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Homocysteine effects classical pathway of GPCR down regulation: $G_{q/11}$, $G_{12/13}$, $G_{i/o}$

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Abstract

G protein-coupled receptors (GPCRs) are known to modulate intracellular effectors involved in cardiac function. We recently reported homocysteine (Hcy)-induced ERK-phosphorylation was suppressed by pertussis toxin (PTX), which suggested the involvement of GPCRs in initiating signal transduction. An activated GPCR undergoes down regulation via a known mechanism involving ERK, GRK2, β -arrestin1: ERK activity increases; GRK2 activity increases; β -arrestin1 is degraded. We hypothesized that Hcy treatment leads to GPCR activation and down regulation. Microvascular endothelial cells were treated with Hcy. Expression of phospho-ERK1 and phospho-GRK2 was determined using Western blot, standardized to ERK1, GRK2, and β -actin. Hcy was shown to dephosphorylate GRK2, thereby enhancing the activity. The results provided further evidence that Hcy acts as an agonist to activate GPCRs, followed by their down regulation. Hcy was also shown to decrease the content of the following G proteins and other proteins: β -arrestin1, $G_{q/11}$, $G_{12/13}$, $G_{i/o}$.

Keywords

G protein; GPCR; GRK2; ERK1; β -Arrestin1; c-Src; Desensitization; Heart failure; Hcy; Homocysteine

Introduction

Homocysteine (Hcy) is a homologue of cysteine and only differs in its side chain that contains an additional methylene group before the thiol group [1]. High plasma levels of Hcy are an independent risk factor in coronary artery disease [1,2]. Moreover, elevated Hcy levels in CHF patients were also correlated with severity of disease and mortality [1,3]. Another study showed that the presence of elevated Hcy ($>12 \mu\text{M}$) predicts progression of coronary plaque burden [4]. Moreover, HHcy exacerbated cardiac remodeling in response to hypertension, making Hcy a powerful stimulus for ventricular hypertrophy [5]. The Women's Antioxidant and Folic Acid Cardiovascular Study (WAFACS) clinical study was conducted to examine the effects of administering relevant vitamin (B6, B12) co-factors that aid in converting Hcy into benign metabolic intermediates; although this study was unable to lower Hcy levels, it did not undermine the independent risk factor that Hcy imposes. Rather, it warrants further investigation for a solution to lowering elevated plasma Hcy levels.

ERK-phosphorylation was suppressed by pertussis toxin (PTX), which suggested that GPCRs were activated by Hcy that leads to ERK1 activation [6]. G protein-coupled receptor (GPCR)

is a large class of receptors that can detect extracellular molecules and activate signal transduction pathways that lead to cellular responses [7]. In context of the cardiovascular, GPCRs can also mediate function via regulation of calcium; this is true for the G_{α_q} pathways that stimulates PLC- β to produce intracellular mediators of calcium, inositol triphosphate (IP3) and diacylglycerol (DAG) [7]. This classical model of activation of GPCRs involves release of heterotrimeric G proteins that act as secondary messengers to activate other intracellular effectors. ERK1 was activated via this classical model of activation that could come from the G_{α_q} class of G proteins [8].

There is a dampening of this signaling cascade via desensitization of GPCR receptors, leading to an uncoupling of the agonist-induced signal [9,10]. One group of kinases, GPCR kinases (GRKs) only phosphorylate GPCRs that are agonist-bound [9,11,12]. After this process, referred to as desensitization, receptors can be removed from the cells' surface. This process is referred to as endocytosis, sequestration, internalization, or down regulation of GPCR receptors; this is, in part, mediated by β -arrestins [9,13].

β -arrestins are proteins found in the cytosol that migrate to the membrane and bind activated, phosphorylated GPCRs [9,14,15]. Binding of β -arrestins to the GPCRs provides a scaffold for the activation of MAPKS (mitogen-activated protein kinases), such as ERK 1/2 [9], and *c*-Src, an intracellular tyrosine kinase [9,15,16]. The β -arrestin1/*c*-SRC interaction is important for receptor internalization since *c*-Src is found to phosphorylate primary endocytic intermediates, such as dynamin [17]. β -arrestins can target receptors to clathrin-coated pits (CCPs) through binding with clathrin and clathrin adapter 2 (AP-2) complex [18]. GPCRs couple to specific classes of heterotrimeric G proteins [9]. When an agonist binds to a GPCR, the receptor shows an active conformation, and G proteins dissociate to further transduce the signal to other intracellular effectors [9].

