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Cardiomyocyte specific overexpression of a 37 amino acid domain of regulator of G protein signalling 2 inhibits cardiac hypertrophy and improves function in response to pressure overload in mice

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ABSTRACT

Regulator of G protein signalling 2 (RGS2) is known to play a protective role in maladaptive cardiac hypertrophy and heart failure *via* its ability to inhibit G_q- and G_s- mediated GPCR signalling. We previously demonstrated that RGS2 can also inhibit protein translation and can thereby attenuate cell growth. This G protein-independent inhibitory effect has been mapped to a 37 amino acid domain (RGS2^{eb}) within RGS2 that binds to eukaryotic initiation factor 2B (eIF2B). When expressed in neonatal rat cardiomyocytes, RGS2^{eb} attenuates both protein synthesis and hypertrophy induced by G_q- and G_s- activating agents. In the current study, we investigated the potential cardioprotective role of RGS2^{eb} by determining whether RGS2^{eb} transgenic (RGS2^{eb} TG) mice with cardiomyocyte specific overexpression of RGS2^{eb} show resistance to the development of hypertrophy in comparison to wild-type (WT) controls. Using transverse aortic constriction (TAC) in a pressure-overload hypertrophy model, we demonstrated that cardiac hypertrophy was inhibited in RGS2^{eb} TG mice compared to WT controls following four weeks of TAC. Expression of the hypertrophic markers atrial natriuretic peptide (ANP) and β-myosin heavy chain (MHC-β) was also reduced in RGS2^{eb} TG compared to WT TAC animals. Furthermore, cardiac function in RGS2^{eb} TG TAC mice was significantly improved compared to WT TAC mice. Notably, cardiomyocyte cell size was significantly decreased in TG compared to WT TAC mice. These results suggest that RGS2 may limit pathological cardiac hypertrophy at least in part *via* the function of its eIF2B-binding domain.

1. Introduction

Pathological cardiac hypertrophy is a maladaptive growth response of the heart to a variety of disease stimuli. Induced by factors such as hypertension or valvular diseases, prolonged pathological hypertrophy has been associated with an increased risk of sudden death, as well as myocardial infarctions and arrhythmias [1–3]. Moreover, maladaptive hypertrophy is a major risk factor for heart failure [3]. Given the high mortality rates following heart failure diagnoses and the current lack of a cure, reducing risk factors such as pathological cardiac hypertrophy may prove therapeutically beneficial.

G protein-coupled receptors (GPCRs) which signal *via* heterotrimeric $G\alpha_q$ and $G\alpha_s$ proteins are well established as critical players in the induction of pathological hypertrophy [3–5]. Clinically effective treatments for heart failure, such as angiotensin II converting enzyme (ACE) inhibitors and beta-adrenergic receptor antagonists, demonstrate the effectiveness of targeting G_q- and G_s- coupled receptors [6]. However, the effectiveness of these drugs is limited to slowing, rather than reversing, the progression of heart failure. Regulator of G protein signalling 2 (RGS2) is a GTPase accelerating protein (GAP) found ubiquitously throughout the body. RGS2 selectively inhibits G_q- and G_s-mediated signalling (some effects on $G_{i/o}$ signalling have also been reported [7,8]), thus making it an important target in the study of cardiovascular disease [9–11]. Studies *in vivo* and in cardiomyocytes have shown that hypertrophy caused by prolonged G_q-coupled receptor stimulation, such as that induced by phenylephrine, can be blocked by the overexpression of RGS2 [12,13]. A similar effect has been seen against G_s-mediated cardiomyocyte hypertrophy induced by isoproterenol [14]. These observations suggest that RGS2 plays an important role in the regulation of hypertrophy; this has been further demonstrated in knockout animal studies. In RGS2 null mice, experimentally induced pressure overload causes marked hypertrophy, heart failure, and death, as well as increased expression of cardiac fetal genes [15]. Thus, RGS2 would appear to be an essential element in the prevention of pathological cardiac hypertrophy.

