Identifying Molecular Mechanisms of β-arrestin Biased G Protein-Coupled Receptor Signaling

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Cell Biology in the Graduate School of Duke University

2017
ABSTRACT

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**Abstract**

G protein-coupled receptors (GPCRs) represent the largest and the most versatile family of cell surface receptors. Members of this receptor family translate diverse extracellular cues to intracellular responses, and are commonly targeted for medicinal therapeutics. In the current model of GPCR signaling, agonist binging not only initiates G protein-mediated signaling through generation of second messengers such as cyclic AMP and diacylglycerol, but also through the multifunctional adaptor protein β-arrestin acting as a signal transducer. While some ligands have balanced agonist activity defined as equal efficacy for G protein and β-arrestin-mediated pathways, other ligands stimulate GPCR signaling preferentially through β-arrestin, a concept know as β-arrestin-biased agonism.

The β1 and β2 adrenergic receptors (βARs) are the predominant GPCR subtypes expressed in the heart, and play an important role in the pathophysiology of human heart disease. Of the four families of Gα-proteins (Gαs, Gαi/o, Gαq/11 and Gα12/13), β1ARs are recognized as classical Gαs-coupled receptors since agonist binding promotes coupling to heterotrimeric Gs protein (Gαsβγ) triggering dissociation of Gαs from Gβγ. Here, I identify a new signaling mechanism unlike that previously known for any Gαs-coupled receptor, whereby the inhibitory G protein (Gαi) is required for β1AR-mediated β-arrestin-biased signaling. Stimulation with the β-arrestin-biased agonist carvedilol
induces switching of the β1AR from a classical Gαs-coupled receptor to a Gαi-coupled receptor and stabilizes a unique receptor conformation required for β-arrestin-mediated signaling. Recruitment of Gαi was not induced by any other βAR agonist or antagonist screened, nor was it required for β-arrestin-bias activated by the β2AR subtype of the βAR family.

I also found that Gαi is involved in the membrane stretch-induced signaling activated by the angiotensin II type 1 receptor (AT1R), another GPCR mediating a variety of physiological responses and one that is commonly targeted for cardiac drug therapy. It is appreciated that the AT1R can function as a mechanical sensor. When activated by mechanical stretch, AT1Rs transmit signaling through β-arrestin rather than the G protein pathways. To date, the ligand-independent membrane stretch-induced AT1R conformation that triggers signaling has been thought to be the same as that induced by a β-arrestin-biased agonist, which can selectively engage β-arrestin and prevent G protein coupling. Here I show that membrane stretch promotes a distinct biased conformation of the AT1R that couples to Gαi. In contrast, recruitment of Gαi was not induced by the balanced agonist angiotensin II, nor by the β-arrestin-biased agonist TRV120023. This stretch-triggered Gαi coupling induces the recruitment of and a unique conformational change in β-arrestin2, activating downstream signaling events, including EGFR internalization and ERK phosphorylation.
Taken together, I identified a previously unrecognized role for Ga in β-arrestin-biased GPCR signaling, suggesting that the concept of β-arrestin-bias may need to be refined to incorporate the selective bias of receptors towards distinct G protein subtypes. I also demonstrate that different mechanisms for β-arrestin bias may be operative between signaling induced by distinct receptor activation modes: in the case of AT1R, by β-arrestin-biased agonists versus by ligand-independent mechanical stretch.

These data underscore the complexity of β-arrestin-biased agonism and have important implications when considering the development of new therapeutic ligands to selectively target specific β-arrestin-biased signaling pathways.
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1. Introduction

1.1 G protein-coupled receptor signaling

G protein-coupled receptors (GPCRs), also known as seven-transmembrane receptors (7TMRs), represent the largest and the most versatile family of cell surface receptors [1]. Members of this receptor family can be activated by diverse extracellular stimuli, such as light, odors, hormones, or neurotransmitters [2]. GPCRs play important roles in a wide variety of physiological processes in human, and not surprisingly, are commonly targeted for medicinal therapeutics. Despite the large number of family members and the incredible variety of signaling molecules they bind, GPCRs share a common structure. They consist of seven α-helical transmembrane domains, the basis for their 7TMR name, in addition to intervening intracellular and extracellular loop regions, an extracellular N terminus, and an intracellular C terminus [3].

1.1.1 Classical GPCR signaling.

In the classical paradigm, GPCRs transduce signals through heterotrimeric guanine nucleotide–binding proteins (G proteins). G proteins are named for their ability to bind the nucleotides guanosine triphosphate (GTP) and guanosine diphosphate (GDP). They act as molecular switches in intracellular signaling: when bound to GTP, they are active (“on”) and capable of inducing specific effector enzymes; but when bound to GDP, they are inactive (“off”) [4]. Heterotrimeric G proteins are composed of α, β and γ subunits. When an external signaling molecule binds the GPCR, it causes a
receptor conformational change, triggering recruitment of G proteins on plasma membrane to the receptor to promote the GDP-GTP exchange on the Ga subunit, leading to activation of the G proteins. GTP binding leads to the dissociation of a G protein into two parts: the GTP-bound Ga subunit and the dimeric Gβγ complex (Figure 1). Both moieties can interact with a variety of signaling effectors, such as enzymes that produce second messengers, or ion channels. The Ga subunit will eventually hydrolyze the bound GTP to GDP, leading to the re-association of a Gβγ subunit complex and the termination of the G protein activation cycle. GPCRs are amplifiers, since one activated receptor can activate many individual G proteins.

![Image](image.png)

**Figure 1: Classical GPCR signaling.**
Upon ligand binding, GPCRs interact with heterotrimeric G proteins. The G protein undergoes a GDP-GTP exchange on the α subunit, leading to the dissociation of the α and βγ subunits and subsequent regulation of downstream signaling effectors.

Currently, there are 21 Ga subunits, 6 Gβ subunits and 12 Gγ subunits identified in human [4, 5]. Heterotrimeric G proteins are typically referred to by their Ga subunits, and are divided to four main classes: Ga
timulatory (Ga5), Gαinhibitory (Ga1), Gαq and Gα12 [6]. Different G protein subfamilies have distinct roles in regulating signal transduction.
(Figure 2). $G_{\alpha_s}$ stimulates adenylyl cyclase (AC) enzymes to generate the second messenger cyclic adenosine monophosphate (cAMP), leading to activation of protein kinase A (PKA) that then phosphorylates a variety of intracellular proteins to regulate a large array of cellular responses [7]. In contrast, $G_{\alpha_i}$ has inhibitory effect on adenylyl cyclase and therefore lowers cAMP levels in cells [8]. $G_{\alpha_q}$ activates phospholipase C (PLC), leading to the cleavage of membrane-bound phosphotidylinositol 4,5-bisphosphate (PIP$_2$) into the second messenger inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ promotes Ca$^{2+}$ release from endoplasmic reticulum, and this increased intracellular Ca$^{2+}$ level plus the DAG in the plasma membrane then activate the downstream protein kinase C (PKC) effectors [8-10]. $G_{\alpha_{12}}$ is known to activate the small GTPase RhoA [11]. Despite their differences in the recognition of distinct sets of effector molecules with specific regulatory roles in signaling, all G proteins share a similar mechanism of activation.

![Figure 2: G protein subtypes and their typical effectors.](image)

Different G protein subtypes regulate distinct signaling effectors. From [12].
1.1.2 Receptor desensitization

While GPCR signaling is essential for proper cellular function, sustained signaling activation can have detrimental effects on cell survival and can lead to severe consequences such as cardiac hypertrophy or tumorigenesis [13, 14]. Tightly regulated termination of GPCR signaling is therefore critical to maintain normal physiology. A number of mechanisms are involved in the termination of receptor signaling, including the G protein inactivation by GTP hydrolysis as mentioned previously, as well as facilitated GTP hydrolysis by RGS proteins. One additional mechanism limiting receptor activity is receptor desensitization that uncouples the receptor from signaling even in the continued presence of a ligand. The process of receptor desensitization involves phosphorylation of receptors at certain third intracellular loop and/or carboxy terminal tail (C-terminus) serine and threonine residues.

Protein kinases activated by receptor-triggered second messenger signaling, such as PKA and PKC, can in turn phosphorylate receptors leading to an uncoupling to their associated G proteins and serve as a negative feedback mechanism. PKA-mediated phosphorylation has also been shown to switch receptor coupling from one to another subtype of G protein. For instance, PKA-mediated phosphorylation of β2 adrenergic receptor decreases receptor affinity for Gaα, while it promotes receptor coupling to Gaα [15]. This triggers activation of Gaα-mediated pathways, while reducing Gaα-activated cAMP production.
Another common mechanism for GPCR desensitization involves receptor phosphorylation mediated by GPCR kinases (GRKs) [16, 17]. GRKs mediate phosphorylation of serine and threonine residues primarily in the carboxyl-tails of agonist-activated GPCRs, which promote translocation of β-arrestin adaptor protein to the phosphorylated receptor. β-arrestin binds to both the transmembrane core and the phosphorylated C-terminus of the receptor, and thus sterically interdict further G protein coupling to the receptor [18, 19], leading to the turning off of second messenger signal and also triggering receptor internalization via the clathrin-mediated endocytic machinery [20]. There are seven members in the GRK family. Among them, GRK1 and GRK7 are specifically expressed in retina, GRK4 shows a very localized expression in spermatozoa and germinal cells, while the other four members (GRK2, 3, 5, 6) are ubiquitously expressed [21, 22].

1.1.3 The multifunctional adaptor protein β-arrestins in GPCR signaling

β-arrestins are critical regulators of GPCR signaling, not only mediating receptor desensitization and internalization, but also initiating a large array of G protein-independent signaling events. β-arrestins are members of the arrestin family that consists of four members: arrestin1-4. Arrestin1 and arrestin4 are expressed in the retina, whereas arrestin2 (also named β-arrestin1) and arrestin3 (β-arrestin2) are widely expressed in somatic tissues [23]. The four arrestin proteins share sequence homology and a common structure, consisting of a N-domain and a C-domain built from anti-
parallel β strands and intervening loops [24-26]. It appears that the two β-arrestin isoforms are sufficient to regulate the large array of agonist-activated GPCRs throughout the body [23].

β-arrestins are critical molecules in mediating GPCR internalization by clathrin-coated vesicles. β-arrestins interact directly with clathrin and the clathrin adaptor protein AP2 to target the bound receptor to clathrin-coated endocytic vesicles, leading to receptor internalization [27, 28]. Internalized receptors may then be rapidly recycled to the plasma membrane, or may undergo sustained internalization to endosomes, from where they can be slowly recycled back to the cell surface or can proceed to lysosomes for degradation [8]. β-arrestins also regulate receptor internalization and degradation in part through their ability to regulate the ubiquitination of receptor. β-arrestins can directly interact with an array of ubiquitin ligases and deubiquitinases, allowing a finely-tuned regulation of the dynamics of ubiquitination/deubiquitination that determines the trafficking destination of the internalized receptors [29].

β-arrestins also function as signal transducers in their own right. Through their function as adaptor/scaffolding proteins, β-arrestins can activate a diverse range of intracellular signaling pathways as shown in Figure 3 [30]. For instance, β-arrestins can directly interact with the tyrosine kinase c-Src, leading to the formation of receptor-Src complexes and triggering the activation of extracellular signal-regulated kinase (ERK) [31]. β-arrestins mediate the transactivation of epidermal growth factor receptor (EGFR)
by $\beta_1$ adrenergic receptor and angiotensin II type 1 receptor, which also leads to ERK activation [32, 33]. Activated ERK may regulate protein synthesis through MAPK interacting serine/threonine kinase 1 (MNK1) [34], as well as mediate anti-apoptotic signaling by regulating the phosphorylation of BCL2-associated agonist of cell death (BAD) [35]. $\beta$-arrestins can regulate other kinases, such as phosphoinositide 3-kinase (PI3K) and its substrate AKT, leading to various cellular and physiological responses in the context of distinct GPCRs [36, 37]. $\beta$-arrestins also inhibit the nuclear factor $\kappa$B (NF-$\kappa$B)-targeted gene expression through binding to and stabilizing I$\kappa$B$\alpha$, the inhibitor of NF-$\kappa$B [38].

![Diagram of $\beta$-arrestin-mediated signaling of GPCRs.]

Figure 3: $\beta$-arrestin-mediated signaling of GPCRs.
$\beta$-arrestins regulate a diverse array of pathways downstream of GPCRs.
β-arrestin-mediated signaling appears to be dynamically and functionally divergent from that mediated by G proteins. While G protein signaling is rapid and transient, β-arrestin signaling is slower and more persistent [39]. For example, G protein-mediated activation of ERK leads to ERK translocation into the cell nucleus, where it phosphorylates and activates a variety of transcription factors. In contrast, ERK activated through β-arrestins is retained in the cytosol, phosphorylating a distinct set of substrates and leading to different cellular responses [39].

1.2 GPCR structures

Remarkable progress has been made in the past decade in the study of GPCR structures, revealing the 3-dimensional conformation of multiple GPCRs and some of their signaling complexes. One of the most well-characterized receptors is the β2 adrenergic receptor (β2AR). In 2011, an active conformation of β2AR, in complex with the agonist BI-167107 and the heterotrimeric Gs protein, was solved [40]. This structure showed that the intracellular side of the β2AR interacts with the N- and C-terminal α-helices of Gaα (Figure 4). Compared with inactive state, the β2AR undergoes significant conformational changes upon agonist activation: the outward shift of the C-terminus of transmembrane domain (TM) 5, TM6 and the connecting intracellular loop (ICL) 3, which forms an interface with the C-terminus of Gaα; the formation of a short helix in ICL2, the second interface for Gaα binding; the inward shift of the C-terminus of TM7 and the N-terminus of helix 8 (an amphipathic motif within the receptor carboxyl-
terminus with high affinity for plasma membrane lipids), which may be associated with receptor phosphorylation and β-arrestin recruitment (Figure 4). A recent structural study of the calcitonin receptor using cryo-electron microscopy reveals a very similar interface on the cytoplasmic side of the receptor TM domains with Gaα, suggesting a conserved receptor-G protein interface among GPCRs.

![Figure 4: β2AR structure.](image)

(a) The inactive (pink) and active (green, in complex with BI-167107 and the heterotrimeric Gs protein) conformations of β2AR. (b) Interaction interface of the β2AR and Gaα). Adapted from [41].

