Differential expression of $G_s$ in a murine model of homocysteinemic heart failure

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Abstract: High plasma homocysteine levels are a known risk factor in heart failure and sudden cardiac death. The G proteins, $G_s$ (stimulatory) and $G_i$ (inhibitory), are involved in calcium regulation; overexpression has pathological consequences. The aims of this study were to examine the differential expression of $G_s$ protein and $G_i$ in the hearts of hyperhomocysteinemic (Hhcy) mice, and to determine if homocysteine (Hcy) acts as an agonist in cell culture to mediate the change in G protein isoforms. To create Hhcy, heterozygous cystathionine-$\beta$-synthase (CBS) knockout (KO) mice were used. Mice were sacrificed, hearts were excised, cardiac tissue homogenates were prepared, and Western blots were performed. The results suggested that $G_s$ G protein was downregulated in cardiac tissue of heterozygous CBS KO mice to 46% that of control hearts. However, the intracellular $G_i$ G protein content remained the same in heterozygous CBS KO mice. Transformed cardiomyocyte HL-1 cells were treated with varying concentrations of homocysteine. The results suggested no detectable differential $G_s$ and $G_i$ expression. This suggested that Hcy did not act as an agonist in vitro to alter G protein content, but that Hcy produced some other in vivo effects to incur these results.

Keywords: homocysteine, G proteins, heart failure, GPCR, HL-1

Introduction

Homocysteine (Hcy) is a nonprotein amino acid. It differs from cysteine only in its side chain that contains an additional methylene group before the thiol group. Moreover, Hcy is the result of methionine metabolism and is converted to cystathionine by cystathionine-$\beta$-synthase (CBS); an established model for creating hyperhomocysteinemia (Hhcy) is with heterozygous CBS knockout mice (CBS KO mice) since these mice are unable to convert Hcy to cystathionine, resulting in excess levels of plasma Hcy. A high plasma Hcy level has recently been suggested to be a major risk factor surrounding chronic heart failure (CHF). Recently, Hcy has been shown to increase ERK phosphorylation in cultured microvascular endothelial cells (MVEC). Hcy-induced ERK phosphorylation was suppressed by pertussis toxin (PTX). Since PTX is known to block $G_i$ and $G_q$-mediated pathways, this suggested the involvement of G protein-coupled receptors (GPCRs) in initiating signal transduction by Hcy that leads to ERK activation. GPCRs are integral membrane-spanning proteins that have G proteins attached intracellularly; upon activation of a GPCR by an agonist, the G-proteins detach from the receptor and proceed to activate other effectors. There is a large class of GPCRs that utilize a great variety of different G proteins. Cardiac-adrenergic receptors are an example of GPCRs that are important regulators of cardiac function.

Stimulation of GPCRs can occur with the following: sympathetic or parasympathetic neuronal activation, by circulating catecholamines, by adrenergic agonists that increase heart rate (chronotropism), force of cardiac contraction (inotropism), rate of cardiac relaxation (lusitropism), and automaticity. The prominent $\beta_1$ subtype is mainly responsible for positive chronotropic and inotropic effects of catecholamines.
Activation of a GPCR coupled to $G_s$ increases the activity of adenylyl cyclase and increases intracellular calcium concentration.$^{12}$ Calcium is a known modulator of cardiac function.

Net $G$ protein content available for signaling is imperative for maintaining homeostasis. Many studies indicate cardiomyopathy from overexpression of $G$ proteins. Cultured neonatal cardiac myocytes from transgenic mice overexpressing cardiac $G$ showed a five-fold higher $I_{\text{calcium}}$ compared with wild type controls.$^{14}$ In Tg mice overexpressing the $G_s$ subunit, heart rate and left ventricular ejection fraction (LVEF) was elevated compared with littermate wild-type mice.\(^\text{15}\)

In summary, there is an association of high plasma Hcy levels and heart failure.$^{4,9}$

Moreover, calcium regulation is crucial to cardiac function. Evidence from our lab suggested the involvement of GPCRs in modulating calcium-induced ERK activation in MVEC.$^{10}$ Moreover, $G_s$ and $G_i$ are fundamental effectors that regulate calcium homeostasis in cardiomyocytes; overexpression can result in pathology.$^{12,14,15}$ We, therefore, hypothesized that high plasma Hcy levels could modulate net $G$ protein content available for signaling in cardiomyocytes in vitro and in vivo, thereby modulating cardiac homeostasis. Moreover, we were unsure whether content would increase or decrease since our results could indicate a contribution or mitigation to cardiomyopathy. Cultured HL-1 transformed cardiomyocytes were used as models for cardiac response to determine if homocysteine acts as an agonist in isolation to alter $G$ protein content and if these results could be replicated in a more complex living system.

