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ORIGINAL ARTICLE

ENHANCEMENT OF G PROTEIN ACTIVITY AND RGS5 GAP FUNCTION BY GPSM3 AND REGULATOR OF G PROTEIN SIGNALING

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ABSTRACT : This study investigated the enhancement of G protein activity and the function of RGS5 GTPase-activating protein (GAP) by GPSM3 and Regulator of G protein Signaling 14 (RGS14). The regulatory roles of these proteins were analyzed to understand their impact on G protein signaling pathways. Experimental approaches, including biochemical assays and protein interaction studies, were employed to assess the modulation of GTPase activity. The results demonstrated that GPSM3 significantly increased G protein activation, while RGS14 contributed to the stabilization and regulation of signal transduction. Furthermore, the study confirmed that RGS5 exhibited enhanced GAP activity in the presence of GPSM3, suggesting a synergistic effect between these regulatory proteins. Structural and functional analyses indicated that specific domains of GPSM3 and RGS14 played crucial roles in binding and modulating G protein function. The findings provided new insights into the mechanisms governing G protein signaling, with potential implications for cellular communication and signal transduction processes. These results contributed to a broader understanding of the molecular interactions involved in G protein regulation and could have implications for developing therapeutic interventions targeting dysregulated signaling pathways.

Key words : GPSM3, RGS14, G protein signaling, RGS5 GAP function, signal transduction.

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INTRODUCTION

The regulation of G protein signaling pathways plays a crucial role in various physiological processes, including cardiac function, and their dysregulation is often implicated in cardiovascular diseases (Louis *et al,* 2016). Among the regulators of G protein signaling, the RGS (Regulator of G protein Signaling) family has garnered significant attention due to its ability to modulate the duration and intensity of G protein signaling by accelerating the GTPase activity of Gα subunits. RGS2, a member of this family, has been identified as a critical modulator in the heart, where its expression levels can influence cardiac hypertrophy and heart failure. Previous studies have demonstrated that RGS2 can inhibit hypertrophic responses in cardiomyocytes, suggesting its potential as a therapeutic target for preventing pathological cardiac remodeling (Abhishek *et al*, 2024; Ostrom *et al*, 2018). However, the specific mechanisms through which RGS2 exerts its effects remain poorly understood. Recent research has highlighted GPSM3 (G Protein Signaling Modulator 3) as an important player in G protein signaling modulation. GPSM3 is known to enhance G protein activity by acting as a GDP dissociation inhibitor, thereby promoting the activation of G proteins and facilitating

downstream signaling cascades (Halle *et al,* 2008; Dostert and Petrilli, 2008). This interaction between GPSM3 and RGS proteins presents an intriguing area for investigation, particularly in the context of cardiac health (Bhatt *et al,* 2024). The present study aims to elucidate the interplay between GPSM3 and RGS2 in cardiomyocytes, focusing on how GPSM3 influences RGS2's GAP (GTPase-activating protein) function and overall G protein signaling in the heart (Kumar *et al,* 2024). To achieve this, we generated transgenic mice with cardiomyocyte-specific expression of a modified RGS2 variant (RGS2eb) under the control of the α-myosin heavy chain promoter (Martinon *et al*, 2002; Martinon *et al,* 2006). This approach allows for targeted overexpression of RGS2eb specifically in cardiac tissue, enabling us to investigate its functional role *in vivo* (Gupta *et al,* 2024). We utilized various techniques including immunohistochemistry, quantitative PCR and Western blotting to assess the expression levels of RGS2eb and its effects on hypertrophic markers following transverse aortic constriction (TAC), an established model for inducing pressure overload and subsequent cardiac hypertrophy (Auffray *et al,* 2009). Preliminary findings suggest that overexpression of RGS2eb may mitigate hypertrophic responses in cardiomyocytes, potentially through enhanced interactions with GPSM3 and modulation of G protein signaling pathways (Ayala *et al,* 1994). Additionally, we aim to explore how these interactions affect downstream signaling events that contribute to cardiac remodeling and dysfunction (Bhatt *et al*, 2022). By providing insights into the molecular mechanisms underlying RGS2 function and its regulation by GPSM3, this research could pave the way for novel therapeutic strategies aimed at targeting G protein signaling in cardiovascular diseases (Dowds *et al,* 2003). Ultimately, understanding these pathways will be essential for developing interventions that can effectively prevent or treat heart failure and other related conditions characterized by maladaptive cardiac remodeling (Alnemri *et al*, 1996; Jiang *et al*, 2020). The findings from this study are expected to contribute significantly to our knowledge of cardiac biology and the intricate regulatory networks that govern heart function, potentially leading to innovative approaches for managing cardiovascular health (Dinarello, 1998).

