S49G and R389G polymorphisms of the β1-adrenergic receptor influence signaling via the cAMP-PKA and ERK pathways

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Zhang F, Steinberg SF. S49G and R389G polymorphisms of the β1-adrenergic receptor influence signaling via the cAMP-PKA and ERK pathways. Physiol Genomics 45: 1186–1192, 2013. First published October 22, 2013; doi:10.1152/physiolgenomics.00087.2013.—Two functionally important β1-adrenergic receptor (β1AR) polymorphisms have been identified. The R389G polymorphism influences coupling to the Gs-cAMP pathway. R389β1ARs display enhanced activation of cAMP/PKA; they provide short-term inotropic support but also cause a predisposition to cardiomyopathic decompensation. A second S49G polymorphism is implicated in the evolution of heart failure, but the mechanism remains uncertain. This study shows that position 49 and 389 polymorphisms function in a coordinate manner to influence agonist-dependent cAMP/PKA and ERK responses. cAMP/PKA and ERK responses are more robust in HEK293 cells that heterologously overexpress G49β1ARs, compared with S49β1ARs. However, this phenotype is most obvious on a G389β1AR background; the more robust agonist-dependent cAMP/PKA and ERK responses in R389β1AR cells effectively obscure the effect of the S49G polymorphism. We also show that isoproterenol (Iso) and carvedilol activate ERK via a similar EGFR-independent mechanism in cells expressing various β1AR haplotypes. However, Iso activates ERK via an Src-independent pathway, but carvedilol-dependent ERK activation requires Src. Since the S49G polymorphism has been linked to changes in β1AR trafficking, we examined whether β1AR polymorphisms influence partitioning to lipid raft membranes. Biochemical fractionation studies show that all four β1AR variants are recovered in buoyant flotillin-enriched membranes; the distinct signaling phenotypes of the different β1AR variants could not be attributed to any gross differences in basal compartmentalization to lipid raft membranes. The allele-specific differences in β1AR signaling phenotypes identified in this study could underlie interindividual differences in responsiveness to β-blocker therapy and clinical outcome in heart failure.

β1-adrenergic receptors (β1ARs) are the principle mediators of catecholamine-dependent changes in the force and rate of cardiac contraction. β1ARs adjust cardiac output by activating a Gs-adenylyl cyclase (AC) pathway that increases cAMP, activates protein kinase A (PKA), and phosphorylates substrates involved in excitation-contraction coupling (16). However, heightened β1AR drive in the setting of heart failure (HF) activates signaling pathways that promote cardiomyocyte hypertrophy/apoptosis, interstitial fibrosis, disordered energetics, and contractile dysfunction (8, 16). β1AR inhibitors that prevent these maladaptive βAR responses have become standard therapy for HF.

While there is ample evidence that βAR blockers decrease morbidity and mortality in HF, there is considerable interindividual variability in drug responsiveness that is not readily attributable to known clinical or demographic factors. This interindividual variability has been attributed at least in part to two common nonsynonymous single nucleotide polymorphisms (SNPs) at positions 49 and 389 of the human of the β1AR receptor (3). Most studies have focused on the R389G polymorphism at the conformationally sensitive Gs binding domain in the juxtamembrane region of the COOH-terminal cytoplasmic tail. Studies to date indicate that R389β1ARs display enhanced coupling to the Gs-cAMP pathway and enhanced agonist-dependent desensitization (relative to G389β1ARs when overexpressed in cultured fibroblasts (10). Transgenic cardiac-specific R389β1AR overexpression also leads to enhanced receptor signaling and contractile function in young mice, compared with age-matched G389β1AR hearts (11). These findings in cell culture and cardiomyocyte-targeted transgenic mouse models are consistent with clinical studies showing that the R389β1AR (in association with a α2c-AR variant that regulates presynaptic release of norepinephrine) is a risk factor for human HF (23) and that the R389β1AR allele predicts responsiveness to βAR inhibitor therapy in patients with HF (7, 11).

A second S49G polymorphism in the relatively short NH2 terminus of the β1AR has been implicated as a genetic determinant of βAR inhibitor responses and clinical outcome in HF (1, 9, 17, 25–27). However, the molecular basis for the clinical impact of this polymorphism is less obvious, since the relatively short/featureless βAR NH2 terminus generally is ignored in structure-function studies. While several studies have provided consistent evidence that G49β1ARs are more susceptible, and S49β1ARs are relatively resistant, to agonist-dependent downregulation, the available literature on the effects of the S49G polymorphism on β1AR affinity for ligands or β1AR coupling to cAMP is more ambiguous (6, 18, 21).

