Heart Failure

Negative Impact of β-Arrestin-1 on Post-Myocardial Infarction Heart Failure via Cardiac and Adrenal-Dependent Neurohormonal Mechanisms

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Abstract—β-Arrestin (βarr)-1 and β-arrestin-2 (βarrs) are universal G-protein–coupled receptor adapter proteins that negatively regulate cardiac β-adrenergic receptor (βAR) function via βAR desensitization and downregulation. In addition, they mediate G-protein–independent βAR signaling, which might be beneficial, for example, antiapoptotic, for the heart. However, the specific role(s) of each βarr isoform in cardiac βAR dysfunction, the molecular hallmark of chronic heart failure (HF), remains unknown. Furthermore, adrenal βarr1 exacerbates HF by chronically enhancing adrenal production and hence circulating levels of aldosterone and catecholamines. Herein, we sought to delineate specific roles of βarr1 in post–myocardial infarction (MI) HF by testing the effects of βarr1 genetic deletion on normal and post-MI cardiac function and morphology. We studied βarr1 knockout (βarr1KO) mice alongside wild-type controls under normal conditions and after surgical MI. Normal (sham-operated) βarr1KO mice display enhanced βAR-dependent contractility and post-MI βarr1KO mice enhanced overall cardiac function (and βAR-dependent contractility) compared with wild type. Post-MI βarr1KO mice also show increased survival and decreased cardiac infarct size, apoptosis, and adverse remodeling, as well as circulating catecholamines and aldosterone, compared with post-MI wild type. The underlying mechanisms, on one hand, improved cardiac βAR signaling and function, as evidenced by increased βAR density and proconstrictive signaling, via reduced cardiac βAR desensitization because of cardiac βarr1 absence, and, on the other hand, decreased production leading to lower circulating levels of catecholamines and aldosterone because of adrenal βarr1 absence. Thus, βarr1, via both cardiac and adrenal effects, is detrimental for cardiac structure and function and significantly exacerbates post-MI HF. (Hypertension. 2014;63:404-412.) • Online Data Supplement

Key Words: β-arrestin-1 ■ aldosterone ■ catecholamines ■ knockout mice

Despite recent advances in its prevention and management, heart disease, such as hypertension and post–myocardial infarction (MI) heart failure (HF), remains the leading cause of death in the Western world and new treatments are needed.1 The molecular hallmark in chronic HF is cardiac β-adrenergic receptor (βAR) dysfunction because of increased receptor desensitization and downregulation.2,4 βARs belong to the superfamily of G-protein–coupled receptors (GPCRs), which, on agonist binding, activate the G protein-adenyl cyclase-cyclic adenosine monophosphate (cAMP)-protein kinase A signaling pathway leading to increased inotropy and chronotropy in the heart.2 In chronic HF, however, there is a selective downregulation of cardiac β1ARs and significant functional desensitization (G protein uncoupling) of the remaining membrane β1ARs and β2ARs, which dramatically diminish cardiac adrenergic and inotropic reserves.3–6 These processes are mediated at the molecular level by the β-arrestins (βarrs), which exist in 2 distinct isoforms (βarr1 and βarr2, also known as arrestin-2 and arrestin-3, respectively) in mammals and are abundantly expressed in the heart.7–9 βarrs bind agonist-activated cardiac βARs, terminating their G-protein–mediated signaling and targeting them for internalization, after receptor phosphorylation by GPCR kinase (GRK)-2 (mainly), the main cardiac GRK that is significantly elevated in HF.2,8–16 Consistent with their crucial role in cardiac βAR desensitization, βarr1 knockout (KO) mice display enhanced cardiac contractile responses to isoproterenol (a standard βAR full agonist) stimulation in vivo.17

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In addition, the βAR-bound βarrs have been shown to elicit a second wave of G-protein–independent signals, some of which might be beneficial for the failing heart, for example, extracellular signal–regulated kinase activation and epidermal growth factor receptor (EGFR) transactivation, both of which can promote cardiac cell survival, proliferation, etc.28–31 However, the actions of the 2 βarrs in vivo are rarely complementary; in fact, they might even oppose each other’s effects in certain tissues/ organs, including in the cardiovascular system (eg, vascular smooth muscle cells and cardiomyocytes).22,23 With regard to the heart, the exact role of each βarr in cardiac βAR desensitization or G-protein–independent signaling is not presently known.

