Curriculum Vitae

LAURA M. SHOOP

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SUMMARY OF QUALIFICATIONS

- Molecular Medicine M.S. degree candidate specializing in pharmacogenomics and receptor biology.
- Over three years of laboratory experience as a graduate research assistant at the University of Maryland.
- Fifteen months of industry experience as a quality inventory associate at Meso-Scale Diagnostics.
- Excellent at independently planning, executing, and troubleshooting experiments.
- Outstanding verbal and written communication skills.
- Strong work ethic and works well in a lab environment

EDUCATION

M.S., Molecular Medicine **(expected Dec 2013)**
University of Maryland, Baltimore, MD

B.S., Biology **(2008)**
University of Virginia, Charlottesville, VA

WORK EXPERIENCE

University of Maryland Liggett Lab **Baltimore, MD**
Graduate Research Assistant **(2009 – 2013)**

- Thesis, "*The Effect of Single Nucleotide Polymorphisms on GPCR Kinase Expression and Function*"
- Located single nucleotide polymorphisms in the promoter region of GRK2 and GRK5 through sequencing of patient DNA and used luciferase reporter assays to determine their effect on expression
- Demonstrated the interaction between GRK4 and the beta-1 adrenergic receptor using whole-cell phosphorylation assays and cAMP assays.
- Generated previously reported variants of GRK4 and the beta-1 adrenergic receptor and tested the effect on the pair's interaction.
- Managed documentation associated with inventory and disposal of radioactive material.
- Created shared database for exchange of protocols and data as well as tracking of radioactive material and liquid nitrogen inventory.
- Gave poster presentation at the 2013 American Thoracic Society conference.

Meso-Scale Diagnostics **Gaithersburg, MD**
Quality Inventory Associate **(2008-2009)**

- Responsible for the management of accurate inventory and controlled documents.
- Exhibited efficiency and self-motivation by entering over 5,000 lines of data in only three weeks while maintaining other responsibilities.
- Undertook overhaul of batch record and certificate of analysis/compliance organization.
- Created Microsoft SharePoint website for internal access to controlled documents.

SKILLS

- **Cell Culture-** Aseptic technique, cell maintenance and plating, transient transfections, mycoplasma testing, HEK293, HEK293T, CHO, CHW, HL-1, GloSensor™ HEK293
- **Molecular**- PCR and dye attachment (for sequencing), DNA/RNA purification, cloning, site-directed mutagenesis, western blotting, immunofluorescence, radioligand binding, radiometric cAMP accumulation assays, radiometric whole-cell phosphorylation assays, luciferase reporter assays
- **Animal-** Mouse and rat handling, perfusion, cryostat/microtome sectioning
- **Computer-** Microsoft office, Graphpad Prism, Sequencher alignment software, NCBI resources

PRESENTATIONS

- Laura Shoop, Danielle Y. Lee, Arda Akoluk, Arum Yoon, Reynold A. Panettieri, Stephen B. Liggett, Steven S. An, Wayne C.H. Wang (2013). *Efficacy of β-agonist treatment following chronic TAS2R activation in airway smooth muscle*. Poster presented at the American Thoracic Society Conference, Philadelphia, PA.

Abstract

Title of Thesis: The Effect of Single Nucleotide Polymorphisms on G Protein-Coupled Receptor Kinase Expression and Function

Laura M. Shoop, Master of Science, 2013

Thesis Directed by: Stephen B. Liggett, M.D., Associate Vice President for Personalized Medicine, Associate Dean for Personalized Medicine & Genomics, Professor of Internal Medicine and Molecular Pharmacology & Physiology, University of South Florida

The beta-1 adrenergic receptor (β_1AR) and beta-2 adrenergic receptor (β_2AR) are two prototypic G protein-coupled receptors (GPCR) targeted in the treatment of a number of diseases, including heart failure, hypertension, and asthma. However, responses to beta adrenergic-targeted therapies vary from individual to individual, making it difficult to predict which patients will benefit from which medication.

One potential explanation for this variable response is the presence of genetic variants, such as single nucleotide polymorphisms (SNPs) in the genes encoding beta adrenergic signaling cascade components. Our study focused on G protein-coupled receptor kinases (GRKs), which recognize and phosphorylate agonist-occupied GPCRs, thereby initiating the process of receptor desensitization. We sought to evaluate the role of SNPs in GRKs in two projects. In the first project, we identified SNPs in the promoters of GRK2 and GRK5, two ubiquitously expressed GRKs known to act on both β_1AR and β_2AR. We then characterized their effect on promoter activity under basal and ligand-treated conditions. In the second project, we sought to explain the biochemical basis for the reduced response to β_1AR antagonists seen in patients with certain GRK4 SNPs. We

did so by evaluating the interaction of β_1AR and GRK4 and the effect of SNPs on this interaction.

Deep resequencing of African American and Caucasian cohorts revealed multiple SNPs in both GRK2 and GRK5 promoters. These SNPs were organized into haplotypes based on linkage disequilibrium. We used *in vitro* luciferase reporter assays to determine differences in promoter activity between haplotypes. In the presence of both β_1AR and β_2AR, we found variation in promoter activity (as measured by luciferase expression) among haplotypes for both GRK2 and GRK5 under basal conditions. The magnitude of change between basal and ligand-treated conditions was not statistically significant across GRK2 or GRK5 haplotypes in the presence of either β_1AR or β_2AR. We did find that only certain GRK5 promoter haplotypes exhibited an increase in activity following antagonist treatment. Further studies, in model systems more relevant to heart failure and asthma/COPD, are necessary to determine the effect of long-term agonist and antagonist exposure on promoter activity among haplotypes. Further studies are also required to determine the effect that variable expression of GRKs has on beta adrenergic receptor desensitization and if this variation is associated with differential response to β_1AR antagonists and β_2AR agonists in heart failure and asthma/COPD patients, respectively.

In evaluating the interaction of β_1AR and GRK4, we found evidence of increased phosphorylation of agonist-stimulated β_1AR in the presence of GRK4. Comparison of β_1AR desensitization between GRK4 haplotypes did not reveal significant haplotype-specific differences. Future studies will determine functional changes in β_1AR signaling in the presence of different haplotypes of GRK4. Nevertheless, results of these studies

pave the way for further exploration of the biochemical basis for the variable response to β_1AR antagonists seen among patients with different GRK4 SNPs.

The Effect of Single Nucleotide Polymorphisms on G Protein-Coupled Receptor Kinase Expression and Function

By
Laura Marie Shoop

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Master of Science
2013

Table of Contents

List of Tables

List of Figures

List of Abbreviations

β_1AR: Beta-1 adrenergic receptor

β_2AR: beta-2 adrenergic receptor

βAR: beta adrenergic receptor

cAMP: Cyclic adenosine monophosphate

CHO: Chinese hamster ovary

COPD: chronic obstructive pulmonary disease

CPM: Counts per minute

dbSNP: Single nucleotide polymorphism database

FBS: Fetal bovine serum

GDP: Guanosine diphosphate

GPCR: G protein- coupled receptor

GRK: G protein-coupled receptor kinase

Gs: Stimulatory G-protein

GTP: Guanosine triphosphate

$G\alpha_s$: Stimulatory G-protein alpha subunit

HCl: Hydrochloric acid

HWE: Hardy-Weinberg equilibrium

ISO: Isoproterenol

JG: Juxtaglomerular

LARII: Luciferase assay reagent II

NCBI: National Center for Biotechnology Information

NE: Norepinephrine

ORF: Open reading frame

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PIP_2: Phosphatidylinositol 4,5-bisphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PLB: Passive lysis buffer

RGS: Regulators of G protein signaling

S&G: Stop & Glo reagent

SNP: Single nucleotide polymorphism

1. Background

1.1 Beta adrenergic receptors as treatment targets

The G protein-coupled receptor (GPCR) family is a group of seven-transmembrane receptors that convert extracellular ligand binding into intracellular signals through the activation of a G protein. Encoded by more than 800 human genes and possessing a wide diversity in ligand binding and tissue expression, GPCRs have become the target of 30-50% of all registered therapeutics[1] . The beta adrenergic receptors (βARs), specifically the beta-1 adrenergic (β_1AR) and the beta-2 adrenergic (β_2AR) receptor, are prototypic GPCRs targeted in a number of therapies. β_1AR antagonists (often referred to as "beta blockers") are employed in the treatment of many conditions. Most notably, they are used as the first line of treatment for patients suffering from cardiovascular diseases, especially heart failure and hypertension. β_2AR agonist are also commonly prescribed, but in the treatment of asthma and chronic obstructive pulmonary disease (COPD).

1.1.1 Cardiovascular diseases and adrenergic receptor antagonists

Heart failure is a condition in which the heart cannot pump enough blood to meet the metabolic demands of the body. This condition affects 5.7 million Americans, with 25% of diagnosed individuals dying within the first year, and 40-50% of individuals dying within 5 years[2] . The poor prognosis suggests a critical need for more effective treatments. Currently, heart failure is managed with a combination drugs, including β_1AR antagonists, such as carvedilol and metoprolol. β_1AR antagonists have been used in the treatment of cardiovascular disease since 1973, and they have been repeatedly shown to

reduce mortality in patients. A recent meta-analysis of clinical trials revealed a 27% decrease in mortality with β_1AR antagonist use over an average of 11.5 months[3] .

In heart failure patients, insufficient output from the heart incites a compensatory response from the sympathetic nervous system in the form of increased circulation of norepinephrine (NE), an endogenous adrenergic agonist. The resulting activation of β_1AR initially increases cardiac output through increased force of contraction (inotropy), increased rate of relaxation (lusitropy), and increased heart rate (chronotropy). Over time, β_1AR desensitization and downregulation reduces the heart's response to sympathetic stimulation, thereby reducing cardiac output once again. Elevated NE levels therefore persist, leading to cardiomyocyte apoptosis, depletion of metabolic reserves, cardiac dilation, and hypertrophy[4] . The increasing damage from chronic stimulation coupled with the inability of the heart to meet the body's demand exacerbates the condition, ultimately resulting in death. β_1AR antagonists work by reducing cardiac damage caused by chronic stimulation by NE. However, β_1AR antagonist treatment limits the ability of the sympathetic nervous system to increase inotropy, lusitropy, and chronotropy in situations of increased demand.

β_1AR antagonists are also used in the treatment of hypertension. Hypertension affects approximately 30% of Americans and is defined as having an untreated systolic blood pressure of $\geq$140 mm Hg and/or a diastolic blood pressure of $\geq$90 mm Hg[5] . Hypertensive individuals are at risk of developing vascular and kidney damage, aneurysm, heart failure, ischemic heart disease, and stroke[6] .

There are many underlying causes of hypertension, including both genetic and lifestyle factors. β_1AR antagonists are not necessarily used to reverse the underlying

cause, but rather to alter mechanisms contributing to blood pressure. As mentioned above, β_1AR antagonists reduce inotropy and chronotropy in the heart, thereby reducing blood pressure. When acting on endothelial cells, certain β_1AR antagonists can cause the release of vasodilatory factors[7,8] . Finally, antagonism of β_1AR on juxtaglomerular (JG) cells of the kidney prevents activation of the renin-angiotensin system, which is responsible for increasing arterial pressure[9] .

Considering the ability of β_1AR antagonists to reduce the incidence of mortality in heart failure patients and their ability to alleviate the symptoms of hypertension, it is clear that β_1AR antagonists are of high clinical importance.

1.1.2 Asthma, COPD, and adrenergic receptor agonists

Asthma and COPD are conditions characterized by reduced airflow due to inflammation, smooth muscle constriction, and airway hyper-reactivity[10] . Though asthma affects 26 million Americans, it is a reversible condition and is not necessarily fatal. COPD is less prevalent, affecting 13 million Americans. It is, however, the third leading cause of death in America[11] . The increased mortality in COPD is due to the disease's progression, whereby chronic inflammation causes permanent airway damage and remodeling.

Adrenergic receptors are used as therapeutic targets in both asthma and COPD. The primary subtype of adrenergic receptor expressed in the airways is β_2AR. Stimulation of β_2AR with adrenergic agonists results in relaxation of airway smooth muscle. In both diseases, β_2AR agonists are used to relieve the airway smooth muscle constriction and bronchospasm by promoting relaxation of airway smooth muscle[12] .

1.1.3 Variation in response to βAR-targeted treatments

Despite being well-established treatments, βAR-targeted drugs are not equally effective among individuals. In recent years, the field of pharmacogenomics has attempted to explain this interindividual variation by identifying and characterizing single nucleotide polymorphisms (SNPs) in genes encoding various proteins within the beta adrenergic pathway. Several findings (described below) have accounted for some, but not all, of the variation in drug response. Considering the numerous components in the βAR signaling cascade, it is plausible that SNPs in any of these components could affect the response to βAR-targeted therapies. Therefore, we set out to determine the effect of uncharacterized SNPs in the βAR signaling cascade.

1.2 Beta adrenergic receptor signaling cascade

As previously mentioned, βARs contain seven transmembrane domains and conform to the traditional GPCR paradigm (Figure 1). Signaling begins when the extracellular domains recognize and bind the cognate ligand. The endogenous ligands for βARs are epinephrine and NE. Upon agonist binding, the receptor changes conformation in a manner that allows the interaction of a G protein with the third intracellular loop of the receptor. G proteins are formed by a heterotrimeric complex, containing an alpha, beta, and gamma subunit. In most cases, βAR will associate with Gs, the G protein subtype containing the stimulatory alpha subunit, $G\alpha_s$. The interaction between the G protein and βAR promotes the exchange of GDP for GTP on $G\alpha_s$, which results in its dissociation from the membrane-anchored beta-gamma subunits. $G\alpha_s$ then activates adenylyl cyclase, an integral membrane protein that catalyzes the synthesis of cyclic adenosine monophosphate (cAMP) from ATP. cAMP directly activates protein kinase A

(PKA), an ubiquitously expressed kinase with a multitude of targets. It is the activity of cAMP and PKA that produces the increased inotropy, lusitropy, and chronotropy in heart tissue[13,14] , the increased renin release in JG cells[15] , and the increased relaxation in airway smooth muscle[12] .

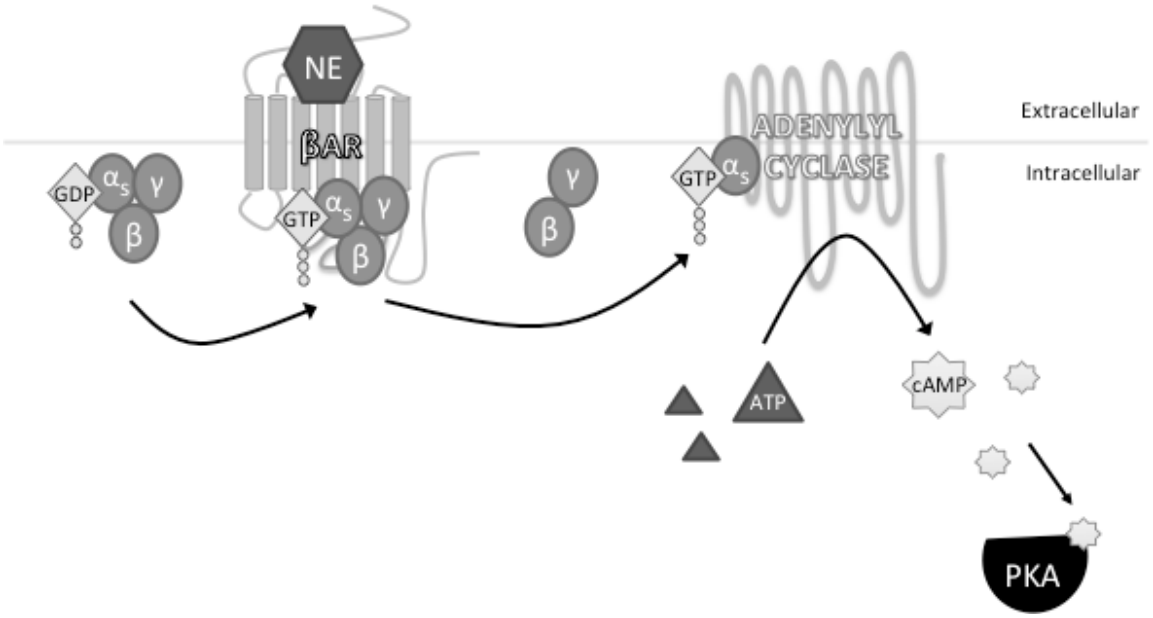

Figure 1. βAR signaling schematic. Upon binding of agonist (in this case, NE), the activated form of the receptor recruits the GDP-bound form of Gs. The interaction promotes the exchange of GDP for GTP on the alpha subunit of Gs. $G\alpha_s$ dissociates from the β/γ subunits and activates adenylyl cyclase. Activated adenylyl cyclase catalyzes the formation of cAMP from ATP. cAMP activates PKA, which goes on to phosphorylate multitude of targets.

1.2.1 Regulation of GPCR activity

GPCR signaling is regulated by a variety of mechanisms. In the case of Gs-coupled GPCR signaling, the ability of $G\alpha_s$ to activate adenylyl cyclase is dependent on its binding of GTP. Regulator of G protein signaling (RGS) proteins activate the GTPase activity of $G\alpha_s$, causing hydrolysis of GTP to GDP and thereby regulating the duration of $G\alpha_s$ activation[16] . Gs-coupled signaling is also dependent on the magnitude and duration

of the cAMP signal, which is regulated by cAMP phosphodiesterases[17] . Finally, GPCR signaling is regulated through the process of desensitization, which can be agonist-dependent (homologous desensitization) or agonist-independent (heterologous desensitization) [18] . Among these mechanisms, desensitization of the receptor is clinically important as the desensitization state of the βAR population can determine responsiveness to antagonist, and long-term exposure to βAR agonists can result in a loss of responsiveness in patients. Types of desensitization and players involved in desensitization are described below.

1.2.2 Homologous desensitization

Persistent stimulation of GPCRs by cognate ligands typically results in a loss of responsiveness, a phenomenon known as "desensitization" (Figure 2). Desensitization is defined as a diminished response despite continued agonist exposure. In the case of "homologous" desensitization, agonist-stimulation of the receptor incites its own desensitization via recruitment of GPCR kinases (GRKs). GRKs recognize and phosphorylate agonist-bound GPCRs, increasing the receptor's affinity for β-arrestin (βarr). βarr's interaction with the receptor prevents the interaction between the receptor and G protein, thereby uncoupling the GPCR from its effector. Additionally, βarr recruits the GPCR to clathrin-coated pits. Subsequent internalization reduces receptor availability at the membrane and is known as "downregulation". Once internalized in clathrin-coated vesicles, the receptor can either be recycled to the membrane (resensitization) or targeted for degradation[19] . The net receptor density on the cell membrane depends upon the rate of resensitization and rate of degradation. In addition, synthesis of new receptor and targeting to the membrane also accounts for receptor density on the plasma membrane[20].