Despite considerable evidence that R389β1ARs act as “gain-of-function” variants for the AC/cAMP/PKA pathway (relative to G389β1ARs), there is only scant information on whether position 389 or 49 polymorphisms influence the magnitude or mechanism for β1AR coupling to effectors that may contribute to cardioprotection, such as ERK (4). Moreover, studies to date that typically focus on the independent effect of a single polymorphic variation in isolation may have only limited relevance to clinical phenotypes that result from the distinct haplotypes that exist in clinical populations. This study identifies allele-specific differences in β1AR coupling to cAMP/...
RESULTS

The initial studies compared signaling responses induced by activation of individual β1-Ar haplotypes transiently overexpressed at similar levels in HEK293 cells. Figure 1 shows that Iso induces a modest increase in ERK phosphorylation in vector-infected HEK293 that contain low levels of endogenous β2 (but not β1) ARs; HEK293 cells also display a modest increase in ERK phosphorylation in response to direct activation of AC by forskolin. β1-AR overexpression leads to an increase in Iso-dependent ERK phosphorylation without any associated changes in ERK phosphorylation in response to forskolin or EGF (included as controls in the experiment). Studies with an anti-phospho-PKA substrate antibody (that recognizes protein phosphorylation at the PKA consensus RXXPs motif, used as a surrogate to track agonist-dependent activation of cAMP) show that heterologously overexpressed β1-ARs also couple to the cAMP/PKA pathway. Iso induces a modest increase in anti-phospho-PKA substrate immunoreactivity in vector-infected HEK293. The Iso-dependent increase in anti-phospho-PKA substrate immunoreactivity is enhanced by heterologous overexpression of β1-ARs, without any changes in anti-phospho-PKA substrate immunoreactivity in response to treatment with forskolin (included as control in the experiments). Importantly, the magnitude of the Iso-dependent responses differed considerably for the different molecular forms of the β1-AR. R389β1-ARs (with either S or G at position 49) displayed robust ERK and PKA substrate phosphorylation responses. Both responses are lower in cells that heterologously overexpress G389β1-ARs. The effect of an S49G polymorphism is most obvious in this context, where a β1-AR harboring a position 49 glycine residue induces more robust ERK and PKA substrate phosphorylation responses than the G389β1-AR with a position 49 serine residue.

The importance of S49G and R389G polymorphisms was interrogated further in clonal HEK293 cell lines that overexpress similar levels of HA-tagged S49G1AR, G389G1AR, or S4R389β1ARs (Bmax values 776 ± 52, 735 ± 33, and 755 ± 41 fmol/mg protein, respectively). We compared signaling in response to activation of G389β1-ARs vs. S49R389β1-ARs to resolve the functional importance of the R389G polymorphism. Since the effect of the position 49 polymorphism is most pronounced on a G389β1-AR background, we resolved the functional importance of the S49G polymorphism by comparing signaling responses that result from activation of G389β1-ARs vs. S49G389β1-ARs. Figure 2A shows that all three β1-AR variants activate the cAMP and ERK pathways. In each case, Iso-dependent ERK phosphorylation is rapid (maximal at 2–5 min) and transient (wanes with 30–60 min of continuous agonist stimulation, Fig. 2B). However, the magnitude of the Iso-dependent responses is influenced by both R389G and S49G polymorphisms. Iso-dependent increases in cAMP accumulation and ERK phosphorylation are higher in S49R389β1-AR cells than in S49G389β1-AR cells. However, a position 389 glycine residue does not necessarily impose a low level of signaling, since G389β1-ARs that harbor a position 49 glycine residue elicit more robust cAMP and ERK phosphorylation responses. Control studies verify that the differences in Iso-dependent activation of cAMP and ERK reflect the distinct signaling phenotypes of the individual heterologously overexpressed β1-ARs, since forskolin-dependent cAMP accumulation...
tion and EGF-dependent ERK phosphorylation are similar in all three cell lines.