The physiological hallmark of chronic HF is the sustained hyperactivity of several neurohormonal systems, in particular the sympathetic nervous and the renin–angiotensin–aldosterone systems, accompanied by elevated circulating levels of the catecholamines (CAs) norepinephrine and epinephrine and of aldosterone, respectively.26,27 All these circulating hormones exert detrimental effects on the failing heart, leading both to diminished function and to accelerated and aggravated adverse remodeling in chronic HF.24,25 The main source of these circulating hormones is the adrenal gland and we have shown in previous studies that βarr1, in particular, is a crucial mediator of adrenocortical aldosterone production,26,27 as well as of adrenomedullary CA production (acting in concert with adrenal GRK2),22,23 both normally and in post-MI HF in vivo.

Given that the specific roles of cardiac βarr1 in βAR (dys) function and signaling in HF have never been investigated and also the crucial role this protein plays in the adrenals in elevating the neurohormonal burden of the failing heart, we sought to investigate, in the present study, the effects of its genetic deletion on cardiac function, both normally and in post-MI HF. To study the (patho)physiological role(s) specifically of this βarr isoform in vivo, we took advantage of the available global βarr1KO mouse model17 and studied these mice alongside age-matched male WT mice. We first examined the cardiac function parameters of these mice, both in sham and in post-MI groups. Echocardiography revealed that, although similar between the sham groups (a finding consistent with the results of a previous study on these mice),17 (basal) ejection fraction was severely diminished in WT mice at 4 weeks after MI, as expected, but significantly higher in the post-MI βarr1KO mice at the same time point (Figure 1A; Table S1). Consistent with these findings, on in vivo catheterization for hemodynamic measurements, post-MI βarr1KO mice also show significantly enhanced cardiac contractility, both basally and in response to isoproterenol stimulation, compared with post-MI WT mice (Figure 1B; Table S1) although left ventricular end-systolic and end-diastolic pressures are indicated. For most 3-group statistical comparisons, Dunnett test using SAS version 8.2 software was used, as well. For all tests, a P value of <0.05 was generally considered to be significant.

**Results**

**Cardiac Function of Normal and Post-MI βarr1KO Mice**

To investigate the impact on cardiac function of the genetic deletion exclusively of βarr1, we used the available global βarr1KO mouse model (Figure S1 in the online-only Data Supplement). These mice breed normally, without any gross abnormalities and present no overt cardiovascular or other phenotype.17 To induce HF, 3-month-old male mice underwent surgical MI and were studied alongside age-matched male WT mice. We first examined the cardiac function parameters of these mice, both in sham and in post-MI groups. Echocardiography revealed that, although similar between the sham groups (a finding consistent with the results of a previous study on these mice),17 (basal) ejection fraction was severely diminished in WT mice at 4 weeks after MI, as expected, but significantly higher in the post-MI βarr1KO mice at the same time point (Figure 1A; Table S1). Consistent with these findings, on in vivo catheterization for hemodynamic measurements, post-MI βarr1KO mice also show significantly enhanced cardiac contractility, both basally and in response to isoproterenol stimulation, compared with post-MI WT mice (Figure 1B; Table S1) although left ventricular end-systolic and end-diastolic pressures are

**Materials and Methods**

**Experimental Animals and Surgical Procedures**

The animals in this study were handled according to the animal welfare regulations and protocols approved by the authors’ institutional review boards. An expanded Materials and Methods section is available in the online-only Data Supplement, which includes detailed methods on the following: animals and surgical procedures, echocardiography and hemodynamics, ELISA measurements of hormones and cytokines, infarct size measurements, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) and Masson-Trichrome staining, real-time polymerase chain reaction, βAR density, cAMP and sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA) activity measurements, and Western blotting.

**Statistical Analyses**

Data are generally expressed as mean±SEM. Unpaired 2-tailed Student’s t test and 1- or 2-way ANOVA with Bonferroni test were generally performed for statistical comparisons, unless otherwise
similar between the 2 post-MI groups on maximal isoproterenol challenge (Table S1). Notably, isoproterenol-induced contractility is elevated also in sham (normal) βarr1KO mice compared with sham WT (Figure 1B; Table S1), which is again consistent with the results of a previous study on these mice.17 Taken together, these findings strongly indicate that cardiac βarr1 is a major negative regulator of βAR-dependent contractility in vivo and that its absence leads to significant attenuation of cardiac dysfunction after MI.