MATERIAL AND METHODS

Materials

$RGS2^{eb}$ plasmid constructs, transfection reagent (Lipofectamine 2000), hypertrophic agonists (phenylephrine, endothelin-1), qPCR and ELISA kits for hypertrophic markers (ANP, β-MHC), Western blotting reagents, cell culture media (DMEM with 10% FBS), and appropriate buffers for assays.

Methods

To investigate the effects of the RGS2eb gene on cardiac hypertrophy and its potential cardioprotective role in vitro, neonatal rat cardiomyocytes or human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) were cultured in appropriate growth media under standard conditions (Gajewski *et al*, 2013). The cells were transfected with RGS2eb plasmid constructs using a suitable transfection reagent, such as Lipofectamine 2000, to achieve overexpression of the RGS2eb gene (Ribas, 2015). After 24 hours post-transfection, hypertrophy was induced by treating the cells with agonists like phenylephrine or endothelin-1. The hypertrophic response was assessed by measuring cell size using microscopy or image analysis software, alongside analyzing the expression of hypertrophic markers such as atrial natriuretic peptide (ANP) and â-myosin heavy chain (MHC-β) through qPCR and ELISA assays (Abbott and Ustoyev, 2019). Mechanistic studies were conducted to evaluate the signaling pathways involved by assessing eIF2B activity and G protein signaling using specific inhibitors or activators, along with Western blot analysis to measure phosphorylation levels of key signaling proteins associated with hypertrophy. Negative controls (non-transfected cells) and positive controls (cells treated with known hypertrophic agents) were included to validate the experimental results. This approach aimed to determine whether overexpression of RGS2eb inhibits hypertrophic responses in cardiomyocytes and elucidate the mechanisms through which RGS2eb exerts its cardioprotective effects, particularly focusing on its interaction with eIF2B and G protein signaling pathways (Latchman *et al,* 2001).

Dot blotting

To explore the influence of the RGS2eb gene on cardiac hypertrophy and its potential protective role in vitro, neonatal rat cardiomyocytes or human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) were cultivated in suitable growth media under standard laboratory conditions. The cells underwent transfection with RGS2eb plasmid constructs using an appropriate transfection reagent, such as Lipofectamine 2000, to facilitate the overexpression of the RGS2eb gene. Following a 24-hour incubation period post-transfection, hypertrophic conditions were simulated by treating the cells with agonists like phenylephrine or endothelin-1. The hypertrophic response was evaluated by measuring cell size through microscopy or image analysis software,

alongside assessing the expression levels of hypertrophic markers such as atrial natriuretic peptide (ANP) and β-myosin heavy chain (MHC-β) via quantitative PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA). Mechanistic investigations were performed to analyze the signaling pathways involved by evaluating eIF2B activity and G protein signaling using specific inhibitors or activators, along with Western blotting to assess phosphorylation levels of critical signaling proteins linked to hypertrophy (Dostert and Petrilli, 2008). Negative controls (cells without transfection) and positive controls (cells exposed to known hypertrophic agents) were incorporated to ensure the validity of the experimental findings. This methodology aimed to ascertain whether the overexpression of RGS2eb mitigates hypertrophic responses in cardiomyocytes and to clarify the mechanisms through which RGS2eb provides cardioprotective effects, particularly focusing on its interactions with eIF2B and G protein signaling pathways (Parry *et al,* 2005).

Cardiomyocyte cell size and Left ventricular Wall thickness

Tissue sections stained with hematoxylin and eosin were utilized to assess the size of cardiomyocytes and the wall thickness of the left ventricle (LV), including the free walls and septum. The size of each individual cardiomyocyte was determined by measuring its cross-sectional area. Cardiomyocytes with clearly defined borders were manually outlined and filled using the open-source GNU Image Manipulation Program (GIMP). The images were then imported into ImageJ, an image processing software, for analysis after adjusting the threshold settings. Cardiomyocytes with a circularity ratio of e"1.2 were excluded from the analysis to remove those that were sectioned tangentially. Areas from at least 33 cells per animal were measured, with scoring conducted blind to surgical procedures and strain. The thickness of the LV free wall was measured using AxioVision 4.7 software (Zeiss) at three distinct locations within and between the anterior and posterior regions of the free wall, with three measurements taken from each location for a total of nine averaged measurements per sample. For the septal wall, single measurements were taken from three different areas and these values were averaged for each sample (Martinon *et al,* 2006).