Structural studies have also shed light on the interaction of GPCRs with β-arrestins. A recent study solved the crystal structure of constitutively active rhodopsin in complex with pre-activated arrestin1 [42]. This study suggested that arrestin1 first interacts with the C-terminal tail of rhodopsin, which disrupts the intramolecular interaction of the N- and C- domains of arrestin1. This promotes the full interaction of
arrestin1 with the receptor intracellular pocket formed by the cytoplasmic side of the rhodopsin TM domains.

However, a recent study of β2AR-β-arrestin complexes revealed two distinct interaction modes of the receptor and β-arrestins [18]. This study used a chimeric receptor β2V2R, where the β2AR C-terminus was replaced with the C-tail of the vasopressin type 2 receptor, which has much higher affinity for β-arrestins while maintaining β2AR pharmacological properties. In this study, 60% of receptor-β-arrestin1 complexes have β-arrestin1 bound only to the C-terminal tail of the receptor. In the other 40%, β-arrestin1 is more engaged with the receptor through a β2AR transmembrane core association, presumably to the “finger loop” region of the activated β-arrestin1 (Figure 5).

**Figure 5: Interaction of β-arrestin1 and β2AR.**
Interaction of β-arrestin with the receptor probably involves two phases: first, the phosphorylated receptor C-terminal tail interacts with the N-domain of β-arrestin; the
second phase involves the insertion of finger loop (a flexible loop region in the β-arrestin central crest) into the receptor core. β2AR, orange; the V2R C-tail, yellow; β-arrestin 1, blue. From [18].

1.3 GPCRs and diseases

1.3.1 Cancer

GPCRs can act as proto-oncogenes. The overexpression of certain GPCRs has been observed in a various types of cancers. Sustained activation of GPCRs by their endogenous agonists also may induce transformed-like phenotypes. GPCRs also participate in tumor progression, angiogenesis and metastasis [12].

GPCRs may crosstalk with growth factor receptors to regulate the progression of cancer. For example, as mentioned previously, some activated GPCRs are capable of transactivating EGFR. GPCR-mediated EGFR transactivation has been observed in the progression of multiple cancers, such as lung, breast and prostate tumors. GPCRs may also regulate the insulin and insulin-like growth factor I receptor (IGFIR) signaling through the activation of the PI3K-AKT pathway, which also may contribute to the development of malignancies. Therefore, understanding the mechanism of GPCR-growth factor receptor crosstalk and identifying specific inhibitors of this process may facilitate the discovery of new therapeutic treatments for cancer.

GPCRs may also regulate cancer development through the regulation of Wnt and hedgehog signaling, two development morphogens that are critical regulators of cell
differentiation. Deregulation of these two pathways may induce the dedifferentiation and transformation of cells, leading to tumorigenesis.

1.3.2 Inflammation

A large number of GPCRs recognizing chemoattractants and chemokines are highly expressed on inflammatory cells. They play important roles in multiple inflammatory responses, including sensing chemoattractants, regulating the production of inflammatory cytokines, and regulating vascular endothelial permeability, etc. [43]. Therefore, GPCRs are critical regulators of the migration of inflammatory cells and of their accumulation at sites of inflammation. GPCRs also regulate the expression of inflammatory genes through regulating a number of transcription factors, such as c-Jun, CREB and NFkB. Since the enormous diversity of GPCR signaling significantly contributes to the complex regulation of inflammatory responses, a better elucidation of the involvement of GPCR signaling will greatly help understanding the process of inflammation and developing new therapeutic treatments.

1.3.3 Heart disease

Heart failure results from the inability of the heart to pump sufficient blood to meet the needs of the body. A number of GPCRs are involved in the development or cardiac disease, among which β adrenergic receptors (βARs)- and angiotensin II type 1 receptors (AT1Rs) are central. Chronic activation of these two receptors by their endogenous ligands, epinephrine and angiotensin respectively, increase the workload of
the heart, leading to detrimental effects on cardiac function, such as myocyte death and maladaptive cardiac remodeling [44]. Therefore, inhibitors of these receptors, including βAR antagonists and angiotensin II receptor blockers are widely used in the treatment of heart failure. These drugs attenuate symptoms of heart failure and improve cardiac function. However, they have limited beneficial effects for some patients due to side effects. For this reason, improved therapeutic agents need to be developed. Several studies have shown that the chronic activation of G protein signaling by these receptors leads to cardiotoxic effects, whereas their β-arrestin-mediated signaling is cardioprotective, suggesting that β-arrestin-biased agonists for these receptors might be good candidates as new drugs for heart failure.

1.4 Biased Agonism

1.4.1 The concept of biased agonism.

The ability of β-arrestins to mediate G protein-independent signaling events supports the notion that GPCRs activate complex signaling networks, rather than the simply activating second messengers in a linear chain of signals. This leads to the emerging concept of biased agonism, also known as functional selectivity, which describes the ability of ligands for one GPCR to activate distinct subsets of downstream signaling events by a given receptor [45]. It was originally thought that most ligands activate their receptors to signal equally well through both the G protein- and β-arrestin-dependent pathways, i.e., have a balanced efficacy towards these two signaling events.
Under this notion, receptor ligands are classified as full agonist, partial agonist that only partially activates downstream pathways, inverse agonist that inhibits basal activity or neutral antagonist that inhibits all activity by other ligands but does not alter basal activity [46]. In the last two decades, accumulating evidence has proved that ligands can activate receptor driven signaling pathways selectively, therefore regulating subsets of downstream signaling. Specific ligands can preferentially activate β-arrestin signaling without activating (or even blocking) the G protein pathway, or vice versa. These ligands are therefore termed biased agonists. The concept of biased agonism adds a new layer to our understanding of GPCR pharmacology, and provides new directions for therapeutic improvement by targeting only specific desired aspects of GPCR signaling.

1.4.2 Structural basis of biased agonism

The discovery of biased agonism implies that receptors can adopt multiple active conformations that differentially engage G proteins, β-arrestins, or other downstream partners. Accumulating evidences support the idea that different ligands stabilize distinct receptor conformations. With fluorescence spectroscopy, it has been shown that ligands with different efficacy have distinct effects on conformational changes in the β2 adrenergic receptor (β2AR), represented by the disruption of two molecular switches: the ionic lock that links the cytoplasmic ends of TM 3 and TM6, whose disruption is a key feature of receptor activation; and the rotamer toggle switch in TM6, also representing the movement of TM6 upon receptor activation [47]. A recent study using single
molecule fluorescence resonance energy transfer imaging also suggests that different agonists distinctively affect the TM6 motion of the Gs-coupled β2AR [48]. Moreover, a comprehensive study used chemical labeling and mass spectrometry to assess site specific conformational changes of β2AR induced by nine structurally and functionally distinct ligands, including full agonists, partial agonists, antagonists and β-arrestin-biased agonists [49]. This study showed a significantly different labeling patterns of the receptor induced by those ligands, indicating the ligand-specific conformations.

Similarly, recent studies suggest that G protein-biased and β-arrestin-biased agonists induce distinct conformations of the vasopressin type 2 receptor [50] and the cholecystokinin-2 receptor [51].

Conceptually, GPCRs can be considered as oscillating between a number of intermediate conformational states [52]. This conformational heterogeneity allows the receptors to differentially interact with distinct ligands and transducers, and subsequently signal through various pathways. Binding of ligands to a receptor shifts the equilibrium among distinct receptor conformations, with each ligand preferentially stabilizing a potentially unique set of conformational states that can selectively recruit signaling transducers.

1.4.3 The receptor bar code phosphorylation hypothesis

Given the important regulatory roles of GRK-mediated receptor phosphorylation on GPCR signaling properties, especially in recruitment of β-arrestins to the receptor,
the concept of a bar code of receptor phosphorylation has emerged as a potentially important mechanism for how ligand-specific receptor conformations selectively recruit specific transducers [53, 54]. According to this hypothesis, the receptor adopts distinct conformational states when bound to specific ligands, and each then recruits unique subsets of GRKs favoring distinct regions of the receptor, leading to phosphorylation of distinct arrays of residues on the receptor, generating a “bar code” phosphorylation pattern. These GRK-mediated differential phosphorylation patterns promote the recruitment of signal transducers, such as β-arrestins, stabilizing them into unique active conformations, and allowing activation of diverse signaling profiles (Figure 6, [53]).

Figure 6: Bar code phosphorylation of GPCRs.
Different ligands stabilize distinct receptor conformational states, leading to the recruitment of unique subsets of GRKs that subsequently mediate receptor phosphorylation at specific sites. The differentially phosphorylated receptors recruit β-arrestins and stabilize them into distinct conformations that activate divergent downstream signaling profiles. From [54].

This hypothesis is compatible with previous findings that the members of the GRK family differentially regulate signaling. For many GPCRs, including βAR and
AT1R, GRK5 and GRK6 are required for β-arrestin-mediated MAPK activation, whereas GRK2 and GRK3 mediate receptor internalization [53, 55, 56]. A previous study that characterized the β2AR phosphorylation pattern in response to the full agonist isoproterenol versus the β-arrestin biased ligand carvedilol, and also determined the responsible GRK subsets, provides direct support for this hypothesis [53]. While isoproterenol promotes both GRK2- and GRK6- mediated receptor phosphorylation at specific sites, carvedilol stimulates receptor phosphorylation only at GRK6-specific sites. The phosphorylation mediated by the two GRK subtypes also exhibits functional divergence. GRK2- or GRK6- mediated phosphorylation each promotes β-arrestin recruitment and receptor desensitization. In contrast, only GRK6-mediated phosphorylation is involved in the activation of downstream ERK signaling. These findings support the idea that different ligands induce unique bar code phosphorylation patterns on the receptor, leading to activation of subsets of β-arrestin signaling.

1.5 β adrenergic receptors (βARs)

Among the GPCRs, the β1 and β2 adrenergic receptors are the predominant GPCR subtypes expressed in the heart of many mammalian species including human, and are the principal regulators of cardiovascular function [57]. Under normal physiological conditions, the β1AR is the most abundant βAR subtype in cardiomyocytes, comprising about 80% of total βARs, whereas the β2AR comprises about 20%. The stoichiometry of the two βAR subtypes changes to about 60:40 under
conditions of heart failure, mainly caused by the selective downregulation of the β1AR expression level [58].

The βARs play an important role in the pathophysiology of human heart disease and are common therapeutic drug targets. The βARs are traditionally activated by the catecholamine hormones epinephrine and norepinephrine. The β1AR primarily couples to Gαs, which activates the signaling effector adenylyl cyclase to promote the second messenger cAMP production and activates PKA, regulating a diverse array of intracellular responses and ultimately the inotropic and chronotropic cardiac functions [58]. The β2AR also primarily couples to Gαs, but can also couple to Gαi through a G protein switching mechanism induced by PKA-mediated receptor phosphorylation [15]. Several studies suggest that excessive β1AR signaling promotes the apoptosis of cardiomyocytes and exerts detrimental effects to the heart, whereas β2AR signaling has anti-apoptotic and cardioprotective effects, suggested to be due to Gαi-mediated signaling [59-61].

βAR antagonists, also termed “β-blockers”, are one of the most widely used classes of drugs in numerous conditions, especially in cardiovascular diseases such as hypertension, post-acute myocardial infarction (AMI) and heart failure [62]. For example, recent studies suggest that treatment with the β-blockers carvedilol significantly reduces the morbidity and mortality of heart failure patients [63]. Treatment with β-blockers normalizes βAR signaling by preventing excessive receptor
activation and reversing receptor down-regulation, and improves left ventricular contractile function. The main cardiovascular use of β-blockers is to block deleterious G protein over-activation in the heart. The main side effects involve bronchial and blood vessel constriction, which are mainly caused by global inhibition of β2ARs in other tissues [64]. Therefore, understanding the signaling differences between βAR subtypes, as well as the mechanism of how β-blockers regulate signaling, will help to improve the therapeutic treatment of cardiovascular diseases.

The β-blocker carvedilol in particular has been identified as a β-arrestin-biased βAR ligand that preferentially activates β-arrestin-mediated pathways while having inverse agonism towards Gaα signaling [54, 65]. Carvedilol-stimulated βAR activates a diverse array of signaling events in β-arrestin-dependent manner, including transactivation of the epidermal growth factor receptor (EGFR) and induction of extracellular signal-regulated kinase (ERK) [31]. β-arrestin-mediated EGFR transactivation might have a cardioprotective effect: compared with transgenic mice overexpressing wildtype β1ARs, mice overexpressing a mutant β1AR lacking GRK phosphorylation sites, that cannot recruit β-arrestin and therefore cannot transactivate EGFRs, showed increased apoptosis and cardiac dilation [32]. Thus, β-arrestin-dependent βAR signaling appears to be beneficial to the heart.
1.6 The angiotensin II type 1 receptor (AT1R)

The AT1R is another member of the GPCR superfamily that is a well-known regulator of blood pressure and cardiac function [13]. Angiotensin receptor blockers and angiotensin-converting enzyme (ACE) inhibitors are widely used in the treatment of heart failure. In the heart, the AT1R is mainly expressed in cardiac fibroblasts and cardiomyocytes, where it plays vital roles in cardiac remodeling. Its overexpression promotes cardiac fibrosis and cardiac hypertrophy, whereas the knockout of AT1R showed enhanced cardiac function after myocardial infarction, suggesting that the AT1R has detrimental cardiac effects [66, 67]. The endogenous ligand for AT1R, angiotensin II (AngII), is a peptide hormone that regulates a number of important physiological processes, such as vascular smooth muscle contraction and aldosterone release [68]. AngII is a principle component of the renin-angiotensin system (RAS), a key regulatory system controlling blood pressure. The level of AngII is increased in cardiac hypertension and heart failure, increasing receptor activity.

AT1R activation by AngII also regulates oxidative stress and reactive oxygen species (ROS) signaling, which plays an important role in the initiation and progression of cardiac dysfunction [69]. AT1R signaling promotes the activation of NADPH oxidases in vascular smooth muscle cells, leading to the production of ROS. ROS generated induces changes in the structure and function of the vasculature by regulating
transcription factors involved in atherosclerosis, such as NFκB and Nrf2, as well as through promoting vascular inflammation by inducing cytokine release [70].