Our experiment did not aim to discover the specific signaling pathway of Hcy on GPCR effectors (which would require many blockers), but rather to examine the effects of Hcy on many signaling effectors of an already established classical pathway of GPCR downregulation/desensitization. If there are any variations to how we already understand this pathway to operate, this manuscript could serve as a basis for that further investigation. Moreover, we were primarily interested in net G protein content available for signaling as well as expression/activity of the aforementioned effectors.

Since administration of PTX leads to the inhibition of Hcy-induced ERK-phosphorylation, we hypothesized that Hcy acted as an agonist to GPCRs and will set in motion the classical pathway of GPCR desensitization. We aimed to perform a time-course evaluation for detecting activation of ERK1/2 (a common intermediate in GPCR activation), activation of GRK2, activation of β -arrestin1 and activation of *c*-Src with a standard dose of Hcy using Western blot analysis of treated rat micro-vascular endothelial cells (MVECs). Moreover, we determined net G protein content modulation for the following G proteins involved in calcium regulation: $G_{\alpha_{q/11}}$, $G_{\alpha_{12/13}}$, $G_{i/o}$.

Materials and methods

Chemicals

The antibody against GRK2, *c*-Src, ERK, *p*-ERK1, $G_{\alpha_{q/11}}$, $G_{\alpha_{12/13}}$, $G_{i/o}$, and β -arrestin1 was obtained from Santa Cruz (Santa Cruz, CA); *p*-GRK2, *p*-*c*-Src, *p*- β -arrestin1, and β -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 was 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

Rat MVECs were procured from Cambrex (Walkersville, MD) and cultured in endothelial basal medium 2 supplemented with growth factors as described in the supplier's protocol and 12% (vol/vol) heat-inactivated FCS maintained at 37°C in a 95% O₂-5% CO₂ humidified atmosphere. MVECs were used at *passages 8–13*, grown to near confluence, and serum starved overnight when treated with the indicated reagents for Western blot analysis.

Preparation of samples, Western blot analysis, and immunodetection

After treatment, medium was aspirated from six-well culture dishes and MVECs were washed twice with ice-cold 1x PBS. Ice-cold lysis buffer (in mM: 50 Tris HCl, pH 7.4, 150 NaCl, 1% Triton X-100, and 1 EGTA) and freshly prepared inhibitors (1 mM PMSF, 1 µg/ml leupeptin, 200 µM sodium orthovanadate, and 1 µg/ml aprotinin) were added to each well. Plates were scraped on ice, and the supernatant containing cytosolic protein was collected and centrifuged at 5,500 g for 10 min at 4°C, and resolved by SDS-PAGE on 10% polyacrylamide gels and blotted onto a polyvinylidene difluoride membrane. After being transferred, blots were washed with Tris-buffered saline (TBS) for 5 min at room temperature and incubated in blocking buffer for 1 h at room temperature. The blots were then incubated with the indicated primary antibodies [appropriate dilutions in 3% Milk solution of TBST, (0.1% Tween 20 + TBS: TBST)] overnight at 4°C using gentle agitation. The blots were washed four times (5-min wash each time) with TBST and incubated with HRP-conjugated secondary antibody (1:3,000 dilution in 3% Milk-TBST). After being washed with TBST 4 times (10 min wash each time), the proteins of interest were detected using an ECL plus kit (Amersham Biosciences, Piscataway, NJ). The membranes were then stripped using 0.2 M NaOH solution for 30 min at room temperature and reprobed with a standard: β -actin. ERK was normalized to β -actin, and *p*-ERK was normalized to ERK. Similarly, GRK2 was normalized to β -actin; *p*-GRK2 was normalized to GRK2. All other proteins were normalized to β -actin.

Data analysis and statistics

Values are means $\pm$ SE from at least three different experiments. The data was analyzed by Student's *t*-test for comparison of the results between various treatment groups. $P < 0.05$ was considered to indicate statistical significance. GRK2 activity was determined as the reciprocal of phospho-GRK2 expression.

Results

Figure 1a shows that at a standard time point of 60 min, ERK1 showed the greatest increase at 10 µM and 100 µM treatments; significance was reached at only 10 µM. However, there was a trend of increasing ERK activation at 100 µM. We selected the higher concentration of 100 µM to perform our time-course evaluation of ERK activation for the following time points: 0, 15, 30, 45, 60, 120 min (Fig. 1b). There was a 1.3-fold increasing trend at 15 min. Significance was reached at 30 min with a 1.4-fold increase and returned to control levels at 45 min. At 60 min, there again was a 1.3-fold increasing trend. At 2 h, ERK1 activation returned to control levels.