Survival and Neurohormonal Status of Post-MI βarr1KO Mice

Next, we sought to further examine the phenotype of the post-MI βarr1KO mice. Kaplan–Meier survival curves indicated a markedly lower (overall) mortality of the post-MI βarr1KO mice compared with post-MI WT controls (P=0.012; Figure 2A). This was accompanied by significantly reduced elevations in the plasma circulating levels of the CAs norepinephrine and epinephrine in post-MI βarr1KO mice compared with post-MI WT (Figure 2B), indicative of reduced overall sympathetic activation in post-MI HF βarr1KO mice. As for the other major cardiotoxic hormone, aldosterone, post-MI βarr1KO mice, remarkably, failed to exhibit any hyperaldosteronism (ie, elevation of circulating aldosterone levels) whatsoever, in marked contrast to post-MI WTs, which, as expected, display severe hyperaldosteronism (Figure 2C). This finding strongly corroborates the essential role of adrenal βarr1 in post-MI HF-associated hyperaldosteronism as we have previously reported.26,27 Finally, and consistent with these effects on circulating levels of CAs and aldosterone, post-MI βarr1KO mice also display significantly lower mean arterial blood pressure (125±4 mm Hg) than post-MI WT (141±5 mm Hg) at 4 weeks after MI (P<0.05; n=5).

Cardiac Apoptosis, Dilatation, and Infarct Size in Post-MI βarr1KO Mice

The increased survival of the post-MI βarr1KO mice prompted us to investigate the cardiac apoptosis in these mice. Cardiac cellular apoptosis, as measured with the TUNEL assay in the hearts of the post-MI mice, was found significantly reduced in βarr1KO mice compared with WT mice at 24 hours after MI, which is consistent with the Kaplan–Meier survival results (Figure 3A). In addition, cardiac dilatation was also decreased in the βarr1KO mice compared with WT controls at 4 weeks after MI (Figure 3B; Table S1), and, importantly, infarct size was also significantly reduced in the βarr1KO hearts compared with control WT hearts at the same post-MI time point (4 weeks; Figure 3C and 3D). To exclude the possibility that differences in the extent of the initial injury inflicted by our surgical MI method on the 2 lines might have been responsible for this result, infarct size at 24 hours after MI was also measured and found indistinguishable between the 2 groups (Figure S2A and S2B). Taken together, these results indicate that post-MI βarr1KO hearts have decreased apoptosis, dilatation, and infarct size, which might underlie their favorable overall survival phenotype.

Cardiac Inflammation, Fibrosis, and Adverse Remodeling Markers in Post-MI βarr1KO Mice

Next, we examined markers of post-MI cardiac inflammation and adverse remodeling in the 2 animal groups. Levels of all 3 major proinflammatory cytokines, tumor necrosis factor-α (Figure 4A), interleukin-6 (Figure 4B), and interleukin-1β (Figure 4C), were found significantly decreased in post-MI βarr1KO hearts compared with control WT hearts at 4 weeks after MI, indicating decreased overall cardiac inflammation in the post-MI βarr1KO mice. Moreover, cardiac fibrosis, as measured with Masson–Trichrome staining, was also markedly decreased in the post-MI βarr1KO mice compared with post-MI WT controls (Figure 4D and 4E). Finally, mRNA expression of all major adverse remodeling-associated biomarkers, that is, collagen synthesis (Figure 4F), B-type natriuretic peptide (Figure 4G), atrial natriuretic factor (Figure S3A), and transforming growth factor-β (Figure S3B), was significantly reduced in the post-MI βarr1KO hearts compared with post-MI WT hearts, strongly suggesting that adverse remodeling is markedly attenuated in post-MI βarr1KO hearts.