1.2.2.1 GRK subtypes and cell type distribution

GRKs are a family of serine/threonine kinases that phosphorylate the third intracellular loop or C-terminus of agonist-bound GPCRs[20] . All seven GRKs contain a centrally located catalytic domain and a fairly homologous amino-terminal domain, hypothesized to be involved in receptor recognition[21] . Despite this similarity, GRKs have been divided into three subfamilies based on sequence and functional similarities. GRK1 and GRK7 are expressed only in visual tissue and exclusively target the retinal opsins[22] . GRK2 and GRK3 are both widely expressed and have approximately 85% sequence similarity[20] . Unlike the other GRK's, GRK2 and GRK3 contain a pleckstrin homology domain that allows them to interact with the membrane-associated beta-gamma subunits of activated G proteins[20] .

The GRK4, 5, and 6 subfamily shares approximately 70% sequence homology[20] . GRK5 and 6 are both widely expressed, but GRK4 expression is more limited[20] . GRK4 is also the only GRK to undergo alternative splicing, resulting in four splice variants[23] . GRK4 and GRK6 are constitutively anchored in the membrane through their palmitoylated C-terminus. GRK5 is unique in that it has a PIP_2-binding domain that allows it to associate with the membrane[24] . The differing mechanisms of membrane association coupled with the highly variable carboxyl-terminal domain have been hypothesized as the underlying causes of differential receptor targeting[20] .

The importance and distribution of each GRK in all tissues relevant to heart failure, hypertension, and asthma/COPD is incompletely defined. GRK2 expression has been demonstrated in heart, vascular, kidney, and lung tissue[25,26] . Expression of GRK5 has also been confirmed in human heart and lung tissue[27,28] . GRK4 expression has been

demonstrated in kidney tissue, although GRK subtype expression in JG cells has not been reported[25] .

1.2.3 Heterologous desensitization

Desensitization can also occur through the GRK-independent mechanism known as "heterologous" desensitization[18] . The process of heterologous desensitization begins when agonist binding of a GPCR within a cell causes activation of second messenger-dependent kinases, such as PKA or protein kinase C (PKC). These kinases can phosphorylate multiple GPCR subtypes within in the same cell, regardless of whether the targeted receptor is agonist-bound, meaning this process is also responsible for negative feedback on the agonist-bound receptor. Phosphorylation by PKA or PKC occurs at sites differing from those targeted by GRKs and results in markedly reduced receptor function[18] .

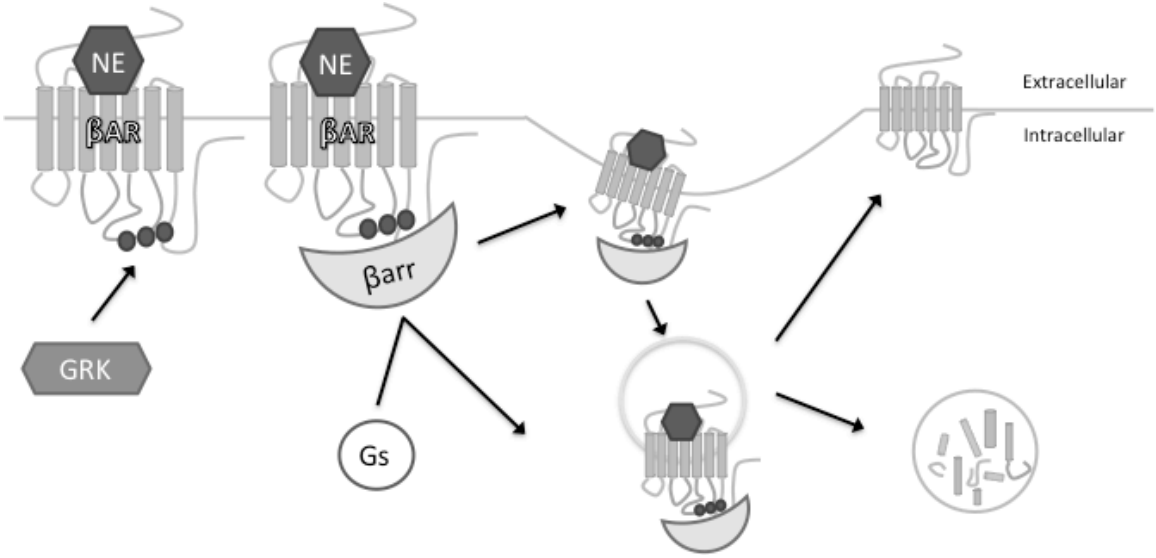

Figure 2. βAR homologous desensitization schematic. GRKs recognize and phosphorylate the agonist-bound form of the receptor. The phosphorylated receptor has an increased affinity for βarr. The interaction between βarr and βAR prevents the interaction of Gs with the receptor. βarr also recruits the receptor complex to clathrin-coated pit, where it is then internalized. Internalized receptors can either be recycled back to the membrane (resensitized) or targeted for degradation.

Enhanced or diminished interactions in any of the aforementioned steps could contribute to variable response to βAR-targeted drugs. Genes encoding proteins involved in both GPCR activation and desensitization are attractive candidates for SNP discovery and characterization. Several components of the βAR signaling cascade have already been sequenced to identify SNPs. In particular, SNPs have been located in the protein-coding region of β_1AR, β_2AR, GRK5, and GRK4. Many of these SNPs have been evaluated for their effect on βAR signaling and desensitization *in vitro*. Additionally, these SNPs have also been evaluated to determine their association with disease risk and drug response.

1.3 Reported effects of SNPs on βAR signaling and βAR-targeted therapies

1.3.1 SNPs in β_1AR

Of the two βARs, β_1AR is primarily responsible for cardiac excitation and renin release from JG cells. As mentioned before, β_1AR antagonists generally produce benefits by blocking β_1AR. In β_1AR, two common SNPs have been identified at amino acid 49 (Ser49Gly) and 389 (Arg389Gly) [29,30] . In comparing Ser49 to Gly49, there is no significant difference in basal or agonist-promoted adenylyl cyclase activity[31] . However, Gly49 displayed enhanced agonist-stimulated downregulation[31] . For Arg389Gly, *in vitro* studies revealed increased Gs coupling in Arg389 compared to Gly389, resulting in increased basal and agonist-stimulated adenylyl cyclase activity[29] . In considering the effect on desensitization, Arg389 showed a greater decrease in adenylyl cyclase activity compared to Gly389[32] . However, the absolute adenylyl cyclase activity of the desensitized Arg389 receptor was equivalent to that of the non-desensitized Gly389 receptor, while the desensitized Gly389 activity was the lowest of all conditions[32] . These

studies suggest that SNPs in β_1AR alter its responsiveness to agonist and the degree of desensitization.

Ser49Gly and Arg389Gly have been extensively studied in their contribution to disease risk and drug response. In the general population, meta-analysis revealed no association between Ser49Gly or Arg389Gly and the risk of heart failure[33] . When stratified by race, however, East Asians carrying the Gly389 allele were found to be more susceptible to heart failure[33] . The opposite was found in Caucasians, where carriers (individuals having at least one copy of the allele) of Gly389 tended to have a decreased risk of developing heart failure[33] . In evaluating the association of β_1AR SNPs with the risk of developing hypertension, one study provides evidence of an increased risk for individuals homozygous for Arg389[34] . No association between Ser49Gly and risk of hypertension was found[34] .

The effect of the β_1AR polymorphisms on β_1AR antagonist response in the context of heart failure and hypertension has been extensively studied. Table 1 provides a selection of these studies and their outcomes. The majority of these studies indicate that Arg389 homozygous individuals are more responsive to β_1AR antagonist therapy for heart failure than those carrying the Gly389 allele[35-39] . Conflicting evidence suggests this may not be true for the treatment of hypertension[40-44] . The effect of Ser49Gly on β_1AR antagonist response is still inconclusive. In heart failure, Gly49 carriers exhibit better response than Ser49 under certain circumstances[39,45] . In treatment of hypertension, however, some studies have shown the Gly49 carriers to be less responsive[41-43]

Table 1: Summary of effect of β_1AR SNPs on β_1AR antagonist response

Reference	Drug	SNP	Subject	Measure of Response	Main Findings
[35]	bucindolol	Arg389Gly	HF, reduced LVEF	reduction in VT/VF	Gly carriers less responsive to bucindolol
[36]	bucindolol	Arg389Gly	HF, reduced LVEF, AF	mortality, hospitalization	Gly389 carriers less responsive to bucindolol
[37]	bucindolol	Arg389Gly	HF	mortality, hospitalization	Gly389 carriers less responsive to bucindolol
[38]	carvedilol, metoprolol	Arg389Gly	HF	NSVT suppression	Gly389 carriers less responsive to β_1AR antagonists
[39]	metoprolol	Arg389Gly	HF	increased LEVF, decreased LVEDD and LVESD	Gly389 carriers less responsive to metoprolol
[40]	metoprolol	Arg389Gly	HTN	reduced diastolic BP	Gly389 carriers less responsive to metoprolol
[41]	metoprolol	Arg389Gly	HTN	time to reach MAP ≤107mm Hg	Gly389 homozygotes less responsive to metoprolol
[42]	bisoprolol	Arg389Gly	HTN	reduced BP	Variants equally responsive
[43]	metoprolol	Arg389Gly	HTN	reduced BP	Gly389 carriers less responsive to metoprolol
[44]	atenolol	Arg389Gly	HTN	reduced BP	Variants equally responsive
[38]	carvedilol, metoprolol	Ser49Gly	HF	NSVT suppression	Variants equally responsive
[39]	metoprolol	Ser49Gly	HF	increased LEVF, decreased LVEDD and LVESD	Gly49 carriers more responsive to metoprolol in LVEDD
[45]	carvedilol, bisoprolol, metoprolol	Ser49Gly	dilated cardio-myopathy	mortality	Gly49 carriers more responsive than Ser49 homozygotes to low doses metoprolol.
[42]	bisoprolol	Ser49Gly	HTN	reduced BP	Ser49 homozygotes more responsive than heterozygotes
[43]	metoprolol	Ser49Gly	HTN	reduced BP	Ser49 homozygotes more responsive than heterozygotes
[44]	atenolol	Ser49Gly	HTN	reduced BP	Variants equally responsive
[41]	metoprolol	Ser49Gly	HTN	Time to time to reah MAP ≤107mm Hg	Gly49 carriers less responsive to metoprolol in obese

Abbreviations: AF= atrial fibriliation, BP=blood pressure, HF=heart failure, HR=heart rate, HTN=hypertension, LVEDD=left ventricle end-diastolic diameter, LVEF=left ventricular ejection fraction, LVESD=left ventricle end-systolic diameter, MAP=mean arterial pressure, NSVT=non-sustained ventricular tachycardia, VT/VD=ventiricular tachycardia/ventricular fibrillation.

1.3.2 SNPs in β_2AR

In β_2AR, three polymorphisms have been characterized at the cellular and clinical level. They are at amino acids 16 (Gly16Arg), 27 (Gln27Glu), and 164 (Thr164Ile). Ile164 was found to have a decreased affinity for the β_2AR agonists isoproterenol (ISO), epinephrine, and NE[46] . Ile164 was also found to have decreased basal and agonist-stimulated adenylyl cyclase activity as well as increased agonist-promoted desensitization compared to Thr164[32,46] . Variations at amino acids 16 and 27 were not shown to affect agonist affinity or G protein coupling[47] . They were, however, shown to effect agonist-promoted downregulation of the receptor. Agonist-promoted downregulation of β_2AR was enhanced in the presence of the Gly16 variant[47] . Downregulation was ablated in the Arg16 variant, but only when the Gln27 variant was also present[47] .

None of the studied β_2AR polymorphisms have been associated with asthma. However, the effect of these SNPs on β_2AR agonist response has been extensively studied and recently reviewed[48] . Most of these studies have examined response in relation to the Gly16Arg polymorphisms. A few studies indicated the Arg16 variant may be associated with better response to short-acting β_2AR agonist treatment in asthmatics[49,50] . Others indicated poorer response to long-acting β_2AR agonists in Arg16 homozygotes[51-53] .

These studies suggest that SNPs in both β_1AR and β_2AR can have a profound effect on receptor function. At the cellular level, SNPs in βARs can affect ligand affinity, magnitude of agonist-promoted signaling, and degree of desensitization and downregulation. At the clinical level, SNPs in these receptors are associated with differential outcomes following βAR-targeted therapies. The effect of polymorphisms on

receptor function suggests that SNPs in the genes encoding other βAR pathway components may too have a profound effect on their function.

In considering other candidates for polymorphism discovery/characterization in the βAR pathway, the proteins involved in desensitization are an attractive choice. The state of desensitization of βAR determines its ability to transduce the signal from the ligand to the G protein effector. Therefore, desensitization could influence the receptor's response to ligands under normal, diseased, and treatment conditions. Since the first step of desensitization involves recognition and phosphorylation of βAR by GRKs, it is possible that polymorphisms that influence GRK expression and activity may result in variable βAR desensitization and thereby response to ligands.

1.3.3 SNPs in GRK2 and GRK5

Several studies have begun to elucidate the role of GRK polymorphisms in disease and treatment outcomes. One such study examined the two GRKs expressed highly in the heart, GRK2 and GRK5[54] . Sequencing revealed no genetic variation in the coding region of GRK2. One common variant was found in GRK5 at amino acid position 41 (Gln41Leu). *In vitro* studies revealed increased desensitization of β_1AR[54] and β_2AR[55] in the presence of Leu41, as evidenced by blunted agonist-stimulated adenylyl cyclase activity. This finding inspired the concept of an "endogenous beta blocker", in which patients expressing the Leu41 variant would have decreased response to β_1AR antagonists due to increased agonist-promoted β_1AR desensitization.

This was tested clinically in heart failure patients undergoing β_1AR antagonist therapy[54] . The Gln41 homozygous individuals showed the typical improvement seen in response to β_1AR antagonists. The Leu41 carriers, however, did not show improvement

in response to β_1AR antagonists. Despite the reduced response, untreated Leu41 carriers had a survival time that mimicked that of treated Gln41 homozygotes. Additionally, Leu41 carrier survival was longer than Gln41 in untreated conditions.

1.3.4 SNPs in GRK4

In GRK4, three common coding polymorphisms have been identified (Table 2). The polymorphisms are located at amino acid 65 (Arg65Leu), amino acid 142 (Ala142Val), and amino acid 486 (Ala486Val). Studies have shown that GRK4 polymorphisms are associated with hypertension[57-61] . Studies have also examined the pharmacogenomics of GRK4 polymorphisms. In a study monitoring African American hypertensive patient response to the β_1AR antagonist metoprolol, men carrying Ala142 showed a reduced response to treatment, but only when also carrying Leu65[62] . This suggests that studying the combination of SNPs inherited together (the "haplotype") may be a more relevant than studying individual SNPs. In response to metoprolol, women, but not men, possessing Val486 showed a significantly decreased response[62] . However, the Val486 variant is difficult to study in African Americans due to its low allele frequency.

Table 2: GRK4 SNPs and minor allele frequencies. Nucleotide and amino acid positions for the three most common GRK4 SNPs are listed with their minor allele frequencies in racial cohorts[56]

Common GRK4 SNPs			**Minor Allele Frequency**			
Position (Nucleotide/ Amino Acid)	**Major Allele** (Nucleotide/ Amino Acid)	**Minor Allele** (Nucleotide/ Amino Acid)	**African American**	**Caucasian**	**Hispanic**	**Asian**
448/65	G/Arg	T/Leu	0.47	0.35	0.25	0.07
679/142	C/Ala	T/Val	0.60	0.40	0.29	0.20
1711/486	C/Ala	T/Val	0.19	0.40	0.28	0.47

Another study evaluated GRK4 polymorphisms and response to the β_1AR antagonist atenolol[63] . In this study, Leu65, Val142, and Val486 carriers showed decreased response to atenolol, measured as a) a lowering in blood pressure or b) reduced incidence of death, non-fatal myocardial infarction, or non-fatal stroke. Val486 analysis was restricted to Caucasians and Hispanics due to its low allele frequency in African Americans. Analysis of Leu65-Val142 haplotype revealed a decrease in blood pressure response to atenolol with each additional copy of Leu65-Val142. The decreased response seen in the Val142 carriers conflicts with the results found in the metoprolol study mentioned above. The investigators suggested this could be due to differences in race (the metoprolol study was in African Americans only, the atenolol study was in African Americans, Caucasians, and Hispanics) or due to the different outcomes measured (the metoprolol study measured time to reach a target blood pressure, the atenolol study measured change in blood pressure).

Knowing that atenolol targets β_1AR, and knowing that SNPs in β_1AR have been shown to influence β_1AR antagonist response, the investigators decided to further stratify their data by β_1AR genotype. In doing so, they hoped to reveal whether the β_1AR genotype could exacerbate or alleviate the reduced response to atenolol seen in carriers of the GRK4 Leu65-Val142 haplotype. Though not statistically significant, they found that the decreased response seen with each additional copy of Leu65-Val142 appeared to be true only in the presence of β_1AR Arg389 homozygotes.

These studies investigating polymorphisms in GRK5 and GRK4 suggest that variation within GRKs can affect patient response to β_1AR antagonists in patients. In GRK5, evidence suggests that this is due to differential desensitization of βARs[54,55] . The

underlying mechanism for reduced response to β_1AR antagonists seen in carriers of certain GRK4 SNPs has yet to be defined.

In this study, we sought to further investigate the effect of SNPs in the aforementioned GRKs in an effort understand the underlying mechanisms responsible for interindividual variation in response to βAR-targeted treatment. We did so in two separate projects, outlined below.

1.4 Project 1: Genetic variation in GRK2 & GRK5 promoters and their effect on promoter activity

1.4.1 Rationale

GRK2 and GRK5 are both expressed in heart[27,64] and lung[26,28] tissue. They are both also capable of desensitizing β_1AR[54] and β_2AR[65] . Therefore, the outcome of this study has implications in both heart failure and asthma/COPD. Several studies have already examined GRK2 expression in the context of heart failure. Expression of GRK2 is increased in human failing myocardium, presumably contributing to the increased desensitization of β_1AR seen in heart failure[27,66,67] . Studies have shown therapeutic effects of limiting GRK2 activity in cardiac tissue in heart failure models[68] . This can be attained partially through treatment with β_1AR antagonists, which decreases GRK2 expression[27] . However, ablation of GRK2 in murine cardiac tissue accelerates the progression of heart failure [69] .

Like GRK2, human GRK5 expression is upregulated in heart failure and can be decreased by β_1AR antagonist treatment[27,70] . Transgenic mouse studies have shown that overexpression of GRK5 reduces cardiac responsiveness to βAR agonist[71] . However, GRK5 knockout studies performed in mice did not reveal a cardiac phenotype[72] .

