus 2.5 μ g of CMV β -gal, as described previously [29]. Myocytes were maintained in serum-free media for 24 h prior to PE addition and assessed by immunofluo-rescent microscopy or luciferase reporter gene assay 24 h post-transfection. To control for potential differences in transfection efficiency among different myocyte cultures, luciferase activity was normalized to β -galactosidase activity and expressed as relative light units. Data were obtained from at least three independent cell cultures using 3 replicate wells per condition tested.

2.6. Western blotting

Cell lysates were prepared in lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 200 μ M Na₃VO₄, 10 mM Na₄P₂O₇, 40 mM β -glycerophosphate, 10 μ g/ml leupeptin, 1 μ M pepstatin A, 1 mM PMSF, and 1 μ M calyculin A). Phospho-ERK, phospho-JNK, phospho-p38 and phospho-c-Jun were determined by using specific antibodies (1:1000 dilution for each) purchased from Cell Signaling Technology Inc (Beverly, MA). A total of 30 μ g cell lysates were separated in SDS–PAGE and transferred to a nitrocellulose membrane, and then analyzed for phosphorylated protein by Western blotting. The membrane was stripped at 45 °C



Fig. 1. RGS1–5 mRNA levels in cardiomyocytes (A). Cardiomyocytes from neonatal rats were serum-starved for 24 h, followed by treatment with 10 μ M PE for up to 24 h. Total RNA was isolated from each well and then assayed using real-time PCR to evaluate the mRNA expression levels of RGS1–5. The mRNA levels at each time point were normalized to 18S rRNA levels and compared to control values taken at time zero. The data from 10 to 12 independent experiments performed in duplicate are shown as means±S.E.M. *p<0.05 (PE-treated vs. vehicle-treated group). The *inset* shows the effects over the same time period of the phorbol ester PMA (white bars) or its inactive analogue 4 α -PMA (black bars). *p<0.05 (PMA- or 4 α -PMA-treated vs. vehicle-treated). (B) RGS2 expression in neonatal rat cardiomyocytes. Cardiomyocyte cultures were treated with vehicle or 10 μ M PE for 3 h and then fixed, followed by immunostaining using an anti-RGS2 antibody. After treatment with PE, cardiomyocytes expressed visually detectable levels of RGS2 protein, with immunostaining dispersed throughout the cytosol. All images were captured using Zeiss LSM 410 confocal software, at 640× magnification and a resolution of 512×512. The images shown are representative of three independent experiments.

for 15 min in 62.5 mM Tris–HCl (pH 6.8) containing 1% SDS, 100 mM β mercaptoethanol [30] and reprobed with the anti-total ERK, JNK and p38 antibodies (1:2000 dilution), purchased from Santa Cruz Biotechnology. The independent experiments were performed six times. Blots were analyzed by densitometric scanning (Bio-Rad, model GS-700, Midland, ON, Canada) and were normalized for differences in protein loading per lane.

2.7. Statistical analysis

Group data are presented as means \pm S.E.M. Data were compared by one-way ANOVA. Differences were further evaluated by Fisher's protected least squares differences, which were considered significant at a value of p < 0.05. For MAP kinase activation, the enhancement of phosphorylated ERK, JNK and p38 in RGS2 overexpression versus vector alone was compared using Student's *t* test.

3. Results

3.1. PE increases RGS levels

To investigate the effects of GPCR-dependent hypertrophic factors on the intracellular RGS mRNA levels in cardiomyocytes, cells were treated with PE for up to 24 h. The expression levels of RGS1, RGS2, RGS3, RGS4 and RGS5 mRNA were examined by real-time PCR. As shown in Fig. 1A, PE produced nearly a ten-fold increase in expression of RGS2 which peaked within the first hour of treatment and then gradually decreased back to control values. Fig. 1B demonstrates confocal images of cardiomyocytes 3 h after PE addition which demonstrates a marked increase in RGS2 immunostaining which was found to be uniformly dispersed throughout the cytosol. Interestingly, none of the other RGS proteins studied appeared to be affected by PE addition.