Cardiac βAR Density, Signaling, and Function in Post-MI βarr1KO Mice

In an effort to dissect the molecular mechanisms underlying the dramatic effect of the absence of βarr1 on cardiac contractile...
function, we also investigated several aspects of cardiac βAR signaling and function in the 2 post-MI groups. Consistent with the phenotypic and functional data, total βAR density in the post-MI βarr1KO hearts was significantly higher compared with post-MI WT hearts, which display marked βAR downregulation after MI, as expected (Figure 5A). This attenuated βAR downregulation in post-MI βarr1KO hearts was accompanied by preserved total cAMP levels, again in sharp contrast with the WT hearts whose cAMP content is dramatically diminished after MI (Figure 5B). Given that cAMP is the most critical mediator of cardiac βAR procontractile signaling, this result strongly indicates that the absence of cardiac βarr1 is sufficient to restore βAR-dependent contractility in post-MI HF. Another important component of the cardiac contractile machinery regulated by βARs is SERCA-2a. SERCA activity was also found to be significantly increased in post-MI βarr1KO hearts compared with post-MI WT hearts (Figure 5C), thus providing another line of evidence for the increased contractile function of post-MI βarr1KO hearts. Of note, however, cardiac SERCA activity of βarr1KO mice seems elevated also in the normal (sham) groups (Figure 5C), indicating that cardiac βarr1 might affect the cardiac contractile machinery even under normal conditions (no HF). Finally, because cardiac βarrs, and in particular βarr2, have been shown to mediate EGFR transactivation from the β1AR upon the latter’s phosphorylation by GRK5, an effect that is considered antiapoptotic in the heart, we also examined levels of EGFR transactivation in the post-MI hearts of βarr1KO mice and WT after acute stimulation with isoproterenol in the presence of ICI-118,551 (a β2-AR-selective antagonist) in vivo (to activate the β1ARs only). Western blotting for phospho-Tyr845-EGFR (active EGFR) in cardiac extracts from these mice revealed significantly elevated EGFR transactivation in βarr1KO hearts compared with WT hearts at 24 hours after MI (and also in sham hearts; Figure 5D and 5E), suggesting that cardiac βarr1 inhibits (rather than promotes) this β1AR-induced EGFR transactivation in the heart. Finally, in an effort to better dissect the relative contributions of cardiac versus adrenal βarr1 on the observed phenotypes of the mice of the study, we overexpressed the known βarr1 inhibitor mini-gene βarr1ct27 specifically in the hearts of post-MI WT mice, to inhibit cardiac (only) βarr1 in vivo, and studied these hearts alongside control post-MI WT hearts receiving adenovirus encoding for green fluorescent protein at the time of MI. As shown in Figure S4, cardiac βarr1 inhibition with βarr1ct led to significant improvements in cardiac βAR density (Figure S4A) and cAMP accumulation (Figure S4B) at 4 weeks after MI, indicating improved cardiac adrenergic and inotropic reserves, as expected; however, no significant changes in circulating CAs of the post-MI WT mice were observed with cardiac βarr1ct at 4 weeks after MI (Figure S4C), indicating that CA production/secretion is mediated by adrenal (rather than cardiac) βarr1.
Discussion

βarr1 (and to a lesser extent βarr2) is abundantly expressed throughout the cardiovascular system7–9 and regulates the majority of cardiovascular GPCRs, including cardiac βARs, cardiac and adrenocortical angiotensin II type 1 receptors, adrenal and central sympathetic nervous system α2-ARs, etc.9,32,33 The importance of βarrs in cardiovascular (and other systems) biology is evidenced by the fact that the double βarr1/2 KO mouse is embryonic lethal.32,33 Their actions on the heart have been attributed to both their functions as receptor
desensitizers/internalizers and as G-protein–independent signal transducers. However, and especially for their latter function, the exact in vivo roles of each cardiac βarr isoform in GPCR-dependent signal transduction are currently unknown. In the present study, we were able to delineate the cardiovascular phenotype of βarr1KO mice in post-MI HF, along with its associated underlying molecular signaling mechanisms. Specifically, we found that the absence of βarr1 from the heart leads to significantly better overall cardiac function in post-MI HF, dramatically increases cardiac βAR-dependent cardiac function both physiologically and in post-MI HF, and significantly attenuates adverse remodeling, apoptosis, inflammation, and infarct size, leading to improved overall survival post-MI. At the same time, the absence of βarr1 from the adrenal gland leads to a dramatically improved neurohormonal profile of the failing post-MI heart, with reduced circulating CA and aldosterone levels. Finally, cardiac βAR signaling and function are dramatically elevated in the post-MI βarr1KO mice, translating into markedly improved cardiac adrenergic and inotropic reserves.

Thus, βarr1 seems to be the single most important negative regulator of βAR-dependent procontractile signaling and function, via the classical processes of desensitization and down-regulation of the cardiomyocyte βAR content, and its absence results in dramatically elevated βAR-dependent contractility, both normally and after MI, and in almost restored functional βAR number and cAMP-mediated signaling post-MI, that is, in dramatically improved β-adrenergic and inotropic reserves of the failing heart. Consistent with this, cardiac SERCA activity is also markedly elevated in post-MI βarr1KO mice compared with control WT mice. Of note however, SERCA activity is also significantly elevated in normal (sham) βarr1KO mice, suggesting that the effects of cardiac βarr1 on SERCA activity extend beyond (merely) βAR desensitization. Indeed, current investigations suggest that cardiac βarr2 directly interacts with SERCA-2a, promoting its SUMOylation and increasing its activity, and that this is actually opposed by βarr1 (A. Lymperopoulos, unpublished data). However, βarr2 seems unable to compensate for the absence of βarr1 for any of these effects in vivo (and it is not compensatively upregulated,}

