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Expression of RGS3, RGS4 and Gi alpha 2 in acutely failing donor hearts and end-stage heart failure

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Background Regulators of G-protein Signalling (RGS) proteins have been shown to limit in vitro signalling of G proteins. In common with end-stage heart failure, we have recently shown that upregulation of the inhibitory G-protein, Gi*α*, occurs in acutely failing donor hearts unused for transplantation due to severe myocardial dysfunction. In light of recent data on RGS proteins, we have evaluated mRNA and protein expression of RGS3, RGS4 and Gi*α*2 in the myocardium from normal, end-stage failing and acutely failing unused donor hearts.

Methods and Results Myocardial samples were obtained from end-stage failing hearts explanted prior to transplantation (n=19), unused donor hearts with ejection fractions <30% (n=14) and used donor hearts with good function (ejection fraction >60%) (n=4–7). mRNA levels were quantified using quantitative reverse transcriptase polymerase chain reaction. Levels of RGS3 and RGS4 mRNA were found to be significantly upregulated in unused donor and end-stage failing myocardium ($P<0{\cdot}05$ and 0.01, and $P<0{\cdot}05$ and 0.02, respectively) compared to non-failing hearts. Protein abundance of RGS3 and RGS4 was found to be higher in myocardium from end-stage failing hearts, and relative RGS4 expression higher in unused donor hearts.

Conclusions We show here that RGS3 and RGS4 mRNA and protein expression is upregulated in human heart failure. These observations suggest that RGS4 may be induced in the heart to regulate cell signalling pathways in response to hypertrophy, and support the existence of a negative feedback loop for the long-term regulation of hypertrophy.
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Key Words: Donor hearts, end-stage heart failure, G-proteins, RGS proteins, mRNA.

Introduction

Myocardium from end-stage failing hearts has been found to be functionally impaired in vitro[1]. Recently, we have shown that a subset of potential donor hearts from brain-dead patients display in vivo and in vitro contractile characteristics similar to myocardium from end-stage heart failure[2]. In vivo these hearts have poor ejection fractions and impaired haemodynamics. In vitro, at both a multicellular level and a single cell level, they also display impaired contraction. Furthermore, we have previously demonstrated increased activity of the inhibitory G-protein, Gi*α*, in unused donor hearts[2] to similar levels seen in end-stage heart failure[3].

Recently, a family of proteins termed RGS (for regulator of G protein signalling) have been described[4,5]. Although RGS proteins have been shown to have other functions (for review see[6]) the majority of work has concentrated on their ability to negatively regulate signalling effected through heterotrimeric G-proteins. In essence, RGS proteins are thought to 'turn off' active alpha subunits of G*α*i and G*α*q families by way of their inherent GTPase activity and their ability to bind to G*α*[7]. This action not only inhibits signalling mediated by affected G-proteins but also increases the $\beta\gamma$ subunits that would otherwise re-associate with *α*, and in this way mediate $\beta\gamma$ signalling[7]. So far no RGS proteins have been identified which affect Gs, but Gi and Gq have both been shown to be modulated by RGS proteins[8,9]. It has been found that RGS gene expression is enhanced in cardiac myocyte hypertrophy induced either in vitro or in vivo by pressure overload[10]. Additionally a recent transgenic model has clearly implicated the involvement of RGS proteins in the cardiac response of hypertrophy[11]. However, studies in vitro have also demonstrated that overexpression of RGS4 can block the hypertrophic response mediated by Gi and Gq signalling pathways[12]. As suggested by De Vries and colleagues[13], these data might imply that RGS4 is induced in the heart to keep

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the process of hypertrophy in check following exposure to hypertrophic stimuli, and suggest the existence of a negative feed-back loop for the long-term regulation of cardiac hypertrophy.

We and others have shown that the activity of Gi*α* is increased in myocardium from acutely dysfunctional unused donor hearts and end-stage failing hearts[2], which may contribute to the impaired myocardial function seen in vivo. Support for this hypothesis comes from experiments in transgenic mice over-expressing Gi*α*2 that display severe myocardial dysfunction[14].

Considering the potential influence of RGS proteins on the activity of Gi*α*, and in turn on myocardial function, we have examined, for the first time, the relative abundance of both mRNA and protein expression of Gi*α*, RGS3 and RGS4 in the same control and pathological human myocardial samples. It is hoped an examination of the relative expression levels of these proteins in these two distinct classes of human heart failure, acute and end-stage, will aid our understanding of the level of influence exerted on the regulation of RGS proteins and their relationship with G-protein signalling in these disease states.

