Myocardial β₁-adrenergic receptor polymorphisms affect functional recovery after ischemic injury

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In vitro studies in transfected fibroblasts have demonstrated that the phenotype of the Arg389 receptor is an enhanced receptor-Gₛ interaction, manifested by increased high-affinity agonist binding and increased activation of adenylyl cyclase (12) and enhanced short-term agonist-promoted desensitization (18). Subsequent studies have assessed the potential role of these β₁-AR variants in the development of heart failure by utilizing transgenic mice with cardiac-specific expression of the human Arg389 or Gly389 β₁-AR under the control of the α-myosin heavy chain promoter (17). The Arg389 mice have enhanced basal and β-agonist stimulated contractility compared with Gly389 at 3 mo of age, and both are significantly greater than nontransgenic (NTG) controls. Interestingly, at 6 mo of age, Arg389 mice maintained elevated ejection fractions and contractility and were anatomically normal but failed to respond to β-agonist stimulation (17). The mechanisms behind this marked physiological desensitization of the Arg389 β₁-AR are not fully understood but include allele-specific alterations in expression of Gₛα, and adenylyl cyclase as well as phospholamban and sarco(endo)plasmic reticulum Ca²⁺-ATPase 2A. The blunted signaling phenotype of these older Arg389 ventricles was also found with human end-stage heart failure ventricles stratified by genotype (17), but the impact of this genetic variation, even in model systems, has not been extensively explored. Of particular relevance to the current report is the potential effect that β₁-AR polymorphisms may have on recovery after myocardial injury due to ischemia. Several animal models using overexpression methods have been utilized for ischemia-reperfusion (I/R) studies relative to β-AR signaling. Overexpression of the β₂-AR subtype appears to be deleterious to recovery after I/R injury (4), whereas overexpression of the G protein-coupled receptor kinase-2 (GRK2, also termed β-ARK1), which desensitizes agonist-occupied β₁- and β₂-ARs, improves functional recovery after I/R injury (4). Signaling through both β₁- and β₂-AR subtypes can activate mitogen-activated protein kinases (MAPKs), including ERK1 and ERK2 (6, 8). ERK2 deficiency, observed in ERK2 heterozygous knockout (−/−) mice, is associated with increased infarct size and decreased ventricular function (11). In contrast, MEK1-overexpressing transgenic mice (equivalent to a gain of ERK1/2 function) had substantial protection from injury (11). The current study was undertaken to ascertain the potential effects of genotypic variation of the β₁-AR at position 389 on recovery of cardiac function after I/R by using transgenic mice

In 3-mo-old mice of either genotype, there was poor recovery after I/R at baseline and after 20 min of ex vivo ischemia and reperfusion (I/R). In Arg389 hearts, agonist-promoted adenylyl cyclase activities were depressed by ~35% at 6 mo of age, and G protein-coupled receptor kinase (GRK) activity was increased by approximately twofold compared with Gly389. Furthermore, I/R evoked an approximately threefold increase in ERK2 phosphorylation in Arg389 but an approximately twofold decrease in Gly389 hearts. Individually, these changes have been shown to mitigate I/R injury; thus the Arg389 β₁-AR uniquely evokes specialized pathways that act to protect against I/R injury. The improved recovery of function after I/R in Arg389 hearts relative to Gly389 appears to be due to an adaptive multimechanism program with allele-specific alterations in receptor signaling, GRK activity, and ERK2. Thus genetic variation of the human β₁-AR may play a role in cardiac functional recovery after ischemic injury.

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expressing the human β₁-AR variants and to explore potential mechanisms of allele-specific phenotypes.

**METHODS**

**Experimental animals.** Transgenic mice (FVB/N strain) with cardiac-specific overexpression of either the Arg389 or Gly389 β₁-AR were utilized in this study and have been previously described (17). Transgenes were targeted to the myocardium by using the α-myosin heavy chain promoter. Both transgenic lines had equivalent expression (~1 nmol/mg) of the two receptors in the heart as previously documented by radioligand binding. NTG littermates were studied in parallel. All animals were male and either 3 or 6 mo of age as specified. The study was approved by the Animal Use and Care Committee of the University of Cincinnati (Cincinnati, OH).