Figure 5. A, β-Adrenergic receptor (βAR) density in cardiac plasma membranes of sham-operated (Sham) or of 4-week post-myocardial infarction (MI) β-arrestin-1 knockout (βarr1KO) and wild-type (WT) mice. *P<0.05 vs all other groups; n=5 hearts/group. B, Steady-state total cAMP levels in cardiac homogenates purified from these mice. *P<0.05 vs all other groups; n=5 hearts/group. C, Cardiac sarco(endo)plasmic reticulum Ca2+-ATPase activity measured in membrane homogenates from the hearts of these mice. *P<0.05 vs WT Sham; **P<0.05 vs WT MI; n=5 hearts/group. D and E, Cardiac epidermal growth factor receptor (EGFR) transactivation in these mice. Representative Western blots in total cardiac protein extracts for phospho-Tyr845-EGFR or total EGFR (loading control) are shown in D and their densitometric measurement in E. Relative EGFR transactivation was calculated by comparing the ratios of phospho-EGFR to total EGFR for each sample. *P<0.05 vs either WT; **P<0.05 vs KO Sham; n=5 hearts/group.
either; data not shown”). Taken together, these phenotypic findings strongly suggest that the βarr1 isoform is the salient βarr responsible for βAR desensitization and downregulation in the heart (Figure 6), processes whose dramatic elevation constitutes a molecular hallmark of chronic HF.\(^\text{1,2,3,5}\) Therefore, prevention of the interaction of βarr1 with cardiac βARs, which is achieved (indirectly) via cardiac GRK2 inhibition with βARKct (β-adrenergic receptor kinase C-terminus) for instance,\(^\text{1,3,15,35}\) might be beneficial as a positive inotropic therapy for HF (Figure 6).

It is now well known and documented that βarrs, in addition to the original biological role ascribed to them as terminators of G-protein–dependent signaling by GPCRs, can also serve as signal transducers in their own right independently of G proteins.\(^\text{32,33}\) Indeed, specifically in the heart, βarrs have been shown to mediate beneficial, antiapoptotic, and proliferation-promoting signals from the β,α AR (upon its phosphorylation by GRK5 and not GRK2 though) and from the mechanical stretch-activated angiotensin II type 1 receptor via EGFR transactivation and subsequent extracellular signal–regulated kinase phosphorylation/activation.\(^\text{18,36}\) In addition, they might even mediate procontractile signaling from the angiotensin II type 1 receptor in cardiac myocytes, an effect ascribed to βarr2 and presumably inhibited by GRK2 and βarr1.\(^\text{21}\) We found that β1,AR-induced EGFR transactivation is significantly elevated in βarr1KO hearts in vivo, both in normal (sham) conditions and (even more so) in post-MI HF, consistent with the apoptotic phenotype of the βarr1KO hearts in vivo. This strongly suggests that cardiac βarr2 normally mediates this beneficial, antiapoptotic β1,AR-dependent EGFR transactivation, whereas cardiac βarr1 opposes this signaling mechanism (Figure 6). Of course, studies in βarr2KO mice are warranted to further validate this scenario. Another important aspect of HF pathophysiology, in which the 2 βarr isoforms might exert differential or even opposing effects, is peri-infarct area neoangiogenesis;\(^\text{37}\) in fact, given the difference observed in infarct size at 4 weeks after MI (Figure 3C and 3D), this is quite likely and certainly warrants further investigation in the future.

Finally, the neurohormonal profile of the post-MI βarr1KO mice confirms the essential roles of adrenal βarr1 in (1) chronically elevated CA secretion in post-MI HF (via chronic desensitization/downregulation of the sympatho-inhibitory α2ARs in the chromaffin cells of the adrenal medulla; Figure 6), and (2) post-MI HF-associated hyperaldosteronism (via increased angiotensin II–dependent aldosterone synthesis and secretion in adrenocortical zona glomerulosa cells; Figure 6), which we have uncovered during the past several years.\(^\text{26-30}\)

Admittedly, the biggest limitation of the present study is the use of a global KO model that precludes any safe conclusions on the relative contributions of adrenal versus cardiac βarr1 on the observed phenotypic effects of the post-MI βarr1KO mice. Only development of tissue-specific KO mice can provide definitive answers to this important question. However, our data on post-MI WT mice having cardiac-specific blockade of βarr1 courtesy of cardiac-specific βarr1ct overexpression strongly suggest that cardiac βarr1 absence is responsible for the observed improvements in the adrenergic and inotropic reserves of the post-MI βarr1KO mice, whereas their vastly improved neurohormonal profile (ie, lower circulating CA levels and complete absence of hyperaldosteronism) is (mainly) because of βarr1 absence from the adrenal glands. Given the extensive cross-talk between the 2 organs (heart-adrenals) and that aldosterone and catecholamines can be produced also in the heart per se however,\(^\text{38,39}\) drawing of these conclusions warrants extreme caution.