Abbreviations: (eIF2), eukaryotic initiation factor 2; (eIF2B), eukaryotic initiation factor 2B; (GPCRs), G protein-coupled receptors; (HWT/BWT), heart weight/body weight; (LV), left ventricle; (RGS2), regulator of G protein signalling 2; (RGS2^{eb}), RGS2-eIF2B binding domain; (TG), transgenic; (TAC), Transverse aortic constriction; (WT), wildtype.

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While the G protein inhibitory effects of RGS2 are well established, studies have also shown that RGS2 can bind to and regulate other targets, including TRPV6 calcium channels and tubulin [16–18]. We have previously shown that RGS2 can bind to the epsilon subunit of eukaryotic initiation factor 2B (eIF2B), a component of the rate-limiting step of the initiation of mRNA translation [19]. By interacting with eIF2Bε, RGS2 limits GDP dissociation on eukaryotic initiation factor 2 (eIF2), which ultimately leads to the attenuation of *de novo* protein synthesis. This property of RGS2 has been mapped to a 37 amino acid domain (residues 79–115) termed RGS2^{eb}, that is homologous to a region in the beta subunit of eIF2 [19].

Since the heart is considered to be a post-mitotic organ [20], hypertrophic growth is thought to be dependent on the enlargement of a pre-existing cardiomyocyte population rather than cell division [21]. Therefore, regardless of the initial stimuli and receptors involved, all hypertrophic signals will ultimately result in increased mRNA translation and *de novo* protein synthesis. Our previous studies have shown that RGS2^{eb} expression in cultured neonatal cardiomyocytes is able to inhibit both protein synthesis and agonist induced hypertrophy at levels comparable to full-length RGS2 [22]. Based on these previous findings, we hypothesized that the *in vivo* expression of RGS2^{eb} in the murine heart could attenuate the development of pathological cardiac hypertrophy. To determine whether RGS2^{eb} could act as an *in vivo* anti-hypertrophic agent, we developed a line of transgenic mice with cardiomyocyte-specific overexpression of RGS2^{eb}, and used transverse aortic constriction (TAC) to induce pressure overload on the heart. Here we report that following 4 weeks of aortic constriction, RGS2^{eb} transgenic mice were protected against pressure overload-induced cardiac hypertrophy, and also were able to maintain heart function at significantly improved levels compared to WT TAC mice. Moreover, reactivation of the "fetal gene program", an indicator of hypertrophy and heart failure, was suppressed. Notably, cardiomyocyte size was decreased in RGS2^{eb} TG compared to WT TAC controls, further supporting our earlier *in vitro* studies which showed RGS2^{eb} inhibition of *de novo* protein synthesis. Together, these findings suggest that in addition to its G-protein inhibitory actions, the RGS2^{eb} region may be contributing to the cardioprotective effects of full-length RGS2 *in vivo via* the inhibition of *de novo* protein synthesis.

2. Materials and methods

2.1. Generation of myosin heavy chain promoter (MHC) - RGS2^{eb} transgenic mice

The RGS2^{eb} gene was targeted to the heart using the mouse α-MHC promoter (kindly provided by Jeffrey Robbins, Cincinnati Children's Hospital Medical Center) [23]. Transgenic mice were generated in the FVB background (London Regional Transgenic and Gene Targeting Facility) and identified by polymerase chain reaction (PCR). Briefly, ear biopsies were taken from three-week old mice and purified for genomic DNA using the QIAquick PCR purification kit (Qiagen). PCR was performed using DreamTaq Green PCR Master Mix (Thermo Scientific). Primers for detection of transgenic mice (CTGCTAGCCAGCAAATATGGTC forward, CCTACAGGTTGTCTTCCCAACT reverse) and control primers for endogenous RGS2 expression (CCGAGTTCTGTGAAGAAAACATTG forward, ATGCTACATGAGACCAGGAGTCCC reverse) were designed using the OligoPerfect Designer (ThermoFisher), OligoCalc [24], and Primer-BLAST (NCBI) programs, resulting in a 293 bp and 342 bp fragment, respectively. RGS2^{eb} TG transgenic mice were back-crossed with C57Bl/6 mice (Charles River) for at least seven generations before animal experiments were performed. Animals were maintained in accordance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals. These studies were approved by the Council on Animal Care at the University of Western Ontario, and complied with the guidelines of the Canadian Council on Animal Care.