**Ischemic injury and recovery.** Hearts were isolated and perfused in the Langendorff mode as described previously (17). All hearts were perfused for 30 min before being subjected to 20 min of normothermic, no-flow ischemia and 40 min of reperfusion. Contractile function was measured as left ventricular (LV) developed pressure (LVDP) and the peak rate of increase of pressure (+dP/dt). Diastolic function was assessed as −dP/dt and measurement of LV end-diastolic pressure (EDP). Creatine kinase (CK) activity was utilized as a marker of myocyte injury, which was assayed in the perfusate by a standard method (Sigma). Samples were obtained at baseline, 10 min after initiation of ischemia, and 30 min after reperfusion.

**Adenylyl cyclase activity.** Cardiac sarcolemmal membranes (~20 μg of protein) were incubated in a reaction mixture consisting of 2 mM Tris (pH 7.4), 0.8 mM EGTA, 5 mM MgCl₂, 2.8 mM phosphoenolpyruvate, 0.06 mM GTP, 0.12 mM ATP, 0.1 mM cAMP, 4 U/ml myokinase, 10 U/ml pyruvate kinase, 0.1 mM ascorbic acid, and 3 x 10⁵ counts/min [α-³²P]ATP. Activities were determined in the presence of water (basal) and 10 μM isoproterenol (a concentration that elicits a maximal response). Reactions were performed at 37°C for 5 min. [³²P]cAMP was separated by chromatography over alumina columns, and recovery was normalized to a [¹H]cAMP tracer.

**Protein immunoblotting.** Hearts were homogenized in ice-cold lysis buffer [25 mM Tris-HCl (pH 7.5), 5 mM/l EDTA, 5 mM/l EGTA, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM/l phenylmethylsulfonyl fluoride]. Nuclei and tissue were separated by centrifugation at 800 g for 20 min. The crude supernatant was then centrifuged at 20,000 g for 20 min. The supernatant from this centrifugation was considered the cytosolic fraction. Sedimented proteins (membrane fraction) were resuspended in 50 mM/l HEPES (pH 7.3) and 5 mM/l MgCl₂. The immunodetection of myocardial levels of GRK2 utilized a polyclonal antibody (Santa Cruz Biotechnology) and was performed on an equal amount of protein from cytosolic and membrane extracts (80 μg) electrophoresed through 12% Tris-glycine gels and transferred to nitrocellulose. A polyclonal ERK1/2 antibody (Santa Cruz Biotechnology) and a polyclonal phosphorylated ERK1/2 (pERK1/2) antibody (Cell Signaling) were used to determine the expression of these peptides in whole heart extracts (100 μg of protein). Membranes were blocked in 5% nonfat dried milk in 0.1% Tween 20 in PBS for 1 h at room temperature. The protein was visualized by using a horseradish peroxidase-linked secondary antibody and ECL detection (Amersham).

**GRK activity by rhodopsin phosphorylation.** The supernatants of the myocardial extracts that contained the soluble kinases were used to determine GRK activity. Extracts (100–150 μg of protein) were incubated with rhodopsin-enriched rod outer-segment membranes in a reaction buffer containing the following (in mmol/l): 10 MgCl₂, 20 Tris·HCl, 2 EDTA, 5 EGTA, 0.1 ATP, and [γ-³²P]ATP, as previously described (1). After the reactions were incubated in white light for 15 min at room temperature, they were quenched with ice-cold lysis buffer and centrifuged for 15 min at 13,000 g. Sedimented proteins were resuspended in 25 μl of protein gel-loading dye and subjected to 12% SDS-PAGE. Phosphorylated rhodopsin was visualized by autoradiography of dried polyacrylamide gels and quantified by using a Molecular Dynamics PhosphorImager.

**Statistical analysis.** Results are expressed as means ± SE. Experimental groups were compared by using Student’s t-test or one-way ANOVA, as appropriate. The Bonferroni test was applied to all significant ANOVA results with the use of SigmaStat software. A value of P < 0.05 was considered significant.