We also examined whether signaling events downstream of Gq might account for the observed increases in RGS2 levels upon receptor activation by determining the effects of the PKC activator PMA in order to mimic Gq signaling downstream to α_1 -receptor activation. As shown in Fig. 1 (inset), there was a significant increase in RGS2 mRNA expression observed within 1 h after PMA addition although the response differed from that observed with PE in that the increase in RGS2 expression was delayed and the peak effect was observed substantially later than that observed with PE. Although the reason for this is uncertain, it may reflect the persistence of PMA within the cell membrane due to the lipophilicity of the compound. The inactive phorbol ester 4α -PMA, in contrast, had no effect on RGS2 mRNA levels over the same time period. Irrespective of the precise basis for the different profiles of RGS2 upregulation with PE versus PMA, when taken together, our results suggest that RGS2 upregulation can occur subsequent to Gq activation.



Fig. 2. Effects of RGS2 overexpression on cell size of cardiomyocytes. Rat neonatal cardiomyocytes infected for 24 h with either control (–) or RGS2-encoding adenovirus (+) were treated with vehicle (control) or PE (10 μ M) for 24 h, and then photographed and assessed for cell surface area. (A) Cardiomyocytes treated as indicated were immunostained to reveal sarcomeric myosin heavy chain. (B) The surface areas of 50 or more cells per condition in each of three independent experiments were estimated as described in Materials and methods, and presented as means±S.E.M. *p<0.05 (RGS2 vs. control adenovirus group).



Fig. 3. Effects of RGS2 overexpression on ANP and NHE1 synthesis. Cultured cardiomyocytes were infected with control (white bars) or RGS2-adenovirus (black bars) for 24 h followed by treatment with vehicle (control) or PE (10 μ M) for a further 24 h. The expression levels of ANP and NHE1 mRNA were determined by real-time PCR and changes were calculated as in Fig. 1. The data from 10 to 12 independent experiments performed in duplicate are shown as means±S.E.M. *p<0.05 (PE-treated vs. vehicle-treated group) and #p<0.05 (RGS2 vs. control adenovirus group).

3.2. RGS2 overexpression attenuates hypertrophic gene expression and the development of cardiomyocyte hypertrophy in response to PE

A major goal of the present study was to determine the possible physiological relevance of RGS2 upregulation, particularly with respect to whether it can modulate the hypertrophic effects of PE. To determine this, we studied the effect of PE on cardiomyocytes infected with either adenovirus encoding RGS2 or control virus in terms of total cell surface area and also expression of key molecular markers associated with the hypertrophic phenotype. A 24-h exposure of cardiomyocytes to PE resulted in a substantial increase in surface area; however, as shown in Fig. 2, this was completely abrogated in cells infected with the RGS2 adenovirus. In parallel experiments, we also found that the PE-induced upregulation of ANP as well as NHE-1 as determined by real-time PCR was prevented by RGS2 overexpression (Fig. 3). To further demonstrate an antihypertrophic effect of RGS2

luciferase reporter DNA constructs for hypertrophy-associated genes including α -S.actin, α MHC and β MHC were transfected into cultured myocytes in concert with infection with adenovirus either lacking or containing RGS2 cDNA. As shown in Fig. 4, the reporter activities of all three hypertrophic genes were significantly increased in control cells by PE. However, this effect was completely abrogated in cells over-expressing RGS2.

3.3. Modulation of MAP kinase pathways by RGS2

RGS proteins are known to govern the effects of GPCRs and G proteins on MAP kinases including ERK1/2 [18,31,32], JNK [33] and p38 [34], and the activation of these pathways is likely to be of importance for the development of cardiomyocyte hypertrophy. However, the relative contribution of various pathways of the MAP kinase system to cardiac hypertrophy is