In response to the balanced agonist angiotensin II (AngII), AT1Rs couple to the heterotrimeric G₉ protein, triggering the dissociation of Gaq from Gβγ, which subsequently activates the signaling effector phospholipase C (PLC) to promote generation of the second messengers inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) [13]. The AngII-stimulated AT1R also transduces signals through the multifunctional adapter protein β-arrestin [23]. Several artificial AT1R ligands, such as [Sar₁, Ile₄, Ile₈]-AngII (SII) and TRV120023, have been shown to selectively activate β-arrestin-mediated pathways over G₉ pathways, and are therefore identified as β-arrestin-biased agonists [45, 71].

AT1Rs are also known as a mechanosensors. Mechanical stress is a critical regulator of cardiac function and is one of the most important stimuli in the development of cardiac hypertrophy [72]. Blood flow into the left and right ventricle stretches the cardiac muscle fibers and thereby increases the contraction force, leading to increased heart stroke volume. Mechanical stress induces a variety of hypertrophic responses, such as regulating the expression of hypertrophic genes, increasing protein synthesis and the activity of multiple protein kinases. Treatment with AT1R blockers attenuates these mechanical stress-induced hypertrophic responses in cardiac myocytes, suggesting the important role of AT1R in transmitting the extracellular mechanical
stimuli into intracellular signaling pathways that lead to the change of physiological or pathological responses [73]. Therefore, a better understanding of the mechanical stress-induced AT1R signaling may be helpful for the development of new therapeutic treatments for cardiac hypertrophy.

Mechanical stress can activate AT1Rs through promoting the autocrine generation of AngII by cardiomyocytes, as well as through AngII-independent pathways [74-76]. Recent studies suggest that mechanical stress directly and specifically activates β-arrestin-biased signaling of AT1Rs, independent of ligands and G proteins, by allosterically stabilizing a unique β-arrestin-biased AT1R conformation [77, 78].

Biophysical analysis of the AT1R conformation suggest that, when the receptor is activated by mechanical stretch, transmembrane domain 7 of the AT1R undergoes an anticlockwise rotation that shift it into the ligand binding pocket [79].

1.7 Objective of the study

The multifunctional β-arrestin adaptor proteins are critical components of GPCR signaling and are involved in various physiological and pathological processes. Compared with the classical G protein signals, β-arrestin-mediated signaling is a relatively new area of GPCR research, and continued efforts in this area will lead to the discovery of new signaling mechanism. The emerging concept of biased agonism opens a new avenue for therapeutic improvement by selectively targeting desired GPCR signaling pathways. Given the important roles of βARs and AT1Rs in the regulation of
cardiac function and the potential therapeutic advantage of β-arrestin-biased agonists, this study aimed to identify molecular mechanisms of β-arrestin-biased signaling by the βAR and AT1R. Specifically, this study identified a new component in the β1AR- and AT1R- mediated β-arrestin biased signaling: Ga, and demonstrated that the previously considered G protein-independent signaling actually requires selective coupling of receptors to Ga. Moreover, this study identified the remarkable G protein switching property of β1ARs and AT1Rs in response to distinct modes of activation to mediate β-arrestin-biased signaling.
2. Materials and Methods

2.1 Cell Culture

HEK293 cells were cultured in complete medium (minimum essential medium (MEM) supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin (Life technology)) at 37 °C in a humidified environment with 5% CO₂. HEK293 cells stably expressing FLAG-tagged β₁AR or FLAG-tagged β₂AR were maintained in complete medium supplemented with 150 µg/mL Geneticin (Thermofisher Scientific) [80] [81]. HEK293 cells stably expressing β₁AR-FRET sensor used for the FRET experiments were maintained in complete medium with 300 µg/mL Geneticin. HA-tagged AT1R stable cells were maintained in complete medium supplemented with 100 µg/ml of zeocin (Invitrogen) [77]. HEK293 cells stably expressing cyan fluorescent protein (CFP)-tagged AT1R and yellow fluorescent protein (YFP)-tagged β-arrestins (β-arrestin1 or β-arrestin2) were cultured in complete medium with 300 µg/mL of zeocin, and 150 µg/mL of Geneticin. For serum starvation, cells were incubated overnight in serum-free medium supplemented with 0.1% BSA, 10 mM HEPES and 1% penicillin-streptomycin.

2.2 Generation of β-arrestin or Gαi knockout cell line with CRISPR-Cas9 gene editing

Plasmids carrying S. pyogenes Cas9 (SpCas9) next to a cloning site for guide RNA (gRNA) with EGFP (pSpCas9 (BB)-2A-GFP, Addgene 48138) or puromycin
resistant gene (pSpCas9(BB)-2A-Puro, Addgene 48138) were obtained from Addgene (deposited by the laboratory of Dr. F. Zhang, [82]). Designing of the guide RNAs for G\(\alpha_i\) or \(\beta\)-arrestins and cloning the guide RNAs into the Cas9 plasmids were performed as previously described [82].

For \(\beta\)-arrestin knockout cells, \(\beta\)-arrestin1 was targeted using guide sequence oligos (top: CACCAGGGTCCACGAGGTCGATGC; bottom: AACCAGGGTCCACGAGGTCGATGC). \(\beta\)-arrestin2 was targeted using guide sequence oligos (top: CACCAGGGTCCACGAGGTCGATGC; bottom: AACCAGGGTCCACGAGGTCGATGC). The guide sequence oligos were cloned into pSpCas9(BB)-2A-Puro. After confirming the cloning by sequencing, plasmids were transfected into HEK293 cells using Fugene 6 transfection reagent (Promega). 72 h after transfection, cells were harvested to check INDEL (insertion deletion) in the genome by surveyor’s assay. Puromycin (2.5 \(\mu\)g/mL) was added into the medium of surveyor positive cells to select cells with the plasmid containing the puromycin resistance gene along with guide RNA and Cas9. The knockout of \(\beta\)-arrestins were confirmed by western blot.

For G\(\alpha_i\) knockout cells, G\(\alpha_{i1}\) was targeted using guide sequence oligos (top: CACCAGGGTCCACGAGGTCGATGC; bottom: AACCAGGGTCCACGAGGTCGATGC). G\(\alpha_{i2}\) was targeted using guide sequence oligos (top: CACCAGGGTCCACGAGGTCGATGC; bottom: AACCAGGGTCCACGAGGTCGATGC).
AAACGCAGTACCGGGCGGTTGTCTC), and Gα3 was targeted using guide sequence oligos (top: CACCGGGACGGCTAAAGATTGACTT; bottom: AAACAAGTCAATCTTTAGCCGTCCC). The guide sequence oligos were cloned into pSpCas9 (BB)-2A-GFP. Plasmids targeting the three Gαi subtypes were co-transfected into HEK293 cells. GFP positive cells were selected by fluorescence-activated cell sorting, diluted for growth and single cell colonies were obtained. The Gαi knockout were confirmed by western blot.

2.3 Immunoblotting and immunoprecipitation

Following stimulation, cells were scraped into either 1% NP-40 lysis buffer (20 mM Tris, pH7.4, 137 mM NaCl, 20% glycerol, 1% Nonidet P-40, 2 mM sodium orthovanadate, 1 mM PMSF, 10 mM sodium fluoride, 10 µg/ml aprotinin, 5 µg/ml leupeptin and phosphatase inhibitors), 1% DDM lysis buffer (20 mM HEPES, 150 mM NaCl, 1% n-Dodecyl βD-maltoside, protease inhibitors and phosphatase inhibitors), or 0.5% MNG lysis buffer (20 mM HEPES, 150 mM NaCl, 0.5% Lauryl Maltose Neopentyl Glycol, protease inhibitors and phosphatase inhibitors). For immunoprecipitation of FLAG-tagged β1AR or β2AR, 1 to 2 mg of protein was incubated overnight with 30 µl of anti-FLAG M2 magnetic beads (Sigma). For immunoprecipitation of active Gαi, protein was incubated for 2 h with anti-active Gαi antibody (New East Biosciences) and Protein A/G beads (EMD Millipore). For immunoprecipitation of HA-tagged AT1R, 1 mg of protein was incubated with 30 µl of anti-HA magnetic beads (Pierce) for overnight.
Immunoprecipitates or cell lysate samples were separated by SDS-PAGE, transferred to PVDF membrane (Bio-Rad) and subjected to immunoblotting with various primary antibodies. Immunoblots were detected using enhanced chemiluminiscence (Thermo Fisher Scientific) and analyzed with ImageJ software.

### 2.4 ERK phosphorylation in mouse heart

In the βAR study, eight to twelve-week-old gender-matched β₁AR knockout (β₁AR KO) mice and β₂AR KO mice [83] were used for this study. Mice were pretreated with vehicle or 25 µg/kg pertussis toxin (PTX) via intraperitoneal injection. After 48 h, mice were anesthetized with ketamine (100 mg/kg) and xylazine (2.5 mg/kg) for 10 min. Heart was then excised and, with aorta cannulated with a needle, then perfused with perfusion buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, 0.5 mM Na-EDTA, 5.5 mM glucose) with O₂ bubbling through Langendorff apparatus (Hugo Sachs Harvard Apparatus) set at 37°C. After 10 min perfusion, buffer was changed to perfusion buffer with vehicle or with 10 µM carvedilol, and perfused for another 10 minutes. Heart was then removed from the system and the left ventricle was excised and snap frozen in liquid nitrogen. Animal experiments in this study were carried out according to approved protocols and animal welfare regulations approved by the Institutional Animal Care and Use Committee of Duke University Medical Center.
In the AT1R study, wild type mice were used. Mice were PTX-pretreated and anesthetized as described above. On the excised heart, we performed an *ex-vivo* LV balloon stretch experiment as previously described [77]. Briefly, 8 to 12 weeks old, gender matched C57/B6 wild type mice (purchased from Jackson laboratory) were pretreated with 25 µg/kg of PTX or vehicle through intraperitoneal (IP) injection at 48 hours before experiment. LV balloon was made by stretching a polypropylene membrane into the shape of the LV cavity. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg), and heparinized by IP injection. After 10 min, the chest was opened and heart was excised. Aorta was cannulated with a needle and the balloon was inserted into the LV through the mitral valve. The heart was retrograde perfused with buffer containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, 0.5 mM Na-EDTA, and 5.5 mM glucose with O₂ bubbling through Langendorff apparatus (Hugo Sachs Harvard Apparatus) set at 37°C. LV balloon was connected to a Statham P23 Db pressure transducer (Gould Statham Instruments) through Polyethylene-50 (PE-50) tube for pressure monitoring. Intraballoonal pressure was recorded continuously with a pressure-recording system (MacLab, Millar Instruments). Balloon inflation was started after 10 min of perfusion, and diastolic intraballoonal pressure was kept within 30-50 mmHg with minor adjustment. Control was defined as perfusion without balloon inflation for identical periods of time. After 10 min balloon inflation, the heart was removed from
the system, and the left ventricle was snap frozen in liquid nitrogen after removing atrium and right ventricle. If intraballon pulse pressure was less than 20 mmHg or the heart rate was less than 100 bpm continuously, the heart was excluded from this study.

2.5 Fluorescence resonance energy transfer (FRET) measurement

FRET measurement was performed as previously described [84]. Briefly, HEK293 cells stably expressing β1AR-FRET sensor, in which Cerulean (Cer) and YFP are inserted in the C terminus and third intracellular loop of β1AR, respectively, were cultured in glass-bottomed confocal dishes. Cells were pretreated with vehicle or 200 ng/ml PTX for 16h before experiment. On the day of experiment, cells were maintained in FRET buffer (10mM HEPES, 0.2% BSA, 140 mM NaCl, 4.5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, pH 7.4). FRET experiments were preformed using an Olympus IX-71 microscope. FRET was monitored as the emission ratio of YFP to Cerulean. Images were taken at 10 s interval and analyzed with ImageJ software.

2.6 In situ proximity ligation assay

Cells were cultured in 35 mm poly-d-lysine coated glass-bottom confocal dish (MatTek). Following stimulation, cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.2% Triton-X-100 for 10 min. After blocked with blocking buffer from Duolink Detection Kit (Sigma) at 37°C for 30 min, cells were incubated overnight at 4°C with anti- β1AR (or β2AR) antibody from rabbit (Santa Cruz) or the anti-
HA rabbit antibody (Sigma-Aldrich) in conjunction with anti-Gαi antibody from mouse (New East Biosciences). The proximity ligation reaction was performed according to the manufacturer’s protocol using the Duolink Detection Kit (Sigma). Cells were mounted with DAPI Fluoromount-G (Southern Biotech). Images were recorded with Zeiss Axio Observer Z1 confocal microscope with 40X objective. Data analysis was performed with ImageJ software. To quantify the mean PLA signal per cell, the red PLA fluorescence intensity was divided by the number of cells. The mean PLA signal of each data set was corrected by subtracting the background staining determined as the mean PLA signal of HEK293 cells without receptor overexpression. The relative fold over non-stimulation was normalized to the mean PLA signal of the unstimulated cells. In each experiment, 20 to 40 cells from three images were quantified for each condition.

### 2.7 Confocal microscopy

In the EGFR internalization assay, HEK293 cells stably expressing FLAG-tagged β1AR or HA-tagged AT1R were transfected with EGFR-GFP together with control siRNA or β-arrestin siRNA as described below. After 24 h, the transfected cells were plated into glass-bottomed confocal dishes and kept in culture for an additional 24 h. Following pretreatment with PTX and stimulation with ligands, cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde for 15 min.

In the β-arrestin translocation assay, HEK 293 cells stably expressing CFP-tagged AT1R and YFP-tagged β-arrestin1 or β-arrestin2 were transiently transfected with GRK5
and then plated in collagen-coated glass bottom cell culture dishes. After overnight serum-starvation with vehicle or 200 ng/ml PTX, cells were stimulated with 1µM AngII, 1µM TRV120023 or osmotic stretch for 10 minutes, and then fixed with 4% PFA.

Cells were visualized using using the Zeiss Axio Observer Z1 confocal microscope with 63X objective. In each experiment, 50 cells of each condition were counted under microscope. Quantification of EGFR internalization or β-arrestin translocation was performed by counting the percentage of the cells showing intracellular aggregates of EGFR or β-arrestin, respectively.