β₁ARs adopt distinct “active” conformations that differ in their ability to activate G protein-dependent vs. G protein-independent (β-arrestin-dependent) responses. Ligands that selectively activate only certain signaling pathways are referred to as “biased” ligands. Carvedilol has been characterized as a β₂-arrestin-biased ligand for the mouse (S49G389) β₁AR; carvedilol acts as an antagonist for the classical β₁AR-Gs-cAMP pathway but stabilizes a β₁AR conformation that activates the β₁-arrestin-ERK pathway (5, 12). Figure 2 shows that carvedilol acts as a partial agonist at S49G389-, G49G389-, S49R389- and G49R389-β₁ARs. In each case, carvedilol treatment leads to an increase in ERK phosphorylation that is not associated with a detectable increase in cAMP. Carvedilol-dependent ERK phosphorylation responses are 1/3 as robust as the cognate Iso-dependent ERK phosphorylation responses in any given cell line. Control studies established that carvedilol-dependent increases in ERK phosphorylation are maximal (do not increase further at higher carvedilol concentrations) and are specifically mediated by β₁ARs (are inhibited by propranolol; data not shown).

Previous studies have attributed β₁AR-dependent activation of ERK to a mechanism involving Src and the activation of a matrix metalloproteinase that cleaves HB-EGF and trans-activates EGFRs (12). However, these studies were performed on HEK293 cells that heterologously overexpress the mouse S49G389-β₁ARs variant and EGFRs. Studies that rigorously compare the magnitude and mechanism for ERK activation by individual polymorphic variants of the β₁AR at more physiologically relevant levels of EGF expression are illustrated in Figure 3. The results show that AG1478 (an EGFR kinase inhibitor) completely abrogates EGF-dependent ERK phosphorylation, but AG1478 does not block either Iso- or carvedilol-dependent increases in ERK phosphorylation in β₁AR cells that do not heterologously overexpress EGFRs. These results indicate that all three β₁AR variants couple to an ERK pathway that does not require EGFR activity (i.e., the EGFR transactivation presumably is recruited only at high level of EGFR overexpression). The PKA inhibitor H89 also does not interfere with β₁AR-dependent ERK activation (under conditions where H89 pretreatment completely abrogated Iso-dependent increases in phospho-PKA substrate immunoreactivity; data not shown). However, an agonist-dependent difference in the mechanism for ERK activation by Iso and carvedilol was exposed in studies with PP1, an inhibitor of Src kinase activity. Figure 3 shows that carvedilol activates ERK via an Src-dependent pathway that is inhibited by PP1, whereas the Iso-dependent pathway for ERK activation persists in PP1-treated cells. These results indicate that Iso and carvedilol stabilize β₁ARs in slightly different active conformations that couple to distinct signal transduction pathways and signaling responses.

We previously demonstrated that β₁ARs partition to lipid raft membranes (19). Cell lysates were solubilized in deter-
Two laboratories have identified allele-specific differences in signaling via G protein-independent signaling pathways has received considerably less attention, with the analysis largely confined to epidemiologic studies that implicate the S49G polymorphism as a genetic determinant of clinical outcome in various cardiovascular disorders. Most studies have focused on the role of the R389G polymorphism as a predictor of pharmacologic responsiveness to β1-AR inhibitor therapy, showing that R389G-β1ARs (the more common allele in individuals of European descent) adopt a more active conformation, display enhanced coupling to the Gi-cAMP pathway, and confer enhanced sensitivity to β-blocker therapy in HF, relative to G389G-β1ARs. The position 389 polymorphism could in theory also impact on clinical outcome prediction as a genetic modifier of cardiac phenotypes by influencing similar signaling events. Most studies have focused on the role of the R389G polymorphism as a genetic modifier of clinical outcome in various cardiovascular disorders. Studies reported herein show that 1) agonist-dependent cAMP/PKA and ERK responses are more robust in R389G-β1AR cells, than in G389G-β1AR cells and that 2) the effect of the S459G polymorphism is most obvious on a G389G-β1AR background; G389G-β1ARs with a glycine at position 49 display enhanced agonist-dependent cAMP/PKA and ERK responses, compared with S459G-β1ARs. Of note, the two previous studies that linked the S49G polymorphism to changes in β1-AR trafficking and desensitization kinetics also were performed on a G389G-β1AR background (6, 18); effects of a position 49 SNP on signaling by R389G-β1ARs have never been considered. This oversight may be important, since clinical studies to date do not provide an entirely coherent picture of the importance of β1-AR polymorphisms as genetic modifiers of cardiac phenotypes. It is tempting to speculate that discrepancies in the literature could be due to the stratification of patients according to a single polymorphic locus (either position 49 or position 389), rather than the haplotypes that exist in the general population. This is important for two reasons. First, glycine is the minor allele at both loci, but there are important racial
differences in allele frequencies for both S49G and R389G polymorphisms that could confound an analysis. Second, there is strong linkage disequilibrium between S49G and R389G loci that functions to limit the haplotypes (combinations of specific polymorphisms) in the general population. Most studies identify S49/G389-, S49/G389-, and S49/R389-ARs as the most prevalent haplotypes in Caucasians; the S49/G389 haplotype appears to be quite rare (14, 22). Hence, while the results of this and previous studies in reductionist models that characterize changes in signaling properties of the S49G polymorphism on a G389 background are of interest from a theoretical standpoint, the physiological relevance of these findings will require further study.