2.2. Transverse aortic constriction (TAC)

TAC was used to induce pressure overload on the hearts of 12 week old male C57BL/6 wild-type mice and RGS2^{eb} TG littermates. Mice were anaesthetized with a ketamine (50 mg/kg) and xylazine (12.5 mg/kg) cocktail intramuscularly, intubated, and ventilated with a respirator (SAR-830, CWE, Ardmore, PA, USA). To access the chest cavity, thoracotomy was performed at the second intercostal space under a surgical microscope [25]. A 6–0 silk suture was placed between the brachiocephalic and left carotid arteries. Two knots were tied against a 25-gauge blunt needle placed parallel to the transverse aorta. The needle was removed immediately after the second tied knot followed by closure of the chest. Control WT and RGS2^{eb} TG mice were subjected to sham operations without aortic constriction.

2.3. Assessment of cardiac function

Hemodynamic measurements were performed as previously described [26,27]: four weeks post-surgery, mice were again anaesthetized with a ketamine and xylazine cocktail and ventilated. A Millar micro-tip pressure catheter was inserted into the left carotid artery to assess carotid artery pressure, followed by removal of the catheter and insertion into the right carotid artery for pressure readings, and then advanced into the left ventricle (LV) to measure LV pressures, volumes, and heart rate at steady state and during transient preload reduction *via* mechanical occlusion of the inferior vena cava. All data were recorded using a PowerLab data acquisition system and analyzed by LabChart 7.0 (ADInstruments) and PVAN 3.4 software (Millar).

2.4. Heart weight/body weight ratios

Upon completion of hemodynamic recordings, mice were immediately euthanized *via* a 10% KCl injection into the left jugular vein to ensure cardiac arrest in the diastolic state. Hearts were excised, weighed after removal of the atria, then cut transversely into three equal sections, with the middle section reserved for histological analysis, and the remaining tissue sections stored at −80 °C for subsequent RNA isolation.

2.5. Histological analysis

Heart samples were fixed in 4% paraformaldehyde overnight at 4 °C, dehydrated, and paraffin embedded. Samples were sectioned into 5 μm thick slices with a Leica RM2255 microtome, mounted onto positively charged slides, and then stained with haematoxylin and eosin. Images of left ventricles were captured at 400× magnification with a Zeiss Observer D1 microscope using AxioVision 4.7 software (Zeiss) for cardiomyocyte size measurements and immunohistochemical imaging; images of the LV for wall thickness measurements were captured at 100× magnification.

2.6. Cardiomyocyte cell size and LV wall thickness

Haematoxylin and eosin stained tissue sections were used to determine left ventricular cardiomyocyte cell size and wall thickness of LV free walls and septum. The size of an individual cardiomyocyte was determined by measuring its cross-sectional area. All cardiomyocytes with a well-defined border were manually outlined and then filled in the open source GNU Image Manipulation Program (GIMP). Images were then opened in the image processing program ImageJ and analyzed after setting the threshold. Cardiomyocytes with a circularity ratio of ≥1.2 were excluded to eliminate cells sectioned tangentially [28]. Areas of at least 33 cells per animal were measured, and were scored blind to surgeries and strain.

Heart wall thickness of the LV free wall was measured using AxioVision 4.7 software (Zeiss) in three distinct areas within and between the