It is clear from these studies that GRK2 and GRK5 expression vary depending on disease and treatment state, and mechanisms reducing GRK expression in target tissue may improve patient prognosis in heart failure. This suggests that SNPs affecting expression of GRK2 and GRK5 under basal conditions and in response to β_1AR ligands could impact the development, progression, and treatment of heart failure, presumably by altering the state of desensitization of the β_1AR population in cardiac tissue.

The role of GRK2 and GRK5 expression in asthma is not well defined. One study found that β_2AR-mediated relaxation of precontracted tracheal smooth muscle was reduced in GRK5 knockout mice[28] . Aside from this finding, β_2AR desensitization has long been suspected of contributing to reduced responsiveness to long-acting β_2AR agonists used in the treatment of asthma[73] . Therefore, differential expression of these two GRKs in the lung may contribute to the potency and efficacy of β_2AR agonists for long-term asthma treatment.

Based on the aforementioned studies, it is clear that GRK2 and GRK5 expression plays a role in the state of desensitization of βARs and therefore may influence response to β_1AR antagonists and β_2AR agonists. Therefore, SNPs that cause differential expression in GRK2 and GRK5 may cause differential desensitization of the βAR population and differential response to β_1AR antagonists and β_2AR agonists.

There are multiple factors that influence gene expression, but the elements that initiate transcription are of particular interest. Non-coding gene promoters, located 5' of the open reading frame (ORF), contain sequences that bind elements that promote or repress gene expression. It is possible that SNPs within this region may significantly

modulate expression of these GRKs. For this reason, we chose the promoter region of GRK2 and GRK5 for our study.

1.4.2 Specific aims

In this study, **we hypothesized** that SNPs exist in the promoter of both GRK2 and GRK5. Furthermore, **we hypothesized** that the identified SNPs, when organized into haplotypes, would result in differential gene expression under basal, agonist, and antagonist treated conditions. We addressed these hypotheses in three specific aims:

Specific Aim 1: Identify polymorphisms within the 2,000 base pair sequence upstream of the translational start site of GRK2 and GRK5 ORFs.

Specific Aim 2: Analyze the effect of GRK2 and GRK5 promoter haplotypes on gene expression capacity under basal conditions.

Specific Aim 3: Analyze the effect of βAR agonists and antagonists on the activity of GRK2 and GRK5 promoter haplotypes.

1.5 Project 2: Biochemical basis for reduced response to β_1AR antagonists in GRK4 haplotypes

1.5.1 Rationale

The association of the GRK4 gene locus and its polymorphisms with hypertension has recently incited interest in this understudied GRK[74-77] . Initial studies indicated expression of GRK4 was limited to the testes, brain, and skeletal muscle[23] . More sensitive methods have recently revealed wider expression, including expression in the kidney[78] and heart[79] . For reasons other than its limited expression, GRK4 is unusual

amongst the other GRKs. As previously mentioned, GRK4 is the only GRK with splice variants[23] . Studies have shown that GRK4 is also unique in that it is capable of phosphorylating target receptors in an agonist-independent manner[65,80] . These studies also indicated that agonist-promoted phosphorylation by GRK4 is to a lesser degree than the more widely expressed GRKs (GRK2, GRK3, GRK5, and GRK6).

Three common polymorphisms have been identified in the coding region of GRK4 (Table 2). Two are located in a putative GPCR-interacting domain: Arg65Leu and Ala14Val[81] . The other, Ala486Val, is located in the autophosphorylation site in the membrane targeting domain[81] . These SNPs have been previously organized into haplotypes based on genotyping data using the program PHASE[56] . GRK4 haplotypes constructed from the three common SNPs and their frequencies in different races are outlined in Table 3.

Table 3: GRK4 haplotypes and frequencies. The three most common GRK4 SNPs were organized into haplotypes using the program PHASE[56] . Haplotype frequencies are listed by race.

Variant at Amino Acid Position			Haplotype Frequency in Racial Cohorts			
65	**142**	**486**	**African American**	**Caucasian**	**Hispanic**	**Asian**
Leu	Ala	Ala	0.06	0.04	0.00	0.00
Leu	Val	Ala	0.11	0.01	0.03	0.00
Leu	Val	Val	0.12	0.23	0.17	0.01
Arg	Ala	Ala	0.28	0.43	0.59	0.33
Arg	Ala	Val	0.01	0.15	0.08	0.37
Arg	Val	Ala	0.00	0.00	0.00	0.07
Arg	Val	Val	0.10	0.04	0.03	0.02

Two studies have investigated the association of GRK4 SNPs with response to β_1AR antagonists as treatments for hypertension and are outlined in detail above. As previously mentioned, a study evaluating response to the β_1AR antagonist metoprolol in

African American male hypertension patients revealed a decrease in response in carriers of the Ala142 variant, but only when also carrying the Leu65 allele[62] . Somewhat conflicting results were found in a separate study evaluating the response of hypertension patients to the β_1AR antagonist atenolol. In the atenolol study, investigators found reduced response, as measured by reduction in blood pressure, with each additional copy of the Leu65-Val142 haplotype[63] . Val486 was also associated with decreased response to atenolol in Caucasians, but its low frequency in African Americans limited investigators' ability to determine the effect in African Americans[63] .

The variation in response to β_1AR antagonist based on GRK4 SNPs suggests an interaction between β_1AR and GRK4. The atenolol study added further evidence by stratifying their data by β_1AR genotype at amino acid position 389. Investigators found that the reduced response to atenolol seen in carriers of GRK4 Leu65-Val142 tends to exist only in β_1AR Arg389 homozygous individuals[63] . If the two proteins do interact, it is possible that GRK4 Leu65-Val142 may have increased activity, causing increased desensitization of β_1AR and therefore decreased response to β_1AR antagonists. This concept is similar to that of the GRK5 Leu41 variant, in which its increased activity reduced responsiveness to the β_1AR antagonist bucindolol in heart failure patients (leading to the concept of an "endogenous beta blocker") [54] . The atenolol study suggests, however, that the increased activity of GRK4 Leu65-Val142 would exist to a greater extent with the β_1AR Arg389 variant versus the β_1AR Gly389 variant. These studies further suggest that GRK interaction with β_1AR is also dependent on β_1AR genotype.

Despite the pharmacogenomic studies involving β_1AR antagonists and GRK4 SNPs, the interaction between β_1AR and GRK4 has yet to be demonstrated *in vitro*. The

interaction between β_1AR and GRK4 is even more likely when considering two additional factors. β_1AR and GRK4 share common tissue distribution in tissues relevant to hypertension- the heart and kidney. Additionally, GRK4 has been shown to phosphorylate β_2AR[65] . It is critical to determine whether GRK4 and β_1AR interact and to determine the effect of SNPs in both proteins on receptor desensitization. In doing so, we can better understand the underlying causes of variable response to β_1AR antagonists seen in hypertension patients with certain GRK4 and β_1AR SNPs.

1.5.2 Specific aims

In this study, we **hypothesized** that GRK4 phosphorylates β_1AR, resulting in desensitization of the receptor. Furthermore, **we hypothesized** that the GRK4 haplotypes would cause differential desensitization of the receptor, depending on β_1AR genotype at amino acid 389. We addressed these hypotheses in three specific aims:

Specific Aim 1: Determine phosphorylation state of β_1AR in the presence of GRK4.

Specific Aim 2: Assess agonist-promoted desensitization of β_1AR in the presence of GRK4.

Specific Aim 3: Analyze the effects of GRK4 haplotypes on desensitization of the β_1AR Arg389 variant and the β_1AR Gly389 variant.

2. Methods

Promoter Sequencing. Genomic DNA was isolated from immortalized B-lymphocytes obtained from the Human Variation Collection of the Coriell Institute (Camden, New Jersey, USA). PCR was performed using the Promega GoTaq system (Madison, Wisconsin, USA). Highly repetitive, GC-rich regions were amplified using the Invitrogen PCRx system (Life Technologies, Grand Island, New York, USA). Reaction conditions for both are listed in Tables 4 and 5. Primers used for both PCR and sequencing were designed using MacVector software (MacVector Inc, Cary, North Carolina, USA). Primers are listed in Tables 6 and 7 with their respective annealing temperature and PCR conditions. PCR-amplified DNA segments were bidirectionally sequenced using the Applied Biosystems 3730XL 96-capillary high-throughput sequencer (Life Technologies, Grand Island, New York, USA). The dye-attachment protocol used for highly GC-rich regions was previously reported[82] . Sequencing chromatograms were individually trimmed and aligned to a reference sequence using Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Variants found in only one or two individuals were re-amplified and re-sequenced to eliminate the possibility of experimental error.

Table 4. Promega GoTaq Reaction Conditions

Component	Concentration per Reaction
GoTaq Flexi Buffer	1x
$MgCl_2$	2 mM
dNTPs	0.2 mM each
Forward Primer	0.5 µM
Reverse Primer	0.5 µM
GoTaq DNA Polymerase	0.025 Units/µl
DNA	0.1 ng/µl

Table 5. Invitrogen PCRx Enhancer System	
Component	**Concentration per Reaction**
Enhancer Buffer	1-2x
Amplification Buffer	1x
$MgSO_4$	1.5 mM
dNTPs	0.2 mM each
Forward Primer	0.3 μM
Reverse Primer	0.3 μM
Platinum Taq DNA Polymerase	0.05 Units/μl
DNA	0.1 ng/μl

Table 6. GRK2 Promoter Primers				
Forward Primer	**Reverse Primer**	**Amplicon location**	**PCR Annealing Temperature**	**PCR Conditions**
5'-ggatggtagcgaaacaaactgg-3'	5'-tgtctcaaggcagcattcctatc-3'	-2106 to -1452	58°C	Promega GoTaq
5'-agagctgcttctgggtgcca-3'	5'-gcgcagttacaggcttccc-3'	-1946 to -1114	58°C	Invitrogen PCRx Enhnacer System
5'-cacctggctttccccactct-3'	5'-acctctgaacaccatcctg-3'	-1431 to -619	58°C	Invitrogen PCRx Enhancer System
5'-tctgaggtggtgggccaagg-3'	5'-ggcgtggccttgctcttctc-3'	-757 to +71	58°C	Invitrogen PCRx Enhancer System

Table 7. GRK5 Promoter Primers				
Forward Primer	**Reverse Primer**	**Amplicon location**	**PCR Annealing Temperature**	**PCR Conditions**
5'-ttttggttcggagtctcgctc-3'	5'-gggggcaacaacagtttacctg-3'	-2459 to -1834	58°C	Promega GoTaq
5'-tttaggggtctcgctgtctg-3'	5'-aagaacaatgaggcctttgc-3'	-2012 to -1493	56°C	Promega GoTaq
5'-ccccaggaaagcaggtaatcttg-3'	5'-taaaaacgcagacggagtcg-3'	-1749 to -1199	55°C	Promega GoTaq
5'-tgaccgaagacagcccatttc-3'	5'-ccgacaggcgactcaaagg-3'	-1439 to -552	58°C	Invitrogen PCRx Enhancer System
5'-cgactccgtctgcgtttttattac-3'	5'-gcgtgtctctgtcactccattc-3'	-1218 to -279	63°C	Promega GoTaq
5'-tgcccgccttcgctttcg-3'	5'-gcaaccttctcagcccaagtc-3'	-569 to +262	60°C	Invitrogen PCRx Enhancer System

Cell Culture and Transient Transfection. All cell lines were expanded, maintained, and transfected in an environment of 95% O_2/5% CO_2 at 37°C. HEK-293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. CHO cells were maintained in F-12 medium supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 1 mM glutamine. B-lymphocytes were maintained in upright T-75 cell culture flasks in RPMI-1640 medium supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Transfections were performed using Lipofectamine 2000 (Life Technologies, Grand Island, New York, USA). Transfections in HEK-293T and CHO cells were performed with a 2:1 and 3:1 ratio of µg DNA:µl Lipofectamine 2000, respectively. Cells were transfected in Opti-MEM reduced-serum medium (Life Technologies, Grand Island, New

York, USA). Four to six hours following transfection, cells were supplemented with medium containing FBS to reach a final concentration of 10%.

Construct Generation and Transfection for Luciferase Assay. Individual genotypes were analyzed by the haplotype reconstruction software PHASE[83] . Haplotypes occurring in our population at a frequency of ≥0.05 were selected for promoter activity analysis. Each haplotype was synthesized within the 2,000 base pair sequence upstream of both the GRK2 and GRK5 translational start site. Synthesis of each polynucleotide was performed by GeneArt (Life Technologies, Grand Island, New York, USA), and the 2,000 base pair fragment was cloned into the pGL4.10[*luc2*] firefly luciferase reporter vector (Promega, Madison, Wisconsin, USA). HEK-293T cells were plated on poly D-lysine pre-coated 24-well plates and transiently transfected at 90% confluency (as recommended by the Lipofectamine 2000 product technical manual). Each well received 250 ng of a GRK2 or GRK5 promoter vector, 250 ng of either β_1AR (in pFLAG-cmv3, Sigma Aldrich, St. Louis, Missouri, USA) or β_2AR (in pcDNA3.1(+), Life Technologies, Grand Island, New York, USA), and 2 ng of pGL4.73[*hRluc/SV40*] *Renilla* luciferase reporter vector (Promega, Madison, Wisconsin, USA). For each experiment, two wells were transfected for each vector combination.

Luciferase Assay. 18 hours prior to the experiment, cells were changed to serum-free medium with 100 μM ascorbic acid alone (as a vehicle; protects catecholamines from degradation in medium) or with and the indicated drug. Passive lysis buffer (PLB), Luciferase Assay Reagent II (LARII), and Stop & Glo Reagent (S&G) were prepared

according to the Dual Luciferase manufacturer's instructions (Promega, Madison, Wisconsin, USA). Cells were washed twice with 500 µl room temperature PBS pH 7.4. 100 µl of PLB was added to each well, and plates were rocked at 4°C for 15 minutes. 20 µl of lysate from each well of the 24-well plate was assayed in duplicate in a 96-well plate using a dual injector Victor3 1420 multilabel counter (Perkin Elmer, Waltham, Massachusetts, USA). Samples were assayed individually using an automated program. 100 µl LARII (firefly luciferase substrate) was injected followed by a 2 second shake and 20 second incubation. Luminescence was read over a 10 second interval. 100 µl S&G was then injected to stop the firefly luciferase reaction and initiate the *Renilla* luciferase reaction. After a 2 second shake and 20 second incubation, luminescence was again counted over a 10 second interval. Firefly counts-per-minute (CPM) were normalized to *Renilla* CPM for each well. These values were subsequently normalized to basal value for the most common haplotype (2H4 for GRK2 promoter haplotypes, 5H6 for GRK5 promoter haplotypes) to account for experiment-to-experiment variability.

Western Blotting. B-lymphocytes from Human Variation Collection of the Coriell Institute (Camden, New Jersey, USA) were passaged at a density of $2x10^5$ cell/ml and allowed to expand to approximately $1x10^6$ cells/ml. Cells were then harvested and lysed in a solution of 1% IGEPAL, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate in PBS. Electrophoresis was performed using the Nupage system (Life Technologies, Grand Island, New York, USA). 30 µg of each sample was prepared for electrophoresis in 1x NuPage reducing agent and 1x NuPage LDS buffer. Samples were heated to 70°C for 10 minutes and immediately run on NuPage 4-12% bis-tris gels.

Proteins were subsequently transferred to nitrocellulose paper and blocked in Superblock PBS (Thermo Scientific, Waltham, Massachusetts, USA) with 0.05% Tween-20 (SB-PBS+T). The following primary antibodies were used in 10% SB-PBS+T: 1:200 rabbit anti-GRK5 (Santa Cruz, Dallas, Texas, USA, cat#sc-565), 1:500 rabbit anti-GRK2 (Santa Cruz, Dallas, Texas, USA, cat#sc-562), and 1:50,000 mouse anti-β-actin (Sigma, St. Louis, Missouri, USA, cat#A5441). The following horseradish peroxidase-conjugated secondary antibodies were used in 10% SB-PBS+T: 1:2,000 anti-rabbit (Cell Signaling, Danvers, Massachusetts, USA, cat#7074) and 1:5,000 anti-mouse (Santa Cruz, Dallas, Texas, USA, cat#sc-2005). Visualization was performed using SuperSignal West Pico Chemiluminescenct substrate (Thermo Scientific, Waltham, Massachusetts, USA) and the FujiFilm LAS 3000 image reader (Tokyo, Japan). Band and background intensity were determined, and background intensity was subtracted out for each band. Expression of GRK was normalized to expression of β-actin to account for loading error and sample 118 (included on each gel) to account for gel-to-gel variation. Antibody specificity was confirmed for the anti-GRK2 and anti-GRK5 antibodies in separate optimization experiments by analyzing non-transfected, GRK2 transiently-transfected, and GRK5 transiently-transfected HEK-293T lysates (data not shown).

Whole-cell Phosphorylation Assay. HEK-293T cells were transfected in 100 mm dishes at 60-70% confluency. Cells received 5 μg FLAG-β_1AR R389 (in pFLAG-cmv3, Sigma Aldrich, St. Louis, Missouri, USA) and 10 μg of either empty vector (pcDNA3.1), GRK2 (in pCMV6-2L5, OriGene, Rockville, Maryland, USA), or GRK4α (in SL1180, courtesy of Richard Premont, Duke University). The following day, cells were disassociated using

1 mg/ml collagenase (to protect FLAG peptide cleavage at β_1AR amino-terminal trypsin site) and then transferred onto poly D-lysine precoated 6-well dishes in serum-free medium. Cells for each transfection were also replated to a 100 mm dish for ^{125}I-CYP saturation binding. After cells attached to the plate, medium was changed to serum-free medium overnight. On the day of the experiment, cells were changed to medium containing 100 μCi/ml ^{32}P-orthophosphoric acid (Perkin Elmer, Waltham, Massachusetts, USA). Cells were incubated for 2 hours at 37°C in the presence of 95% O_2/5% CO_2. Cells were then treated with 100 μM ascorbic acid alone or with 10 μM NE. Cells were lysed and equal amounts of protein for each condition were used to isolate FLAG-β_1AR. FLAG immunoprecipitation was performed using a FLAG immunoprecipitation kit (Sigma-Aldrich, St. Louis, Missouri, USA). Equal amounts of receptor, measured using protein concentration and ^{125}I-CYP saturation binding, were run on a 10% polyacrylamide gel. The gel was then dried onto chromatography paper and exposed to X-ray film for 72 hours. Films were developed using Carestream Kodak autoradiography GBX developer and fixer (Rochester, New York, USA). Films were submerged in developer for five minutes, followed by water for three minutes, and then fixer for five minutes.