## Materials and methods

### Animals

All animal procedures were in accordance with the National Institute of Health Guidelines for animal research and were approved by the Institutional Animal Care and Use Committee of the University Of Louisville School Of Medicine. There were 4 mice used in each of 2 groups: control, CBS KO. The CBS KO mice used were also used in a previous study: 3-Deazaadenosine mitigates arterial remodeling and hypertension in hyperhomocysteinemic mice.\(^\text{16}\) Wild-type (WT; B6.129PF2/J) and breeding pairs of CBS heterozygous animals with plasma Hcy levels between 25 and 30 $\mu M$, a well established model of HHcy. Each group was obtained from Jackson Laboratories (Bar Harbor, ME). Mice were bred at the breeding facility of University of Louisville School of Medicine. Homozygous mutants, completely lacking CBS, have 40- to 50-fold higher plasma Hcy levels and a very short life span; hence, they were not used for the study. In this study, we used male heterozygous animals with plasma Hcy levels between 25 and 30 $\mu M$, a well established model of HHcy. Each group received a standard lab chow for that duration. After 12 weeks of treatment, mice were sacrificed, hearts were excised and stored at $-70^\circ$C until further experimentation. Homogenates were prepared from ventricle of the hearts.

### Plasma Hcy measurements

The level of Hcy in plasma was quantitatively analyzed with the Bio-Rad microplate enzyme immunoassay homocysteine assay (Bio-Rad Laboratories, Hercules, CA) and a SpectraMax M2 Analyzer (Molecular Devices, Sunnyvale, CA) according to the manufacturers’ instructions to ensure Hcy levels of CBS KO were between 25 and 30 $\mu M$.

### Chemicals

The antibodies against $G_s$ and $G_i$ were obtained from Santa Cruz (Santa Cruz, CA). The antibody against-actin was obtained from Sigma (St. Louis, MO). Plain DMEM/F-12 50/50 medium was obtained from Mediatech, Inc (Herndon, VA). Fetal Bovine Serum was acquired from Gemini Bio-Products, (West Sacramento, CA). Claycomb medium with penicillin, streptomycin, trypsin-EDTA, and Hanks’ balanced salt solution (HBSS) were purchased from GIBCO-BRL (Grand Island, NY); DL-Hcy, NaCl, sodium orthovanadate, Tris, EDTA, EGTA, dithiothreitol, NP-40, protease inhibitor cocktail, fibronectin, agarose, from Sigma (St. Louis, MO); D,L-homocysteine was obtained from Sigma (St. Louis, MO).

### Cell culture and treatments

Cultured HL-1 cells were received as a gift from LSU, (New Orleans, LA) The complete Claycomb medium was supplemented with sterile 100 $\mu M$ norepinephrine bitartrate (10 mL/L of a 10 mM norepinephrine stock solution), 4 mM L-glutamine (Catalog No. 59202C) and 10% FBS prior to use. A T-25 flask was coated with fibronectin overnight before plating cells. Cells were grown to near confluence, and serum starved overnight when treated with the indicated reagents for Western blot analysis. Claycomb Medium is designed for use in a 5% CO$_2$ humid environment. HL-1 cells require higher than normal cell densities for optimal growth and behavior.

### Preparation of samples, Western blot analysis, and immunodetection

After treatment, medium was aspirated from six-well culture dishes and HL-1 were washed twice with ice-cold
Decrease of G\(\alpha\) in Hhcy mice

**Statistical analysis**

Statistical analysis was performed using an unpaired Student’s t-test for comparison between control, Hcy, and other pharmacological treatment groups. \(P < 0.05\) was considered statistically significant. All results are averages \(\pm SE\) of at least three different experiments.

**Results**

We report that G\(\alpha\) expression in left ventricular heart tissue decreased to 46% of the control value of expression (\(P < 0.01\), \(n = 4\)) (Figure 1). G\(\alpha\) expression remained unchanged in heart homogenates of both CBS KO mice and wild type mice. Equal protein concentration was determined using Bradford dye experiment, while equal loading was confirmed using -actin.

The level of G\(\alpha\) expression and G\(\alpha\) expression remained the same in HL-1 transformed cardiomyocytes cultured cells treated with the following concentrations of Hcy for 24 hours: 0 \(\mu M\), 25 \(\mu M\), 50 \(\mu M\), 100 \(\mu M\), 500 \(\mu M\). There were no significant changes in expression (\(P < 0.05\), \(n = 3\)) (Figure 2).

**Discussion**

There is a consistent association of high plasma Hcy with clinical and echocardiographic measures of CHF.\(^6\) In fact,
Vacek et al. data suggested that Hhcy is not only associated with an increased incidence of CHF, but also with severity of disease. Moreover, Hhcy could evoke cardiac remodeling that features interstitial and perivascular fibrosis. The presence of elevated plasma Hcy is also an independent predictor of progression of coronary plaque load that results in atherosclerosis. In heart failure, the sympathetic system is activated, cardiac receptor number and function are decreased, and downstream mechanisms are altered. Our CBS KO mice demonstrated indicators of heart failure such as hypertension, hypertrophy, and arterial remodeling. It is important to note that metabolic, structural and functional effects on the heart may differ fundamentally if Hhcy is induced by methionine, homocystine or B-vitamin deficiency.