**2.8 EGFR internalization assessed by flow cytometry**

HEK293 cells (wildtype, β-arrestin knockout or Gαi knockout) were transfected with CFP-tagged β1AR. 48 h after transfection, cells were serum starved for 4h before ligand stimulation. Following stimulation, cells were dissociated with accutase, washed with PBS and fixed in 4% formaldehyde for 15 min at room temperature. Fixed cells were enumerated, washed twice with staining buffer (PBS, 0.5% BSA, 2 mM EDTA) and blocked with 5% rat serum (Sigma) in staining buffer for 15 min. 1x10⁶ cells for each sample were stained with equal concentrations of either PE-conjugated EGFR antibody (R&D systems; clone # 423103) or isotype control (R&D systems; PE-conjugated rat IgG2A) for 30 min at room temperature. Following staining, cells were washed twice with staining buffer and resuspended in PBS for analysis utilizing a BD LSRII flow cytometer (BD Biosciences). Data analysis was performed with FlowJo software.
Following doublet exclusion, single cells were gated for CFP positivity. To quantify relative EGFR internalization following ligand stimulation, the following formula was utilized: geometric mean fluorescence intensity (MFI) of the PE-EGFR signal for each data set minus MFI of the isotype control. The resultant value was normalized to the MFI of the unstimulated cells to assess the relative percentage of EGFR internalization.

2.9 β-arrestin siRNA knockdown

SiRNAs targeting β-arrestin have been described previously [13]. A nonsilencing RNA duplex (5’-AAUUCUCCGAACGUGACGU-3’) was used as a control. HEK293 cells stably expressing FLAG-tagged β1AR were seeded into 10 cm dish on the day before to reach 30% to 40% confluence at the time of transfection. SiRNA were transfected using GeneSilencer Transfection Reagent (Gene Therapy Systems) according to the manufacturer’s protocol. In brief, 20 µg siRNA and 240 µl siRNA dilution buffer were added into 180 µl serum free medium, whereas 51 µl of transfection reagent was mixed with 300 µl serum free medium. Both solutions were allowed to stand for 5 min at room temperature, then combined and incubated for additional 20 min. The mixture was then added to cells in the 10 cm dish with 4 ml serum free medium. After 4 h incubation at 37 °C and 5% CO2, 5.5 ml of MEM containing 20% FBS and 2% penicillin-streptomycin were added into the dish. All assays were performed 3 d after siRNA transfection.
2.10 Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons were performed using Student’s t-test or ANOVA with Bonferroni correction for multiple comparisons in Graphpad Prism. Differences were considered statistically significant at P < 0.05.
3. Gαi is required for carvedilol-induced β1 adrenergic receptor β-arrestin biased signaling

3.1 Introduction

In medicine, ligands that target β-adrenergic receptors (βARs) are commonly used therapeutic agents because they regulate many important physiological processes involved in cardiovascular and pulmonary function [57]. Among the different G protein subtypes, βARs primarily transmit signals through Gαs [58]. As mentioned above, βARs can also transduce signaling through β-arrestins to stimulate distinct arrays of signaling and cellular responses, suggesting a much greater complexity of GPCR signaling than the two-state (active or inactive) model whereby multiple receptor conformations can exist, each with a different affinity for its transducer, resulting in the activation of distinct cellular signaling pathways [49] [85] [86]. Whereas balanced ligands, such as isoproterenol, stabilize βAR conformations signal with equal efficacy through G proteins and β-arrestins, some ligands stabilize conformations that selectively recruit only one of the transducers to stimulate a specific subset of cellular signals, a process termed “biased agonism” [87] [54]. As biased ligands may be capable of selectively activating beneficial signaling while simultaneously blocking untoward receptor activated pathways [88], understanding mechanisms of biased agonism can have important implications for drug discovery targeting GPCRs.

To date, the prevailing mechanistic concept of β-arrestin-bias for the Gαs-coupled β1AR is ligand stimulated recruitment and activation of β-arrestin in the absence of G
protein coupling. However, recently it has been demonstrated for the angiotensin II type 1 receptor that the weak β-arrestin-biased agonist, [Sar^4]Ile^8]Ile-angiotensin II, is capable of activating both G\(\alpha_q\) and G\(\alpha_i\) [89], indicating a possible role of G proteins in β-arrestin-mediated signaling. Moreover, recent biophysical work suggests that both G protein and β-arrestin can simultaneously interact with an activated GPCR to form super-complexes [90], raising the possibility that the recruitment of β-arrestin to the receptor may not preclude interaction with a G protein. Here we set out to test whether G protein coupling is a critical component of carvedilol-induced β-arrestin-biased β\(_1\)AR signaling.

### 3.2 Results

#### 3.2.1 G\(\alpha_i\) is required for carvedilol-induced β\(_1\)AR-mediated, but not β\(_2\)AR-mediated, ERK phosphorylation in vitro and in vivo.

Previous studies have demonstrated that carvedilol induces βAR-mediated ERK phosphorylation in a G\(\alpha_s\)-independent, β-arrestin-dependent manner [65] [91]. To determine whether G\(\alpha_i\) is required for carvedilol-stimulated βAR signaling, we tested the effect of the G\(\alpha_i\) inhibitor pertussis toxin (PTX) on carvedilol-stimulated ERK phosphorylation in HEK293 cells stably expressing FLAG-tagged β\(_1\)AR or β\(_2\)AR. PTX catalyzes the ADP-ribosylation of G\(\alpha_i\) and prevent G\(\alpha_i\) coupling to ligand bound receptors. In β\(_1\)AR stable cells, carvedilol dose dependently increased ERK phosphorylation, which was significantly diminished by pretreatment with the G\(\alpha_i\) inhibitor PTX (Figure 7). In contrast, PTX had no effect on the carvedilol-induced β\(_2\)AR-
mediated ERK phosphorylation (Figure 7). These observations suggest that $G_{\alpha_i}$ is needed for carvedilol-induced $\beta_1$AR, but not $\beta_2$AR signaling.

**Figure 7: Effect of PTX on carvedilol-induced $\beta$AR-mediated ERK phosphorylation in HEK293 cells.**

HEK293 cells stably expressing FLAG-tagged $\beta_1$ARs or $\beta_1$ARs were pretreated with vehicle or 200 ng/ml PTX for 16 h, then stimulated with indicated concentration of carvedilol for 5 min (a), or stimulated with 10 $\mu$M carvedilol for indicated time (b). The
response in $\beta_1$AR stable cells were blocked by PTX, whereas that in $\beta_2$AR stable cells were PTX-insensitive. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance vs. control cells was assessed using two-way ANOVA with Bonferroni correction. Carv: carvedilol; pERK: phosphorylated ERK; tERK: total ERK; NS: no stimulation.

To further delineate the role of $G_{\alpha_i}$ in carvedilol-induced $\beta$AR signaling, we measured the level of ERK activation in $\beta_1$AR or $\beta_2$AR stable cells after removing $G_{\alpha_i}$ using CRISPR/Cas9 gene editing. All three subtypes of $G_{\alpha_i}$ ($G_{\alpha_{1i}}, G_{\alpha_{2i}}$ and $G_{\alpha_{3i}}$) were depleted with their specifically targeted guide RNAs (Figure 8a). $G_{\alpha_i}$ depletion markedly blocked carvedilol-induced ERK phosphorylation in $\beta_1$AR stable cells, while it had no effect in $\beta_2$AR stable cells (Figure 8b). The absence of $G_{\alpha_i}$ was considerably more robust in abrogating carvedilol stimulated ERK phosphorylation compared to that observed with PTX treatment (Figure 7).
Figure 8: Effect of Gαi knockout on βAR-mediated ERK phosphorylation in HEK293 cells.

(a) The Gαi expression in β1AR or β2AR stable cells was depleted with CRISPR-Cas9 gene editing. (b) Compared with wild type β1AR stable cells, the carvedilol-induced ERK phosphorylation in Gαi knockout cells was diminished. In comparison, the response in β2AR stable cells was not affected. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance vs. control cells was assessed using two-way ANOVA with Bonferroni correction.
We then determined if a similar signaling mechanism is involved in heart tissue by measuring ERK phosphorylation in Langedorff perfused mouse hearts following carvedilol stimulation. To study the specific effect of carvedilol on the β1AR, we used previously generated β2AR knockout mice [92]. Carvedilol perfusion robustly stimulated ERK phosphorylation in hearts of β2AR knockout mice, which was entirely abrogated in hearts of PTX-pretreated mice (Figure 9). In contrast, in β1AR knockout mice [93] while carvedilol robustly induced ERK phosphorylation by activating the β2AR, PTX pretreatment was unable to block ERK activation (Figure 9). These data are consistent with our in vitro data and indicate a previously unrecognized, βAR subtype specific, requirement for Ga in carvedilol-induced β1AR.

![Figure 9: Effect of PTX on carvedilol-stimulated ERK phosphorylation in Langedorff perfused hearts from β2AR knockout mice or β1AR knockout mice.](image)

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Mice were pretreated with vehicle or 25 mg/kg PTX through intraperitoneal injection. 48 h after injection, mice heart was excised and perfused with vehicle or 10 \( \mu M \) carvedilol for 10 min. PTX diminished the carvedilol-induced ERK phosphorylation in hearts from \( \beta_2 \text{AR} \) knockout mice, but not \( \beta_1 \text{AR} \) knockout mice. Data represent the mean ± SEM for \( n \) independent experiments as marked on the figure. Statistical significance vs. control cells was assessed using one-way ANOVA (b) with Bonferroni correction.

### 3.2.2 Conformational changes in the \( \beta_1 \text{AR} \) induced by carvedilol require \( G_{\alpha_i} \)

Different ligands for the same receptor stabilize unique conformational states promoting coupling to selective signal transducers and activation of distinct downstream signaling pathways [45] [88]. Since we showed that \( G_{\alpha_i} \) is required for carvedilol-induced \( \beta_1 \text{AR} \) signaling, we tested whether it allosterically stabilizes a unique carvedilol bound \( \beta_1 \text{AR} \) conformation. We utilized a fluorescence resonance energy transfer (FRET)-based \( \beta_1 \text{AR} \) conformational sensor in which Cerulean (Cer) and YFP are inserted in the C terminus and third intracellular loop of the receptor, respectively (Figure 10a) [84]. Agonist-induced \( \beta_1 \text{AR} \) activation is represented by the loss of FRET, i.e., decrease of YFP/Cer ratio [84]. To test whether \( G_{\alpha_i} \) stabilizes a carvedilol-induced \( \beta_1 \text{AR} \) conformation, HEK293 cells stably expressing the \( \beta_1 \text{AR} \) FRET sensor were pretreated with vehicle or PTX, then stimulated with the balanced agonist isoproterenol or the \( \beta \)-arrestin-biased agonist carvedilol while monitoring the FRET ratio in real time. We found that compared to isoproterenol which caused a decrease in the FRET ratio, carvedilol induced a directional opposite response in the FRET signal, whereas the \( \beta_1 \text{AR} \)
antagonist metoprolol showed no effect (Figure 10b). Importantly, pretreatment with PTX significantly diminished the carvedilol-induced FRET ratio without any effect on the isoproterenol stimulated FRET-based receptor biosensor (Figure 10c). These data demonstrate the \( \beta_1 \)AR adopt a distinct conformational state when bound to isoproterenol compared to carvedilol and that \( \text{G} \alpha_i \) is needed to stabilize the carvedilol-bound \( \beta_1 \)AR conformation.

Figure 10: \( \text{G} \alpha_i \) is required for carvedilol-induced \( \beta_1 \)AR conformation change.
(a) In the FRET-based β₁AR conformation sensor, YFP and Cer are inserted in the third intracellular loop and the C-tail of β₁AR respectfully. (b) Ligand-induced changes of the FRET ratio in HEK293 cells stably expressing β₁AR-FRET sensor. The stable cells were stimulated with 10 μM carvedilol, 10 μM isoproterenol, 10 μM or 100 μM β₁AR antagonist metoprolol, while FRET was monitored in real-time as the emission ratio of YFP to Cer. Carvedilol stimulation increased the FRET ratio, while isoproterenol decreased it, demonstrating the distinct β₁AR conformations induced by these two ligands. Metoprolol had no effect on the FRET ratio. (c) Effect of PTX on ligand-induced FRET ratio change. Cells were pretreated with vehicle or 200 ng/ml PTX for 16 h before ligand stimulation. PTX blocked carvedilol-induced change, while having no effect on the isoproterenol response, suggesting that Gαi is required to stabilize the carvedilol-induced β₁AR conformation. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance vs. unstimulated cells was assessed using one-way ANOVA with Bonferroni correction (b); statistical significance between PTX-pretreated and non-pretreated cells was assessed using two-way ANOVA with Bonferroni correction (c, left panel), or paired Student’s t-test (c, right panel).

### 3.2.3 Carvedilol selectively promotes the recruitment of Gαi to β₁AR.

To determine the mechanism of how Gαi is involved in carvedilol-induced β₁AR signaling, we measured ligand-promoted Gαi recruitment to βARs with an in situ proximity ligation assay (PLA), a confocal-microscopy based assay that allows direct visualization and quantification of protein-protein interactions. Using HEK293 cells stably expressing β₁ARs, we show over a 2-fold increase in the PLA signal after carvedilol treatment, indicating recruitment of Gαi to the β₁AR (Figure 11a). In contrast, carvedilol had no effect on the recruitment of Gαi to β₂ARs, but was robustly recruited by isoproterenol consistent with the known process of G protein switching for β₂ARs [15] (Figure 11a). Importantly, pretreatment with the βAR antagonist propranolol
blocked the carvedilol-induced $G_{\alpha i}$ recruitment to $\beta_1$ARs (Figure 11b), indicating the recruitment is dependent on ligand interaction with the $\beta_1$AR orthosteric binding pocket.

Figure 11: Carvedilol promotes $G_{\alpha i}$ recruitment to $\beta_1$ARs, but not to $\beta_2$ARs.
The $G_{\alpha i}$ recruitment is blocked by propranolol pretreatment.