^{3}H-Adenine cAMP Accumulation Assay. For CHO cell transfections, cells were transiently transfected in 150 mm dishes at 60-70% confluency. Cells received 20 μg of β_1AR Arg389 (in pCMV-XL5, OriGene, Rockville, Maryland, USA) and 10 μg of either an empty vector (pcDNA3.1) or a GRK4α haplotype (in SL1180, courtesy of Richard Premont, Duke University). The day following transfection, cells were detached and

replated onto 24-well plates. Cells for each transfection were also replated to a 100 mm dish for ^{125}I-CYP saturation binding. The experiment was performed 18 hours after replating.

On the day of the experiment, medium was changed to serum-free medium containing 2 μCi/ml ^{3}H-adenine (Perkin Elmer, Waltham, Massachusetts, USA). Cells were incubated for 2 hours at 37°C in the presence of 95% O_2/5% CO_2. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine was added to reach a final concentration of 100 μM 1.25 hours into incubation. Cells were then washed three times with room temperature PBS. Drug treatments were performed in serum-free medium containing 100 μM ascorbic acid and either 100 μM Forskolin or 10 μM ISO. Drug treatments were carried out for 30 minutes at 37°C in the presence of 95% O_2/5% CO_2. Treatment was terminated by adding 100 μl of 2.2 N HCl. Basal conditions were determined by adding 100 μl of 2.2 N HCl to wells instead of drug treatment. Three replicates of each transfection were performed for each basal and drug-treated condition. cAMP was isolated using acidic alumina columns as previously described[84] . Eluted cAMP was collected in 20 mL high-density polyethylene vials (Research Products International Corp., Mount Prospect, Illinois, USA), and 5 ml of Ultima Gold Scintillation Cocktail (Perkin Elmer, Waltham, Massachusetts, USA) was added to each vial. ^{3}H-cAMP counts were determined using the LS6500 beta counter (Beckman Coulter, Brea, California, USA).

^{125}I-CYP Saturation Binding. Transiently-transfected cells set aside for saturation binding were washed three times with ice-cold PBS. Cells were scraped in an ice-cold 5/2

solution, pH 7.4 (5 mM Tris and 2 mM EDTA) supplemented with a cOmplete protease inhibitor cocktail tablets (Roche, Basel, Switzerland). Scraped cells were centrifuged at 19,000 rpm for 10 minutes at 4°C. The supernatant was aspirated, and the pellet was resuspended in an ice-cold 75/12/2 solution, pH 7.4 (75 mM Tris, 12.5 mM MgCl2, and 2 mM EDTA). The resuspended pellet was subsequently homogenized using a Polytron homogenizer (Kinematica, Bohemia, New York, USA). Samples were assayed in triplicate for both specific and non-specific binding. ^{125}I-CYP counts/µl were determined prior to each experiment to account for decay. Specific tubes received 25 µl homogenized sample, 200 µl 75/12/2 pH 7.4, and 25 µl of 20,000 counts/µl ^{125}I-CYP. Non-specific tubes received 25 µl homogenized sample, 175 µl of 75/12/2 pH 7.4, 25 µl of 20,000 counts/µl ^{125}I-CYP, and 25 µl 10 µM propranolol. Samples were covered, vortexed, and incubated for two hours at room temperature in a lead-lined box. Samples were then passed through a filter paper using a protein harvester. Filter paper was washed three times with 10 mM Tris to remove unbound ^{125}I-CYP. Filters for each sample were counted using the Cobra II Auto-Gamma counter (GMI Inc., Ramsey, Minnesota, USA). Non-specific binding was subtracted from specific binding. Homogenized sample protein concentrations were determined using the BCA protein assay kit (Thermo Scientific, Rockford, Illinois, USA). Receptor expression was calculated using the specific activity of ^{125}I-CYP (2200 Ci/mmol) and divided by protein concentration to determine the fmol of receptor per µg of protein.

Statistical Analysis. Hardy-Weinberg equilibrium was tested using Fisher's exact test as previously outlined[85] . In comparison of GRK2 and GRK5 haplotypes, the differences in

means were determined using an unpaired two-tailed t-test. In comparing GRK4 haplotypes, we used a one-way ANOVA to determine statistical differences. In all cases, means are considered significantly different when p-values are less than 0.05.

3. Results

3.1 Genetic variation in GRK2 & GRK5 promoter and their effect on promoter activity

3.1.1 SNP discovery

We began our search for promoter polymorphisms by sequencing 2,000 base pairs upstream of the translational start site for both GRK2 and GRK5. Genomic DNA from healthy, unrelated individuals was obtained from the Coriell Institute Human Variation Collection. In total, 32 African Americans and 31 Caucasians were sequenced. Power analysis done prior to sequencing indicated n=30 individuals for each race would be sufficient for SNP discovery.

In the GRK2 promoter, we identified six "common" SNPs, defined as having an allele frequency of ≥ 0.05 in at least one of the cohorts (Table 8). We also found five "rare" variants, defined as being present in only one or two individuals (Table 8). All but two of the variants (-136 G/T and -238-240 GCG/deletion) had been previously reported to the US National Center for Biotechnology Information (NCBI) Single Nucleotide Polymorphisms database (dbSNP). In the GRK5 promoter, we identified five common SNPs at a frequency of ≥0.05 and nine rare variants (Table 9). All but five of the rare variants (-61 G/deletion, -517 C/A, -837 G/C, -981 C/T, and -1057 C/G) had been previously reported to NCBI dbSNP. SNP location, NCBI dbSNP identification number (rs #), and minor allele frequency for GRK2 and GRK5 are listed in Tables 8 and 9, respectively.

Table 8: GRK2 Promoter SNPs. SNPs identified within 2,000 base pair upstream of the GRK2 translational start site were analyzed for allele frequency in both African American and Caucasian cohorts. Heterozygote probability values provide the probability of obtaining our observed number of heterozygotes given our observed umber of alleles. A probability < 0.05 indicates deviation from Hardy Weinberg Equilibrium.

Position relative to translational start site	NCBI dbSNP identification number (rs #)	Major Allele	Minor Allele	Frequency of Minor Allele in African Americans	Heterozygote Probability (African Americans)	Frequency of Minor Allele in Caucasians	Heterozygote Probability (Caucasians)
-43	rs182084609	G	A	0	1	0.08	<0.01
-939	rs61758504	T	C	0.31	0.09	0.02	1
-1022	rs12286664	G	A	0.28	0.13	0.02	1
-1095	rs11605263	C	T	0.02	1	0.05	0.95
-1,138	rs10791901	G	A	0.08	0.84	0	1
-1,147	rs12286610	G	A	0.11	0.03	0	1
Rare Variants							
-136		G	T	0	1	0.02	1
-238 TO -240		GCG	deletion	0.03	0.98	0	1
-325	rs139825050	C	T	0.03	0.98	0	1
-694	rs61758506	A	G	0	1	0.02	1
-839	rs61758505	C	T	0	1	0.02	1

Table 9: GRK5 Promoter SNPs. SNPs identified within 2,000 base pair upstream of the GRK5 translational start site were analyzed for allele frequency in both African American and Caucasian cohorts. Heterozygote probability values provide the probability of obtaining our observed number of heterozygotes given our observed umber of alleles. A probability < 0.05 indicates deviation from Hardy Weinberg Equilibrium.

Position relative to translational start site	NCBI dbSNP identification number (rs #)	Major Allele	Minor Allele	Frequency of Minor Allele in African Americans	Heterozygote Probability (African Americans)	Frequency of Minor Allele in Caucasians	Heterozygote Probability (Caucasians)
-54	rs2230347	C	T	0.20	.28	0.01	0.20
-394	rs10886423	T	G	0.08	0.85	0	
-678	rs61876593	C	T	0.14	<0.01	0.73	0.22
-1435	rs7923896	C	T	0.17	0.174	0.73	0.22
-1437	rs10787929	A	G	0.30	0.289	0.73	0.22
Rare Variants							
-61		G	deletion	0.03	.98	0	1
-184-189	rs76422636	deletion	CGGCGG	0.02	1	0	1
-321	rs112475529	C	T	0.02	1	0	1
-517		C	A	0.02	1	0	1
-790	rs10886422	C	T	0.02	.98	0	1
-837		G	C	0.02	1	0	1
-981		C	T	0.02	1	0	1
-1057		C	G	0.02	1	0	1
-1091	rs1397615	C	T	0.02	1	0	1

Fisher's exact test was used to determine whether the observed genotypes are within Hardy-Weinberg equilibrium (HWE). The heterozygote probability values listed in Tables 8 and 9 represent the probability of obtaining the observed number of heterozygous individuals given the observed minor allele frequency. A probability < 0.05 would indicate deviation from HWE. We found that genotypes for several SNPs deviated from HWE (GRK2: -1147 G/A in African Americans, -43 G/A in Caucasians; GRK5: -678 C/T in African Americans, -54 C/T in Caucasians). This deviation from HWE may indicate experimental error, a small sample size, or a sampling population that does not

meet the Hardy-Weinberg assumption that genetic drift, migration, mutation, natural selection, and assortatitve mating are negligible in the population[85] . Therefore our observed genotypic frequencies may not be an accurate representation of the true population for these loci.

3.1.2 Haplotype construction

In order to analyze the effect of these polymorphisms in a relevant manner, we organized the common SNPs into haplotypes. The term "haplotype" in this study refers to the 2,000 base pair sequence upstream of either GRK2 or GRK5 containing a specific combination of SNPs. To create haplotypes based on our sequencing data, we used the software PHASE, which analyzes the frequency with which variants are inherited together to produce haplotypes[83] . PHASE produced seven GRK2 haplotypes (Table 10) and seven GRK5 haplotypes (Table 11). Despite their low frequency, we also included polymorphisms that resulted in nucleotide insertions or deletions (GRK2: -238-240 GCG/deletion; GRK5: -61 G/deletion, -184-189 deletion/CGGCGG). GRK2 and GRK5 haplotypes and their frequencies are listed in Tables 10 and 11, respectively. Haplotypes are denoted by either "2H" for GRK2 and "5H" for GRK5, followed by the haplotype number (e.g. GRK2 haplotype #1 = 2H1).

Table 10. GRK2 promoter haplotypes. GRK2 SNPs with an allele frequency ≥0.05 and the rare deletion at -238 to -240 were organized into haplotypes using PHASE. Haplotypes with frequencies ≥0.05 and the rare haplotype were synthesized and cloned into luciferase reporter vectors for promoter activity analysis.

Haplotype ID	-1147	-1138	-1095	-1022	-939	-43	Allele Frequency in African Americans	Allele Frequency in Caucasians
2H1	A	G	C	A	C	G	0.11	0
2H2	G	G	C	A	C	G	0.17	0.02
2H3	G	G	C	G	C	G	0.03	0
2H4	G	G	C	G	T	G	0.59	0.85
2H5	G	A	C	G	T	G	0.08	0
2H6	G	G	T	G	T	G	0.02	0.05
2H7	G	G	C	G	T	A	0	0.08

RARE:

Haplotype ID	-1147	-1138	-1095	-1022	-939	-238 to -240	-43
2H8	G	G	C	G	T	deletion	G

Table 11. GRK5 promoter haplotypes. GRK5 SNPs with an allele frequency ≥0.05, the rare deletion at -61, and the rare insertion at -184 to -189 were organized into haplotypes using PHASE. Haplotypes with frequencies ≥0.05 and the rare haplotypes were synthesized and cloned into luciferase reporter vectors for promoter activity analysis.

Haplotype ID	-1437	-1435	-678	-394	-54	Allele Frequency in African Americans	Allele Frequency in Caucasians
5H1	A	C	C	G	C	0.08	0
5H2	A	C	C	T	C	0.05	0
5H3	G	C	C	T	C	0.48	0.15
5H4	A	T	C	T	C	0.05	0
5H5	G	C	T	T	C	0.02	0
5H6	A	T	T	T	C	0.12	0.73
5H7	G	C	C	T	T	0.20	0.13

RARE:

Haplotype ID	-1437	-1435	-678	-394	**-61**	-54
5H8	G	C	C	T	deletion	C

Haplotype ID	-1437	-1435	-678	-394	**-184 to -189**	-54
5H9	A	C	C	G	CGGCGG	C

3.1.3 Basal activity of promoter haplotypes

3.1.3.1 Basal activity of GRK2 promoter haplotypes

Haplotypes present in the population at a frequency of ≥0.05 and haplotypes containing the insertion/deletion rare variants were chosen for analysis using luciferase reporter assays. The 2,000 base pair sequence containing each haplotype was cloned into the pGL4.10 firefly luciferase reporter vector, upstream of the firefly luciferase ORF. Promoter activity could therefore be assessed by measuring expression of firefly luciferase. Firefly luciferase expression is measured as bioluminescent light produced from the reaction of luciferase with its substrate.

Each haplotype construct was co-transfected into HEK-293T cells with either β_1AR or β_2AR. In addition, cells were co-transfected with a *Renilla* luciferase vector to control for variations in expression caused by transfection in individual cells. Basal promoter activity was determined by measuring luciferase expression following an 18-hour period of serum starvation.

Firefly luciferase expression driven by GRK2 haplotypes in the presence of β_1AR can be found in Figure 3, and expression in the presence of β_2AR can be found in Figure 4. For GRK2, the haplotypes that tend to drive the highest expression are 2H4, the most common haplotype, and 2H1. 2H5 appears to drive the lowest expression. This conclusion is based on the observation that both 2H4 and 2H1 levels are significantly higher than 2H5 in the presence of both β_1AR and β_2AR. 2H4 levels are also significantly higher than 2H2 when co-expressed with β_1AR and significantly higher than 2H6 and 2H8 when co-expressed with β_2AR.

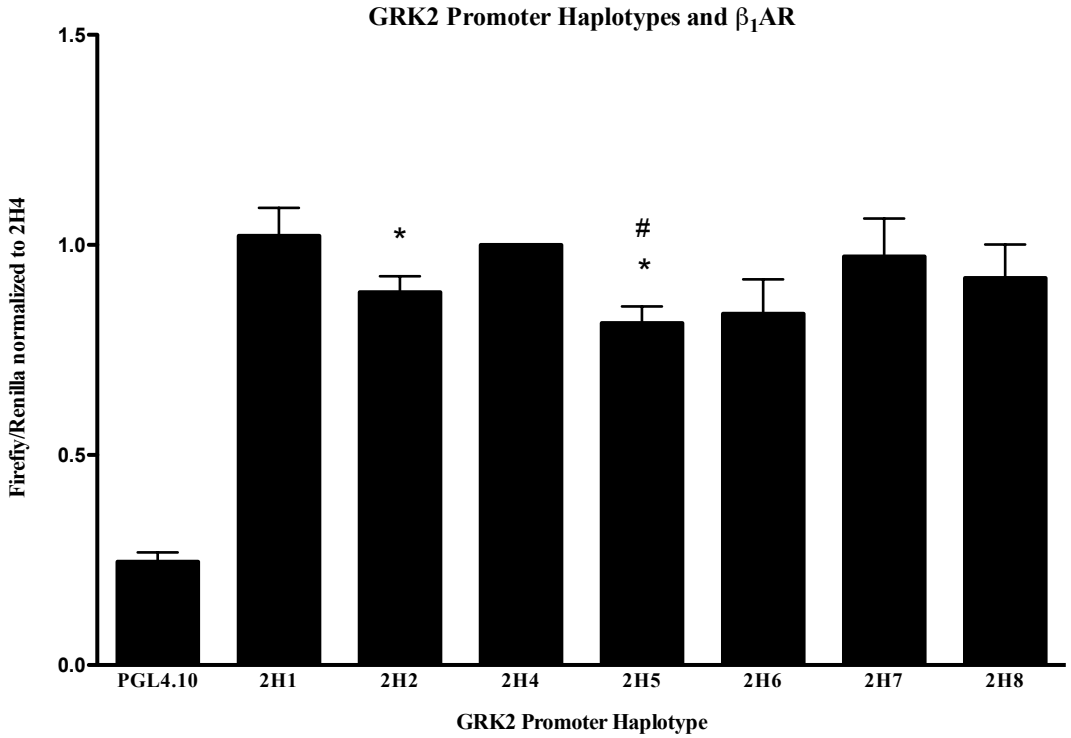

Figure 3. Basal activity of GRK2 promoter haplotypes in the presence of β_1AR. GRK2 promoter haplotypes (in firefly luciferase reporter vector) and the empty vector (PGL4.10) were each co-transfected into HEK-293T cells with β_1AR and a *Renilla* luciferase transfection control. Promoter activity was assessed as its ability to drive firefly luciferase expression. Firefly luciferase expression was measured as light output produced by its interaction with substrate. Cells were assayed for luciferase expression following and 18-hour incubation in 100 μM ascorbic acid in the absence of serum. Expression is shown as the ratio of firefly luciferase activity to *Renilla* luciferase activity normalized to the most common haplotype (2H4) to account for variability between experiments (n=11). Error bars represent standard error of the mean. *, $P < 0.05$ vs 2H4. #, $P<0.05$ vs 2H1.