Nevertheless, all of the above findings are clinically important: we report for the first time that βarr1 is the βarr isoform mainly (if not exclusively) responsible for cardiac βAR desensitization and downregulation in vivo, a hallmark abnormality in chronic HF. In addition, βarr1 promotes elevation of the neurohormonal burden of the failing heart through its actions in the adrenal gland and seems to oppose several beneficial signaling effects of its counterpart, βarr2, in the myocardium, such as EGFR transactivation and extracellular signal–regulated kinase signaling, which lead to inhibition of apoptosis and inflammation and to promotion of cardiac survival. Thus, βarr1 removal from both the heart and the adrenals results in significantly improved cardiac function, structural remodeling, β-adrenergic and inotropic reserves, reduced neurohormonal burden, and increased cardiac and overall survival in

Figure 6. Schematic illustration of the cardiac (left) and adrenal (right) signaling mechanisms underlying the effects of β-arrestin-1 (βarr1) on cardiovascular function. α,AR indicates α2-adrenergic receptor; AT,R, angiotensin II type 1 receptor; CA, catecholamine; EGFR, epidermal growth factor receptor; G, stimulatory G protein; P, phosphorylation; and PKA, protein kinase A. See text for details and all other molecular acronym descriptions.
post-MI HF. These findings strongly suggest that inhibition of βarr1 activity toward βARs in the heart and βarr1 blockade in the adrenals, either directly (with a pharmacological βarr1-specific inhibitor or via small interfering RNA knockdown) or indirectly (eg, via blockade of GRK2, which promotes association of βarr1 with cardiac βARs) might be a viable therapeutic strategy for chronic post-MI HF treatment, with the potential also of complementing or enhancing the benefits of β-blocker therapy, which is hampered by the adverse effect of negative inotropy.

Perspectives

The present study reports for the first time that cardiac βarr1 is the βarr isoform responsible for cardiac βAR desensitization and downregulation in vivo, a hallmark molecular abnormality in chronic HF. In addition, adrenal βarr1 promotes elevation of the neurohormonal burden of the failing heart by mediating CA secretion and aldosterone synthesis and secretion in the adrenal medulla and cortex, respectively. Finally, βarr1 seems to oppose several beneficial signaling effects of its counterpart, βarr2, in the myocardium, such as EGFR transactivation and extracellular signal–regulated kinase signaling, which lead to inhibition of apoptosis and inflammation and to promotion of cardiac survival. Thus, βarr1 removal from both the heart and the adrenals results in significantly improved cardiac function, halted adverse remodeling, elevated β-adrenergic and inotropic reserves, reduced neurohormonal burden, and increased cardiac and overall survival in post-MI HF. These findings strongly suggest that inhibition of βarr1 activity toward βARs in the heart and βarr1 blockade in the adrenals, either directly (with a pharmacological βarr1-specific inhibitor or via small interfering RNA knockdown) or indirectly (eg, via blockade of GRK2, which promotes association of βarr1 with receptors) might be a viable therapeutic strategy for chronic post-MI HF treatment.

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Disclosures

None.

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What Is New?

- β-arrestin (βarr1; rather than βarr2) is the βarr isoform responsible for β-adrenergic receptor desensitization and downregulation in vivo in the heart, a hallmark molecular abnormality in chronic heart failure.
- βarr1 in the adrenals is absolutely essential for post–myocardial infarction hyperaldosteronism and catecholamine elevation in vivo, thereby heightening the neurohormonal burden of the failing heart.
- Cardiac βarr1 also counters some beneficial effects of βarr2 in the myocardium, for example, antiapoptosis and suppression of inflammation.

What Is Relevant?

- Adrenal βarr1 can promote hyperaldosteronism and sympathetic hyperactivity. 2 neurohormonal mechanisms that contribute significantly to the development of hypertension and heart failure.
- Pharmacological or genetic βarr1 inhibition in vivo, at least in the adrenals and in the heart, might be a valid antihypertensive and anti–heart failure treatment strategy.