**RESULTS**

Cardiac function and recovery after I/R is dependent on β₁-AR genotype in an age-dependent manner. Baseline systolic cardiac function at 3 mo of age was significantly greater in both Arg389 and Gly389 hearts (+dP/dt = 5.05 ± 209 and 4.092 ± 304 mmHg/s, respectively) compared with NTG controls (3.201 ± 260 mmHg/s). The results of 20 min of normothermic no-flow ischemia and 40 min of reperfusion are shown in Fig. 1. The systolic recovery from I/R (Fig.1A) was significantly depressed for both Arg389 and Gly389 hearts (~38% recovery compared with ~68% for NTG). This level of dysfunction was equivalent between the two transgenic mice. Recovery of diastolic function was also equivalently depressed in the 3-mo-old Arg- and Gly389 hearts compared with NTG (Fig. 1B). In marked contrast, hearts from the 6-mo-old mice revealed an allele-specific effect in functional recovery (Fig. 2). The preschismic levels of systolic function revealed elevated +dP/dt for both Arg- and Gly389 mice (4.193 ± 216 and 5.129 ± 316 mmHg/s, respectively) compared with NTG (3.425 ± 185 mmHg/s). As with the 3-mo-old mice, after I/R, the Gly389 hearts had depressed recovery (31 ± 5.0%). However, Arg389 hearts recovered to the same extent as NTG (52 ± 1.8% vs. 57 ± 8.1%, respectively). A similar pattern showing significant recovery of diastolic function for Arg, but not Gly, ventricles was also found (Fig. 2B). CK activity of 6-mo-old mice at baseline, before I/R, was higher in Gly389 hearts. During the ischemic period and after reperfusion, CK levels were markedly increased in the Gly389 hearts, amounting to a ~20-fold difference compared with Arg389 (P < 0.005; Fig. 3). Interestingly, the NTG CK activities were somewhat higher than Arg389, particularly during reperfusion (28 ± 5.2 vs. 6 ± 2.5 IU).

Myocardial β-AR functional coupling is minimally affected by I/R and decreases in older Arg389 β₁-AR hearts. We assessed functional β-AR coupling by studying the basal and agonist-stimulated activity of adenylyl cyclase in myocardial sarcolemmal membranes under baseline conditions and after I/R. These experiments were performed to define a potential role of β-AR functional coupling to adenylyl cyclase in the recovery of myocardial function after an ischemic insult and were limited to the 6-mo-old mice, where allele-specific physiological effects were found (Figs. 1 and 2). Basal activities were not significantly different between NTG, Gly389, and Arg389 in 6-mo-old mice before I/R (Fig. 4). Maximal isoproterenol-stimulated activities pre-I/R were similar between 6-mo-old NTG and Arg389 mice; however, isoproterenol-stimulated activities for Gly389 mice remained greater than either. After I/R injury, there was a trend toward a small decrease in isoproterenol-stimulated activities in NTG and Gly389 membranes; nevertheless, Gly389 activities remained greater than those of Arg389 and NTG. Thus myocardial I/R injury did not have a significant, acute impact on functional coupling of any of the β₁-AR genotypes in these mice. Impor-
tantly, even though β-AR coupling was enhanced in 6-mo-old Gly389 hearts (compared with Arg389), functional recovery was poor. This could be due to 1) enhanced metabolic demand imposed by the Gly389 receptor compared with the Arg389 receptor or 2) additional genetically defined signaling pathways that are altered accounting for the phenotypic differences.

Allele-specific alterations of ventricular GRK and ERK1/2 activities. To further define a potential mechanism of improved myocardial recovery after I/R injury in the Arg389 mice compared with Gly389, we examined myocardial GRK expression and activity. There was no difference in myocardial GRK2 expression in 6-mo-old Arg389 and Gly389 mice before or after I/R injury (Fig. 5A). However, myocardial sarcolemmal membrane GRK functional activity, as assessed by using a rhodopsin phosphorylation assay, was increased approximately twofold in the 6-mo-old Arg389 mice compared with Gly389 both pre- and post-I/R injury (Fig. 5, B and C). In 3-mo-old hearts, though, GRK activities were not different between the two transgenic hearts (data not shown). Studies assessing myocardial total ERK1 and ERK2 expression revealed no difference between 6-mo-old Arg389 and Gly389 mice both at baseline and after I/R injury (data not shown). In contrast, several differences in phosphorylated (activated) ERK1 and ERK2 expression were noted. pERK1 levels were equivalent before I/R but were modestly reduced after I/R only in Gly

Fig. 1. Functional recovery from ischemia-reperfusion (I/R) injury is not different between Arg389 and Gly389 β₁-adrenergic receptor (β₁-AR) hearts at 3 mo of age. Shown are results from work-performing heart preparations (n = 5–7), with data collected at baseline and at indicated times after 20 min of ischemia. Neither systolic (A) nor diastolic (B) functional recovery was different between Arg and Gly mice, and both had less recovery than non-transgenic (NTG). +dP/dt and −dP/dt, peak rate of increase and decrease of left ventricular pressure. *P < 0.01 vs. NTG.