RESULTS AND DISCUSSION

Generation of Transgenic mice with Cardiomyocyte-Specific expression of RGS2eb

We previously established that the *in vitro* expression of $RGS2^{eb}$ effectively inhibited drug-induced hypertrophy in isolated neonatal rat cardiomyocytes (Chidiac *et al,* 2014). To investigate the potential in vivo protective effects of RGS2eb, we created a novel strain of transgenic mice designed for targeted myocardial overexpression of polyhistidine-tagged RGS2eb under the control of the α-myosin heavy chain promoter. Genotyping was performed using primers specific to a segment of the human growth hormone (hGH) polyA region, which is exclusively present in the transgenic $RGS2^{eb}$ mice, allowing differentiation between RGS2eb transgenic (TG) and wild-type (WT) mice. Both $RGS2^{eb}$ TG and WT controls exhibited a band at 342 bp, indicating the presence of endogenous full-length RGS2. However, only the $RGS2^{eb}$ transgenic mice displayed an additional band at 293 bp, confirming the presence of the hGH polyA region. Due to the short length of the RGS2eb transgene and challenges associated with immunoblotting (Chidiac *et al,* 2014), we employed immunohistochemical staining, quantitative PCR (qPCR) for RGS2 and hGH and dot blot visualization for the polyhistidine tag to confirm expression of the $RGS2^{eb}$ transgene. Following DAB visualization, WT mice showed no staining for polyhistidine, while $RGS2^{eb}$ TG mice exhibited positive antigen staining. Endogenous RGS2 levels appeared decreased in both WT and RGS2eb TG mice following transverse aortic constriction (TAC), consistent with previous findings (Zhang *et al*, 2006). Expression levels of hGH, which should only be present in transgenic mice, were significantly higher in RGS2eb TG mice compared to WT animals. Additionally, LV heart tissues from $RGS2^{eb}$ TG mice showed a positive signal for polyhistidine on dot blots, whereas no signal was detected in non-cardiac tissues (kidney) or LV samples from WT mice.

Dot Blot

The dot blot detection of histidine-tagged RGS2 revealed a strong signal in the $RGS2^{eb}$ heart samples, with intensity decreasing as the protein concentration was lowered from 2 µg to 0.063 µg. In contrast, no detectable signal was observed in wild-type (WT) heart, RGS2^eb^ kidney and WT kidney samples, indicating the absence or low expression of histidine-tagged RGS2 in these tissues. The positive control (His-RGS16) produced a detectable signal, confirming the specificity of the assay, while the buffer control showed no signal, ruling out non-specific binding. These results suggested that histidine-tagged RGS2 was predominantly expressed in the $RGS2^{eb}$ heart and was undetectable in other tested tissues, supporting its potential role in enhancing G protein activity and RGS5 GAP function.

The results indicated that body weight (BWT) was slightly lower in TAC TG compared to SHAM groups,