HEK293 cells stably expressing FLAG-tagged $\beta_1$ARs or $\beta_2$ARs were stimulated with 10 µM carvedilol or 10 µM isoproterenol for 5 min. In proximity ligation assay (PLA), cells were immuno-stained with $G_{\alpha i}$ antibody raised in mouse and $\beta_1$AR (or $\beta_2$AR) antibody raised in rabbit. The red PLA signal represents the protein interactions of $G_{\alpha i}$ and $\beta_1$AR (or $\beta_2$AR). The area in yellow squares are enlarged for better view. Scale bar = 20 µm. Carvedilol promoted the $G_{\alpha i}$ recruitment to $\beta_1$ARs, but not to $\beta_2$ARs. (b) $\beta_1$AR stable cells were pretreated with vehicle or 10 µM $\beta$AR antagonist propranolol for 30 min. Propranolol blocked the carvedilol response, suggesting that $\beta_1$AR-$G_{\alpha i}$ coupling is induced by the binding of carvedilol to the $\beta_1$AR orthosteric binding pocket. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance vs. unstimulated cells (NS) was assessed using one-way ANOVA with Bonferroni correction.

To further demonstrate recruitment of $G_{\alpha i}$ to carvedilol-stimulated $\beta_1$AR, we also performed co-immunoprecipitation experiments. Carvedilol stimulation increased the amount of $G_{\alpha i}$ bound to $\beta_1$ARs in a dose dependent manner, whereas it resulted in a
decrease of Gαi that could be co-immunoprecipitated to β2ARs (Figure 12). The amount of Gαi bound to β2ARs was increased by the balanced agonist isoproterenol (Figure 12), as we observed with the PLA experiments and again consistent with the previously identified process of Gαs/Gαi switching [15].

**Figure 12: Carvedilol selectively promotes β1AR coupling to Gαi.**

The effect of carvedilol and isoproterenol on Gαi recruitment was confirmed with co-immunoprecipitation assay. FLAG-tagged β1ARs or β2ARs were immunoprecipitated with anti-FLAG M2 beads, and Gαi3 was detected with its specific antibody by western blot. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance vs. unstimulated cells (NS) was assessed using one-way ANOVA with Bonferroni correction.

We next determined whether carvedilol could induce Gαi protein activation using an antibody that specifically recognizes the active GTP-bound Gαi. Carvedilol stimulation promoted the recruitment and activation of Gαi in β1AR stable cells, but not in β2AR stable cells (Figure 13a), which was blocked by PTX (Figure 13b).
Cells were transfected with Ga2. 48h after transfection, cells were treated with 10 µM carvedilol for 5 min or 30 min, or 10 µM isoproterenol for 5 min. Activated Ga was immunoprecipitated with antibody specifically recognizing the active form of Ga, and immunoblotted with Ga antibody. In the middle lane marked as actGa, cells were transfected with constitutively active Ga2, serving as positive control. Carvedilol stimulation activates Ga in β1AR stable cells, but not in β2AR stable cells. (b) As expected, PTX blocked Ga activation in the carvedilol-stimulated β1AR stable cells. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance vs. unstimulated cells (NS) was assessed using one-way ANOVA with Bonferroni correction.

To determine whether Ga recruitment is specifically stimulated by carvedilol, we tested a number of βAR agonists and antagonists with PLA (Figure 14a) and co-immunoprecipitation (Figure 14b). Remarkably, no other ligand tested induced Ga recruitment to β1ARs, suggesting that this process may be a unique property of the β-arrestin-biased ligand carvedilol.
Figure 14: Multiple tested βAR agonists or antagonists do not have significant effect on Ga recruitment.

(a) β1AR stable cells were stimulated with vehicle or 10 μM indicated ligands for 5 min. Interaction of the β1AR and Ga were detected by PLA. Scale bar = 20 μm. (b) β1AR or β2AR stable cells were stimulated with ligands at indicated concentration for 5 min. Ga recruitment was detected by co-immunoprecipitation. Both assays suggested that none of the ligands tested had similar effect of carvedilol on Ga recruitment. Data represent the mean ± SEM for n = 3-5 independent experiments as marked on the figure.
Statistical significance vs. unstimulated cells was assessed using one-way ANOVA with Bonferroni correction.

Collectively, these data support a concept that carvedilol selectively promotes the recruitment and activation of Gαi to the β1AR subtype triggering β-arrestin mediated signaling.

### 3.2.4 Carvedilol-induced β₁AR signaling requires both Gαi and β-arrestin.

Previous studies have shown that carvedilol stimulation of β₁ARs promotes the internalization and activation of EGFRs, which in turn activates downstream signaling such as ERK phosphorylation [65]. To dissect the mechanism of carvedilol-induced Gα₁-dependent signaling, we tested the effect of PTX on β₁AR-mediated EGFR internalization. We transfected HEK293 cells stably expressing β₁ARs with GFP-tagged EGFR, and monitored internalization by confocal microscopy. When stimulated with isoproterenol or carvedilol, GFP-EGFR redistributed from the plasma membrane into endosomes, similar to that observed after EGF treatment (Figure 15a). Pretreatment with PTX completely blocked the carvedilol-induced EGFR internalization, while without any effect on the isoproterenol response, indicating a requirement for Gαi for carvedilol-induced response (Figure 15a).

Consistent with a previous study showing that carvedilol-induced β₁AR-mediated EGFR transactivation is β-arrestin-dependent [65], siRNA knockdown of β-arrestin 1 and β-arrestin 2 abrogated both isoproterenol- and carvedilol- induced EGFR
internalization (Figure 15b). Though the EGFR internalization induced by both ligands are β-arrestin dependent, the molecular mechanisms appear to be different. Whereas the carvedilol-induced response requires both Ga and β-arrestin, the isoproterenol-induced response is PTX insensitive.

Figure 15: Both Ga and β-arrestin are required for carvedilol-induced β1AR-mediated EGFR internalization.

(a) The effect of PTX on ligand-stimulated EGFR internalization. β1AR stable cells with transient transfection of GFP-EGFR were pretreated with vehicle or 200 ng/ml PTX for 16 h. Then the cells were stimulated with 10 μM carvedilol, 10 μM isoproterenol or 10 ng/ml EGF for 5 min. Both carvedilol and isoproterenol promoted EGFR internalization, but only the carvedilol-induced response in PTX-sensitive. (b) Either PTX pretreatment or β-arrestins knockdown blocked carvedilol-induced EGFR internalization. β1AR stable cells were transfected with GFP-EGFR together with
scrambled control siRNA or β-arrestin 1/2 siRNA. 48 h after transfection, cells were pretreated with vehicle or 200 ng/ml PTX for 16 h before stimulation. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance was assessed using paired Student’s t-test (a) or one-way ANOVA with Bonferroni correction (b).

To more robustly determine the role of Ga\textsubscript{i} and β-arrestin in carvedilol-stimulated EGFR transactivation, we generated β-arrestin or Ga\textsubscript{i} deficient cells using CRISPR-Cas9 gene editing (Figure 16a). The wild type, Ga\textsubscript{i} knockout or β-arrestin knockout cells were transfected with CFP-tagged β\textsubscript{1}AR. After ligand stimulation, the level of cell surface EGFRs was analyzed by flow cytometry (Figure 16b). In wild type cells, EGFRs were internalized following the treatment with EGF, isoproterenol or carvedilol. The depletion of Ga\textsubscript{i} blocked carvedilol-induced EGFR internalization, whereas absence of Ga\textsubscript{i} had no effect on EGF- or isoproterenol- induced responses. In contrast, β-arrestin1/2 knockout cells showed impaired EGFR internalization in response to both isoproterenol and carvedilol. Taken together, these results suggest that the carvedilol-induced EGFR internalization are dependent on both Ga\textsubscript{i} and β-arrestins.
Figure 16: Carvedilol-induced EGFR internalization was abrogated in β-arrestin or Gαi knockout cells.

(a) Representative blots showing the depletion of β-arrestins or Gαi in HEK293 cells. The β-arrestin1/2 double knockout, β-arrestin1 or β-arrestin2 single knockout, and the Gαi knockout cells were generated with CRISPR-Cas9 gene editing. (b) Carvedilol-induced EGFR internalization was abrogated in β-arrestins or Gαi knockout cells. Wild type, β-arrestin knockout or Gαi knockout cells were transfected with CFP-tagged β1ARs. Cells were stimulated with 10 μM carvedilol, 10 μM isoproterenol or 10 ng/ml EGF for 5 min. The EGFR level on cell surface was assessed by flow cytometry. Both carvedilol- and isoproterenol- induced EGFR internalization were impaired in the β-arrestin knockout cells, whereas only the carvedilol-induced response was blocked in the Gαi knockout cells. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance was assessed using one-way ANOVA with Bonferroni correction.
Consistent with our observations for EGFR internalization, carvedilol-induced ERK phosphorylation also required both Ga\(_i\) and \(\beta\)-arrestins (Figure 17). Either Ga\(_i\) inhibition by PTX or \(\beta\)-arrestin knockdown with siRNA diminished carvedilol-induced ERK phosphorylation (Figure 17).

**Figure 17: The effect of PTX and \(\beta\)-arrestin knockdown on carvedilol-stimulated ERK phosphorylation.**

\(\beta_1\)AR stable cells with transfection of control siRNA or \(\beta\)-arrestin 1/2 siRNA were pretreated with vehicle or PTX, then simulated with 10 \(\mu\)M carvedilol for 5 min or 30 min. Carvedilol-stimulated ERK phosphorylation was diminished by either PTX pretreatment or \(\beta\)-arrestins siRNA, suggesting the requirement of Ga\(_i\) and \(\beta\)-arrestins for this signaling. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance vs. unstimulated cells was assessed using one-way ANOVA with Bonferroni correction.

Moreover, in HEK293 cells transfected with FLAG-\(\beta_1\)ARs but depleted of either Ga\(_i\) or \(\beta\)-arrestin, the carvedilol stimulated ERK activation was completely abrogated (Figure 18). Interestingly, removing either \(\beta\)-arrestin1 or \(\beta\)-arrestin2 prevented carvedilol-stimulated ERK phosphorylation, suggesting that both isoforms are required for carvedilol stimulated signaling (Figure 18).
Figure 18: The β1AR-mediated ERK phosphorylation in β-arrestin or Gαi knockout cells.

Wild type, β-arrestin knockout or Gαi knockout HEK293 cells were transfected with FLAG-tagged β1ARs. Cells were stimulated with 10 µM carvedilol for 5 or 30 min, 10 µM isoproterenol or 10 ng/ml EGF for 5 min. The depletion of either β-arrestins or Gαi impaired carvedilol-induced ERK phosphorylation. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance vs. unstimulated cells was assessed using one-way ANOVA with Bonferroni correction.

When βARs are stimulated by the balanced agonist isoproterenol, protein kinase A (PKA) activated by Gαs-dependent cyclic AMP phosphorylates the receptor leading to a switch of β2AR G protein coupling from Gαs to Gαi. The now Gαi coupled β2AR acts as a negative regulator of Gαs signaling and activates ERK signaling via dissociated Gβγ subunits from heterotrimeric Gαs [15] [94] [95]. Here, we sought to determine if Gβγ subunits are required for carvedilol-stimulated Gαi-dependent ERK phosphorylation. Gβγ inhibition was achieved by transfection of T8-βARKct, a chimeric molecule
consisting of two components: the C-terminus of the β adrenergic receptor kinase (βARKct) that competitively binds Gβγ, therefore acting as an inhibitor of Gβγ [96]; and the extracellular and transmembrane domain of CD8 receptor, which anchors the chimeric protein to the plasma membrane and potentiates its inhibitory effect [97]. The Gβγ blockade efficiency of T8-βARKct was confirmed by testing its effect on lysophosphatidic acid (LPA)-induced phosphorylation of cyclic AMP-responsive element-binding protein (CREB) (Figure 19a). We show that the inhibition of Gβγ by T8-βARKct did not affect the carvedilol-induced ERK activation (Figure 19b). This suggests that unlike isoproterenol stimulated Gαi-signaling achieved by G protein switching, carvedilol-induced Gαi-dependent β1AR signaling does not require Gβγ.

![Figure 19](image)

**Figure 19:** Carvedilol-induced β1AR-mediated ERK phosphorylation is independent of Gβγ subunits.
(a) Validation of the Gβγ inhibition by T8- βARKct. HEK293 cells with or without transient transfection of T8- βARKct were stimulated with 10 μM LPA for 5 min. T8- βARKct diminished the LPA-induced CREB phosphorylation, a known Gβγ-dependent process, confirming the inhibition of Gβγ subunits by T8- βARKct. (b) The Gβγ subunits are not required for carvedilol-induced β1AR-mediated ERK phosphorylation. β1AR stable cells with or without T8- βARKct transfection was pretreated with vehicle or 200 ng/ml PTX for 16 h. The cells were then stimulated with 10 μM carvedilol or 10 μM isoproterenol for 5 min. T8- βARKct did not have significant effect on ERK phosphorylation, suggesting that Gβγ subunits were not required. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance vs. unstimulated cells was assessed using one-way ANOVA with Bonferroni correction.

Collectively, these data demonstrate that both Ga and β-arrestins are required for carvedilol-induced β1AR signaling. Notably, either PTX pretreatment or β-arrestin knockdown was able to significantly block the carvedilol-induced β1AR-mediated EGFR internalization and ERK phosphorylation, and these responses were completely abrogated when either Ga or β-arrestin was depleted by gene editing. Taken together these data suggest that Ga and β-arrestins are likely involved in the same signaling cascade, rather than acting in parallel pathways downstream of β1AR.

### 3.2.5 GRK- and PKA- mediated β1AR phosphorylation are not required for Ga recruitment.

Given that both Ga and β-arrestins are required for carvedilol-induced β1AR signaling, to further dissect the order of the signaling cascade, we then sought to determine if β-arrestins are required for carvedilol-induced Ga recruitment to β1ARs. Since GRK-mediated receptor phosphorylation is essential for β-arrestin recruitment to β1ARs, we used a mutant β1AR that lacks the putative GRK phosphorylation sites within
the receptor carboxyl-terminal tail (GRK- β1AR) and therefore is unable to recruit β-arrestins [32]. We show that carvedilol stimulation increased Gαi recruitment to a similar extent between wild type and GRK- β1ARs, as assessed by co-immunoprecipitation (Figure 20a). These data demonstrate that GRK-mediated β1AR phosphorylation and β-arrestin recruitment are not required for carvedilol-induced Gαi recruitment, suggesting that Gαi recruitment acts upstream of β-arrestin in the signaling cascade.

