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

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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 R389G-β1ARs display enhanced coupling to the Gs-cAMP pathway and enhanced agonist-dependent desensitization (relative to G389G-β1ARs) when overexpressed in cultured fibroblasts (10). Transgenic cardiac-specific R389G-β1AR overexpression also leads to enhanced receptor signaling and contractile function in young mice, compared with age-matched G389G-β1AR hearts (11). These findings in cell culture and cardiomyocyte-targeted transgenic mouse models are consistent with clinical studies showing that the R389G-β1AR (in association with a α2c-AR variant that regulates presynaptic release of norepinephrine) is a risk factor for human HF (23) and that the R389G-β1AR 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 relative short/featureless βAR NH2 terminus generally is ignored in structure-function studies. While several studies have provided consistent evidence that G49S-β1ARs are more susceptible, and S49S-β1ARs 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 R389G-β1ARs act as “gain-of-function” variants for the AC/cAMP/PKA pathway (relative to G389G-β1ARs), 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/PKA andcontractile dysfunction (8, 16). βAR inhibitors that prevent these maladaptive βAR responses have become standard therapy for HF.
PKA and ERK pathways that have implications for the pathogenesis and treatment of clinical HF phenotypes.

MATERIALS AND METHODS

Materials. Antibodies were from the following sources: β1-AR and flotillin-1 were from Santa Cruz Biotechnology. ERK1/2, p-ERK1/2, and p-PAK substrate antibodies were from Cell Signaling Technology (Danvers, MA). Isoproterenol (Iso), forskolin, and EGF were from Sigma (St. Louis, MO). All other chemicals were reagent grade.

Plasmids. A plasmid that drives expression of the human S49R389-G49R389-β1-AR, with an NH2-terminal Flag, was obtained from Addgene. Site-directed mutagenesis of β1-AR-Flag with the QuikChange mutagenesis system (Agilent Technologies) was performed to generate G49R389-β1-ARs, S49G389-β1-ARs, or G49G389-β1-ARs. All constructs were confirmed by sequencing.

Cell culture and transfection. HEK293 cells were cultured in DMEM containing 10% FCS, and 100 units/ml penicillin-streptomycin. Cells were transfected with plasmids that drive expression of human β1-AR polymorphic variants with the Effectene Transfection reagent (Qiagen) according to the instruction manual. Stable cell lines were generated by selection with G418 (400 μg/ml).

Western blotting. Immunoblotting was performed on cell extracts according to methods described previously or manufacturer’s instructions. All panels in each figure represent the results of a single gel (exposed for a uniform duration); detection was with enhanced chemiluminescence. All results were replicated in at least three experiments on separate culture preparations.

Preparation of caveolar membranes. Caveolin-enriched fractions were prepared according to a detergent-free purification scheme described previously (20). All steps were carried out at 4°C. In brief, cells from five 100 mm diameter dishes were washed twice with ice-cold phosphate-buffered saline and then scraped into 0.5 m sodium carbonate, pH 11.0 (0.5 ml/dish). Cells from five dishes were combined for each preparation. The extract was sequentially disrupted by homogenization with a loose-fitting Dounce homogenizer (10 strokes), a Polytron tissue grinder (three 10 s bursts), and a tip sonicator (three 20 s bursts). The homogenate was then adjusted to 40% sucrose by adding an equal volume of 80% sucrose prepared in MES-buffered saline (25 mm MES, pH 6.5, and 0.15 m NaCl), placed on the bottom of an ultracentrifuge tube, overlaid with a 5–30% discontinuous sucrose gradient (3 ml of 5% sucrose and 4 ml of 35% sucrose, both in MES-buffered saline containing 0.25 m sodium carbonate), and centrifuged at 260,000 g for 16–18 h in a SW40 rotor (Beckman Coulter, Palo Alto, CA). After centrifugation, 12 1 ml fractions were collected. A pooled caveolar fraction (fractions 4–5), a pooled fraction 8–12 (which contains the bulk of the cellular material including the cytosol and most of the particulate membrane fraction), and the insoluble pellet (which is solubilized in SDS-PAGE sample buffer) were subjected to SDS-PAGE and immunoblotting.

Measurements of βAR density and cAMP accumulation. Radioligand binding experiments with [125I]ICYP were performed on membranes prepared from each stable cell line according to published methods (24). cAMP measurements also were performed according to standard methods. In brief, cells were cultured in six-well plates, grown to confluence, and then serum deprived for 12 h. Cells were preincubated with 10 nM theophylline for 60 min and then challenged for 5 min with vehicle, Iso (10−6 M), or forskolin (10−5 M) as indicated. Assays were terminated by aspiration of the incubation buffer and addition of 0.5 ml of 100% ice-cold ethanol to each well. Cell lysates were dried in a spin vacuum, and cAMP in the residue was determined with a commercially available enzyme-linked immunosorbent assay kit (R & D Systems, Minneapolis, MN) according to manufacturer’s instructions.

Statistics. Results are shown as means ± SE and were analyzed by Student’s t-test followed by Bonferroni adjustment for multiple comparisons with GraphPad Prism software.