Fig. 4. Effects of RGS2 overexpression on hypertrophic gene expression. Cultured cardiomyocytes were infected with control (white bars) or RGS2adenovirus (black bars) and concurrently transfected with reporter DNA constructs for hypertrophic genes including α -S. actin, α MHC and β MHC for 24 h. This was followed by treatment with vehicle (control) or PE (10 μ M) for a further 24 h. Reporter gene activities were assessed by luminometry relative to an internal standard and normalized to control values (no drug, control adenovirus). Data shown are the means ±S.E.M. of three to five independent experiments performed in triplicate. *p<0.05, **p<0.01 (PE-treated vs. vehicle-treated group). #p<0.05 (RGS2 vs. control adenovirus group).

not well understood. In order to gain mechanistic insights into RGS2-mediated inhibition of hypertrophic responses we determined changes in agonist-induced activation of MAP kinase. As summarized in Fig. 5A, RGS2 infection had no effect on ERK phosphorylation whereas a 10-min PE treatment increased ERK phosphorylation by about three-fold in control cells, an effect which was completely prevented by RGS2 overexpression. The results with JNK and p38 were substantially more surprising since RGS2 overexpression on its own increased phosphorylation of both kinases by approximately three- and seven-fold respectively (Fig. 5B and C). The mechanism(s) underlying such increases are not obvious, but it would appear that RGS2 has effects on these pathways distinct from its ability to deactivate G proteins upstream. The net ability of PE to activate JNK was increased by RGS2 infection although the fold-increase was similar (Fig. 5B), whereas with respect to p38, the net effect of PE was decreased and the relative increase, although significant, was reduced from approximately five-fold to less than two-fold (Fig. 5C). To more precisely dissect the MAPK activation in exogenous RGS2 overexpressing rat cardiomyocytes, we treated the cells with PE for up to 60 min and measured kinase phosphorylation at specific intervals as well as the profile of the phosphorylation of the early-inducible gene c-jun, a downstream component of JNK, which plays an important role in transducing signaling from cytoplasm into the nuclear to stimulate transcription activity. Decreased PE-dependent ERK phosphorylation was evident in RGS2 overexpressing cells during the 60-min treatment period (Fig. 6A and B). In contrast, p38, JNK and c-Jun phosphorylation were increased in RGS2 infected myocytes throughout the 60-min treatment period (Fig. 6A, C-E). Thus, when taken together the data suggest that RGS2 is a negative regulator of ERK whereas p38 and JNK activation is enhanced by RGS2 infection.

4. Discussion

This study was carried out to address two primary questions. First, we sought to determine whether cardiomyocyte hypertrophy produced by α_1 -adrenoceptor activation is associated with changes in RGS protein expression. Our second goal was to determine whether overexpression of RGS protein modulates the hypertrophic response and if so, to assess the potential cellular mechanisms for this effect. Our study shows that RGS2 is the only RGS protein studied that was upregulated by PE. Moreover, we have demonstrated that the effect can be replicated by PMA suggesting that PKC activation could represent a key downstream mediator for the increased RGS2 expression seen with α_1 -adrenoceptor stimulation. We further demonstrate that RGS2 is an important negative-regulatory factor in cardiac hypertrophy through complex mechanisms involving modulation of MAP kinase signaling pathways. A role for RGS2 in mitigating PE-induced hypertrophy was supported by the finding that RGS2 overexpression completely



Fig. 5. Effects of RGS2 overexpression on MAP kinase activation. Cultured cardiomyocytes were infected with control (white bars) or RGS2-adenovirus (black bars) for 32 h, and serum-starved for a further 16 h. This was followed by treatment with vehicle (control) or PE (10 μ M) for 10 min. Proteins in cell lysates were separated by SDS–PAGE and transferred to a nitrocellulose membrane, and the phosphorylation of ERK (A), JNK (B) and p38 (C) was detected by Western blotting. The membrane was then stripped and the expression of total ERK (A), JNK (B) and p38 (C) was determined by Western reblotting with anti-total ERK, JNK and p38 antibodies, respectively. The immunoblots shown are representative of six independent experiments, the densitomeric data of which are summarized in the bar graphs in the respective lower panels. **p<0.01 and *p<0.05 (RGS2 vs. control adenovirus group).