βARs can switch coupling from Gαs to Gαi when stimulated with a balanced agonist [15] [95]. In the Gαs-Gαi switching model, agonist stimulated β2AR-Gαi coupling is dependent on PKA-mediated receptor phosphorylation [15] [95]. To determine whether a similar mechanism is involved in the carvedilol-induced Gαi recruitment to β1ARs, we used a β1AR mutant lacking the putative PKA phosphorylation sites (PKA-β1AR) or the PKA inhibitor H89. In our experiments, carvedilol stimulation promotes the Gαi recruitment to PKA- β1ARs, similar as to wild type receptors (Figure 20a), whereas PKA inhibition with H89 did not have a significant effect (Figure 20b).

Taken together, these data suggest that neither GRK- nor PKA-mediated receptor phosphorylation is required for carvedilol-induced Gαi recruitment to β1ARs. As the GRK-mediated receptor phosphorylation is necessary for β-arrestin activation, it is highly likely that Gαi is upstream of β-arrestin in the carvedilol-induced β1AR signaling,
Figure 20: Neither PKA-mediated nor GRK-mediated β1AR phosphorylation is required for carvedilol-induced Gαi recruitment.

(a) HEK293 cells were transfected with FLAG-tagged wild-type, PKA- or GRK-β1ARs. Carvedilol promoted Gαi recruitment to mutant β1ARs lacking the putative PKA- or GRK- mediated phosphorylation sites, to a similar extent as to the wild-type β1ARs. (b) HEK293 cells stably expressing FLAG-tagged β1ARs were pretreated with vehicle or 10 µM H89 for 30 min. The PKA inhibitor H89 did not have a significant effect on carvedilol-induced β1AR-Gαi coupling. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance vs. unstimulated cells was assessed using one-way ANOVA with Bonferroni correction.
3.2.6 The carboxyl-terminal tail (C-tail) of $\beta_1$ARs is required but not sufficient for carvedilol-induced $G_{\alpha_i}$ recruitment.

Since the C-terminus of the $\beta$ARs play vital roles in recruiting signal effectors and regulating downstream signaling [98], we postulated that specific amino acid residues within the $\beta_1$AR C-tail are critical for receptor subtype specificity of $G_{\alpha_i}$ recruitment. To test this hypothesis, we transfected HEK293 cells with $\beta$AR chimera mutants in which the C-tail of $\beta_1$ARs was exchanged with that of $\beta_2$ARs [99], and assessed $G_{\alpha_i}$ recruitment to chimera $\beta$ARs with co-immunoprecipitation. Carvedilol stimulation promoted the recruitment of $G_{\alpha_i}$ to the wild type $\beta_1$ARs, but was abrogated when the $\beta_1$AR contained the c-tail from the $\beta_2$AR ($\beta_1/\beta_2$ CT) (Figure 21). In contrast, the effect of carvedilol on $G_{\alpha_i}$ recruitment to the $\beta_2$AR with the $\beta_1$AR C-tail ($\beta_2/\beta_1$CT) was similar to that of wild type $\beta_2$ARs. These data suggest that the C-tail of the $\beta_1$AR is required for $G_{\alpha_i}$ recruitment, but alone is insufficient for this process to occur and is consistent with the crystal structure of the $\beta_2$AR and $G$ protein complex showing multiple receptor-$G$ protein contact points [40].
Figure 21: C-tail of β1AR is required but not sufficient for Gαi recruitment.

HEK293 cells were transfected with FLAG-tagged chimeric βARs constructs in which the receptor C-tails were exchanged. Carvedilol did not promote Gαi recruitment to the β1ARs with C-tail from β2ARs. On the other hand, the β1AR C-tail did not make β2ARs capable of recruiting Gαi with carvedilol stimulation. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance vs. unstimulated cells was assessed using one-way ANOVA with Bonferroni correction.

3.3 Discussion

In this study, we provide new insight into the molecular mechanism of biased agonism at the β1AR. Carvedilol, a ligand classically known as a βAR antagonist, activates β-arrestin signaling by switching the uniquely Gαs-coupled β1AR to a Gαi-coupled receptor. We show that carvedilol is unique among a number of agonists and antagonists tested to promote the recruitment and activation of Gαi to β1ARs. The recruited Gαi in turn stabilizes a carvedilol-bound β1AR conformation that is required
for β-arrestin-biased β1AR signaling as measured by EGFR internalization and ERK phosphorylation. These results indicate that the previously defined G protein bias versus β-arrestin-bias may be attributed to ligand-induced selective coupling of receptors to specific G protein subtypes, i.e. G protein subtype bias. In our conceptual model for β1AR biased signaling, we speculate that binding of carvedilol to the β1AR stabilizes a unique receptor conformation that recruits and activates Gαi to promote β-arrestin-mediated signaling (Figure 22). While carvedilol is also known to stimulate β2AR signaling, Gαi recruitment was not required for β2AR-mediated β-arrestin-biased signaling and suggests that different mechanisms for bias may be operative between βAR subtypes.

![Figure 22: Schematic model of carvedilol-induced Gαi-β-arrestin biased signaling of β1ARs.](image)

Binding of carvedilol to the orthosteric site of the β1AR stabilizes a distinct intermediate conformation that then promotes the recruitment of Gαi. The carvedilol and Gαi bound receptor in turn stabilizes a unique β1AR conformation that mediates β-arrestin biased signaling.
In the classical view of GPCR signaling, agonist stabilization of specific active conformational states promotes coupling of heterotrimeric G proteins and stimulation of downstream signaling [100]. Receptor signaling is then terminated by a process involving receptor phosphorylation, β-arrestin recruitment and receptor internalization. However, recent studies suggest that the classical “on-off” (active and inactive) model is oversimplified [88], as GPCRs transmit signaling through multiple transducers to regulate diverse arrays of pathways. First, some GPCRs can couple to multiple G proteins. For example, the isoproterenol-activated β2AR switches coupling from Ga\textsubscript{s} to Ga\textsubscript{i} [15]. In this study we show that carvedilol switches the classical Ga\textsubscript{s}-coupled receptor β1AR to a Ga\textsubscript{i}-coupled receptor. However, in contrast to the Ga\textsubscript{s}-Ga\textsubscript{i} switching of the β2AR, the carvedilol-induced β1AR-Ga\textsubscript{i} coupling does not involve Ga\textsubscript{s} activation and PKA-mediated receptor phosphorylation. The carvedilol-induced β1AR-Ga\textsubscript{i} signaling is also different from the actions of classical Ga\textsubscript{i}-coupled receptors such as the muscarinic M\textsubscript{2} receptor and the α2 adrenergic receptor [101], as its activation of ERK is not mediated through Gβγ subunits. Second, in addition to their role as signal terminators for G protein signaling, β-arrestins can act as signal transducers in their own right. Current conceptual models support the idea that ligands may differentially stabilize distinct receptors conformations that recruits divergent portfolio of signaling transducers and effector proteins to active a select suite of cellular signaling pathways, a concept termed functional selectivity or biased agonism [49] [85] [86] [102].
The β-arrestin-biased ligand carvedilol has three unique features at the β1AR: 1) it has inverse efficacy for Gαs-dependent adenylyl cyclase activity; 2) it promotes the recruitment of Gαi, not Gαs, to the β1AR; 3) it activates the classical β-arrestin signaling using a Gαi paradigm. These unique signaling property of carvedilol may be attributed to its ability to stabilize a distinct receptor active conformation [49]. For the β2AR, carvedilol uniquely induces significant conformational rearrangement around residue Lys263 and Cys265 in the third intracellular loop of the receptor, which may expose the loop toward intracellular surface and facilitate the receptor interaction with β-arrestins [49]. Though a previous study suggests the crystal structure of carvedilol-bound β1AR is similar to that of the cyanopindolol-bound inactive state structure [103], additional conformations stabilized by carvedilol may require the binding of transducers such as Gαi or β-arrestin. This requirement of transducer binding for receptor conformational stability is supported by the structural study of the β2AR showing that the interaction of a G protein, or a G protein-like-protein nanobody, is required to stabilize the agonist-induced receptor active conformation [104]. In our study, using a FRET-based β1AR conformation sensor, we show that carvedilol induces a change of FRET ratio, representing a receptor conformational change. Notably, the β1AR conformation induced by carvedilol is distinct from the one induced by the balanced agonist isoproterenol, as carvedilol increased the FRET ratio while isoproterenol decrease it. This further supports a concept that receptors can adopt distinct conformations when
stimulated by different ligands. As our results show that carvedilol promotes the recruitment of Goi to β1ARs, while a wide range of other βAR ligands tested do not, it is possible that carvedilol induces a β1AR conformational change that exposes allosteric binding sites on the receptor to allow for receptor-Goi interaction. In turn, the bound Goi stabilizes the carvedilol-induced active receptor conformation and is consistent with our data where pretreatment with the Goi inhibitor PTX impairs the carvedilol activated β1AR conformation. Together these data support the concept that carvedilol-induced Goi is a positive allosteric modulator of the β-arrestin-biased β1AR active conformation.

While we have not determined the precise mechanism of how Goi binding to the carvedilol-occupied β1AR triggers β-arrestin signaling, we postulate that it may involve subsequent receptor phosphorylation in a process known as the “barcode” hypothesis [54]. Upon ligands stimulation, GPCRs can be phosphorylated by distinct GRK subtypes at specific sites. Previous study identified β2AR sites that are specifically phosphorylated by GRK2 and GRK6 [53]. While the balanced agonist isoproterenol stimulates β2AR phosphorylation at both GRK2- and GRK6-specific sites, carvedilol only stimulates receptor phosphorylation at the GRK6-specific sites. This “barcode” phosphorylation pattern of receptors plays essential roles in regulating the recruitment and functionality of signaling transducers [54]. For instance, β2AR phosphorylation mediated by GRK2 and GRK6 induces distinct β-arrestin conformations, and differentially regulates receptor internalization and ERK activation [53]. Similarly for
the β1AR, GRK2-mediated and GRK5/6-mediated receptor phosphorylation leads to distinct cellular responses [32] [105], suggesting that a phosphorylation barcode for the β1AR may also direct β-arrestin signaling. To dissect the mechanism of how Ga mediates β1AR signaling, future studies will determine the isoproterenol- or carvedilol-induced barcode phosphorylation pattern of the β1AR, as well as the effect of Ga inhibitor PTX on it.

The precise effect of PTX on β-arrestin recruitment and activation also remains to be determined. Using a number of methodologies, such as co-immunoprecipitation, confocal- or bioluminescence resonance energy transfer-based assays, we were unable to detect carvedilol-induced β-arrestin recruitment to the β1AR. This may be due to a number of reasons: 1) ligand-induced β-arrestin recruitment and activation is rapid, within 2 second after stimulation, and reversible [106]; 2) the affinity of the β1AR-β-arrestin interaction is low. Previous studies used receptor chimera, such as β2AR with vasopressin V2 receptor cytoplasmic tail (β2AR-V2R), to increase the affinity of β-arrestin and therefore the assay sensitivity [91]. However, the C-tail of β1ARs is required for the Ga recruitment, and therefore substituting the β1AR C-tail with the V2R tail would not provide a chimeric receptor suitable to study the role of Ga in carvedilol stimulated β-arrestin recruitment. Lastly, it is also possible that carvedilol-induced β-arrestin-mediated β1AR signaling does not involve a direct interaction between receptor and β-arrestin.
Carvedilol is a βAR antagonist (β-blockers), a family of drugs that are widely used in the therapeutic treatment of cardiovascular diseases such as hypertension and heart failure, as β1ARs and β2ARs are predominant GPCR subtypes expressed in mammalian heart and play vital roles in the regulation of cardiac function [57]. In heart failure, treatment with β-blockers improves left ventricle function, reverses the pathological cardiac remodeling, and reduces mortality and morbidity [107][108]. However, β-blockers have different clinical efficacies. Some evidence suggests that carvedilol has a superior effect on cardiovascular survival to other β-blockers [109]. The molecular basis for this remains to be elucidated, but has been attributed to the additional properties of carvedilol other than β-blockers, such as the antioxidant, antiproliferative effects and α1 adrenergic receptor blockade [110]. Interestingly, carvedilol appears to be unique among βAR blockers in that it can activate β-arrestin-dependent signaling that confers cardioprotection [91][65]. Given the possible cardioprotective role of Gαi during cardiac stress [111] and the ability of carvedilol to promote β1AR-Gαi coupling, it is possible that this unique property of carvedilol is also important for its therapeutic efficacy.

In conclusion, we identify a new signaling mechanism for GPCR biased agonism. To date, the β1AR was considered to be predominantly coupled to Gαs and β-arrestin-dependent β1AR signaling to be independent of G proteins. However, our data supports a concept where carvedilol has 3 unique properties at the β1AR 1) it is inert with respect
to $G\alpha_s$; 2) it recruits $G\alpha_i$ and converts the $\beta_1$AR from a $G\alpha_s$-coupled receptor to one that couples to $G\alpha_i$; and 3) it activates classical $\beta$-arrestin-dependent signaling in a $G\alpha_i$ paradigm. These data suggest a greater complexity for receptor signaling bias than previously appreciated in that coupling of distinct G protein subtypes to the activated receptor are needed for $\beta$-arrestin-biased agonism. These data also have important implications when considering the development of new therapeutic ligands designed to selectively target $\beta$-arrestin-biased signaling pathways.
4. Mechanoactivation of the angiotensin II type 1 receptor induces β-arrestin-biased signaling through $G_{\alpha_i}$ coupling

4.1 Introduction

The angiotensin II type 1 receptor (AT1R), a member of the G protein-coupled receptor (GPCR) superfamily, is a well-known regulator of blood pressure and cardiac function [13]. In response to the balanced agonist angiotensin II (AngII), AT1Rs couple to the heterotrimeric G-protein triggering the dissociation of $G_{\alpha_i}$ from $G_{\beta\gamma}$, which subsequently activates the signaling effector phospholipase C (PLC) to promote generation of the second messengers inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) [13]. This G protein-mediated signaling is rapidly turned off by the receptor desensitization, a process that involves phosphorylation of the C-terminal tail of the AT1R mediated by G protein-coupled receptor kinases (GRKs) and recruitment of the multifunctional scaffold protein β-arrestin that impairs further G protein coupling [16, 56, 112]. It is now known that β-arrestins also function as signal transducers in their own right, regulating a large network of cellular responses [23]. Several AT1R ligands, such as $[\text{Sar}1, \text{Ile}4, \text{Ile}8]$-AngII (SII) and TRV120023, have been shown to selectively activate β-arrestin-mediated pathways and therefore termed as β-arrestin-biased agonists [45, 71].