Fig. 2. Allele-specific functional recovery after I/R injury in hearts from 6-mo-old mice. Shown are results from work-performing heart preparations (n = 5–7), with data collected at baseline and indicated times after ischemia. Both systolic (A) and diastolic (B) function of the Arg389 β₁-AR hearts revealed recovery similar to NTG mice, but Gly389 β₁-AR hearts showed poor recovery, which was not different from that observed at 3 mo of age (see Fig. 1). *P < 0.01 Gly vs. NTG or Arg.
hearts (Fig. 6, A and B). Strikingly, there were significant opposite effects of I/R on pERK2 expression in Arg versus Gly hearts. I/R increased pERK2 in Arg389 hearts, whereas it decreased pERK2 in Gly389 hearts (Fig. 6, A and C).

**DISCUSSION**

In the general population, Gly or Arg can be found at amino acid position 389 of the \( \beta_1 \)-AR (12). In Caucasians, the minor allele is Gly, with a frequency of 30%. In African-Americans, the allele frequency for Gly is 45% (13). This amino acid variability occurs in the intracellular cytoplasmic tail near the seventh transmembrane-spanning segment of the human \( \beta_1 \)-AR, within the presumed eighth \( \alpha \)-helix, which is critical in receptor-G protein-coupling (14). In vitro studies have shown that the phenotype of the Arg389 receptor is one of enhanced receptor-G\(_i\) interaction compared with Gly389, which is functionally manifested as increased activation of the downstream effector adenylyl cyclase (12). Cardiac-specific overexpression of the Arg389 \( \beta_1 \)-AR led to enhanced basal and \( \beta_1 \)-agonist-stimulated LV contractility compared with Gly389 mice at 3 mo of age (17). By 6 mo of age, basal LV contractility was still greater in the Arg389 and Gly389 mice, but there was no increase in function in response to dobutamine stimulation in the Arg389 groups, whereas Gly389 \( \beta_1 \)-agonist-stimulated contractility at 6 mo was minimally desensitized (17). These results revealed that Arg389 \( \beta_1 \)-AR, as compared with Gly389 \( \beta_1 \)-AR, causes enhanced signaling at an early age but evokes downstream events that ultimately lead to decreased \( \beta_1 \)-AR signaling. Nevertheless, Arg389 mice showed no functional
or pathological evidence of ventricular failure until 9 mo of age (17).

The role of β₁-AR polymorphisms in myocardial recovery after ischemic injury has not been addressed prior to the current study. We assessed the recovery of systolic and diastolic LV function after I/R injury in transgenic mice with cardiac-specific overexpression of Arg389 or Gly389 β₁-ARs. We recognized that such overexpression might predispose hearts to greater susceptibility to I/R injury, compared with NTG hearts. We thus primarily compared the phenotypes of the two transgenics, which had equivalent expression levels of the two receptors, as a model for genotype-dependent events. There was a significant age-dependent difference in recovery. Young Arg389 and Gly389 mice had poor recovery of cardiac function after 20 min of no-flow normothermic ischemia compared with NTG controls, despite having significantly greater function before I/R. It appears, then, that enhanced signaling through both variants of the β₁-AR is detrimental to recovery of function after ischemia in these mice at an early age. Such findings are not altogether unexpected, given the increased metabolic demand imposed by enhanced β₁-AR signaling and other deleterious effects of β₁-AR activity on myocardial signaling (26).

There was a reversal of the Arg phenotype, however, at 6 mo of age. After I/R injury, 6-mo-old Gly389 mice recovered only ~30% of preischemic LV contractility compared with ~60% in the Arg389 and NTG groups. Recovery of LV diastolic function was also significantly lower in the Gly389 hearts at this age. Consistent with these findings, there was a much greater degree of myocyte damage in the Gly389 hearts after I/R as assessed by CK levels compared with Arg389. This improved recovery was temporally related to the following: 1) depressed inotropic response to β₁-AR stimulation; 2) desensitization of the Arg389 receptor coupling to adenylyl cyclase; 3) enhanced GRK activity in Arg389 β₁-AR mice; and 4) pERK2 that increased in Arg389 hearts and decreased in Gly389 hearts after I/R injury, and pERK1 that decreased in Gly389 hearts. The data obtained in 3-mo-old mice indicate that enhanced β₁-AR signaling (regardless of genotype) leads to worsened recovery of cardiac function after I/R. However, at 6 mo of age, Gly389 β₁-AR coupling was still robust and indeed greater than NTG or Arg as assessed by agonist-stimulated adenylyl cyclase activities. Such activities were not altered by the ischemic insult in either group. Therefore, the lack of Gly389 recovery of function does not appear to be due to a loss of β₁-AR function, and instead, the recovery of Arg389 ventricular function that seems to occur by 6 mo of age is associated with a “normalization” of its agonist-stimulated adenylyl cyclase activities. Thus this genotype is protective against ischemic injury at this age. The Gly389 genotype, on the other hand, continues to have enhanced β₁-AR signaling that appears to remain unfavorable.