Fig. 6. Time course analysis of MAP kinase activation affected by RGS2 overexpression. (A) Cultured cardiomyocytes were infected with control (left panel) or RGS2-adenovirus (right panel) for 32 h, and serum-starved for a further 16 h. This was followed by treatment with PE (10 mM) for up to 60 min, as indicated. Equal amounts of proteins in cell lysates were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The phosphorylation of ERK, p38, JNK and c-Jun was then determined as in Fig. 5. The immunoblots shown are representative of six independent experiments, the desitomeric data of which are summarized in panels B (ERK), C (p38), D (JNK) and E (c-Jun).

abrogated the increased cell surface area and also the upregulation of key hypertrophic markers including α -S.actin, α MHC, β MHC and ANP. Further, we showed that NHE-1 upregulation is also blocked by RGS2 overexpression. Because of the importance of NHE-1 to cardiomyocyte hypertrophic responses [35], these findings suggest that this antiporter likely contributes to PE-induced hypertrophy and that one of the potential intracellular mechanisms for inhibiting PE-induced hypertrophy involves NHE-1 inhibition.

Cardiomyocyte hypertrophy represents a complex phenomenon mediated by a myriad of cell signaling processes, many of which involve G protein-coupled receptors (reviewed by [3]). In terms of its relevance to cardiac pathology, prevention of cardiac hypertrophy likely represents an important therapeutic target for the attenuation of myocardial remodelling and eventual development of heart failure [36].

To our knowledge, this is the first demonstration of 1) RGS2 upregulation in response to α_1 -adrenergic receptor activation and 2) the ability of RGS2 overexpression to block the hypertrophic response. We concentrated our study on this protein since it was the only one affected by PE addition although other RGS proteins may also be involved in regulating the hypertrophic response by inhibiting Gq signaling. For example, RGS4 was observed in a study on transgenic mice overexpressing myocardial Gq, wherein the spontaneous

development of cardiac hypertrophy was found to be delayed when these animals were crossed with a strain overexpressing RGS4 in the same tissue [37]. Taken together, these findings highlight the critical role that RGS proteins can play in longterm receptor signaling. Although RGS2 and RGS4 have similar effects on Gq GTPase activity (W. Cladman and P Chidiac, unpublished data), there is evidence that RGS2 may be the more potent of the two in terms of their relative abilities to inhibit Gq signaling [38]. Moreover, in terms of abundance, RGS2 mRNA levels appear to be roughly 100-fold higher than those of RGS4 in the human myocardium [9,10]. In addition, RGS2 may be better poised to act as a selective attenuator of hypertrophic GPCR signaling due to its unique G protein selectivity. RGS2 is unlike all other RGS proteins in that it generally interacts poorly with members of the Gi subfamily, which do not appear to mediate hypertrophic signals, while it clearly inhibits Gq activity [6].

We studied the potential mechanisms underlying the antihypertrophic effect of RGS2 by concentrating on the MAP kinase family based on other evidence in the literature which describe RGS proteins as inhibitors of MAP kinase activation. For example, RGS proteins 1–5 have all been found to inhibit ERK activation in response to various GPCRs [18,31,32]. Accordingly, our study shows that RGS2 over-expression completely blocked PE-induced ERK activation. In

view of the important role of ERK in the genesis of cardiac hypertrophy [39], inhibition of its activation serves as a logical mechanistic basis for the antihypertrophic effect seen in RGS2 overexpressing cells. While the theoretical considerations noted above suggest that RGS2 would be a more effective antihypertrophic factor than RGS4, a recent study demonstrated equal efficacy of RGS2 and RGS4 overexpression in blocking PE-induced ERK activation although the hypertrophic response was not determined in that study which was done using adult rat cardiomyocytes [40].