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. β1AR 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 β1ARs also couple to the cAMP/PAK pathway. Iso induces a modest increase in anti-phospho-PAK substrate immunoreactivity in vector-infected HEK293. The Iso-dependent increase in anti-phospho-PAK substrate immunoreactivity is enhanced by heterologous overexpression of β1ARs, without any changes in anti-phospho-PAK 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. R389-β1-ARs (with either S or G at position 49) displayed robust ERK and PAK substrate phosphorylation responses. Both responses are lower in cells that heterologously overexpress G389-β1-ARs. The effect of an S49G polymorphism is most obvious in this context, where a β1AR harboring a position 49 glycine residue induces more robust ERK and PAK substrate phosphorylation responses than the G389-β1-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 S49G389-, G49R389-, or S49R389-β1-ARs (with either S or G at position 49) displayed robust ERK and PAK substrate phosphorylation responses. Both responses are lower in cells that heterologously overexpress G389-β1-ARs. The effect of an S49G polymorphism is most obvious in this context, where a β1AR harboring a position 49 glycine residue induces more robust ERK and PAK substrate phosphorylation responses than the G389-β1-AR with a position 49 serine residue.

β1-AR HAPLOTYPES DISPLAY DISTINCT SIGNALING PHENOTYPES

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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 β-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 β-arrestin-ERK pathway (5, 12). Figure 2 shows that carvedilol acts as a partial agonist at S49G389-, G49G389-, and S49R389-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 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 EGFR 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-
HF. Two laboratories have identified allele-specific differences received considerably less attention, with the analysis largely not previously been considered. The S49G polymorphism has of signaling via G protein-independent signaling pathways has ing ERK, but a role for the R389G polymorphism as a modifier by influencing signaling via cardioprotective pathways involv-
morphism could in theory also impact on clinical outcome to polymorphism as a predictor of pharmacologic responsiveness in various cardiovascular disor-

gent-free alkaline sodium carbonate buffers and subjected to isopycnic centrifugation on a discontinuous sucrose gradient to determine whether S49G or G389R polymorphisms alter signaling phenotypes by influencing β1AR partitioning to buoyant lipid raft membranes. Figure 4 shows that β1AR variants are recovered almost exclusively in the light sucrose gradient fraction that contains the bulk of the cellular flotillin immunoreactivity (a lipid raft recovery marker) and excludes >98% of total cell protein (including Golgi and other intracellular organelle markers). We did not identify any gross allele-specific differences in β1AR partitioning to lipid raft microdomains.

DISCUSSION

There is recent evidence that polymorphisms at positions 49 and 389 in the coding region of the β1AR act as genetic modifiers 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 β-AR inhibitor therapy, showing that R389β1ARs (the more common allele in individuals of European descent) adopt a more active conformation, display enhanced coupling to the Gs-cAMP pathway, and confer enhanced sensitivity to β-blocker therapy in HF, relative to G389β1ARs. The position 389 polymorphism could in theory also impact on clinical outcome by influencing signaling via cardioprotective pathways involving ERK, but a role for the R389G polymorphism as a modifier of signaling via G protein-independent signaling pathways has not previously been considered. The S49G polymorphism 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 HF. Two laboratories have identified allele-specific differences in agonist-dependent downregulation kinetics in studies performed in reductionist models (6, 18). A single study from Levin et al. (6) also showed that a position 49 glycine residue confers enhanced basal and agonist-dependent coupling to the AC/cAMP pathway (i.e., the β1AR harboring a position 49 glycine residue is a constitutively active receptor), but this finding was not replicated by others (18). Studies to date also have not rigorously considered a possible interplay between the polymorphisms at positions 49 and 389. Therefore, we examined the signaling profiles of β1AR polymorphic variants harboring either serine or glycine at position 49 and either glycine or arginine at position 389.

Studies reported herein show that 1) agonist-dependent cAMP/PKA and ERK responses are more robust in R389β1AR cells, than in G389β1AR cells and that 2) the effect of the S459G polymorphism is most obvious on a G389β1AR background: G389β1ARs with a glycine at position 49 display enhanced agonist-dependent cAMP/PKA and ERK responses, compared with S459G389β1ARs. Of note, the two previous studies that linked the S49G polymorphism to changes in β1AR trafficking and desensitization kinetics also were performed on a G389 background (6, 18); effects of a position 49 SNP on signaling by R389β1ARs 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 β1AR 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/R389-, S49/G389-, and G49/R389-1ARs as the most prevalent haplotypes in Caucasians; the G49/G389 haplotype appears to be quite rare (14, 22). Hence, while the results of this and previous studies in reductionist models that characterize the 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 β1AR trafficking, and the spatial relationship between the βAR and its downstream effectors can function to either facilitate or restrict signaling responses, we examined whether polymorphic variants of the β1AR compartmentalize differently between lipid raft and nonraft membranes. Lipid rafts are a buoyant membrane fraction that are highly enriched in βARs, 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 the β1AR are recovered exclusively in the buoyant lipid raft membrane fraction; we did not identify an obvious difference in the partitioning of β1AR 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 β1AR trafficking patterns following agonist exposure that would influence signaling responses.

HF is associated with elevated catecholamine levels and chronic/persistent β1AR stimulation; this heightened sympathetic tone contributes to the pathogenesis of various HF syndromes. The cardiotoxic actions of β1AR have been attributed to Gs-dependent activation of AC and enhanced accumulation of cAMP. Drugs that inhibit cardiotoxic β1AR-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, antipro-liferative, 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 β1AR. 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 β1AR-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 β1AR polymorphic 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.

REFERENCES