Figure 2a shows that GRK2 was dephosphorylated and, thereby, activated at the following concentrations with a standard 60 min treatment: A 100 µM Hcy treatment resulted in a 1.4-fold increasing trend of activity; 500 µM Hcy treatment showed a 1.7-fold statistical increase in activity. The time-course evaluation of activity showed an increasing trend at 60 min to 1.2-

fold. Statistical significance was reached with an increase in activity at 120 min to 1.2-fold that of control levels (Fig. 2b). Figures 3a, b and 4a, b show similar results for two different proteins and phosphor-isoforms: *c*-Src and β -arrestin1. There was a statistically significant decrease of *c*-Src, *p*-*c*-Src, *p*- β -arrestin1, and β -arrestin1 with the following results: 60 min treatment *c*-Src (500 μ M, 0.7 that of control values); 60 min treatment *p*-*c*-Src (500 μ M, 0.5 that of control values); 60 min treatment *p*- β -Arrestin1 (500 μ M, 0.7 that of control values); 60 min treatment β -arrestin1 (500 μ M, 0.6 that of control values); Time-course evaluation *p*-*c*-Src (100 μ M, 120 min, 0.7 that of controls); time-course evaluation *c*-Src (100 μ M, 120 min, 0.8 that of controls); time-course evaluation β -arrestin1 (100 μ M, 120 min, 0.7 that of controls); time-course evaluation *p*- β -arrestin1 (100 μ M, 120 min, 0.8 that of controls).

Figure 5 shows that $G\alpha_{q/11}$ was decreased dose-dependently at 24 h with a trend beginning at 50 μ M, and significance reached with the following concentrations: 75,100,500 μ M 0.05. At 48 h, there was a significant decrease of $G\alpha_{q/11}$ at 500 μ M. Figure 6 shows no increase or decrease of $G\alpha_{12/13}$ at 24 h at the following Hcy concentrations: 0,25,50,75,100,500 μ M. Figure 6 shows a significant decrease of $G\alpha_{12/13}$ at 48 h and 500 μ M. At 48 h, there is a significant decrease of $G_{i/o}$ using 500 μ M Hcy treatment (Fig. 7).

Discussion

A vessel consists of both an endothelial layer and smooth muscle layer [19]. The endothelial layer contains many GPCRs that affect signaling of the inner smooth muscle layer [20]. One study has already shown that Hcy increased intracellular calcium within MVEC [6]. Another study has shown that the increased $[Ca^{2+}]_i$ of endothelial cells triggered a release of an endothelium-derived hyperpolarizing factor (EDHF) from the endothelial cells, thereby relaxing the underlying muscle layer [21]. Other studies have linked an increase in endothelial calcium to an increase in Nitric oxide (NO) that may permeate the membrane and relax smooth muscle of vasculature [19]. Since we have shown much of the $G\alpha_{q/11}$ (increases intracellular calcium) available for signaling has decreased in presence of Hcy, this would logically inhibit the ability of the vasculature to relax further with continued presence of Hcy. The continued down regulation of G proteins responsible for increase of calcium in endothelial layer may aggravate hypertension that is known to occur in HHcy Fig. 8.

A GPCR agonist can transduce an increase in ERK1 activity [22]. ERK-phosphorylation was suppressed by PTX (PTX inhibits both $G_{i/o}$ and $G\alpha_q$ G proteins of GPCRs), which suggested that GPCRs were activated by Hcy that lead to ERK1 activation [6]. This suggested that initial binding of agonist to GPCRs activated ERK1 through a classical pathway (e.g. IP3, DAG); further ERK1 activation occurred via scaffolding by GPCR receptor internalization protein species, β -arrestins and *c*-Src [23]. Consistent with GPCR activation, we witnessed an increasing trend of ERK1 activation at 15-min 100 μ M Hcy; at 30 min there was a significant increase with this concentration. Our decision to use the greater Hcy concentration (100 μ M) for ERK when only a trend was seen at the 60 min time point stems from past studies that have used higher concentrations for testing expressions of other proteins, like matrix metalloproteinases and tissue inhibitors of metalloproteinases [24,25]. ERK is a very unique protein that is constantly phosphorylating and dephosphorylating; ERK may be phosphorylated at one time and concentration while dephosphorylated at a further time and same concentration (this can also be seen in the time-course using 100microMolar Hcy). ERK1 activity dropped to control levels at 45 min; however, there was an increasing trend at 60 min with 100 μ M Hcy treatment.