Methods

Tissue retrieval and storage

Non-failing myocardium was obtained from the right ventricular septum of donor hearts used for transplantation (ejection fractions >60%). Samples from the left ventricle of end-stage heart failure (NYHA IV) were taken at the time of transplantation. The aetiology of all heart failure patients was either ischaemic heart disease or idiopathic dilated cardiomyopathy. Ventricular function in potential cardiac donors was assessed by transoesophageal echocardiography as previously described[2]. Those individuals with ejection fractions <30% coupled with poor haemodynamic parameters were not used for transplantation and are referred to as unused donor hearts (see[2] for more detail). No end-stage heart failure patients were on inotropic support and all of the unused donors were on at least one inotropic drug, with just over half of these (8/14) being on two or more inotropes.

Ethical approval was obtained from the Royal Brompton and Harefield Hospital Ethical Committee, and informed, written consent was obtained in all cases.

Western blot analysis

Western blotting was performed as described previously[15]. Primary rabbit polyclonal antibody to RGS3 was kindly supplied by Dr Anthony Muslin, and was used at a dilution of 1 in 500 (v/v) in phosphate buffered saline-T/5% milk protein. Affinity purified RGS4 primary antibody was obtained from Santa Cruz (U.S.A.). Secondary antibodies were obtained from Dako (U.K.). All Western blotting was done at the same time to ensure that all groups could be compared. The intensity of immunoreactive bands was detected using chemiluminescence and the density of the bands was assessed using Image Analysis 1000 software (Alpha Innotech). Blots were stained with Amido Black in order to ensure equal loading of total protein per lane.

Quantitative reverse transcriptase polymerase chain reaction

Gi*α*2, RGS3 and RGS4 mRNA were detected by polymerase chain reaction amplification and quantified by 5′ nuclease assay[16] using fluorescent labelled TaqMan probes analysed using real time quantitative polymerase chain reaction[16]. Total RNA was extracted from tissue using the Qiagen RNeasy mini-column procedure according to the manufacturer's instructions. RNA quality and quantity was assessed by EtBr-agarose gel electrophoresis and by relative absorbance at 260 nm vs 280 nm. Hexamer-primed cDNA synthesis was performed on 150 ng of total RNA in a volume of 10 µl using the PE Biosystems reverse transcriptase kit (Cat #N8080234) and then diluted to 100 µl with sterile dH_2O and stored at −20 °C.

Primers and TaqMan probe for Gi*α*2 (Forward primer (Tm=59 °C) 5′-GCTCAAGGGAATACCAGC TCAA-3′, reverse primer (Tm=60 °C) 5′-GCGGGTCC GTAGCACATCT-3′, TaqMan probe (Tm=70 °C) 5′-ACTCAGCTGCCTACTACCTGAACGACCTGG-3′), RGS3 (Forward primer (Tm=59 °C) 5′-AAGGCAGA CAAAATGATGAAGTCA-3′, reverse primer (Tm=60 °C) 5′-GTGCGAAGGAAGGCTTGGA-3′, TaqMan probe (Tm=69 °C) 5′-TGCTAACCCGTATTTG TGAACCAGCAGCT-3′) and RGS4 (Forward primer (Tm=60 °C) 5′-GACAAAGTGGTTATTTGCCAGA GAGT-3′, reverse primer (Tm=60 °C) 5′-TCCTC ACTATATTCAGACTTCAAGAAAGC-3′, TaqMan probe (Tm=69 °C) 5′-AAGCTGCCAGCCCACATTC ATGACTAATC-3′) were designed using the Primer Express Software (PE Biosystems) from published mRNA sequences (EMBL/GenBank accession numbers X04828, U27655 and U27768, respectively) with flanking primers located on separate exons and the TaqMan probe straddling the exon–exon junction. Predicted amplicon sizes for Gi*α*2, RGS3 and RGS4 were 92 bp, 125 bp and 134 bp, respectively. Internal control 18S rRNA primers and TaqMan probe were provided as a pre-optimized kit (PE Biosystems, Cat #4310893E). Polymerase chain reaction amplifications were performed as previously described[16] using an ABI PRISM 7700 and results analysed using Sequence Detection Software (PE Biosystems). Relative levels of Gi*α*2, RGS3 and RGS4 mRNA were normalized to 18S rRNA and calculated as $2^{-\Delta\Delta CT}$ as outlined in User Bulletin #2 provided by Perkin-Elmer.