Summary

Blockade of βarr1 activity in both the heart and the adrenal gland might be of therapeutic value for halting or even reversing post–myocardial infarction heart failure progression.
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This document contains:

1) The expanded “Materials and Methods” section of the manuscript
2) Supplementary Figures S1-S4 with associated legends
3) Supplementary References
4) Supplementary Table S1 with associated legend
Materials and Methods

Experimental animals and surgical procedures

The animals in this study were handled according to animal welfare regulations and protocols approved by the authors’ Institutional Review (IACUC) Boards. Genetically engineered, 8- to 12-wk-old βarr1KO (on C57BL/6 background) and corresponding C57BL/6 wild type (WT) male mice were used for this study. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg). Animals were placed in the supine position on a heated operation board and a midline cervical incision was made to expose the trachea. Following successful endotracheal intubation, the cannula was connected to a rodent ventilator. The entire left ventricle (i.e. both infarct and non-infarct zone) were used for subsequent histological and biochemical assays. Myocardial infarction was performed as previously described. Briefly, MI was inflicted by ligation of the left anterior descending (LAD) coronary artery. At the end of the procedure, the chest cavity was closed and the mouse was gradually weaned off the respirator, endotracheal tube removed, and the animal was placed in a cage on a heating pad until regaining consciousness. Direct adenoviral injection (Adβarr1ct or AdGFP) into the left ventricular (LV) cavity was done as described previously. Briefly, while the animal’s chest was open for the LAD ligation, 1 × 10⁹ total particles, diluted in 80 µl phosphate-buffered saline (PBS), of the respective adenoviral vectors were rapidly injected via an apically inserted 33-gauge needle. Overexpression of βarr1ct or GFP was confirmed by western blotting in total protein extracts obtained from the hearts of the animals at the end of the study period (4 weeks post-surgery, data not shown).

Echocardiography & in vivo hemodynamics

Transthoracic echocardiography was performed with a linear 30-MHz transducer (VeVo 770 High Resolution Imaging System, VisualSonics, Toronto, ON, Canada), as described. In vivo hemodynamic analysis was again performed as previously described. Briefly, the chest was opened and the pericardium was dissected to expose the heart. A 1.4-Fr pressure-conductance catheter (Millar Instruments, Houston, TX) was used to catheterize the animal and record cardiac hemodynamics. A polyethylene-50 catheter was inserted into the right external jugular vein for isoproterenol infusions.

Measurements of plasma circulating hormones & cardiac cytokines via ELISA

Plasma norepinephrine and epinephrine were determined with the BI-CAT EIA kit from ALPCO Diagnostics (Windham, NH), as described. Serum aldosterone was determined with the Aldosterone EIA kit also from ALPCO Diagnostics, as described. Pro-inflammatory cytokines TNFα, IL-6 and IL-1β were measured in serum obtained from left ventricular blood, immediately prior to heart excision and animal euthanizing, via multiplexed ELISA, as described. The assay was performed using the Mouse Cytokine ELISA Profiling Kit (EA-1091, Signosis), according to the manufacturer’s instructions.

Infarct size measurements

After termination of experiments, hearts were excised and the left ventricle was transversely dissected into two halves. The portion from the base to mid-cavity was snap-frozen and stored for subsequent biochemical studies. The portion from mid-cavity to apex was fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with triphenyltetrazolium chloride (TTC) to determine infarct size, as previously described.
In situ TUNEL & Masson-Trichrome staining

Heart specimens were fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5-µm thickness. DNA fragmentation was detected in situ in deparaffinized sections using the ApopTag Kit (Intergene) and according to manufacturer’s instructions, as described previously. The total number of nuclei was determined by manual counting of DAPI-stained nuclei in six random fields per section. All terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)-positive nuclei were counted in each section. Masson-trichrome staining for cardiac interstitial fibrosis was performed as described.

Real time & RT-PCR

Conventional RT-PCR for the presence of βarr1 transcript in heart mRNA was performed with a specific primer mix for mouse βarr1 mRNA, as described. Real-time PCR for cardiac adverse remodeling markers (ANF, BNP, Collagen-1α1, and TGF-β1) was performed in total heart mRNA, as described. Briefly, quantitative real-time PCR was performed using a MyIQ Single-Color Real-Time PCR detection system (Bio-Rad) using SYBR Green Supermix (Bio-Rad) and 100 nM of gene-specific oligonucleotides. Quantification of mRNA included normalization to 18s rRNA levels. No bands were seen in control reactions in the absence of reverse transcriptase. Primer pairs used were: 5′-CAAGAACCTGCTAGACCACC-3′ & 5′-AGCTGTTGCAAGCTAGTCC-3′ for ANF; 5′-CCAGAGACAGCTTGAAGG-3′ & 5′-TCCGATCCGTCTATCTTG-3′ for BNP; 5′-CAACAATTCTGCGTTACCTTG-3′ & 5′-GAAAGCCCTGTATTCGTCTCCT-3′ for TGF-β1; 5′-CTGCTGGCAAGAGGAGCTGGAGA-3′ & 5′-ACCAGGAAGACCTGGAATC-3′ for Col-1α1; 5′-AAGGGACACGAGTGTCCAAGA-3′ & 5′-ATGCTCGCCAGCTTCTTG-3′ for βarr1; 5′-TCGATGCTTATAGCTGAGTG-3′ & 5′-TGATCGTCTTCCGAACCTCC-3′ for 18S rRNA.