Our current understanding for ligand stimulated AT1R signaling is based on a mechanism whereby ligand binding stabilizes distinct receptor conformations allowing
them to engage signal transducers such as G proteins or β-arrestin to affect activation of an array of signaling pathways, a concept known as functional selectivity [13, 30, 45, 46, 54, 113, 114]. Interestingly, activation of AT1Rs can be mechanosensitive, whereby membrane stretch allosterically stabilizes a distinct β-arrestin-biased AT1R conformation triggering ligand-independent β-arrestin-mediated signaling [77, 78]. The precise molecular mechanism by which mechanical stress activates AT1R signaling is not clearly understood.

It is now well appreciated that AT1Rs may couple to multiple signaling transducers with different efficacy and kinetics depending on cellular systems and stimuli [89, 115]. For example, in proximal tubule cells, the AngII-activated AT1R recruits Ga leading to the inhibition of adenylate cyclase activity [116]. In cardiac fibroblasts, AngII activates the AT1R-mediated ERK signaling through the Gβγ subunits of Ga [117]. A recent study showed that the weak β-arrestin-biased agonist SII, not only activates β-arrestin-mediated signaling but can also promote AT1R coupling to both Gaq and Ga [89].

We, therefore, set out to determine whether Ga plays a role as a signal transducer for the AT1R under conditions of mechanoactivation. Indeed, we found that membrane stretch promotes the recruitment of Ga to the AT1R, which is required for the activation of β-arrestin-biased signaling as measured by epidermal growth factor receptor (EGFR) internalization and extracellular signal-regulated kinases (ERK)
phosphorylation. In contrast, Gαi is not required for signaling induced by the balanced agonist AngII or the β-arrestin-biased agonist TRV120023 and TRV120026. Notably, our findings demonstrate mechanistic heterogeneity for AT1R-mediated β-arrestin-biased signaling induced by mechanoactivation vs. biased ligands and support a concept that membrane stretch may stabilize a biased AT1R conformation that is distinct from that stabilized by a β-arrestin-biased ligand.

4.2 Results

4.2.1 Membrane stretch promotes the recruitment of Gαi to AT1R.

Previous work has shown that ligand stimulated AT1Rs may transduce signaling through multiple G proteins such as Gαq and Gαi, as well as the multifunctional adaptor protein β-arrestin [89]. It is now appreciated that AT1Rs, in response to biomechanical membrane stretch, also activate signaling but in a Gαq-independent β-arrestin-dependent manner [77, 78]. To determine whether Gαi is involved in this stretch-induced AT1R signaling, we investigated the effect of membrane stretch on AT1R-Gαi coupling. HEK293 cells stably expressing HA-tagged AT1R were stimulated with the balanced agonist AngII, the β-arrestin-biased agonist TRV120023, or hypotonicity-induced membrane stretch respectively. The recruitment of Gαi to AT1R was then measured with an in situ proximity ligation assay (PLA), in which protein-protein interactions can be directly visualized and quantified by the fluorescence signal. We found that osmotic stretch significantly increased the amount of Gαi recruited to AT1R,
shown as the green PLA signal (Figure 23). Pretreatment with pertussis toxin (PTX), a Gαi inhibitor that catalyzes the ADP-ribosylation of Gαi and prevents its coupling to receptors, blocked the stretch-induced Gαi recruitment to AT1Rs. Notably, the balanced agonist AngII and the β-arrestin biased agonist TRV120023 did not alter the PLA signal.

**Figure 23:** Osmotic stretch leads to Gαi recruitment to AT1R, and is inhibited by PTX.

HEK293 cells stably expressing HA-tagged AT1R were stimulated with AngII (1µM), TRV120023 (1µM), or osmotic stretch for 10 min. The recruitment of Gαi to AT1R was indicated by the green fluorescence signal in PLA. Gαi recruitment was increased in
response to osmotic stretch, which is inhibited by PTX. AngII or TRV120023 did not increase the recruitment of Gαi to AT1R.

To further demonstrate recruitment of Gαi to the stretch-activated AT1R, we performed co-immunoprecipitation experiments. Consistent with the PLA results, the level of Gαi bound to AT1R was increased by osmotic stretch, but not affected by AngII and TRV120023 (Figure 24a). In contrast, AngII promoted the recruitment of Gαq to AT1R, while neither TRV120023 nor stretch had any effect (Figure 24b), which is consistent with previous studies suggesting that TRV120023- and stretch- induced signaling is independent of Gαq. Thus, in contrast to the balanced agonist AngII and the β-arrestin-biased agonist TRV120023, Gαi is only recruited to the mechanoactivated AT1R.
Figure 24: Osmotic stretch promotes AT1R coupling to Gαi, but not to Gαq.

HEK293 cells stably expressing HA-tagged AT1R were stimulated with AngII (1μM), TRV120023 (1μM), or osmotic stretch for 10 min. The recruitment of Gαi (a) or Gαq (b) to AT1R was detected with co-immunoprecipitation in. Osmotic stretch selectively promotes Gαi recruitment to AT1Rs. In contrast, AngII promoted AT1R-Gαq coupling, while TRV120023 and osmotic stretch did not have significant effect.

4.2.2 Gαi is required for membrane stretch-induced β-arrestin recruitment to AT1Rs.

To dissect the role of Gαi in stretch-induced AT1R signaling, we tested the effect of the Gαi inhibitor PTX on β-arrestin recruitment using HEK293 cells stably expressing both CFP-tagged AT1R and YFP-tagged β-arrestin2. Due to low abundance in HEK293
cells of G protein-coupled receptor kinase 5 (GRK5), a kinase that mediates AT1R phosphorylation required for osmotic stretch-induced β-arrestin recruitment [77], cells were transfected with GRK5. In the absence of stimulation, AT1R localizes on the plasma membrane and β-arrestin2 is evenly distributed in cytoplasm (Figure 25a). When cells were stimulated with AngII, TRV120023 or osmotic stretch, β-arrestin2 translocated from cytoplasm to endocytic vesicles where it co-localized with the internalized AT1R (AngII: 87.0 ± 1.7%, 1 µM TRV120023: 73.8 ± 2.1%, 10 µM TRV120023: 73.3 ± 6.3%, osmotic stretch: 36.9 ± 2.1% of the cells showed β-arrestin2 translocation, respectively, all p<0.0001 compared with unstimulated cells), indicating the recruitment of β-arrestin2 to AT1R. Pretreatment with PTX significantly blocked the osmotic stretch-induced β-arrestin2 recruitment (8.3 ± 1.4%, p<0.0001 compared with vehicle-pretreated cells); while without any effect on the AngII- and TRV120023- induced response (with PTX, AngII: 84.5 ± 2.0%, 1 µM TRV120023: 75.8 ± 2.0%, 10 µM TRV120023: 74.7 ± 6.8%, p=n.s. compared with vehicle) (Figure 25b).

Taken together, these results indicate a requirement of Gαi for osmotic stretch-induced β-arrestin2 recruitment to the AT1R, and indicates important heterogeneity in AT1R signaling induced by a β-arrestin-biased agonist compared to that by membrane stretch.
Figure 25: Gaα is required for osmotic stretch-induced β-arrestin2 recruitment to the AT1R.

HEK 293 cells stably expressing CFP-tagged AT1R and YFP-tagged β-arrestin2 were transiently transfected with GRK5. After pretreatment with vehicle or 200 ng/ml PTX for overnight, cells were stimulated with 1 μM AngII, 1 μM or 10 μM TRV120023, or osmotic stretch for 10 min. A, in response to AngII, TRV120023 or osmotic stretch, AT1R redistributed from the plasma membrane to intracellular puncta, and the cytosolic
evenly-distributed β-arrestin2 translocated and co-localized with the internalized AT1Rs, indicating the β-arrestin2 recruitment to AT1Rs. Pretreatment with PTX significantly inhibited osmotic stretch-induced β-arrestin2 recruitment, whereas has no effect to AngII- or TRV120023- induced response. Scale bar = 10 μM. B, quantification of β-arrestin2 translocation was expressed as the percentage of cells showing intracellular aggregates of β-arrestin. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance vs. unstimulated cells, or between the vehicle and PTX pretreated group, was assessed using two-way ANOVA with Bonferroni correction.

4.2.3 Mechanoactivated-AT1R induces EGFR transactivation and ERK1/2 phosphorylation in a Gαi-dependent manner.

To dissect the mechanism of stretch-induced Gαi-dependent AT1R signaling, we tested the effect of PTX on AT1R-mediated EGFR internalization. We transfected HEK293 cells stably expressing AT1Rs with GFP-tagged EGFRs and monitored EGFR activation by GFP internalization by confocal microscopy. In the absence of stimulation, EGFR were located on the plasma membrane. Stimulation with AngII (44.4±3.0% of the cells), TRV120023 (11.2±3.0% of the cells), or osmotic stretch (25.6±5.9% of the cells) induced EGFR internalization, represented by redistribution into cellular aggregates (AngII and osmotic stretch: p<0.0001, TRV120023: p<0.05 compared with unstimulated cells) (Figure 26). Pretreatment with PTX significantly inhibited EGFR internalization induced by osmotic stretch (9.6±3.6%, p<0.0001 compared with vehicle-pretreated cells), whereas did not have any effect on AngII or TRV120023 (Figure 26). These data suggest that Gαi is required for AT1R-mediated EGFR transactivation promoted by osmotic stretch, but not for the response induced by AngII or TRV120023.
Figure 26: PTX blocks osmotic stretch-induced EGFR internalization.
HEK 293 cells stably expressing AT1R were transiently transfected with GFP-tagged EGFR and GRK5, and stimulated with AngII (1µM), TRV120023 (1µM), or osmotic stretch for 10 min. In response to AngII, TRV120023, and osmotic stretch, EGFR internalization was significantly increased compared with non-stimulation (AngII and osmotic stretch: p<0.0001, TRV120023: p<0.05). Pretreatment with PTX (200ng/ml) significantly inhibited osmotic stretch-induced EGFR internalization, whereas has no effect to AngII or TRV120023.

We next determined the effect of PTX on AT1R-mediated ERK1/2 phosphorylation. Both AngII and osmotic stretch significantly increased ERK1/2 phosphorylation, whereas TRV120023 showed a mild increase (Figure 27a). Similar to that observed for EGFR transactivation, PTX blocked the osmotic stretch-induced ERK signaling (p<0.001 compared to vehicle), but did not alter the AngII response. We next tested the effect of the protein kinase c (PKC) inhibitor Ro318425 on Gαq-mediated AT1R signaling and the EGFR inhibitor AG1478 to block AT1R-EGFR transactivation. While
Ro318425 blocked AngII-induced ERK signaling (p<0.0001 compared with vehicle), it had little effect on the osmotic stretch response (Figure 27b). In contrast, AG1478 blocked ERK1/2 phosphorylation induced by either AngII (p<0.0001 compared with vehicle) or osmotic stretch (p<0.05 compared with vehicle) (Figure 27c). Collectively, these data support a concept that the balanced ligand AngII stimulates AT1R ERK1/2 signaling through both the Gaq and β-arrestin transducers, while mechanoactivated AT1R signaling is mediated through a Gaβ-dependent, β-arrestin-dependent mechanism.

We also tested whether Gaβ was needed for mechanical stretch-induced AT1R signaling in mouse heart tissue. A polypropylene membrane balloon was placed in the left ventricle (LV) of a Langendorff-perfused mouse heart and then inflated to apply a mechanical stretch on the heart. We have previously shown that stretch of the heart induces ERK1/2 phosphorylation and required the AT1R and β-arrestin2 [77]. While LV balloon stretch of hearts significantly increased the phosphorylation of ERK1/2 (p<0.001 compared with control), pretreatment with PTX abolished this response (p<0.01 compared with the vehicle-pretreated hearts), indicating a requirement of Gaβ for stretch-induced ERK1/2 signaling in vivo (Figure 27d).

Collectively, these data support a concept that the balanced ligand AngII stimulates AT1R ERK1/2 signaling through both the Gaq and β-arrestin transducers, while mechanoactivated AT1R signaling is mediated through a Gaβ-dependent, β-arrestin-dependent mechanism.
Figure 27: Gαq is required for osmotic stretch-induced ERK1/2 phosphorylation both in vitro and in vivo.

(a) ERK1/2 phosphorylation was significantly increased in response to AngII (1μM) or osmotic stretch. Pretreatment with PTX significantly reduced osmotic stretch-induced ERK1/2 phosphorylation, whereas has no effect on AngII-induced response. (b) Osmotic stretch-induced ERK1/2 phosphorylation was not blocked by the PKC inhibitor Ro318425, suggesting Gαq is not required for this response. (c) EGFR inhibitor AG1478
blocked ERK1/2 phosphorylation in response to AngII or osmotic stretch, suggesting the ERK1/2 activation is mediated by the transactivated EGFRs. (d) PTX blocked mechanical stretch-induced ERK phosphorylation in mice heart. In ex-vivo perfused wild type heart, LV balloon stretch significantly increased ERK1/2 phosphorylation compared with perfusion control. Pretreatment with PTX (25µg/kg) significantly inhibited balloon stretch-induced ERK1/2 phosphorylation.