Statistical analysis

Statistical analysis was carried out by one-way analysis of variance with a correction for multiple comparisons using Dunnett's critical values. Values are expressed as mean ± SEM.

Results

Expression levels of Giα2 mRNA in failing and non-failing human myocardium

Our analysis of mRNA levels of Gi*α*2 in myocardium shows that it is increased in both acutely failing unused donor hearts (n=14) and in end-stage failing hearts (n=19) compared to non-failing samples (n=7) ($P<0{\cdot}05$ and 0·01, respectively) (Fig. 1(a)). 18S rRNA was used to normalize levels as it has been reported that the commonly used GAPDH increases in cardiac hypertrophy[17]. Figure 1(b) illustrates the efficiency of reverse transcriptase polymerase chain reaction amplification of Gi*α*2 and endogenous control 18S rRNA gene expression. This was tested on serial dilutions of a human ventricular muscle cDNA. Co-amplification of Gi*α*2 and 18S rRNA resulted in a linear response of similar slope, demonstrating identical amplification efficiency over the range tested.

Although Gi*α* mRNA levels in human end-stage failure[18] and animal models of cardiomyopathy[19] have been previously reported, the present study utilizes the recently developed quantitative polymerase chain reaction technique to measure the gene products of interest in the same samples. This, in conjunction with both higher protein expression (data not shown) and activity levels in these same samples[2], suggests that Gi*α*2 expression may principally be regulated at the transcriptional level. The possibility of alterations in mRNA stability and rates of degradation have not been examined in this study.

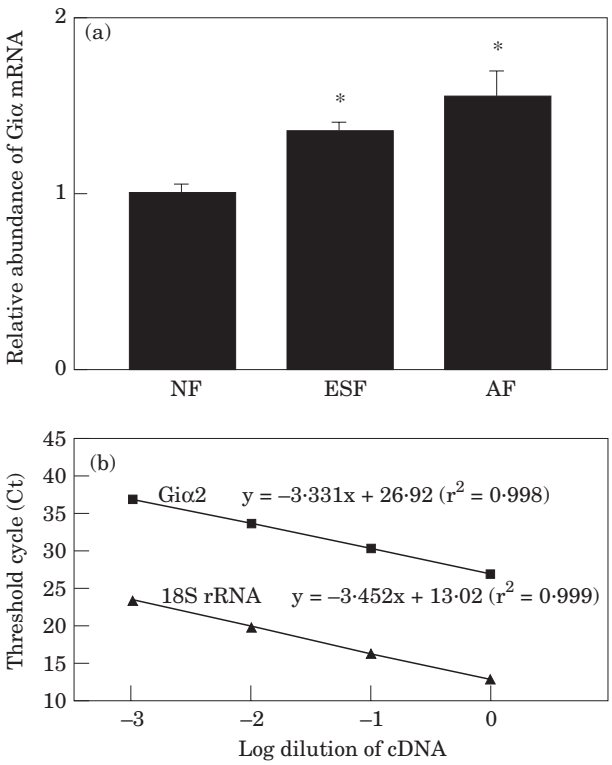

Figure 1 **Gi*α*2 mRNA expression in non-failing (NF) end-stage failing (ESF) and acutely failing unused donor hearts (AF). (a) Gi*α*2 mRNA expression is higher in myocardium from end-stage failing (n=19) and acutely failing unused donor hearts (n=14) compared to non-failing (n=7) (*$P<0{\cdot}02$ and 0·01, respectively). (b) (Inset) The efficiency of reverse transcriptase-polymerase chain reaction amplification of Gi*α*2 and endogenous control 18S rRNA gene expression was tested on serial dilutions of a human ventricular muscle cDNA. Co-amplification of Gi*α*2 and 18S rRNA resulted in curves showing a similar slope, demonstrating identical amplification efficiency over the range tested. Data are shown relative to levels expressed in non-failing myocardium and are shown as mean ± SEM.**

Expression of the RGS3 gene in acutely failing unused donor hearts and end-stage heart failure