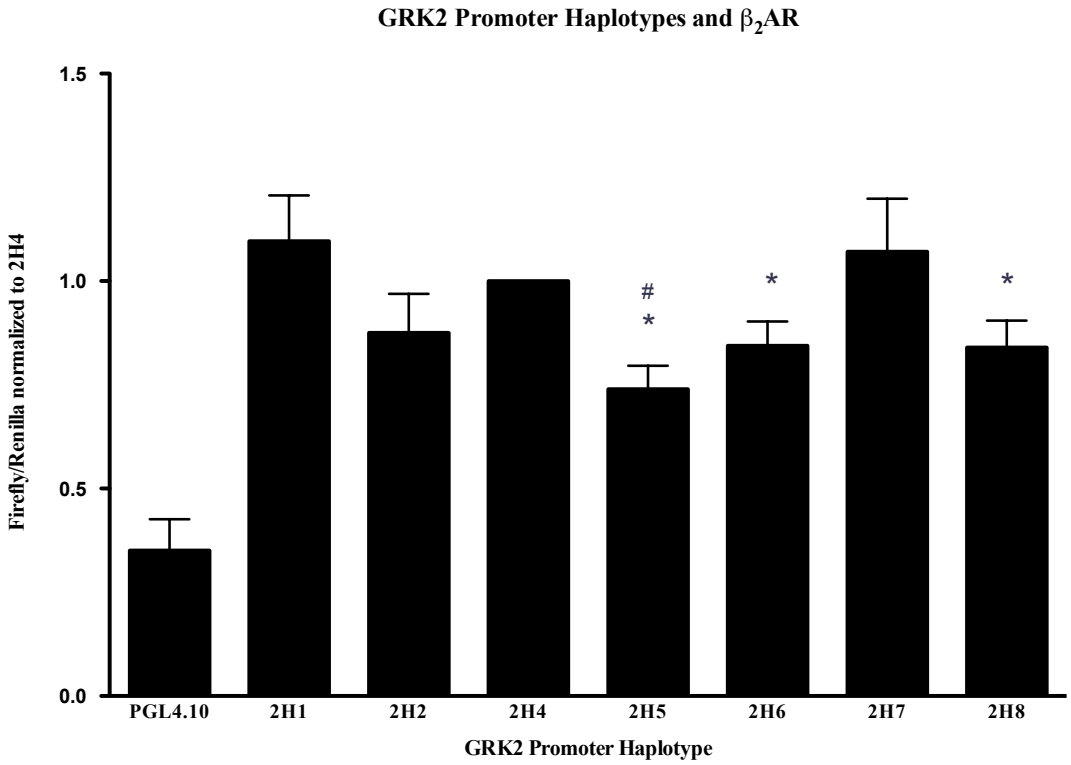

Figure 4. Basal activity of GRK2 promoter haplotypes in the presence of β_2AR. GRK2 promoter haplotypes (in firefly luciferase reporter vector) and the empty vector (PGL4.10) were each co-transfected into HEK-293T cells with β_2AR and a *Renilla* luciferase transfection control. Promoter activity was assessed as its ability to drive firefly luciferase expression. Firefly luciferase expression was measured as light output produced by its interaction with substrate. Cells were assayed for luciferase expression following and 18-hour incubation in 100 μM ascorbic acid in the absence of serum. Expression is shown as the ratio of firefly luciferase activity to *Renilla* luciferase activity normalized to the most common haplotype (2H4) to account for variability between experiments (n=9). Error bars represent standard error of the mean. *, $P < 0.05$ vs 2H4. #, $P < 0.05$ vs 2H1.

3.1.3.2 Basal activity of GRK5 promoter haplotypes

In comparing basal GRK5 promoter haplotype activity, we found that 5H2 stood out as driving the highest luciferase expression among the common haplotypes (Figures 5 and 6). In the presence of both β_1AR and β_2AR, 5H2 drove expression at levels significantly higher than 5H1, 5H3, 5H4, and 5H6. Additionally, 5H2 levels were significantly higher than 5H7 in the presence of β_2AR.

Despite a high standard error, luciferase expression driven by 5H8 was significantly higher than that driven by 5H1 and 5H6 in the presence of β_1AR and β_2AR.

When co-expressed with β_2AR, 5H8 promoter activity was also significantly higher than 5H3 and 5H4.

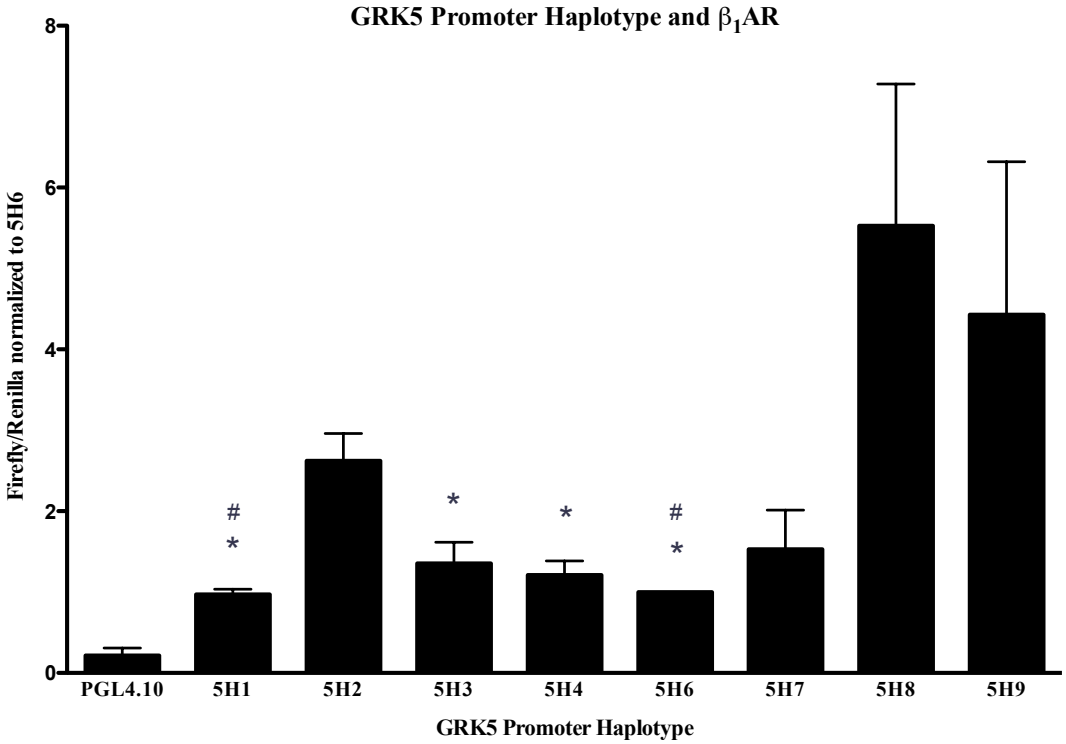

Figure 5. Basal activity of GRK5 promoter haplotypes in the presence of β_1AR. GRK5 promoter haplotypes (in firefly luciferase reporter vector) and the empty vector (PGL4.10) were each co-transfected into HEK-293T cells with β_1AR and a *Renilla* luciferase transfection control. Promoter activity was assessed as its ability to drive firefly luciferase expression. Firefly luciferase expression was measured as light output produced by its interaction with substrate. Cells were assayed for luciferase expression following and 18-hour incubation in 100 μM ascorbic acid in the absence of serum. Expression is shown as the ratio of firefly luciferase activity to *Renilla* luciferase activity normalized to the most common haplotype (5H6) to account for variability between experiments (n=6). Error bars represent standard error of the mean. *, $P < 0.05$ vs 5H2. #, $P < 0.05$ vs 5H8.

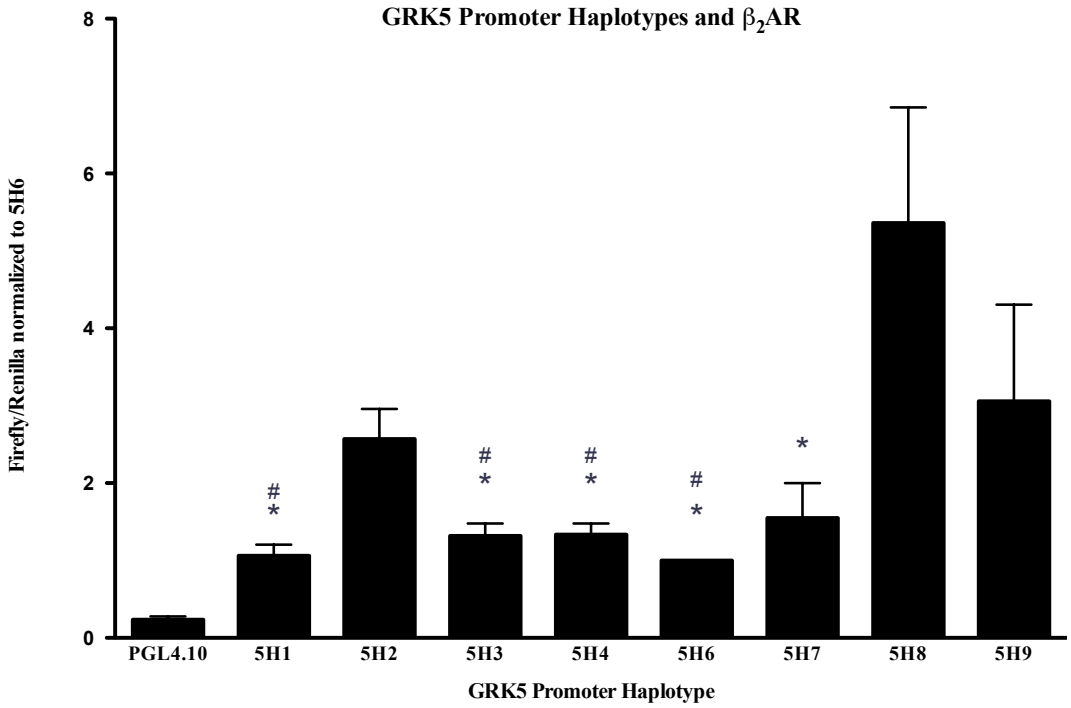

Figure 6. Basal activity of GRK5 promoter haplotypes in the presence of β_2AR. GRK5 promoter haplotypes (in firefly luciferase reporter vector) and the empty vector (PGL4.10) were each co-transfected into HEK-293T cells with β_2AR and a *Renilla* luciferase transfection control. Promoter activity was assessed as its ability to drive firefly luciferase expression. Firefly luciferase expression was measured as light output produced by its interaction with substrate. Cells were assayed for luciferase expression following and 18-hour incubation in 100 μM ascorbic acid in the absence of serum. Expression is shown as the ratio of firefly luciferase activity to *Renilla* luciferase activity normalized to the most common haplotype (5H6) to account for variability between experiments (n=6). Error bars represent standard error of the mean. *, $P < 0.05$.

3.1.4 GRK2 and GRK5 promoter haplotype activity in response to drug treatment

We then determined the effect of βAR agonists and antagonists on the variability in promoter activity across haplotypes. HEK-293T cells were treated with agonist (10 μM NE for β_1AR, 10 μM ISO for β_2AR) or 10 μM of the non-specific βAR antagonist carvedilol in serum-free medium for 18 hours. We calculated response as the percent change from basal conditions. In evaluating agonist and antagonist response by this method, we did not find significant differences in the magnitude of response between

haplotypes for either GRK2 or GRK5 in the presence of either β_1AR or β_2AR (data not shown).

However, we did find that certain haplotypes had a significant increase in promoter activity following carvedilol treatment, whereas others did not. We found these results solely in the GRK5 haplotypes, both in the presence of β_1AR (Figure 7) and β_2AR (Figure 8). When co-expressed with β_1AR and β_2AR, 5H6 showed a significant increase in promoter activity following the 18-hour carvedilol treatment. Carvedilol treatment also significantly increased 5H3 and 5H4 promoter activity in cells co-expressing β_1AR. We did not find significant changes in GRK5 promoter activity following agonist treatment for any conditions. Agonist and antagonist responses for GRK2 haplotypes co-expressed with β_1AR and β_2AR can be found in Appendix Figures A1 and A2.

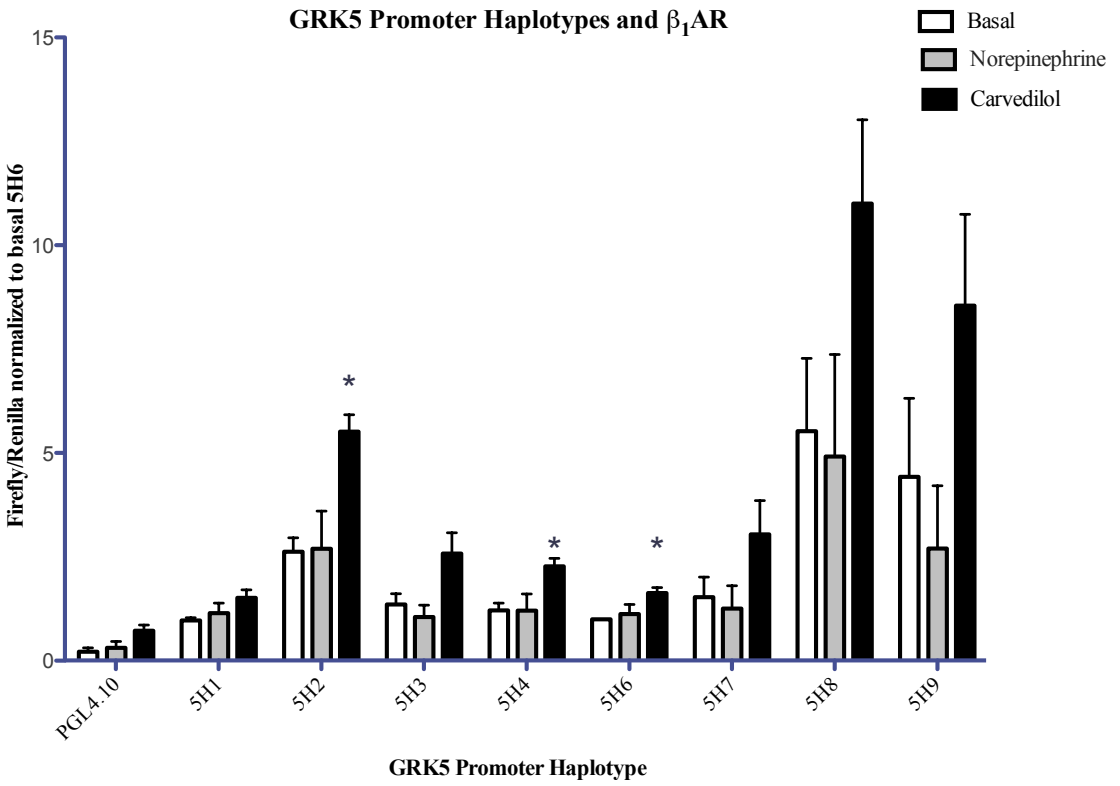

Figure 7. Drug-treated GRK5 promoter haplotype activity in the presence of β_1AR. GRK5 promoter haplotypes (in firefly luciferase reporter vector) and the empty vector (PGL4.10) were each co-transfected into HEK-293T cells with β_1AR and a *Renilla* luciferase transfection control. Promoter activity was assessed as its ability to drive firefly luciferase expression. Firefly luciferase expression was measured as light output produced by its interaction with substrate. Cells were assayed for luciferase expression following and 18-hour incubation in 100 μM ascorbic acid alone (basal, n=6) or with either 10 μM norepinephrine (n=4) or 10 μM carvedilol (n=4) in the absence of serum. Expression is shown as the ratio of firefly luciferase activity to *Renilla* luciferase activity normalized to basal expression of the most common haplotype (5H6) to account for variability between experiments. Error bars represent standard error of the mean. *, $P < 0.05$ vs basal.

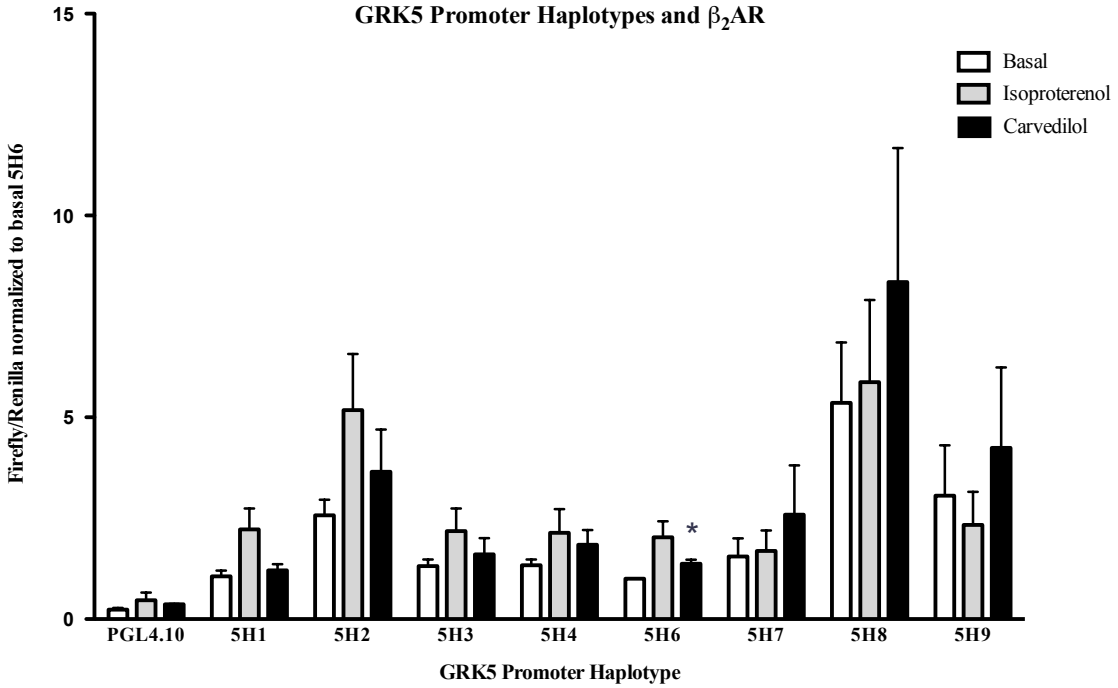

Figure 8. Drug-treated GRK5 promoter haplotype activity in the presence of β_2AR. GRK5 promoter haplotypes (in firefly luciferase reporter vector) and the empty vector (PGL4.10) were each co-transfected into HEK-293T cells with β_1AR and a *Renilla* luciferase transfection control. Promoter activity was assessed as its ability to drive firefly luciferase expression. Firefly luciferase expression was measured as light output produced by its interaction with substrate. Cells were assayed for luciferase expression following and 18-hour incubation in 100 μM ascorbic acid alone (basal, n=6) with either 10 μM norepinephrine (n=4) or 10 μM carvedilol (n=4) in the absence of serum. Expression is shown as the ratio of firefly luciferase activity to *Renilla* luciferase activity normalized to basal expression of the most common haplotype (5H6) to account for variability between experiments (n=4). Error bars represent standard error of the mean. *, $P < 0.05$ vs basal.

3.1.5 Endogenous expression of GRK2 and GRK5 in B-lymphocytes

Our next aim was to determine if the variation between haplotypes held true for endogenously expressed GRK2 and GRK5. To do so, we used B-lymphocytes with known GRK2 and GKR5 haplotypes. GRK2 and GRK5 expression was analyzed by western blot and is shown in Figures 9 and 10, respectively. Quantified results from these blots can be found in Figures 11 and 12. Due to the limited availability of homozygotes, we could not compare all haplotypes. For those haplotypes that were available for

comparison, we did not find any statistically significant differences in expression of GRK2 and GRK5 among haplotypes.

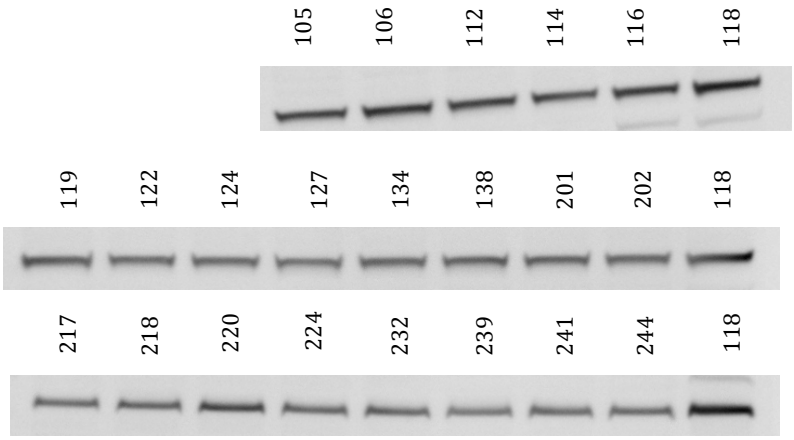

Figure 9. GRK2 lymphocyte expression. 30 µg of B-lymphocyte lysate from previously-genotyped subjects were subjected to western blot analysis using anti-GRK2 antibody. Lanes are identified by sample number. Sample 118 was run on each gel to account for gel-to-gel variation.