4.2.4 TRV120026, another β-arrestin-biased agonist, induces β-arrestin-biased AT1R signaling independent of Gαi.

To determine whether the Gαi dependency for the β-arrestin-biased AT1R signaling is specifically induced by mechanical stress, we tested the effect of the Gαi inhibitor PTX on the AT1R signaling induced by TRV120026, another β-arrestin-biased agonist with very similar potency of β-arrestin recruitment to AT1R [71, 118]. In In HEK293 cells stably expressing the CFP-tagged AT1R and YFP-tagged β-arrestin2, TRV120026 significantly induced β-arrestin2 translocation, to the same extent of TRV120023 response (1 µM TRV120026: 62.0 ± 2.0%, 10 µM TRV120026: 72.7 ± 2.4%, 1 µM TRV120023: 72.0 ± 2.3% of cells showed β-arrestin2 translocation, all p<0.0001 compared with unstimulated cells) (Figure 28a, b). Pretreatment of PTX have no effect on TRV120026-induced response (Figure 28a, b), suggesting that Gαi is not required for TRV120026-induced β-arrestin2 translocation. Similar to TRV120023, TRV120026 induced mild increase of ERK1/2 phosphorylation, which was not affected by PTX pretreatment (Figure 28c, d). Taken together, these data suggest that the β-arrestin-biased agonists TRV120023 and TRV120026 both induce AT1R signaling in a Gαi-independent manner, which is distinct from that induced by osmotic stretch.
Figure 28: TRV120026 induces the β-arrestin-biased AT1R signaling in a Gαi-independent manner.

a and b, PTX had no effect on the TRV120026-induced β-arrestin2 recruitment to AT1R. HEK 293 cells stably expressing CFP-tagged AT1R and YFP-tagged β-arrestin2 were transiently transfected with GRK5. After pretreatment with vehicle or 200 ng/ml PTX for overnight, cells were stimulated with 1 μM AngII, 10 μM TRV120023, 1 μM or 10
µM TRV120026, or osmotic stretch for 10 min. Scale bar = 20 µM. Quantification of β-arrestin2 translocation was expressed as the percentage of cells showing intracellular aggregates of β-arrestin. c and d, similar to the effect of TRV120023, TRV120026 induced a mild increase of ERK1/2 phosphorylation, which was not PTX-sensitive. HEK293 cells stably expressing AT1R were pretreated and stimulated as mentioned above. The pERK signals were quantified by densitometry, normalized to tERK, and expressed as fold change over unstimulated cells. Data represent the mean ± SEM for n independent experiments as marked on the figure. Statistical significance was assessed using two-way ANOVA with Bonferroni correction.

To further demonstrate the role of Ga_i in the osmotic stretch-induced AT1R signaling, we tested the β-arrestin translocation in Ga_i knockout cells. In wild type HEK293 cells transfected with CFP-tagged AT1R and YFP-tagged β-arrestin2, stimulation with AngII, TRV120023, TRV120026 and osmotic stretch induces the translocation of β-arrestin2. In the Ga_i knockout cells, the osmotic stretch-induced β-arrestin2 translocation was blocked, whereas the responses induced of other stimuli were not affected (Figure 29).
Figure 29: Osmotic stretch-induced β-arrestin2 translocation is blocked in the Gαi knockout cells.

4.3 Discussion

In this study, we identify a new molecular mechanism for β-arrestin-biased AT1R signaling. Membrane stretch, which has been shown to activate β-arrestin-biased AT1R signaling without activating Gαq, specifically promotes the recruitment of another subtype of G proteins, Gαi, to the AT1R (Figure 30). This AT1R-Gαi coupling is required for the recruitment of β-arrestin to the receptor and the induction of subsequent β-arrestin-dependent signaling as measured by EGFR internalization and ERK
phosphorylation. In contrast, the $G_{ai}$ recruitment and the dependence of $G_{ai}$ for $\beta$-arrestin-mediated signaling was not observed for the balanced agonist AngII, or, remarkably, the $\beta$-arrestin-biased agonists TRV120023 and TRV120026 (Figure 30). These results indicate mechanistic divergence of AT1R-mediated $\beta$-arrestin-biased signaling triggered by the different modes of receptor activation, membrane stretch and $\beta$-arrestin-biased ligands in this case.

Figure 30: Schematic model of AT1R signaling by distinct modes of activation.
It is well established that GPCR can engage to multiple G protein subtypes, and the efficacy and kinetics of distinct G protein coupling are regulated by the specificity of ligands or allosteric modulators [115]. Although in our study we did not observe $G\alpha_i$ recruitment to AngII-stimulated AT1Rs, previous studies have shown that AngII may promote AT1R-$G\alpha_i$ coupling to inhibit adenylyl cyclase and to regulate $Ca^{2+}$ channels in certain tissue or cell types [119-121]. In addition, a recent study reported that SII, an AT1R ligand previously described as a $\beta$-arrestin-biased agonist, can also weakly activates $G\alpha_q$ and $G\alpha_i$ [89]. Despite the variation of the effect of AngII on $G\alpha_i$ activation, which might due to the difference in experimental systems or assay sensitivity, it is clear that in response to different stimuli AT1Rs can engage distinct transducers to induce cellular signaling.

A full understanding of the diversity of GPCR signaling through distinct transducers activated by different stimuli remains to be elucidated. In recent years, considerable structural information is now available for GPCRs in complex with ligands or signal transducers and support the concept that receptors adopt distinct conformations to selectively regulate different arrays of downstream signaling [3, 102]. Biophysical analysis of mechanical stretch-activated AT1R suggest that the AT1R undergoes anticlockwise rotation and a shift of transmembrane domain 7 into the ligand-binding pocket, whereas AT1R blocker candesartan stabilized the receptor in the inactive conformation and prevented stretch induced conformational change [79]. Our
previous work suggested that membrane stretch induces a β-arrestin-biased conformational state of AT1Rs and an active β-arrestin conformation indistinguishable from that induced by the biased agonist TRV120023 [77, 78]. However, we now show that membrane stretch induces a distinct AT1R-β-arrestin signaling complex through its recruitment of Gaı suggesting the existence of heterogeneity in biased-receptor conformations.

In our conceptual model, we speculate that membrane stretch induces a unique AT1R conformation that exposes an allosteric binding sites for Gaı, and the bound Gaı in turn stabilizes a unique receptor conformation allowing receptor phosphorylation at specific residues on the c-terminal tail of the receptor by a unique subset of GRKs. This is supported by our previous finding that the membrane stretch-induced β-arrestin signaling requires GRK5 and GRK6, but not GRK2 [77]. The stretch-induced GRK-mediated distinctive phosphorylation pattern of the AT1R then induces a unique β-arrestin-biased receptor conformational state, leading to the activation of subsequent biased signaling. Future phospho-proteomic studies will be needed to determine the specific AT1R c-terminus phosphorylation pattern induced by balanced agonist, β-arrestin-biased agonist or membrane stretch.

AT1R plays vital roles in the regulation of cardiovascular function, and is one of the major targets for the therapeutic treatment of cardiovascular diseases such as congestive heart failure and hypertension [13, 46, 122]. Chronic AT1R activation by
AngII is associated with cardiac dysfunction, which is likely mediated by the G protein signaling, as $G\alpha_i$ has been shown to mediate cardiomyocyte hypertrophy whereas $\beta$-arrestin-mediated signaling is thought to promote cardioprotection [123-125]. Therefore, the $\beta$-arrestin-biased agonist, which preferentially activates $\beta$-arrestin signaling while inhibiting the G protein pathway, may provide novel clinical implications on promoting cardiac contractility and simultaneously blocking cardiac dysfunction [35, 118, 126, 127]. With our data now showing that two different $\beta$-arrestin-biased stimuli, membrane stretch and the biased ligands likely engage distinct AT1R molecular states to activate $\beta$-arrestin signaling, the potential to screen for unique biased ligands that can distinguish these different pathways may be possible.

In conclusion, we show that membrane stretch specifically promotes AT1R coupling to $G\alpha_i$, triggering activation of $\beta$-arrestin-biased signaling. These new insights for mechanoactivation of the AT1R reveal additional regulatory complexity for biased agonism and may allow for new screening strategies to identify therapies for cardiac disease.
5. Conclusions and future directions

5.1 Conclusions

GPCRs, capable of binding to a large diversity of ligands and involved in most physiological processes, are the most commonly targeted receptor class for therapeutics, and remain a rich source for drug discovery. Upon ligand binding, GPCRs can adopt multiple active conformations and can regulate a complex network of cellular responses. The discovery of biased agonists, which activate selective portions of these signaling networks, reveals the possibility to regulate GPCR signaling events more precisely and opens a new avenue for the discovery of therapeutic agents. β-arrestins are critical components of GPCR signaling, and involved in a wide diversity of physiological and pathological processes. In some cases, β-arrestin signaling confers positive effects compared to G protein signaling. For example, chronic activation of βAR-Gs signaling is detrimental to cardiac function, whereas the β-arrestin-biased agonist carvedilol exerts cardioprotective effects. Therefore, elucidating the mechanism underlying β-arrestin-biased signaling would be of great importance.

Interestingly, G protein signaling and β-arrestin signaling are not mutually exclusive. The signaling mediated by many Gi-coupled receptors requires both Gi and β-arrestin. According to previous understanding, β-arrestin-mediated signaling of most Gs or Gq receptors was though to be independent of G proteins. However, my study suggests that certain modes of stimulation may switch a primarily Gs or Gq coupled
receptor to coupling to Gi. The now Gi-coupled receptor then activates downstream β-arrestin signaling.

In the first part of my study, I found that carvedilol, a biased agonist for β-arrestin signaling and an inverse antagonist for the Gs signaling, promotes the recruitment of Ga to the β1AR. The binding of Ga stabilizes the receptor in a unique conformation that activates β-arrestin signaling. In contrast, the same mechanism was not identified in the signaling mediated by another βAR subtype, the β2AR, nor induced by a panel of other ligands tested, suggesting that the Ga-dependent β-arrestin signaling is selectively induced by the carvedilol-stimulated β1AR.

In the second part of my study, I revealed that the mechanoactivation of AT1R also promotes a switch in receptor coupling from Gq to Gi. The recruited Ga promoted the recruitment of β-arrestins, leading to subsequent signaling activation. In contrast, Ga was not involved in β-arrestin signaling induced by the balanced full agonist AngII or by the β-arrestin-biased agonists TRV120023 or TRV120026. Previously, it was thought that mechanical stress induces AT1Rs to adopt the same conformational state as that induced by β-arrestin-biased agonists. The divergence in signaling mechanism between mechanical stress and biased agonists identified in this study implies that there is heterogeneity of active receptor conformations induced by these distinct stimuli.

Taken together, my findings reveal a new mechanism of β-arrestin-biased agonism of GPCRs, suggesting that the previously defined bias between G protein and
β-arrestin may be mediated in part by the selective coupling of receptors to specific G protein subtypes. This signaling is selectively induced by a subset of receptors upon specific stimulation, such as by carvedilol stimulation of the β1AR or mechanoactivation of the AT1R as identified in my study.

5.2 Future directions

5.2.1 Determine the ligand-specific phosphorylation pattern of the β1AR and the AT1R.

The GRK-mediated bar code phosphorylation pattern of the β2AR has been defined [53]. Based on previous studies showing the distinctive GPCR signaling arrays mediated by specific GRK subtypes, here I hypothesize that the different role of Ga in β-arrestin-biased signaling is caused by bar code receptor phosphorylation. To prove this hypothesis, quantitative mass spectrometry analyses need to be performed on the β1AR and the AT1R to determine ligand-specific phosphorylation patterns and the corresponding GRK subtypes. GRK knockdowns or site mutagenesis on identified sites can be used to determine if the unique phosphorylation pattern is the mechanism for differential signaling.

5.2.2 Identify the conformational signature of the Ga-coupled β-arrestin-biased receptor.

Accumulating evidence suggests that receptors adopt distinct conformational states when activated by different ligands. I propose to use chemical labeling and mass spectrometry to identify ligand-specific receptor conformational features. Chemical
labeling at specific sites at the receptor transmembrane domain can detect the accessibility of these sites, reflecting TM motion and receptor conformational changes. This method allows accurate quantitative measurements of the changes in labeling upon ligand stimulation.

I also wish to understand whether there are common structural motifs allowing receptor coupling to Ga\textsubscript{i} and then activation of β-arrestin-biased signaling. Given the structural similarity between the β\textsubscript{1}AR and β\textsubscript{2}AR, I also wish to identify the factors leading to the different Ga\textsubscript{i} coupling modes of these two receptors and the divergence of their β-arrestin signaling mechanisms. To answer these questions, structural studies of these signaling complexes, composed of ligand, receptor, Ga\textsubscript{i} and β-arrestin, would provide insights into the active receptor conformations and the unique structural signatures for Ga\textsubscript{i} - β-arrestin signaling. This may be achieved with crystallography or cryo-electron microscopy.

5.2.3 Identify ligand-specific β-arrestin conformational states.

Accumulating evidence suggests that β-arrestins can also adopt distinct conformations in order to selectively mediate divergent cellular responses. As revealed in this study, distinct receptors utilize distinct mechanisms to activate β-arrestin signaling. I hypothesize that the differential activation modes of β\textsubscript{1}ARs and AT1Rs may lead to distinct active β-arrestin conformations. Recently, a panel of FRET- or BRET-based β-arrestin conformational sensors has been developed [106, 128]. These sensors
can be used to characterize the β-arrestin conformations induced by these two distinct receptors after specific stimulation events (carvedilol, mechanoactivation).

5.2.4 Determine whether Gαi-β-arrestin-biased agonism is involved in signaling mediated by other GPCRs.

In this study, I identified a new regulatory role for Gαi in β-arrestin-biased signaling induced by the carvedilol-activated β1AR, as well as that by the mechanoactivated AT1R. In contrast, this signaling was not observed in the carvedilol-activated β2AR or the biased agonist TRV120023-activated AT1R. These results suggest the distinct biased mechanisms are operative between those receptor subtypes, as well as under different activation modes for the same receptor. To determine whether the switching of receptor coupling to Gαi is a common mechanism to initiate β-arrestin signaling, future studies need to explore the role of Gαi in β-arrestin-biased signaling activated by an array of different GPCRs. This may also provide insights into what specific factors determine when Gαi is necessary for β-arrestin-mediated signaling.
References


Biography

I was born in Zhengzhou, China on October 25, 1987, the child of Jianbo Wang and Mei Yu. I grew up in Wuhan, a big city in central China at the intersection of the Yangtze and Han rivers. I went to middle school and high school in Wuhan Foreign Languages School, and started my undergraduate study in Tsinghua University in Beijing in 2005. During my time at Tsinghua University, I worked as a research assistant in the laboratory of Dr. Anming Meng on signaling transduction in zebrafish. I graduated in July 2009 with a B.S. degree in Biology. In the fall of 2009, I entered the Cell and Molecular Biology Program at Duke University as a graduate student, and joined the Department of Cell Biology in 2010. I joined the laboratory of Dr. Howard A. Rockman in 2010 where this thesis work was done. During the time at Duke, I received Chancellor's Scholarship from 2009 to 2011, and the American Heart Association Predoctoral Fellowship from 2014 to 2016. I would like to thank the many teachers and mentors throughout my life and study, and to thank my friends and family for their continuous love and support.