GRK2 is a major player for the regulation of cardiac chronotropic and ionotropic response to catecholamines; in fact, its genetic ablation caused embryonic lethality in the developing embryo [11,26–28]. The decrease in signaling activity of lymphocyte β 2-adrenergic receptors

was associated with an increase in expression of GRK2 [11,28]. The inhibition of GRK2 also allows for enhanced response to circulating catecholamines since this reduces receptor desensitization [11,28]. In vitro studies showed that GRK2 is capable of phosphorylating and desensitizing almost all GPCRs [11]. Overexpression of GRK2 can attenuate G_{α_q} signaling; one example of this is for metabotropic glutamate receptor 1a [9]. In fact, our results show that an increase of activity of GRK2 was associated with a decrease in G_{α_q} content.

Consistent with how GRK2 mediates signaling response, we found that the sympathoexcitatory molecule, Hcy, resulted in an increase in GRK2 activity, thereby decreasing the chronic activation of certain GPCRs. One site of phosphorylation for GRK2 is at a carboxyl-terminal serine residue (Ser⁶⁷⁰) [22]. The phosphorylation at Ser⁶⁷⁰ impaired ability of GRK2 to phosphorylate soluble and membrane-incorporated receptor substrates, thereby inhibiting activity; hence, dephosphorylation of GRK2 at this site indicated an increase in activity of this protein in phosphorylating agonist-bound GPCRs.

One caveat that should be mentioned is that although GRK activity is generally indicative of an agonist-bound GPCR activity, GRK2 can be phosphorylated by PKC and PKA [29]; although these kinases are major players in G protein pathways, PKC can also be activated by NMDA receptor activation [30]. Hcy has been shown to activate NMDA receptors [30]. Of course, this caveat may not be relevant considering there are several isoforms of these kinases that could be specific to a certain protein and pathway.

A classical role of desensitization is the binding of β -arrestin to GRK-phosphorylated receptors; this uncoupled the receptor from its G proteins and prevented further signal transduction [9, 11,12,31]. Dephosphorylation of Ser⁴¹² of β -arrestin1 is required for an association between β -arrestin1 and *c*-Src, as well as β -arrestin1/clathrin association that results in GPCR internalization [32,33]. A decrease of ERK activity attenuated β -arrestin1 phosphorylation and, therefore, increased the concentration of dephosphorylated β -arrestin1 that can proceed in mediating endocytosis of a receptor [34]. We were unable to capture the increase in activity of β -arrestin1 at Ser⁴¹² since baseline levels of β -arrestin1 also decreased at our chosen concentration and time point (100 μ M Hcy 120 min). This is easy to explain in context of how a receptor is down regulated. In the case of class B GPCRs, β -arrestin1 is degraded along with the receptor [35].

One study showed that *c*-Src regulated clathrin adapter protein 2 interaction with β -arrestin during clathrin-mediated internalization; *c*-Src-depleted cells show a delay in receptor internalization [18]. The anti-Tyr⁵²⁹ *c*-SRC was used to investigate the phosphorylation status of the regulatory tyrosine in the carboxyl terminus of *c*-Src [36]. In the inactive Src molecule, the Tyr⁵²⁹ is normally phosphorylated and engaged with the Src homology 2 domain of the amino terminus of Src; hence, dephosphorylation of this residue is typically required for activation of *c*-Src [36]. We had similar difficulty in testing for activity of *c*-Src as we did with β -arrestin1. Since *c*-Src is known to complex with β -arrestin1, this could suggested that *c*-Src is degraded along with the β -arrestin1/*c*-Src complex. However, as it stands, we are only witnessing the degradation of these proteins: *c*-Src, β -arrestin1.

A decrease in receptor number will also correspond with a decrease in the attached G proteins that a receptor utilizes. This was true when performing live cell imaging that shows G_{α_s} and β 2-adrenergic receptor internalizing via clathrin and dynamin mechanism [37]. Another study showed that β -adrenergic receptor stimulation promoted G_{α_s} internalization though lipid rafts for further signaling and eventual degradation in absence of its receptor [38]. In fact, chronic activation of β 2-adrenoceptor by salmeterol downregulated both β 2-adrenoceptor along with the G protein it utilizes: G_{α_s} [39].

The G_{α_q} pathway is characterized by stimulation of PLC, producing intracellular messengers, IP3, and DAG; IP3 evokes a release of calcium from intracellular stores, while DAG recruits protein kinase C (PKC) [7], thereby effecting vasoconstriction. The $G_{i/o}$ pathway is important since these G proteins can modulate K⁺-channels and inhibit cyclic AMP (cAMP) that modulates calcium [7]. Interestingly, it was shown that 500 μ M Hcy treatment for 48 h resulted in a significant decrease in $G_{i/o}$ content in the cell. Consequently, the ability to inhibit calcium influx within the cell is impaired since this signaling molecule was absent under these conditions. Our results showed a significant decrease in $G_{\alpha_{12/13}}$, a protein known to mediate calcium signaling [40–42] content at only 0.5 mM conditions for 48 h. In alignment with our in vitro study, a final study found that G_{α_q} and $G_{\alpha_{12}}$ protein levels decreased significantly in CHF [43].