Levels of RGS3 mRNA expression were significantly increased in both end-stage failing ($P<0{\cdot}01$; n=19) and acutely failing unused donor hearts (Fig. 2(a)) ($P<0{\cdot}05$, n=14) compared to non-failing hearts (n=7). Figure 2(b) illustrates the efficiency of reverse transcriptase polymerase chain reaction amplification of RGS3 and endogenous control 18S rRNA gene expression. Western blot analysis revealed that RGS3 protein levels were significantly increased in end-stage failure by 1·6-fold compared to non-failing myocardium (Fig. 2(c)) ($P<0{\cdot}01$). There was no difference in the expression of RGS3 protein between non-failing and the unused donor group ($P>0{\cdot}05$). There was no difference in the expression of RGS3 mRNA or protein in the myocardium from patients with either ischaemic cardiomyopathy or dilated cardiomyopathy ($P>0{\cdot}05$) (data not shown).

Expression of the RGS4 gene in acutely failing unused donor hearts and end-stage heart failure

The relative abundance of RGS4 mRNA was significantly elevated in both end-stage ($P<0{\cdot}05$) and acutely failing unused donor hearts ($P<0{\cdot}01$) when compared to non-failing myocardium (Fig. 3(a)). No difference was seen in the abundance of RGS4 mRNA levels between either ischaemic cardiomyopathy or dilated cardiomyopathy heart failure. Figure 3(b) illustrates the

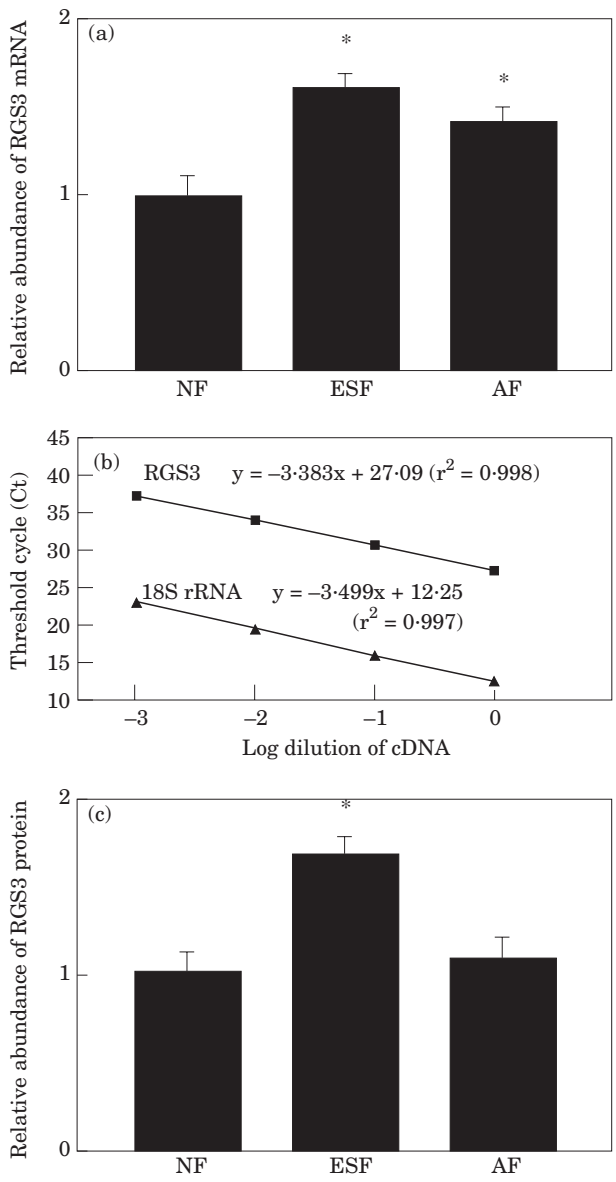

Figure 2 **RGS3 mRNA and protein expression in non-failing (NF) end-stage failing (ESF) and acutely failing unused donor hearts (AF). (a) RGS3 mRNA expression is higher in myocardium from end-stage failing (ESF) (n=19) and acutely failing unused donor hearts (n=14) compared to non-failing (n=7) (*$P<0{\cdot}01$ and $P<0{\cdot}05$, respectively). Quantitative reverse transcriptase polymerase chain reaction was used to analyse levels of mRNA abundance. (b) (Inset) The efficiency of reverse transcriptase polymerase chain reaction amplification of RGS3 and endogenous control 18S rRNA gene expression was tested on serial dilutions of a human ventricular muscle cDNA. Co-amplification of RGS3 and 18S rRNA resulted in curves showing a similar slope, demonstrating identical amplification efficiency over the range tested. (c) The relative abundance of RGS3 protein was significantly higher in end-stage failing hearts (n=19) compared to both non-failing (n=4) and acutely failing unused donor hearts (n=14) (*$P \leq 0{\cdot}01$). Data are shown relative to levels expressed in non-failing myocardium and is shown as mean ± SEM.**