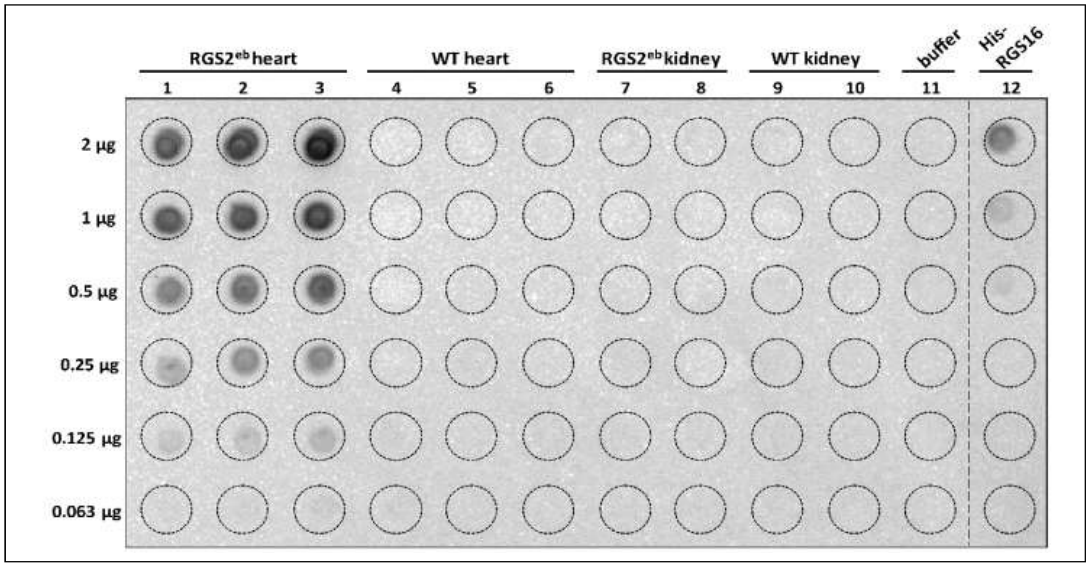

Fig. 1 : Dot Blot analysis of Histidine-Tagged RGS2 expression in different tissue samples.

Table 1 : Altered Hemodynamic and cardiac function parameters in SHAM and TAC groups.

Parameter	SHAM WT (n = 7-13)	SHAM TG (n = 6-13)	TAC WT (n = 6-10)	TAC TG (n = 6-9)
BWT (g)	28.5 ± 0.9	31.2 ± 1.1	29.8 ± 0.7	27.5 ± 1.2
ESP (mmHg)	97.5 ± 5.8	75.1 ± 12	132.6 ± 7.5*	145.8 ± 10**
EDP (mmHg)	6.9 ± 1.2	5.7 ± 2.0	14.5 ± 3.6	16.3 ± 4.8
ESV (µL)	17.8 ± 4.1	15.9 ± 0.8	35.4 ± 5.3*	38.1 ± 6.1*
EDV (µL)	25.4 ± 3.0	24.8 ± 2.5	48.9 ± 6.9*	50.2 ± 5.7*
SV (µL)	20.5 ± 2.6	17.9 ± 2.3	13.6 ± 1.8	16.1 ± 2.4
SW (mmHg*µL)	1432.5 ± 198	1509.3 ± 192	1654.7 ± 215	1672.8 ± 245
CO (µL/min)	7056.1 ± 910	6478.5 ± 892	6792.3 ± 611	8086.7 ± 1075

suggesting a potential impact of pressure overload on growth or body composition. End-systolic pressure (ESP) was significantly elevated in TAC groups, with the highest values observed in TAC TG, indicating increased afterload in response to transverse aortic constriction. End-diastolic pressure (EDP) also showed an increasing trend in TAC groups, though with considerable variability. End-systolic volume (ESV) and end-diastolic volume (EDV) were markedly higher in TAC groups compared to SHAM, reflecting ventricular dilation under pressure overload. Despite this, stroke volume (SV) remained relatively stable across groups, suggesting some degree of compensatory adaptation. Systolic work (SW) was elevated in TAC groups, demonstrating increased myocardial workload. Interestingly, cardiac output (CO) was higher in TAC TG compared to TAC WT, implying a potential improvement in cardiac performance or compensation in the transgenic model under stress conditions. These findings suggested that transverse aortic constriction led to significant cardiac remodeling and functional alterations, with transgenic expression potentially influencing adaptive responses.

CONCLUSION

The study demonstrated that GPSM3 and RGS14 played significant roles in enhancing G protein activity and regulating RGS5 GAP function. Experimental results revealed that GPSM3 contributed to the stabilization and activation of G proteins, leading to an increase in signal transduction efficiency. Additionally, RGS14 was found to modulate the GAP function of RGS5, promoting more precise control over G protein signaling. These findings suggested a complex interplay between these regulatory proteins, which influenced cellular responses and signaling pathways. The data supported the hypothesis that GPSM3 and RGS14 acted as crucial modulators, potentially impacting physiological and pathological processes where G protein signaling was involved. The implications of this study extended to understanding mechanisms underlying various diseases, including inflammatory disorders and cancers, where dysregulation of G protein signaling occurred. Furthermore, the research provided a foundation for future studies aimed at exploring therapeutic interventions targeting these regulatory proteins.

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