Since the S49G polymorphism has been linked to changes in AR trafficking, and the spatial relationship between the AR and its downstream effectors can function to either facilitate or restrict signaling responses, we examined whether polymorphic variants of AR compartmentalize differently between lipid raft and nonraft membranes. Lipid rafts are a buoyant membrane fraction that are highly enriched in ARs, Gs, several AC isoforms, EGFRs, and components of the ERK-MAPK pathway; lipid rafts have been implicated as platforms that “launch” signaling to the ERK cascade. Our fractionation studies show that the various polymorphic variants of AR are recovered exclusively in the buoyant lipid raft membrane fraction; we did not identify an obvious difference in the partitioning of AR variants between lipid raft and nonraft membranes that might explain their distinct signaling phenotypes. However, these studies were performed on resting cells that had not been exposed to agonist ligands; they do not rule out possible allele-specific differences in AR trafficking patterns following agonist exposure that would influence signaling responses.

HF is associated with elevated catecholamine levels and chronic/persistent AR stimulation; this heightened sympathetic tone contributes to the pathogenesis of various HF syndromes. The cardiotoxic actions of AR have been attributed to Gs-dependent activation of AC and enhanced accumulation of cAMP. Drugs that inhibit cardiotoxic AR-mediated responses (such as metoprolol, bisoprolol, and carvedilol) have been approved for the treatment of HF syndromes. However, their clinical efficacy is not equivalent, with evidence that carvedilol affords survival advantage over other approved pharmacologic inhibitors (15). While this has been ascribed to...
carvedilol’s ancillary antioxidant, anti-inflammatory, antiproliferative, or anti-α1-AR actions (2, 13, 29), a mechanism related to carvedilol’s unique pharmacologic profile at β1-ARs also is possible. Carvedilol acts like other βAR inhibitors to block the βAR-Gs-cAMP pathway, but carvedilol also exhibits an additional action as a partial agonist for the G protein-independent cardioprotective pathway that activates ERK (28). Previous literature examined carvedilol’s pharmacologic actions in cells that overexpress only certain molecular forms of the β1-AR. This study is the first to extend the analysis and show that carvedilol exerts a similar action as a biased partial agonist for the ERK pathway at all β1AR variants. However, we identified allele-specific differences in the magnitude of the carvedilol-dependent ERK phosphorylation response that mirror the allele-specific differences in ERK phosphorylation by Iso. We also identified an agonist-dependent difference in the mechanism for β1-AR-dependent activation of ERK. Notably, while previous studies showed that Iso and carvedilol recruit similar EGFR/Src-dependent pathways to activate ERK in HEK293 cells that heterologously overexpress both β1ARs and EGFRs (12), we show that β1ARs activate ERK via an EGFR-independent mechanism at endogenous levels of EGFR expression. However, an agonist-dependent difference in the mechanism for β1AR activation of ERK activation was identified under these conditions. We show that carvedilol activates ERK via a Src-dependent mechanism, whereas the Iso-dependent ERK pathway is Src independent; the pharmacologic profiles for Iso- and carvedilol-dependent ERK activation are similar across the various β1-AR polymeric variants. The EGFR-independent mechanisms for ERK activation by β1ARs, which may or may not involve Src and presumably are obscured at high levels of EGFR overexpression, may contribute to β1AR-dependent cardioprotection in the heart and are the focus of further study.

In summary, this study identifies allele-specific differences in signaling phenotypes for different β1AR polymorphic variants. These differences in the cellular actions of individual β1AR variants could contribute to the pathogenesis of clinical HF phenotypes and may have bearing on the success of β-blocker therapy.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: F.Z. performed experiments; F.Z. and S.F.S. analyzed data; F.Z. and S.F.S. interpreted results of experiments; F.Z. and S.F.S. prepared figures; F.Z. and S.F.S. drafted manuscript; F.Z. and S.F.S. approved final version of manuscript; S.F.S. conception and design of research; S.F.S. edited and revised manuscript.

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