efficiency of reverse transcriptase polymerase chain reaction amplification of RGS4 and endogenous control 18S rRNA gene expression, demonstrating identical amplification efficiency over the range tested. An analysis of the relative abundance of RGS4 protein revealed significantly elevated levels, by 1·7-fold in end-stage and 1·8-fold in acutely failing myocardium ($P<0{\cdot}02$ for each group) compared to non-failing myocardium (Fig. 3(c)).

Discussion

This study documents, for the first time, that the relative abundance of RGS3 and 4 mRNA is significantly increased in both end-stage failing hearts and hearts from brain dead organ donors with acute failure. Additionally, it demonstrates that mRNA levels of the inhibitory G protein, Giα2, are increased in both acutely failing unused donor hearts and end-stage failing human myocardium, suggesting that the abundance of Giα2 is regulated at the transcriptional level. Finally, the relative abundance of RGS4 protein is significantly elevated in both conditions, while RGS3 protein expression is elevated in end-stage heart failure but unaltered in acute heart failure.

There are many hypotheses to account for the increased level of Giα. In light of the existing literature our findings are not surprising. In particular, studies using in vitro systems and animal models have also shown increased transcription of Giα in response to the β-agonist isoprenaline[19], increased levels of tumour necrosis factor alpha[20,21] or a decrease in the level of thyroid hormone (T3)[22]. These findings are of particular interest considering the reported decrease in circulating concentrations of T3 in brain-dead patients showing acute myocardial dysfunction[23]. Furthermore, we have recently shown that tumour necrosis factor alpha levels are significantly elevated in both the myocardium and serum of this group of acutely failing unused donors[24]. Such changes may provide possible mechanisms for the noted increase in expression and activity of Giα, which probably contributes to the myocardial dysfunction seen in both unused donor and end-stage failing hearts.

We have examined RGS gene expression in the pathogenesis of human heart failure in an attempt to identify whether these proteins have a role in progression of this disease. Our findings are consistent with the increase in RGS3 and RGS4 mRNA seen by Zhang *et al.*[10] in an in vitro hypertrophic model, and the increase in RGS4 mRNA observed in their in vivo model of cardiac hypertrophy using pulmonary artery banding. Unfortunately, protein expression was not examined in these models. However, in contrast to our results in human heart failure, Zhang and colleagues' spontaneously hypertensive rat model of congestive heart failure (SHHF) showed a decrease in RGS3 and 4 mRNA and protein[17]. It is known that a relationship exists between inotropes and G-proteins[19], and therefore possibly also between inotropes and RGS protein, (although this has