Compared to ERK1/2, the contributions of other MAP kinase pathways to hypertrophy are less clearly understood, and it appears that the modulation of these pathways by different RGS proteins may be dissimilar. The roles of JNK or p38 in hypertrophy appear to be complex [41], perhaps reflecting distinct roles which depend on the nature of the hypertrophic stimulus. For example, p38 mediates the hypertrophic effect of leptin [22] whereas both JNK and p38 activation mediate the angiotensin II-induced hypertrophic responses [42]. On the other hand, JNK has been shown to antagonize cardiac hypertrophy through cross-talk with calcineurin-nuclear factor of activated T-cells (NFAT) signaling and it has been proposed that both JNK and p38 are negative regulators of NFAT in cultured neonatal rat cardiomyocytes thus acting as antihypertrophic factors [43]. Thus, the present results suggest that RGS2 upregulation could effectively mitigate the hypertrophic response to some factors by inhibition of ERK activation concomitant with JNK and p38 upregulation. In contrast to our findings with RGS2, a previous study showed RGS4 to inhibit PE-stimulated JNK activation in neonatal rat ventricular myocytes [33], while another found RGS4 to inhibit p38 activation in lung epithelial cells [34]. It follows that different RGS proteins may vary in their abilities to selectively attenuate or promote the activation of different MAP kinase pathways, and that the pattern exhibited by RGS2 in the present study may not be common to all RGS proteins.

Due to their abilities to limit G protein-mediated signaling, RGS proteins may limit the development of cardiac hypertrophy due to chronic GPCR activation. In the present study, it was evident that α_1 -adrenergic receptor activation selectively increased expression of RGS2. However, other RGS proteins could also conceivably be upregulated under hypertrophic conditions in vivo, where for example β -adrenergic, endothelin and angiotensin II receptor activation are likely to play contributing roles. Indeed, there is evidence that RGS2, RGS3 and RGS4 all can be upregulated in various forms of human heart failure [10,12,13]. It is also important, however, to distinguish between hypertrophy and heart failure as our finding is likely more relevant towards the understanding of the hypertrophic response per se rather than heart failure. For example, in clinical terminal heart failure caused by either dilated or idiopathic cardiomyopathy, the only significant change observed was an increased expression of RGS4 with RGS2 unaffected, although in terms of content it should be noted that myocardial RGS4 mRNA abundance was less than 5% of that for RGS2 even under upregulated conditions [10]. Although the latter study failed to demonstrate any changes in RGS3 expression, another report showed enhanced RGS3 and RGS4 expression in end stage heart failure (RGS2 was not determined) [13]. Yet, in another study in patients with end stage heart failure, only RGS2 and RGS3 were upregulated whereas, in contrast to the reports just noted, RGS4 was unaffected [12]. Intriguingly, in that study, placing patients on a left ventricular assist device (LVAD) from 29 to 97 days resulted in selective near normalization of RGS2 but not RGS3 protein levels, although the reduction in RGS2 protein levels was not significantly correlated to either the duration of LVAD support or cardiac function in these patients [12]. Thus, important questions remain concerning changes in RGS proteins in terms of the time course of myocardial remodelling or heart failure or the relationship between RGS2 content and cardiac function. While it is possible that upregulation of RGS2 and other RGS proteins could be beneficial in terms of mitigating the hypertrophic response, it is also conceivable that upregulation of these proteins could be counterproductive particularly in terms of their ability to inhibit early compensatory responses.

Indeed, transgenic mice overexpressing RGS4 respond with diminished myocardial hypertrophy due to aortic coarctation although this was associated with increased mortality [44]. Thus while further illustrating an antihypertrophic effect of RGS4 overexpression, the study also suggests that, at least with respect to RGS4, its overabundance may result in diminished compensatory responses to pressure overload. It remains to be determined whether RGS proteins also regulate compensatory responses with respect to other forms of hypertrophy not related to pressure overload especially left ventricular hypertrophy secondary to myocardial infarction. Our study shows that α_1 adrenergic receptor-mediated hypertrophy, which is likely related to pathological hypertrophy associated with postinfarction remodelling, can be completely prevented by RGS2 overexpression. However, in view of the complexity of RGSdependent regulation of cardiac function, it remains to be determined whether the results of our studies can be translated to the in vivo condition. Current work in our laboratories is aimed at extending the current work to investigating the role of RGS2 in the development of cardiac hypertrophy heart failure in vivo.

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