Downregulation of GPCRs can clearly result in differences of G protein content since G proteins are attached to their respective receptors via cytoplasmic C-terminal and cytoloops. Several studies show differential β-adrenergic downregulation in conjunction with the differential G protein expression that we observed in vivo. The decrease resulted from receptor internalization, sequestration, and consumption. In a study using a pacing tachycardia HF model in canines, HF resulted in a uniform reduction in β1-AR density in surface and T-tubule membrane. In a pulmonary in vivo model of β2-adrenoceptor desensitization and internalization, it was found that albuterol or salmeterol induced a downregulation of $G_\alpha_s$. Another fluorescent study of $G_\alpha_s$ showed that internalization of β2 adrenergic receptors and $G_\alpha_s$ subunit occurs simultaneously, providing another link for receptor downregulation occurring in conjunction with differential G protein content.

It is important to note that there are several GPCRs that utilize the $G_\alpha_s$ and $G_\alpha_i$ G proteins other than the β-adrenergic receptors. It is likely that the decrease in $G_\alpha_i$ can be attributed to downregulation of several different GPCRs that utilize this G protein. Western blot analysis showed $G_\alpha_s$ content is decreased to 46% that of control levels in CBS KO mice (Figure 1).

Varying levels of G proteins and GPCRs can influence cardiomyopathy. The result of a disrupted balance of β-ARs can be seen in a study of TG mice overexpressing β1-adrenoceptors that encounter mortality sooner. β-AR blockage by propranolol treatment was salutary in these TG mice, and can prevent the development of cardiomyopathy. The model of volume overload cardiac hypertrophy produced our same results for CBS KO mice: decreased content of $G_\alpha_s$ but not of $G_\alpha_i$ in heart. These results have a clear link to Hcy-induced CHF and hypertrophy; it is already known Hhcyc leads to pathological ventricular hypertrophy in normotensive rats. Clinical and experimental studies have indicated a role of homocysteine in ventricular hypertrophy via angiotensin II mechanism. Moreover, a human study showed an association between plasma homocysteine concentrations and cardiac hypertrophy in end-stage renal disease.

G protein content is important for proper cardiac function. It was found that activation of $G_\alpha_s$ via β-adrenergic receptors
enhanced the activity of cardiac voltage-dependent Ca2+ channels of the L-type. One other study showed TG mice with cardiac Gs overexpression exhibited enhanced inotropic and chronotropic responses to sympathetic stimulation, and TG mice developed cardiomyopathy with age. Other TG models with selective induction of Gi subunit expression showed novel downstream events in hypertrophic signaling that could be critical factors leading to cellular electrophysiological remodeling and cardiac arrhythmias in hypertrophic cardiomyopathy.32

Hcy treatment of HL-1 transformed cardiomyocytes at varying doses for 24 hours produced no detectable changes in intracellular Gs or Gi protein content, which suggested that downregulation of any GPCRs utilizing these G proteins did not occur in isolated treatment of Hcy agonist (Figure 2). It is known that treatment of cells with receptor agonists for many minutes or hours results in a loss of cellular receptors (downregulation).6 Hence, this treatment would have been sufficient to evoke receptor downregulation and variable G protein content if Hcy is, indeed, an agonist to GPCRs utilizing these G proteins. Since this is not the case, this suggested that Hcy does not act as an agonist to evoke GPCR downregulation and modulate Gs content. Therefore, this suggested that Hcy acts via some other mechanism in vivo to evoke differential Gs content.

Conclusion
Results indicated that Gs is the only G protein of interest decreased in vivo, this limits available Gs for calcium signaling. Moreover, our results suggested a compensatory decrease of calcium signaling via G protein pathway in spite of greater chronotropy and ionotropy characteristic of heart failure. Moreover, Hcy did not act as an agonist in vitro to incur differential G protein content, which suggested that Hcy incurs some other in vivo effects that lead to a decrease in Gs in the living system.

Limitations
We did not test whether the levels of expression of G protein isoforms of interest are the same in CBS KO mice at the early stage (normal cardiac function) and at the late stage (heart failure); this could have served as another control to ensure the CBS gene did not affect G protein isoforms. Our interest in this study laid in determining net G protein content available for signaling before discovering which GPCRs that contain the respective G proteins are modulated. We speculate that β1-AR downregulation, which contains Gs G protein, is responsible for our measured decrease in Gs G protein in in vivo data since β1-AR is consistently downregulated in cases of heart failure (Figure 3). Since HL-1 cells are known to demonstrate the same phenotype as primary culture cardiomyocytes, we did not view

![Figure 3 Proposed in vivo model G protein content based on decrease in Gs content. High homocysteine levels created by CBS KO model decreased Gs content available for calcium signaling. A decrease in Gs content decreased chronotropic and ionotropic response to circulating GPCR agonists that utilize this G protein.](https://www.dovepress.com/)

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this as a limitation. The duration of time for treatment in vitro (24 hours) was chosen since it is known that treatment of cells with receptor agonists for many minutes or hours resulted in a loss of cellular receptors (downregulation). If downregulation is going to occur, it is likely to be captured within this period of time.

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References