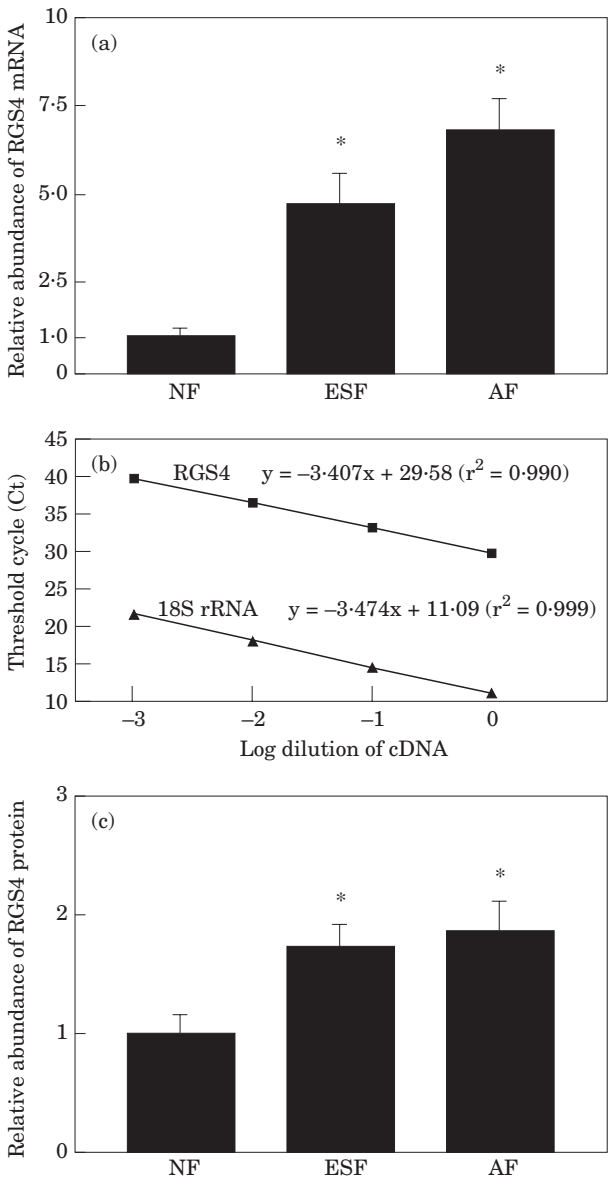

Figure 3 **RGS4 mRNA and protein expression in non-failing (NF) end-stage failing (ESF) and acutely failing unused donor hearts (AF). (a) RGS4 mRNA expression is higher in myocardium from end-stage failing (ESF) (n=19) and acutely failing unused donor hearts (n=14) compared to non-failing (n=7) (*P<0·01 and <0·05, respectively). Quantitative reverse transciptase polymerase chain reaction was used to analyse levels of mRNA abundance. (b) (Inset) The efficiency of reverse transcriptase polymerase chain reaction amplification of RGS4 and endogenous control 18S rRNA gene expression was tested on serial dilutions of a human ventricular muscle cDNA. Co-amplification of RGS3 and 18S rRNA resulted in curves showing a similar slope, demonstrating identical amplification efficiency over the range tested. (c) The relative abundance of RGS4 was significantly higher in myocardium from both end-stage failing (n=19) and acutely failing unused donor hearts compared to non-failing myocardium (n=7) (P<0·02, in both cases). Data are shown relative to levels expressed in non-failing myocardium and is shown as mean ± SEM.**

not been examined in this study). However, looking at our data, it seems unlikely that levels of inotropes could be responsible for the difference between Zhang's results on the SHHF rat model of chronic failure and our results on human heart failure. One reason is that neither the SHHF rats, nor the end-stage heart failure patients, were administered any inotropes. Additionally, both the SHHF rats and human end-stage heart failure patients have endogenously elevated levels of noradrenaline. In the case of the aged SHHF rat, noradrenaline is increased by 25%–35% in the myocardium[25]. So in fact these cases are very similar in respect to inotrope levels but still differ in their RGS expression. We cannot postulate a specific mechanism to explain the difference in RGS expression between the rat SHHF model of chronic failure and human end-stage failure. However, it must be pointed out that although the SHHF rat seems to be a good model of heart failure it can never be entirely comparable to the human situation.

There are other possible physiological effects an increase in RGS protein may have. In addition to the RGS domain acting as GTPase activating proteins, it appears that both RGS3 and 4 increase the concentration of free G$\beta\gamma$. In an in vitro situation, an increase in free G bg subunits has been shown to affect potassium channels by increasing their open probability[7]. RGS3 has a relatively large non-RGS domain portion particularly compared to RGS4, which consists almost entirely of an RGS domain. This N-terminal domain is thought to be responsible for translocation of RGS3 from the cytoplasm to the membrane. As this translocation can also be mediated through a G-protein independent mechanism it has been proposed that the RGS domain itself may have targets distinct from G-proteins and functions other than GTPase activating proteins for Gα subunits[7].

At present, the direct role of RGS proteins in the pathophysiology of heart failure is unclear. We have shown that RGS proteins are deferentially regulated in pathological heart failure. Further in vitro studies examining the effects of agents implicated in the development of hearts failure such as raised tumour necrosis factor alpha and catecholamines will help resolve any influence RGS proteins have on the regulation and expression of G-proteins. However, this finding may prove to be a new potential target for therapeutic intervention leading to improved myocardial function in heart failure.

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