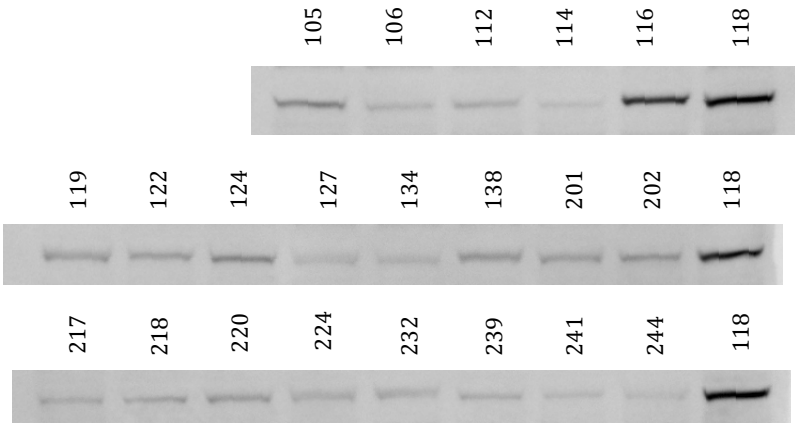

Figure 10. GRK5 lymphocyte expression. 30 µg of protein from lysed B-lymphocytes from previously-genotyped subjects were subjected to western blot analysis using anti-GRK5 antibody. Lanes are identified by sample number. Sample 118 was run on each gel to account for gel-to-gel variation.

Figure 11. Quantified GRK2 expression in lymphocytes. Quantitation of bands of the GRK2 western blot shown in figure 9. GRK2 band intensity was normalized to β-actin band intensity for each sample. The normalized value for each sample was then divided by the normalized value for 118 to account for gel-to-gel variation. ◆, homozygous for 2H1. ★, homozygous for 2H2. ▼, homozygous for 2H4. ■ homozygous for 2H7. ●, heterozygotes.

Figure 12. Quantified GRK5 expression in lymphocytes. Quantitation of bands of the GRK5 western blot shown in figure 10. GRK5 band intensity was normalized to β-actin band intensity for each sample. The normalized value for each sample was then divided by the normalized value for 118 to account for gel-to-gel variation. ◆, homozygous for 5H3. ▼, homozygous for 5H6. ■ homozygous for 5H7 ●, heterozygotes.

3.2 Biochemical basis for reduced response to β_1AR antagonists in GRK4 haplotypes

3.2.1 GRK4 phosphorylation of β_1AR

The first step in GPCR desensitization is GRK-mediated phosphorylation of the agonist-bound receptor. To determine whether the presence of GRK4 increases phosphorylation of β_1AR following agonist treatment, we used a whole-cell phosphorylation assay using a protocol previously established in our laboratory[55] . We used GRK2 as a positive control and an empty vector (pcDNA3.1) as a negative control. As expected, we found an increase in β_1AR phosphorylation following agonist treatment in cells co-expressing GRK2, but not in cells co-expressing pcDNA3.1 (Figure 13).

When analyzing GRK4, we used the full-length isoform, GRK4α, because the distribution of GRK4 isoforms in relevant tissues is unavailable. Qualitative results indicate that co-expression of GRK4α results in increased agonist-stimulated phosphorylation of β_1AR. This supported our hypothesis that GRK4α promotes phosphorylation, and presumably desensitization, of β_1AR.

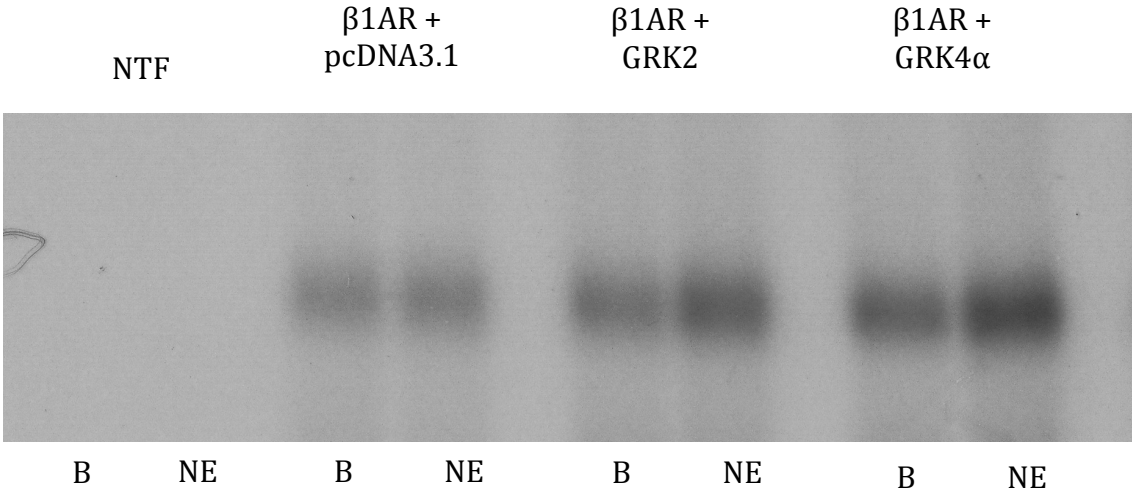

Figure 13. Phosphorylation of β_1AR. HEK-293T cells were transiently transfected with FLAG-β_1AR and either an empty vector (pcDNA3.1), GRK2, or GRK4α. Non-transfected (NTF) HEK-293T cells were included as a control. Cells were exposed to 100μM ascorbic acid alone (considered basal, columns labeled "B") or with 10μM norepinephrine (column labeled "NE") for 15 minutes. FLAG-β_1AR was isolated by immunoprecipitation and separated on a 10% polyacrylamide gel. The dried gel was exposed to film for 72 hours. (n=1)

3.2.2 GRK4-mediated desensitization of β_1AR

To test for the effect of GRK4α on β_1AR desensitization in the presence of GRK4α, we measured the accumulation of cAMP, a second messenger synthesized upon activation β_1AR. We utilized a radiometric method in which ^{3}H-adenine becomes incorporated into cAMP following agonist exposure[84] . The use of a phosphodiesterase inhibitor allowed us to measure the total amount of cAMP accumulated during a thirty-

minute agonist treatment. Decreased cAMP accumulation after thirty minutes would therefore indicate desensitization.

We initially chose CHO cells as our model system due to negligible levels of endogenous βAR expression[86] . The goal of these experiments was to assess desensitization of β_1AR signaling in the presence of GRK4 and different GRK4 haplotypes. Since the variation in atenolol response based on GRK4 haplotype was more pronounced in patients homozygous for β_1AR Arg389, we chose to study that variant of β_1AR first[63] . Cells were transfected with the β_1AR Arg389 variant and either an empty vector (pcDNA3.1) or one of the GRK4 haplotypes present in the population at a frequency ≥0.05 (Table 3). 48-hours post-transfection, cells were stimulated with 10 μM ISO for 30 minutes, as described in the Methods section. Analysis using a one-way ANOVA demonstrated that agonist-induced cAMP accumulation following a 30 minute ISO treatment does not change in the presence of different GRK4α haplotypes (Figure 14). Therefore, we cannot conclude that GRK4 causes desensitization of β_1AR or that SNPs within GRK4 affect its interaction with β_1AR.

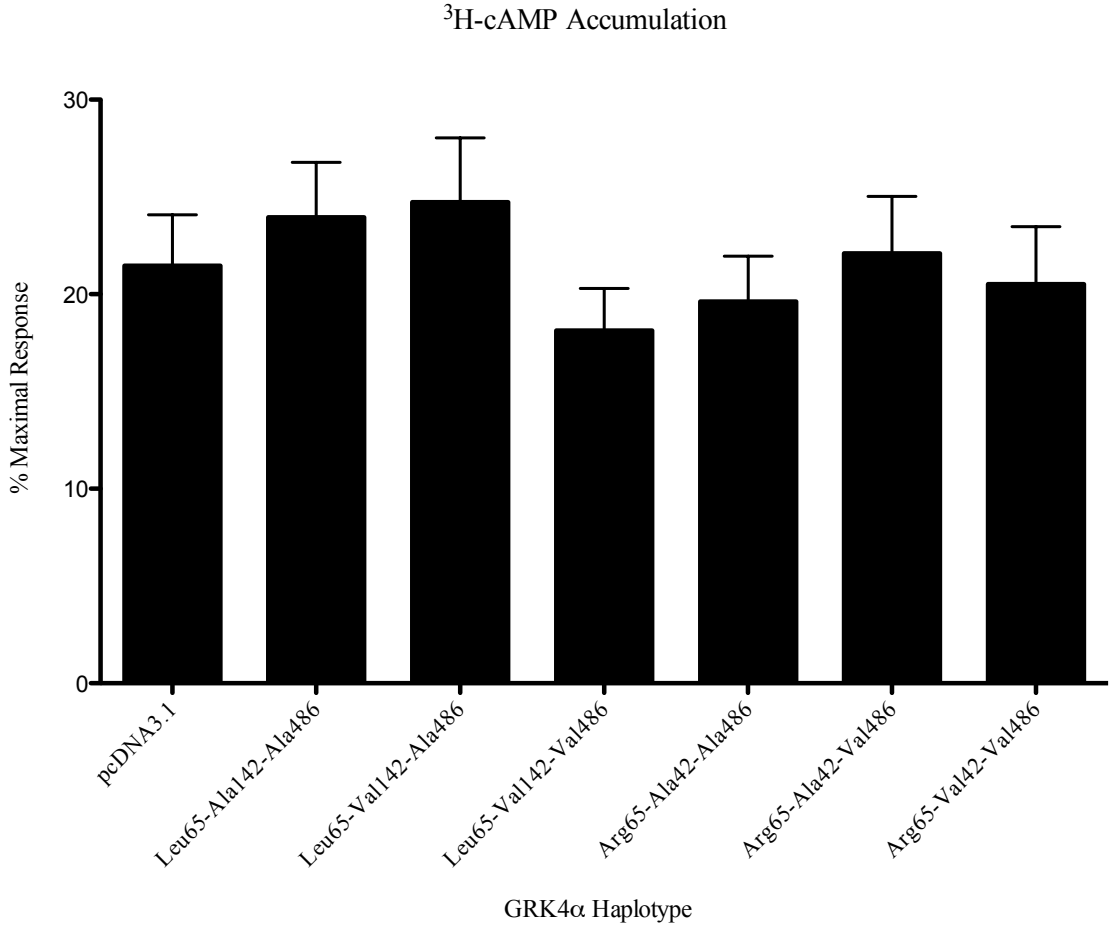

Figure 14. β_1AR desensitization by GRK4α in CHO cells. CHO cells were transiently transfected β_1AR R389 variant and either a GRK4α variant or an empty vector (pcDNA3.1). Cells exposed to ^{3}H-adenine were subjected to a 30-minute drug treatment with 10 μM isoproterenol and 100 μM ascorbic acid. ^{3}H-cAMP accumulation is displayed as the percent of the maximal response (measured using 100 μM forskolin with 100 μM ascorbic acid) after subtraction of the basal level (measured using 100 μM ascorbic acid alone). n=5.

4. Discussion

In this study, we sought to investigate the role of SNPs in non-coding and coding regions of GRKs and assessed functional effects on promoter activity and receptor desensitization. In doing so, we hoped to contribute to the understanding of the pharmacogenomics of β_1AR and β_2AR agonists. We explored GRK SNPs in two separate projects.

4.1 Genetic variation in GRK2 & GRK5 promoters and their effect on promoter activity

In the first project, we explored genetic variation in GRK2 and GRK5 promoters and their effect on promoter activity and expression. We chose GRK2 and GRK5 because they are both expressed in heart and lung and are able to desensitize both β_1AR and β_2AR[26-28,54,64,65] . Therefore, SNPs that affect expression of GRK2 and GRK5 have implications in heart failure, asthma, and COPD.

In heart failure, both GRK2 and GRK5 are upregulated[27,66] . This upregulation has been reportedly reduced with the treatment of β_1AR antagonists[27] . Expression of GRK2 and GRK5 in healthy versus asthmatic/COPD lung tissue has yet to be reported, but β_2AR desensitization contributes to the reduced response seen with chronic use of β_2AR agonist treatments[73] . Therefore, it is possible that SNPs affecting expression of GRK2 and GRK5 could influence the desensitization state of the βAR population in cardiac and lung tissue and contribute to the degree of response to β_1AR antagonists and β_2AR agonists.

Our primary hypothesis was that SNPs in the promoter of GRK2 and GRK5 can result in differential gene expression under basal, agonist, and antagonist-treated

conditions. Our first aim was to identify polymorphisms within the 2,000 base pair sequence upstream of the GRK2 and GRK5 translational start site. Although not specifically explored in this project, this region includes the promoter and encompasses most of the sites for transcriptional factors and regulatory element binding. We achieved this aim by sequencing 32 African Americans and 31 Caucasians over this 2,000 base pair region. In GRK2, we found six common SNPs and five rare SNPs. In GRK5, we found five common SNPs and nine rare variants.

Several SNPs were of particular interest. We found several SNPs in the 5' untranslated region of both GRK2 and GRK5, which encodes a sequence of mRNA that is involved in the regulation of expression. Of particular interest are the rare variants in this region that result in insertions (GRK5 -184 to -189 deletion/CGGCGG) or deletions (GRK2 -238 to -240 GCG/deletion, GRK5 -61 G/deletion). Several other SNPs are worth noting due to their location. In the GRK2 promoter, four polymorphisms fall between -640 base pairs and the translational start site. This is a region meeting the criteria of a CpG island[87] . CpG islands are regions with a high density of the CG dinucleotide, which tend to be unmethylated and are associated with ubiquitously expressed genes[88] . -43 G/A, -136 G/T, -238-240 GCG/deletion, and -325 C/T all result in a loss of a guanine and/or cytosine and thereby a CG dinucleotide. Additionally, regions upstream of the CpG island were shown to contain potent repressive elements in certain cell types[89] . Therefore, it is possible that the remaining SNPs identified beyond -640 may interrupt or enhance the interactions responsible for repression.

SNPs of note upstream of the GRK5 ORF include three of the common SNPs (-54 C/T, -394 T/G, and -678 C/T) and all of the rare variants, which lie within a region

meeting the criteria of a CpG island. We also found a 6-nucleotide insertion between -184 and -189, which increases the length of the CpG island.

Our second aim was to analyze the effect of GRK2 and GRK5 promoter haplotypes on gene expression capacity under basal conditions. We achieved this by first organizing SNPs into haplotypes based on linkage disequilibrium using the computational program PHASE. The haplotypes were cloned into luciferase reporter vectors, driving expression of firefly luciferase. Promoter haplotype activity (measured by luciferase expression) was analyzed under basal conditions in the presence of both β_1AR and β_2AR.

Several haplotypes for both GRK2 and GRK5 stood out as having exceptionally low or exceptionally high promoter activity. In GRK2, 2H5 had the lowest activity of the haplotypes in the presence of both β_1AR and β_2AR. 2H5 is present only in African Americans at a frequency of 0.08. 2H5 is also the only haplotype containing the adenine variant at -1138. It's possible that this unique variant interrupts transcription factor binding to some degree. Future studies will determine whether -1138 falls within a transcription factor-binding motif using transcription factor prediction programs and characterize the effect of the adenine variant at this locus on transcription factor binding.

In our study of GRK5 promoter haplotype activity under basal conditions, we found two haplotypes that drove exceptionally high expression of luciferase. Of the common haplotypes, 5H2 had the highest activity. In the presence of both β_1AR and β_2AR, 5H2-driven luciferase expression was, on average, 2.6-fold higher than that of 5H6, the most common haplotype. 5H2 is present only in African Americans at a

frequency of 0.05. None of the SNPs are unique to 5H2, and therefore it must be the specific combination of SNPs that produces the increased activity.

5H8 also exhibited exceptionally high promoter activity. On average, 5H8 drove expression of luciferase at levels about 5.4-fold higher than that of 5H6. 5H8 is one of the rare haplotypes, containing a deletion in the 5' untranslated region at -61. 5H3 and 5H8 contain the same combination of SNPs, except for the deletion at -61. Since the mean expression driven by 5H3 is approximately 4.2-fold lower than that of 5H8, it's likely that the deletion at -61 is solely responsible for its exceptionally high expression.

These results suggest that SNPs in GRK2 and GRK5 promoters can indeed cause differential promoter activity among haplotypes. Overall, the pattern of basal promoter activity across haplotypes did not differ much between the β_1AR and β_2AR conditions. This was expected, as basal activity of β_1AR and β_2AR is negligible.

We found that 2H5 drives decreased expression of GRK2 compared to other GRK2 promoter haplotypes in HEK-293T cells, suggesting that the decreased expression driven by 2H5 may potentially decrease desensitization of receptor and increase response to agonist. Similarly, 5H2 and 5H8 may drive increased expression of GRK5 compared to other GRK5 promoter haplotypes, potentially causing an increase in receptor desensitization and decreased response to agonist.

Our third aim was to analyze the effect of βAR agonists and antagonists on the activity of GRK2 and GRK5 promoter haplotypes. We addressed this aim by co-transfecting promoter haplotypes with β_1AR or β_2AR and then subjecting the transfected cells to agonist (10 μM NE for β_1AR conditions, 10 μM ISO for β_2AR conditions) or 10 μM of the non-specific antagonist carvedilol.

The magnitude of change between basal and drug-treated conditions was not significantly different across haplotypes. We then evaluated drug response of each haplotype. Several haplotypes in GRK5, 5H2, 5H4, and 5H6 responded differently upon treatment with carvedilol in the presence of β_1AR. In the presence of β_2AR, only 5H6 had a statistically significant increase in luciferase expression following carvedilol treatment. The increase in promoter activity of GRK5 haplotypes was unexpected, considering previous reports demonstrating decreased GRK5 expression with β_1AR antagonist treatment[27] . Collectively, these findings suggest that GRK5 promoter activity varies among haplotypes in response to antagonist treatment, although the magnitude of drug response among haplotypes does not differ.