In summary, we were able to witness portions of the classical pathway of GPCR desensitization: an increase in ERK1 activity characteristic of GPCR activation; activation of GRK2 warranting further evidence that Hcy binds to GPCRs and marks them for down regulation; degradation of β -arrestin1, consistent with how Class B GPCRs are down regulated. This provides further evidence that Hcy binds to GPCRs, marked by increased GRK2 activity to evoke the machinery involved in GPCR desensitization; in fact, net G protein content of the following G proteins decreased with various Hcy treatments: $G_{\alpha_{q/11}}$, $G_{\alpha_{12/13}}$, $G_{i/o}$.

Limitations

We did not detect the migration of β -arr-estin1 to the membrane or the attachment of *c*-Src to β -arrestin1 as a scaffold. However, these proteins are known to migrate to activated, phosphorylated GPCRs. We are also unsure what became of *c*-Src, but speculate it could be degraded along with β -arrestin-1, or is involved in some other pathway with this consequence. The concentrations of homocysteine required to evoke the greatest significant difference in phosphorylation and/or expression of various signaling proteins was 0.5 mM; however, as low as 75 μ M Hcy treatment could produce a significant change in expression of G proteins, such as $G_{\alpha_{q/11}}$. The normal plasma concentration in adults is 5–15 μ M; only in severe cases do levels reach 100 μ M or more.

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Fig. 1.

(a) A significant increase of ERK1 activity at 5 and 100 μM Hcy with a standard 60 min time condition ($P < 0.05$, $n = 3$). (b) A time-course evaluation of ERK1 activity using 100 μM Hcy and an increasing trend at 15 min and 60 min with statistical significance reached at 30 min ($P < 0.05$, $n = 3$)

Fig. 2.

(a) A dose-dependent increase in GRK2 activity: trend at 100 μM Hcy, 60 min, significance 500 μM Hcy, 60 min ($P < 0.05$, $n = 3$). (b) A time-course evaluation was performed with significance at 100 μM Hcy, 120 min ($P < 0.05$, $n = 3$)

Fig. 3.

(a) A dose-dependent decrease of p - β -Arrestin1 and β -arrestin1 content: significance at 500 μ M Hcy, 60 min ($P < 0.05$, $n = 3$). (b) A time-course evaluation was performed showing a significant decrease at 100 μ M Hcy 120 min ($P < 0.05$, $n = 3$)

Fig. 4.

(a) A dose-dependent decrease of *p-c-Src* and *c-Src* content: significance at 500 μ M Hcy, 60 min ($P < 0.05$, $n = 3$). (b) A significant decrease in content of *p-c-Src* and *c-Src* with a time-course evaluation of 100 μ M Hcy 120 min ($P < 0.05$, $n = 3$)

Fig. 5.

Hcy treatment showed a dose-dependent decrease of G α _{q/11} at 24 h for the following concentrations: 0, 25, 50, 75, 100, 500 μ M; significant decreases were found at 75, 100, and 500 μ M ($P < 0.05$, $n = 3$). At 48 h, G α _{q/11} content returned to baseline levels for all doses of Hcy except 500 μ M where there was a significant decrease ($P < 0.05$, $n = 3$).

Fig. 6.

Western blot showed no significant increase or decrease of G $\alpha_{12/13}$ for the following concentrations: 0, 25, 50, 75, 100, 500 μ M at 24 h ($P < 0.05$, $n = 3$). There was a significant decrease of G $\alpha_{12/13}$ using 500 μ M Hcy treatment for 48 h ($P < 0.05$, $n = 3$)

Fig. 7.

Western blot showed no significant increase or decrease of G_{i/o} for the following concentrations: 0, 25, 50, 75, 100, 500 μM at 24 h ($P < 0.05$, $n = 3$). There was a significant decrease of G_{i/o} using 500 μM Hcy treatment for 48 h ($P < 0.05$, $n = 3$)

Fig. 8.

Our results suggested Hcy acts as an agonist to potentiate GPCRs and activate the classical GPCR desensitization pathway. We have shown an increase of ERK1 activity that is characteristic of GPCR activation followed by an increase in GRK2 activity and final degradation of β -arrestin1, which is known to occur in down regulation of Class B GPCRs. Moreover, G $\alpha_{q/11}$, G $\alpha_{12/13}$, and G i/o G proteins that are involved in calcium regulation and attached to GPCRs are down regulated