To assess a potential mechanism of this beneficial effect of Arg389, we assessed myocardial GRK expression and activity. GRK-mediated phosphorylation of β-ARs is a major mechanism of desensitization. Also, the Arg389 β₁-AR appears to be more readily desensitize (compared with Gly389) in recombinant expressing cells, probably due to its more favorable conformation for agonist-promoted GRK binding (15). We considered, then, if long-term Arg389 signaling evoked enhanced cellular expression or activity of myocardial GRKs, that the potential for desensitization would be even further enhanced. At 6 mo of age, when the Arg389 mice have a blunted β₁-agonist inotropic response, there is no difference in myocardial GRK2 expression relative to NTG for either genotype. However, we observed a previously unrecognized mechanism that appears to contribute to the blunted agonist-stimulated β₁-AR signaling in the 6-mo-old Arg389 hearts, which was a significant, and allele-specific, increase in myocardial sarcolemmal membrane GRK activity as assessed by rhodopsin phosphorylation. Taken together with previously reported data that describe improved cardiac functional recovery after I/R injury in transgenic mice with threefold cardiac-specific overexpression of GRK2 (4), we conclude that the improved recovery in Arg389 mice is, in part, due to GRK2-mediated desensitization of this form of the β₁-AR. β₁-ARs couple to activation of the MAPK cascade, and several members of the signal transduction pathway have been implicated in regulating cardiac myocyte survival after I/R injury (22, 25).

Two forms of cell death (necrosis and apoptosis) are important in the pathology of ischemia and infarction (7). The precise contribution of these two forms of cell death in the setting of I/R injury is unclear, but activation of signaling cascades including ERK1/2 has been implicated in cellular survival through their recruitment of antiapoptotic pathways of protection (5). Transgenic mice overexpressing MEK1 (four- to sixfold) in the heart had a 2.5-fold increase in ERK1/2 protein (3). These mice were protected from ischemic damage as assessed by invasive hemodynamics, which showed no decrease in ejection fraction 7 days after 30 min of ischemia (11). In addition, rat neonatal myocytes infected with adenovirus to overexpress MEK1 had a twofold increase in ERK1/2 activation, virtually complete loss of the apoptotic effect of 2-deoxyglucose, and partial protection from caspase 3 activation (3). Cardiomyocytes transfected with cardioprophin 1 had antiapoptotic effects to serum deprivation with concomitant activation of ERK1/2 (21). This antiapoptotic effect of cardioprophin 1 was negated by an ERK1/2 inhibitor (21). In ERK1⁻/⁻ mice (16), the LV infarct size (after 60 min of ischemia and 24 h of reperfusion) was not different from that of wild-type mice (11). In contrast, ERK2⁻/⁻ mice (19) showed a marked increase in infarct area under the same I/R conditions (11). These data suggest a particularly important role for ERK2 in protecting the heart from I/R. These data are consistent with our conclusions that the I/R-promoted increase in ERK2 activation in Arg389 hearts, and its decrease in Gly389 hearts, contributes to the recovery phenotypes observed.

In summary, we have shown that the two human polymorphic forms of the β₁-AR, Arg389 and Gly389, have different responses to I/R. The Arg389 variant has recovery from I/R similar to that of NTG, whereas Gly389 mice show poor recovery. Cardiac Arg389 β₁-ARs evoke a program that results in blunted β₁-AR signaling, enhanced GRK activity, and an I/R-dependent increase in ERK2 activation, which together act to influence I/R recovery in an allele-specific manner. These data strongly suggest that in human cardiac ischemia, β₁-AR genotype could direct different pathways and influence outcome.

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