The interpretation of drug response findings are limited by small n values (in most instances we had n=4). Other potential limitations include level of expression of the receptor and ratio between the receptor and GRK. Future studies are needed to systematically evaluate drug responses among different haplotypes.

These studies provide preliminary evidence that SNPs in the promoters of GRK2 and GRK5 can drive protein expression to different degrees. However, these experiments were performed in HEK-293T cells overexpressing βAR and containing multiple copies of the promoter/luciferase vector. Future studies may include studies using relevant cell culture models (e.g. airway smooth muscle cells and cardiomyocytes). However, our findings suggest that GRK2 and GRK5 promoter activity among haplotypes is worth exploring in future studies to see if the resulting differential expression can indeed impact heart failure and asthmatic/COPD conditions in both treated and untreated states.

In this context, we determined endogenous expression of GRK2 and GRK5 using immortalized B-lymphocytes that had been isolated from peripheral blood of the individuals we had sequenced for the SNP discovery section of our study. However, we did not have enough homozygous individuals to perform statistical comparisons between haplotypes. If this section of the study was expanded to include B-lymphocytes from several individuals homozygous for each haplotype, we may be able to draw conclusions about basal activity of promoter haplotypes.

Promoter activity is dependent on cell type, as evidenced by differential expression of proteins across tissues. Therefore, studying GRK2 and GRK5 promoter haplotype activity in cell types involved in heart failure and asthma/COPD would provide more clinically relevant results compared to studies done in HEK-293T cells. The HL-1 mouse cardiomyocyte cell line would be a better model system for studying promoter haplotype activity in relation to heart failure[90] . Despite being mouse-derived, the protein expression profile and receptor expression levels in HL-1 cells would be similar to that of human cardiomyocytes. For studying promoter haplotype expression in relation to asthma and COPD, human airway smooth muscle cell lines isolated from healthy and asthmatic subjects are commercially available. Being human-derived, these cells are as accurate a model system as possible for *in vitro* study of GRK promoter polymorphism and β_2AR desensitization under normal and airway inflammation conditions. Using either of these cell types for the study of promoter haplotype activity would eliminate the need for co-transfection of βAR, providing more physiologically accurate response to drug treatments.

The study of promoter activity in response to β_1AR antagonists and β_2AR agonists can also be improved with extended drug treatments. In our experiments, we assessed changes in promoter activity following an 18-hour drug treatment. However, in the case of heart failure, NE levels are chronically elevated. In the treatment of both heart failure and asthma/COPD, patients are exposed to chronically elevated levels of β_1AR antagonists and long-acting β_2AR agonists, respectively. Additional studies are needed to systematically assess the effect of drug treatment for extended period of time, conditions that simulate the *in vivo* situations.

Future studies are needed to ascertain the effect of differential expression of GRK haplotypes on βAR desensitization and downregulation. This can be addressed using several of the methods previously mentioned in this paper: ^{3}H-adenine cAMP accumulation assays, whole cell kinase assays, and ^{125}I-CYP saturation binding studies. This study could also be expanded to include the effect of SNPs within β_1AR and β_2AR to see if differential GRK2 and GRK5 expression affects desensitization and downregulation of βARs to different degrees based on the receptor SNPs. These studies can further expanded to include sequencing of GRK2 and GRK5 promoters from previous heart failure and asthma drug response studies to determine if promoter haplotypes are associated with increased or decreased drug response.

4.2 Biochemical basis for reduced response to β_1AR antagonists in GRK4 haplotypes

In our second study, we explored the interaction between GRK4 and β_1AR in an effort to explain the reduced response to β_1AR antagonists seen in individuals with certain GRK4 haplotypes. GRK4 has three common SNPs that have been associated with hypertension: Arg65Leu, Ala142Val, and Ala486Val[57-60] . Two studies have investigated

the association of these SNPs with response to β_1AR antagonists as a treatment for hypertension. One study, evaluating response to metoprolol in African American hypertension patients, demonstrated that individuals carrying the Leu65 allele had reduced response to metoprolol with each additional copy of the Ala142 allele[62] . Another study evaluating response to atenolol in several races found a reduced response to atenolol with each additional copy of the Leu65-Val142 haplotype[63] . Though not significant, this reduced response was limited to individuals homozygous for the β_1AR Arg389 variant[63] .

The variability in response to β_1AR antagonists in hypertensives based on GRK4 SNPs suggests an interaction between β_1AR and GRK4. The reduced response to atenolol seen with increasing copies of the GRK4 Leu65-Val142 haplotype in β_1AR Arg389 homozygous individuals also suggests that the interaction between β_1AR and GRK4 may be affected by SNPs in both proteins.

The interaction between β_1AR and GRK4 was further supported by their co-localization in heart and kidney tissue as well as previous reports that GRK4 phosphorylates β_2AR[65] . For these reasons, we hypothesized that GRK4 is able to phosphorylate β_1AR, causing its desensitization and that SNPs in both GRK4 and β_1AR would alter the interaction between the two proteins.

Our first aim was to determine phosphorylation state of β_1AR in the presence of GRK4. Whole-cell phosphorylation assay revealed an agonist-dependent increase in β_1AR phosphorylation in cells co-transfected with GRK4α. However, the data presented is from one experiment. This experiment needs to be repeated several times with the same conditions to confirm this finding. Additionally, our interpretation of results is

based on band density. Use of a phosphoimager to quantify ^{32}P in the gel bands can increase the validity of these results and should be used in the future studies.

Though the increase in β_1AR phosphorylation in GRK4α co-transfected cells is pronounced in the agonist-treated condition compared to the basal condition, it also appears that β_1AR phosphorylation under basal condition may be higher than the basal condition of cells co-transfected with the empty vector pcDNA3.1 or GRK2. Though this increase needs to be confirmed by quantitation and repeated experiments, there are several other explanations for this observation.

First, it is possible that the amount of receptor loaded in the gel varies between the transfections, though we tried to control for this by determining receptor expression and protein concentration prior to immunoprecipitation. Secondly, GPCRs can spontaneously flip to an active confirmation in the absence of agonist[91] . The increase in basal phosphorylation in GRK-transfected conditions may be a result of increased recognition of this active confirmation due to increased intracellular concentration of GRK. This would explain why the GRK2 co-transfected condition appears to have increased basal phosphorylation also. Finally, the increase in β_1AR basal phosphorylation in the GRK4α co-transfected condition may be due to agonist-independent activity of GRK4, a phenomenon already demonstrated with β_2AR and the D_1 dopamine receptor[65,80] .

Our second aim, to assess agonist-promoted desensitization of β_1AR in the presence of GRK4, and part of our third aim, to analyze the effects of GRK4 haplotypes on desensitization of the β_1AR Arg389 variant and the β_1AR Gly389 variant, were addressed using ^{3}H-adenine cAMP accumulation assays. In these experiments, a decrease in cAMP accumulation over a 30-minute ISO treatement in the presence of

phosphodiesterase inhibitors would indicate increased desensitization of the receptor. We initially used the Arg389 variant of β_1AR in our experiment because the decreased atenolol response of GRK4 Leu65-Val142 carriers was more pronounced in β_1AR Arg389 homozygous individuals versus Gly389 carriers[63] . We analyzed GRK4 haplotypes present in African Americans or Caucasians at a frequency ≥ 0.05[56] .

In CHO cells transiently-transfected with β_1AR Arg389, we did not find statistically significant decreases in cAMP accumulation upon agonist stimulation in cells co-transfected with a GRK4α haplotype compared to cells co-transfected with the empty vector pcDNA3.1. Based on these limited studies, we conclude that GRK4α does not promote agonist-dependent desensitization of β_1AR. However, there are several factors that may have contributed to our failure to demonstrate a functional change due to desensitization of the receptor. Most importantly, the data presented above were from a limited number of studies. Experimental conditions optimal for desensitization need further evaluation. Because these factors need to be addressed and conditions optimized, we were unable to assess GRK4α haplotype activity on the β_1AR Gly389 variant in this study.

In each experiment, GRK4α expression for each haplotype was assessed by western blotting. These experiments showed a failure to consistently and robustly express GRK4α in CHO cells (data not shown). This inconsistent and low expression may be responsible for the lack of desensitization of β_1AR Arg389 seen upon co-expression with GRK4α haplotypes. Expression of GRK4α in HEK-293T cells, however, is much more robust and consistent (data not shown). If low expression of GRK4α is the reason behind

the lack of desensitization seen in our experiments, future studies may benefit from switching to another host cell line such as HEK-293T cells.

Another factor that may have affected our results is the level of β_1AR Arg389 expression. ^{125}I-CYP binding indicated a mean expression for all conditions to be 3.4 pmol/mg protein. This level of expression may be so high that desensitization could not be discerned with the expression of GRK4α achieved by transfection in these experiments. Future studies may benefit from examining desensitization with lower β_1AR Arg389 expression. This could be achieved by transiently transfecting cells with less DNA or by using a stably-transfected line expressing lower levels of receptor.

Once GRK4α expression levels and β_1AR Arg389 expression levels are optimized, an agonist-dependent increase in desensitization may be detected by measuring ^{3}H-adenine cAMP accumulation in cells. Under these conditions, GRK4α haplotype activity with the β_1AR Gly389 variant can also be assessed.

4.3 Summary and conclusions

In this study we aimed at establishing genetic basis for the interindividual differences in responses to βAR agonists and antagonists. Through deep resequencing of African American and Caucasian cohorts, we found SNPs in the promoter region of both GRK2 and GRK5. These SNPs, analyzed in haplotypes, can affect basal promoter activity, as measured by luciferase expression. We also found that although the magnitude of change in promoter activity following βAR agonist and antagonist response does not vary among haplotypes, only certain GRK5 promoter haplotypes exhibited an increase in activity following antagonist treatment. These results indicate that differential GRK2 and GRK5 expression due to promoter SNPs may increase or decrease

desensitization of βAR, affecting response to β_1AR antagonists used in the treatment of heart failure and β_2AR agonists used in the treatment of asthma and COPD. Further studies are required to determine the effect of SNPs on promoter activity in model systems more relevant to heart failure and asthma/COPD and to determine the effect of long-term agonist and antagonist exposure on promoter activity among haplotypes. Future studies are also required to examine the effect of variable expression on β_1AR and β_2AR desensitization and downregulation. Finally, the clinical impact of GRK2 and GRK5 promoter SNPs on β_1AR antagonist and β_2AR agonist therapies need to be analyzed by sequencing of GRK2 and GRK5 promoters in patients previously involved in studies analyzing response to βAR-targeted therapies.

In the analysis of the GRK4and β_1AR interaction, our studies demonstrated an increased phosphorylation of β_1AR in the presence of GRK4. Though this increase in β_1AR phosphorylation suggests increased desensitization, we did not observe statistically significant agonist-promoted desensitization of β_1AR in the presence of GRK4. Additionally, we did not find significant GRK4 haplotype-specific differences in desensitization of β_1AR. Several experimental conditions need to be optimized before these studies can continue. A cell type that produces consistent and robust expression of transiently-transfected GRK4 must be chosen. Additionally, the appropriate ratio of β_1AR to GRK4 expression needs to be determined. Once these conditions are optimized, evaluation of GRK4-mediated desensitization and the effect of SNPs in both GRK4 and β_1AR can be performed. Results from future studies analyzing differential receptor desensitization due to SNPs in GRK4 and β_1AR may elucidate the underlying cause of

the variable response to β_1AR antagonists seen among patients with different GRK4 SNPs.

5. Appendix

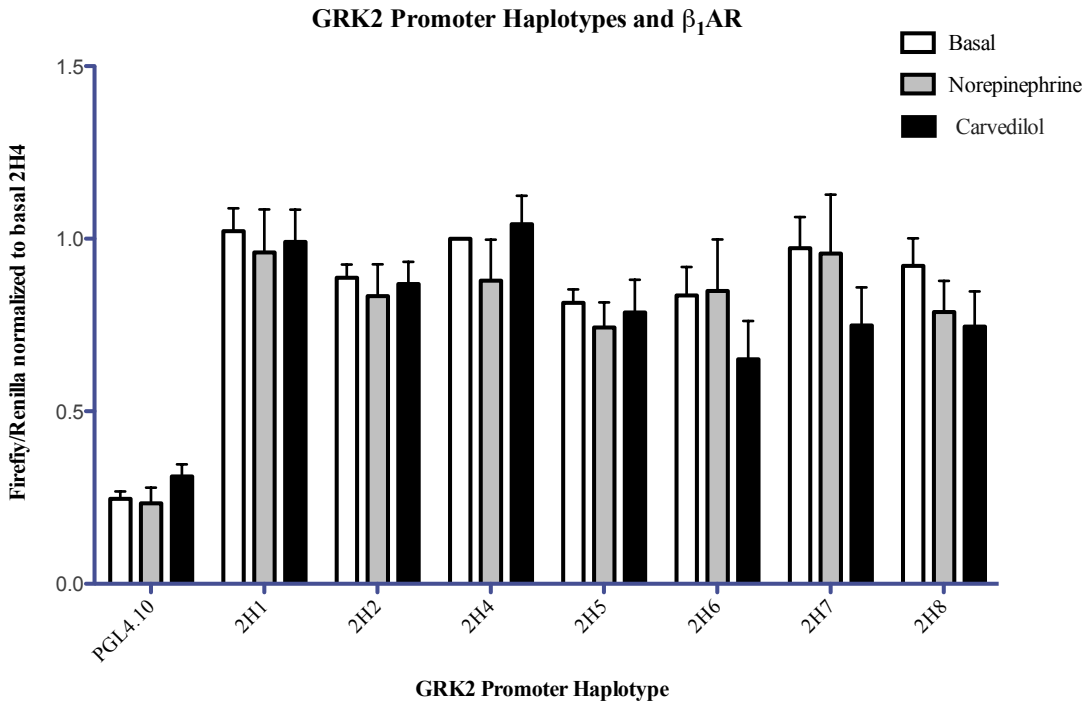

Figure A1. Drug-treated GRK2 promoter haplotype activity in the presence of β_1AR. GRK2 promoter haplotypes (in firefly luciferase reporter vector) and the empty vector (PGL4.10) were each co-transfected into HEK-293T cells with β_1AR and a *Renilla* luciferase transfection control. Promoter activity was assessed as its ability to drive firefly luciferase expression. Firefly luciferase expression was measured as light output produced by its interaction with substrate. Cells were assayed for luciferase expression following and 18-hour incubation in 100 μM ascorbic acid alone (n=11) or with either 10 μM norepinephrine (n=6) or 10 μM carvedilol (n=4) in the absence of serum. Expression is shown as the ratio of firefly luciferase activity to *Renilla* luciferase activity normalized to basal expression of the most common haplotype (2H4) to account for variability between experiments. Error bars represent standard error of the mean.

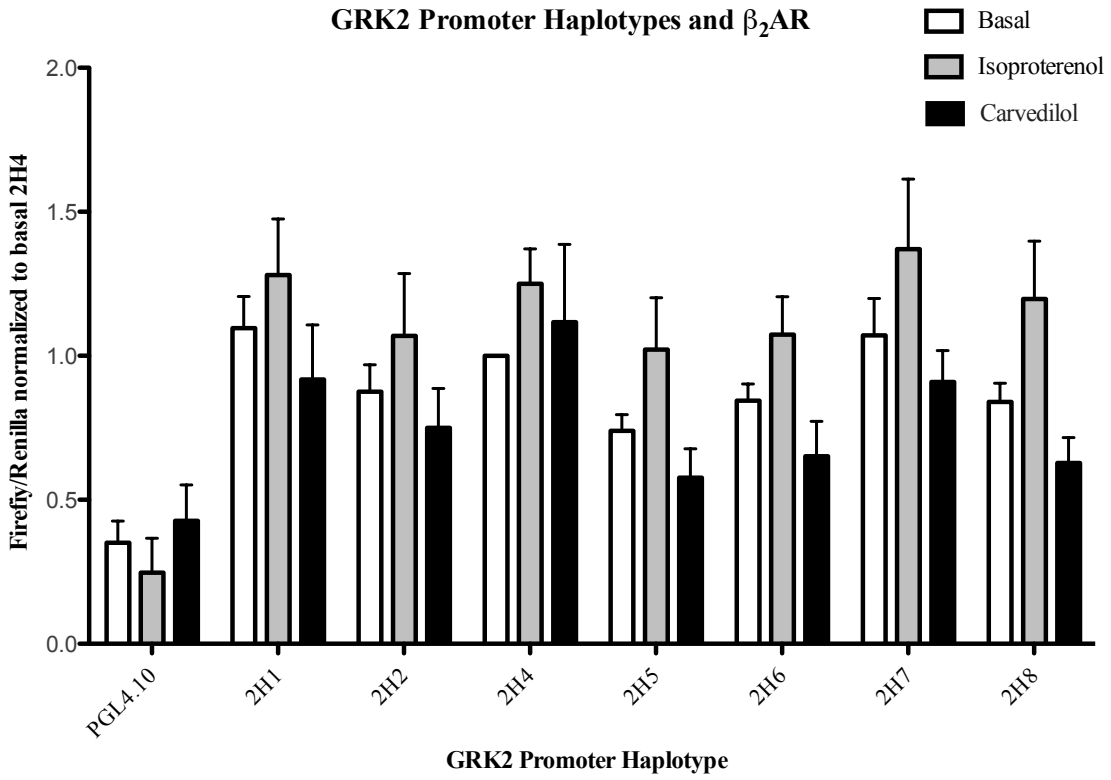

Figure A2. Drug-treated GRK2 promoter haplotype activity in the presence of β_2AR. GRK2 promoter haplotypes (in firefly luciferase reporter vector) and the empty vector (PGL4.10) were each co-transfected into HEK-293T cells with β_1AR and a *Renilla* luciferase transfection control. Promoter activity was assessed as its ability to drive firefly luciferase expression. Firefly luciferase expression was measured as light output produced by its interaction with substrate. Cells were assayed for luciferase expression following and 18-hour incubation in 100 μM ascorbic acid alone (n=9) or with either 10 μM norepinephrine (n=4) or 10 μM carvedilol (n=4) in the absence of serum. Expression is shown as the ratio of firefly luciferase activity to *Renilla* luciferase activity normalized to basal expression of the most common haplotype (2H4) to account for variability between experiments. Error bars represent standard error of the mean.

6. References

1. Gruber CW, Muttenthaler M, Freissmuth M. Ligand-based peptide design and combinatorial peptide libraries to target G protein-coupled receptors. *Curr Pharm Des*. 2010;16:3071-3088.

2. Go AS, Mozaffarian D, Roger VL, et al. Heart disease and stroke statistics--2013 update: A report from the american heart association. *Circulation*. 2013;127:e6-e245.

3. Al-Gobari M, El Khatib C, Pillon F, Gueyffier F. Beta-blockers for the prevention of sudden cardiac death in heart failure patients: A meta-analysis of randomized controlled trials. *BMC Cardiovasc Disord*. 2103;13:52.

4. Dorn GW, 2nd, Liggett SB.
Mechanisms of pharmacogenomic effects of genetic variation within the cardiac adrenergic network in heartfailure
. *Mol Pharmacol*. 2009;76:466-480.

5. Crim MT, Yoon SS, Ortiz E, et al. National surveillance definitions for hypertension prevalence and control among adults. *Circ Cardiovasc Qual Outcomes*. 2012;5:343-351.

6. Lewington S, Clarke R, Qizilbash N, Peto R, Collins R, Prospective Studies Collaboration. Age-specific relevance of usual blood pressure to vascular mortality: A meta-analysis of individual data for onemillion adults in 61 prospective studies. *Lancet*. 2002;360:1903-1913.

7. Pedersen ME CJ. The vasodilatory beta-blockers. *Curr Hypertens Rep*. 2007;9:269-277.

8. Rath G, Balligand JL, Dessy C. Vasodilatory mechanisms of beta receptor blockade. *Curr Hypertens Rep*. 2012;14:310-317.

9. Wilkinson R. Beta-blockers and renal function. *Drugs*. 1982;23:195-206.

10. Yawn BP.
Differential assessment and management of asthma vs chronic obstructive pulmonary dis ease. *Medscape J Med*. 2009;11:20.

11. Heron M, Hoyert DL, Murphy SL, Xu J, Kochanek KD, Tejada-Vera B. Deaths: final data for 2006. *Natl Vital Stat Rep*. 2009;57:1-134.

12. Billington CK, Penn RB. Signaling and regulation of G protein-coupled receptors in airway smooth muscle. *Respir Res*. 2003;4.

13. Kho C, Lee A, Hajjar RJ. Altered sarcoplasmic reticulum calcium cycling--targets for heart failure therapy. *Nat Rev Cardiol*. 2012;9:717-733.

14. Vinogradova TM, Lakatta EG.
Regulation of basal and reserve cardiac pacemaker function by interactions of cAMP-mediated PKA-dependent Ca2+ cycling with surface membrane channels. *J Mol Cell Cardiol*. 2009;47:456-474.

15. Friis UG, Madsen K, Stubbe J, et al.
Regulation of renin secretion by renal juxtaglomerular cells. *Pflugers Arch*. 2013;465:25-37.

16. Kach J, Sethakorn N, Dulin NO. A finer tuning of G-protein signaling through regulated control of RGS proteins. *Am J Physiol Heart Circ Physiol*. 2012;303:H19-H35.

17. Knight WE, Yan C. Cardiac cyclic nucleotide phosphodiesterases: Function, regulation, and therapeutic prospects. *Horm Metab Res*. 2012;44:766-775.

18. Chuang TT, Iacovelli L, Sallese M, De Blasi A. G protein-coupled receptors: Heterologous regulation of homologous desensitization and its implications. *Trends Pharmacol Sci*. 1996;17:416-421.

19. Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci*. 2002;115:455-465.

20. Pitcher JA, Freedman NJ, Lefkowitz RJ. G protein-coupled receptor kinases. *Annu Rev Biochem*. 1998;67:653-692.

21. Inglese J, Freedman NJ, Koch WJ, Lefkowitz RJ. Structure and mechanism of the G protein-coupled receptor kinases. *J Biol Chem*. 1993;268:23735-23738.

22. Osawa S, Weiss ER. A tale of two kinases in rods and cones. *Adv Exp Med Biol*. 2012;723:821-827.

23. Premont RT, Macrae AD, Stoffel RH, et al. Characterization of the G protein-coupled receptor kinase GRK4: Identification of four splice variants. *J Biol Chem*. 1996;271:6403-6410.

24. Pitcher JA, Fredericks ZL, Stone WC, et al. Phosphatidylinositol 4,5-bisphosphate (PIP2)-enhanced G protein-
coupled receptor kinase (GRK) activity. Location, structure, and regulation of the PIP2 binding site distinguishes theGRK subfamilies. *J Biol Chem*. 1996;271:24907-24913.

25. Kamal FA, Travers JG, Blaxall BC. G protein-coupled receptor kinases in cardiovascular disease: Why "where" matters. *Trends Cardiovasc Med*. 2012;22:213-219.

26. McGraw DW, Liggett SB. Heterogeneity in beta-adrenergic receptor kinase expression in the lung accounts for cell-specific desensitization of the beta2-adrenergic receptor. *J Biol Chem*. 1997;272:7338-7344.

27. Agüero J, Almenar L, Montó F, et al. Myocardial G protein receptor-coupled kinase expression correlates with functional parameters and clinical severity in advanced heart failure. *J Card Fail*. 2012;18:53-61.

28. Walker JK, Gainetdinov RR, Feldman DS, et al. G protein-coupled receptor kinase 5 regulates airway responses induced by muscarinic receptor activation. *Am J Physiol Lung Cell Mol Physiol*. 2004;286:312-319.

29. Mason DA, Moore JD, Green SA, Liggett SB. A gain-of-function polymorphism in a G-protein coupling domain of the human beta1-adrenergic receptor. *J Biol Chem*. 1999;274:12670-12674.

30. Moore JD, Mason DA, Green SA, Hsu J, Liggett SB. Racial differences in the frequencies of cardiac beta(1)-adrenergic receptor polymorphisms: Analysis of c145A>G and c1165G>C. *Hum Mutat*. 1999;14:271.

31. Rathz DA, Brown KM, Kramer LA, Liggett SB. Amino acid 49 polymorphisms of the human beta1-adrenergic receptor affect agonist-promoted trafficking. *J Cardiovasc Pharmacol*. 2002;39:155-160.

32. Rathz DA, Gregory KN, Fang Y, Brown KM, Liggett SB. Hierarchy of polymorphic variation and desensitization permutations relative to beta1- and beta2-adrenergic receptor signaling. *J Biol Chem*. 2003;278:10784-10789.

33. Liu WN, Fu KL, Gao HY, et al. Beta1-adrenergic receptor polymorphisms and heart failure: A meta-analysis on susceptibility, response to beta-blocker therapy and prognosis. *PLoS One*. 2012;7:e37659.

34. Bengtsson K, Melander O, Orho-Melander M, et al. Polymorphism in the beta1-adrenergic receptor gene and hypertension. *Circulation*. 2001;104:187-190.

35. Aleong RG, Sauer WH, Robertson AD, Liggett SB, Bristow MR. Adrenergic receptor polymorphisms and prevention of ventricular arrhythmias with bucin dolol in patients with chronic heart failure
. *Circ Arrhythm Electrophysiol*. 2013;6:137-143.

36. Kao DP, Davis G, Aleong R, et al. Effect of bucindolol on heart failure outcomes and heart rate response in patients with reduced ejection fraction heart failure and atrial fibrillation. *Eur J Heart Fail*. 2013;15:324-333.

37. Liggett SB, Mialet-Perez J, Thaneemit-Chen S, et al. A polymorphism within a conserved beta1-adrenergic receptor motif alters cardiacfunction and beta-

blocker response in human heart failure.. *Proc Natl Acad Sci U S A*. 2006;103:11288-11293.

38. Biolo A, Clausell N, Santos KG, et al. Impact of beta1-adrenergic receptor polymorphisms on susceptibility to heart failure, arrhythmogenesis, prognosis, and response to beta-blocker therapy. *Am J Cardiol*. 2008;102:726-732.

39. Terra SG, Hamilton KK, Pauly DF, et al. Beta1-adrenergic receptor polymorphisms and left ventricular remodeling changes in response to beta-blocker therapy. *Pharmacogenet Genomics*. 2005;15:227-234.

40. Johnson JA, Zineh I, Puckett BJ, McGorray SP, Yarandi HN, Pauly DF. Beta1-adrenergic receptor polymorphisms and antihypertensive response to metoprolol. *Clin Pharmacol Ther*. 2003;74:44-52.

41. Lee J, Aziz H, Liu L, et al. beta1-adrenergic receptor polymorphisms and response to beta-blockade in the african-american study of kidney disease and hypertension (AASK).. *Am J Hypertens*. 2011;24:694-700.

42. Suonsyrjä T, Donner K, Hannila-Handelberg T, Fodstad H, Kontula K, Hiltunen TP. Common genetic variation of beta1- and beta2-adrenergic receptor and response to four classes of antihypertensive treatment. *Pharmacogenet Genomics*. 2010;20:342-345.

43. Liu J, Liu ZQ, Yu BN, et al. Beta1-adrenergic
receptor polymorphisms influence the response to metoprolol monotherapy in patients wi
th essential hypertension. *Clin Pharmacol Ther*. 2006;80:23-32.

44. Karlsson J, Lind L, Hallberg P, et al. Beta1-
adrenergic receptor gene polymorphisms and response to beta1-
adrenergic receptor blockade in patients with essential hypertension
. *Clin Cardiol*. 2004;27:347-250.

45. Magnusson Y, Levin MC, Eggertsen R, et al. Ser49Gly of beta1-adrenergic receptor is associated with effective beta-blocker dose in dilated cardiomyopathy. *Clin Pharmacol Ther*. 2005;78:221-231.

46. Green SA, Cole G, Jacinto M, Innis M, Liggett SB. A polymorphism of the human beta 2-adrenergic receptor within the fourth transmembrane domain alters ligand binding and functional properties of the receptor. *J Biol Chem*. 1993;268:23116-23121.

47. Green SA, Turki J, Innis M, Liggett SB. Amino-terminal polymorphisms of the human beta2-adrenergic receptor impart distinct agonist-promoted regulatory properties. *Biochemistry*. 1994;33:9414-9419.

48. Hizawa N. Beta-2 adrenergic receptor genetic polymorphisms and asthma. *J Clin Pharm Ther*. 2009;34:631-643.

49. Martinez FD, Graves PE, Baldini M, Solomon S, Erickson R. Association between genetic polymorphisms of the beta2-adrenoceptor and response to albuterol in children with and without a history of wheezing. *J Clin Invest*. 1997;100:3184-3188.

50. Tan S, Hall IP, Dewar J, Dow E, Lipworth B. Association between beta2-adrenoceptor polymorphism and susceptibility to bronchodilator desensitisation in moderately severe stable asthmatics. *Lancet*. 1997;350:995-999.

51. Israel E, Chinchilli VM, Ford JG, et al. Use
of regularly scheduled albuterol treatment in asthma: genotype-stratified, randomised, placebo-controlled cross-over trial. *Lancet*. 2004;364:1505-1512.

52. Wechsler ME, Lehman E, Lazarus SC, et al. Beta-adrenergic receptor polymorphisms and response to salmeterol. *Am J Respir Crit Care Med*. 2006;173:519-526.

53. Isada A, Hizawa N, Shimizu K, et al. The Arg16Gly beta2-adrenergic receptor polymorphism influences long term clinicalresponses to beta2-agonist. *Aerugi*. 2008;57:713-721.

54. Liggett SB, Cresci S, Kelly RJ, et al. A GRK5 polymorphism that inhibits beta-adrenergic receptor signaling is protective in heart failure. *Nat Med*. 2008;14:510-517.

55. Wang WC, Mihlbachler KA, Bleecker ER, Weiss ST, Liggett SB. A polymorphism of G-protein coupled receptor kinase 5 alters agonist-promoted desensitization of beta2-adrenergic receptors. *Pharmacogenet Genomics*. 2008;18:729-732.

56. Lohmueller KE, Wong LJ, Mauney MM, et al. Patterns of genetic variation in the hypertension candidate gene GRK4: Ethnic variation and haplotype structure. *Ann Hum Genet*. 2006;70:27-41.

57. Gu D, Su S, Ge D, et al. Association study with 33 single-nucleotide polymorphisms in 11 candidategenes for hypertension in chinese. *Hypertension*. 2006;47:1147-1154.

58. Wang Y, Li B, Zhao W, et al. Association study of G protein-coupled receptor kinase 4 gene variants withessential hypertension in northern Han Chine se. *Ann Hum Genet*. 2006;70:778-782.

59. Speirs HJ, Katyk K, Kumar NN, Benjafield AV, Wang WY, Morris BJ. Association of G-protein-coupled receptor kinase 4 haplotypes, but not HSD3B1 or PTP1B polymorphisms, with essential hypertension. *J Hypertens*. 2004;22:931-936.

60. Wang Z, Armando I, Asico LD, Escano C, Wang X, Lu Q, Felder RA, Schnackenberg CG, Sibley DR, Eisner GM, Jose PA.
The elevated blood pressure of human GRK4gamma A142V transgenic mice is

not associated with increased ROS production. *Am J Physiol Heart Circ Physiol*. 2007;292:H2083-H2092.

61. Leineweber K, Rohe P, Beilfuss A, et al. G-protein-coupled receptor kinase activity in human heart failure: Effects of beta-adrenoceptor blockade. *Cardiovasc Res*. 2005;66:512-519.

62. Bhatnagar V, O'Connor DT, Brophy VH, et al. G-protein-coupled receptor kinase 4 polymorphisms and blood pressure response to metoprolol among african americans: Sex-specificity and interactions. *Am J Hypertens*. 2009;22:332-338.

63. Vandell AG, Lobmeyer MT, Gawronski BE, et al. G protein receptor kinase 4 polymorphisms: Beta-blocker pharmacogenetics and treatment-related outcomes in hypertension. *Hypertension*. 2012;60:957-964.

64. Premont RT, Koch WJ, Inglese J, Lefkowitz RJ. Identification, purification, and characterization of GRK5, a member of the family of G protein-coupled receptor kinases. *J Biol Chem*. 1994;269:6832-6841.

65. Ménard L, Ferguson SS, Barak LS, et al. Members of the G protein-coupled receptor kinase family that phosphorylate the beta2-adrenergicreceptor facilitate sequestration. *Biochemistry*. 1996;35:4155-4160.

66. Ungerer M, Böhm M, Elce JS, Erdmann E, Lohse MJ. Altered expression of beta-adrenergic receptor kinase and beta1-adrenergic receptors in the failing human heart. *Circulation*. 1993;87:454-463.

67. Petrofski JA, Koch WJ. The beta-adrenergic receptor kinase in heart failure. *J Mol Cell Cardiol*. 2003;35:1167-1174.

68. Rockman HA, Koch WJ, Lefkowitz RJ. Seven-transmembrane-spanning receptors and heart function
. *Nature*. 2002;415:206-212.

69. Matkovich SJ, Diwan A, Klanke JL, et al. Cardiac-specific ablation of G-protein receptor kinase 2 redefines its roles in heartdevelopment and beta-adrenergic signaling
. *Circ Res*. 2006;99:996-1003.

70. Dzimiri N, Muiya P, Andres E, Al-Halees Z.
Differential functional expression of human myocardial G protein receptor kinases in left ventricular cardiac diseases
. *Eur J Parmacol*. 2004;489:167-177.

71. Rockman HA, Choi DJ, Rahman NU, Akhter SA, Lefkowitz RJ, Koch WJ. Receptor-specific in vivo desensitization by the G protein-coupled receptor kinase-5 in transgenic mice. *Proc Natl Acad Sci U S A*. 1996;93:9954-9959.

72. Gainetdinov RR, Bohn LM, Walker JK, et al. Muscarinic supersensitivity and impaired receptor desensitization in G protein-coupled receptor kinase 5-deficient mice. *Neuron*. 1999;24:1029-1036.

73. Lipworth BJ. Airway subsensitivity with long-acting beta2-agonists. is there cause for concern?. *Drug Saf*. 1997;16:295-308.

74. Allayee H, de Bruin TW, Michelle Dominguez K, et al. Genome scan for blood pressure in dutch dyslipidemic families reveals linkage to a locus on chromosome 4p. *Hypertension*. 2001;38:773-778.

75. Province MA, Arnett DK, Hunt SC, et al. Association between the alpha-adducin gene and hypertension in the HyperGEN study. *Am J Hypertens*. 2000;13:710-718.

76. Chen W, Li S, Srinivasan SR, Boerwinkle E, Berenson GS. Autosomal genome scan for loci linked to blood pressure levels and trends since childhood: The bogalusa heart study. *Hypertension*. 2005;45:954-959.

77. Casari G, Barlassina C, Cusi D, et al. Association of the alpha-adducin locus with essential hypertension. *Hypertension*. 1995;25:360-362.

78. Villar VA, Jones JE, Armando I, et al. G protein-coupled receptor kinase 4 (GRK4) regulates the phosphorylation and function of the dopamine D3 receptor. *J Biol Chem*. 2009;284:1425-1434.

79. Dzimiri N, Muiya P, Andres E, Al-Halees Z. Differential functional expression of human myocardial G protein receptor kinases inleft ventricular cardiac diseases.. *Eur J Pharmacol*. 2004;489:167-177.

80. Rankin ML, Marinec PS, Cabrera DM, Wang Z, Jose PA, Sibley DR. The D1 dopamine receptor is constitutively phosphorylated by G protein-coupledreceptor kinase 4. *Mol Pharmacol*. 2006;69:759-769.

81. Harris RC. Abnormalities in renal dopamine signaling and hypertension: The role of GRK4. *Curr Opin Nephrol Hypertens*. 2012;21:61-65.

82. Hawes JW, Knudtson KL, Escobar H, et al. Evaluation of methods for sequence analysis of highly repetitive DNA templates. *J Biomol Tech*. 2006;17:138-144.

83. Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet*. 2001;68:978-989.

84. Alvarez R, Daniels DV. A separation method for the assay of adenylyl cyclase, intracellular cyclic AMP, and cyclic-AMP phosphodiesterase using tritium-labeled substrates. *Anal Biochem*. 1992;203:76-82.

85. Wigginton JE, Cutler DJ, Abecasis GR. A note on exact tests of Hardy-weinberg equilibrium
. *Am J Hum Genet*. 2005;76:887-893.

86. Hoffmann C, Leitz MR, Oberdorf-Maass S, Lohse MJ, Klotz KN. Comparative pharmacology of human beta-adrenergic receptor subtypes--characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch Pharmacol*. 2004;369:151-159.

87. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc Natl Acad Sci U S A*. 2002;99:3740-3745.

88. Vinson C, Chatterjee R. CG methylation. *Epigenomics*. 2012;4:655-663.

89. Ramos-Ruiz R, Penela P, Penn RB, Mayor F, Jr. Analysis of the human G protein-coupled receptor kinase 2 (GRK2) gene promoter: regulation by signal transduction syste ms in aortic smooth muscle cells
. *Circulation*. 2000;101:2083-2089.

90. Claycomb WC, Lanson NA, Jr, Stallworth BS, et al. HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci U S A*. 1998;95:2979-2984.

91. de Ligt RA, Kourounakis AP, IJzerman AP. Inverse agonism at G protein-coupled receptors: (patho)physiological relevance andimplications for drug discovery. *Br J Pharmacol*. 2000;130:1-12.