Cardiac βAR density, cAMP & SERCA activity measurements

βAR density was measured in isolated cardiac plasma membranes using 125I-CYP (Iodocyanopindolol), as described. Cardiac cAMP levels were measured with the BIOMOL Cyclic AMP PLUS EIA kit (Biomol, Plymouth Meeting, PA), as described. Cardiac (maximal) SERCA activity was measured as described. Briefly, crude ventricular membranes were prepared and assayed in 10 mM Tris, pH 7.5, 100 mM KCl, 5 mM MgCl2, 5 mM Na2ATP, 0.1 mM CaCl2, 0.2 mM NADH, 1.5 mM trisodium phosphoanolypruvate, 15 units/ml pyruvate kinase, and 36 units/ml lactate dehydrogenase. Total ATPase activity was assayed by monitoring the rate of loss of A340 after addition of the membrane preparation to a thermostatically controlled (37 °C) cuvette in a spectrophotometer. Background ATPase activity was determined in the absence of ATP. Ca2+-independent ATPase activity was assayed in the presence of 10 mM EGTA instead of Ca2+ and subtracted from the total ATPase activity to derive the Ca2+-dependent ATPase (SERCA) activity. SERCA activity is expressed as nmol of inorganic phosphate (P_i) produced per min per mg of protein.

Western blotting for EGFR transactivation
EGFR transactivation was measured by western blotting in extracts obtained from hearts excised from the mice 30 min after injection with a solution containing 333 ng/kg of body weight isoproterenol in the presence of 25 μM ICI-118,551 (to stimulate β1ARs only). Cardiac extracts were prepared in a 20 mM Tris pH 7.4 buffer, containing 137 mM NaCl, 1% Nonidet P-40, 20% glycerol, 10 mM PMSF, 1 mM Na3VO4, 10 mM NaF, 2.5 μg/ml aprotinin, and 2.5 μg/ml leupeptin. After homogenization, protein concentration was determined and equal amounts of protein per sample were loaded on SDS-PAGE gels for electrophoretic separation, as described previously. Total EGFR and phosphoTyr845-EGFR were detected by using anti-EGFR (sc-03) and anti-phosphoTyr845-EGFR (sc-101669) antibodies, both from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoblots were revealed by enhanced chemiluminescence (ECL, Amersham Biosciences) and visualized in the FluorChem E Digital Darkroom (Cell Biosciences). Densitometry was performed with the AlphaView software (Cell Biosciences) in the linear range of signal detection (on non-saturated bands). The ratios of the phosphoEGFR-to-total EGFR for each sample were compared to determine EGFR activation.

**Figure S1**

RT-PCR in total cardiac mRNA

<table>
<thead>
<tr>
<th>βarr1 (321 bp)</th>
<th>WT</th>
<th>βarr1KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA (503 bp)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure S1:** RT-PCR in cardiac mRNA isolated from βarr1KO or WT mice for confirmation of the absence of βarr1 transcript.
Figure S2: Infarct size in βarr1KO (KO) and WT mice at 24 hrs post-MI. Sham hearts are also shown as negative controls. (A) Representative triphenyltetrazolium chloride (TTC)–stained cardiac cross-sections. (B) Average left ventricular (LV) infarct size (n=5 for each group). No significant difference between the MI groups was observed (p=0.05).
Figure S3: Heart mRNA levels of (A) atrial natriuretic factor (ANF) (expected PCR product size: 326 bp) and (B) transforming growth factor (TGF)-β1 (expected PCR product size: 127 bp) in sham-operated or 4-week post-MI βarr1KO (KO) and WT mice. All values were standardized to amplified 18S rRNA. Data are presented as mean ± SEM and plotted as fold of WT-Sham values. *, p<0.05, vs. WT MI, n=6 hearts/group.
Figure S4: Cardiac plasma membrane βAR density (A), cardiac steady-state total cAMP levels (B), and plasma circulating NE & Epi (catecholamine) levels (C) of age-matched WT (C57BL/6) mice at 4 weeks post-MI and post-intracardiac gene delivery of adenovirus encoding for either GFP (control, AdGFP) or the βarr1 inhibitor mini-gene βarr1ct (Adβarr1ct). *, p<0.05, n=5 mice/group. No statistically significant differences between the two groups were observed in (C) (n=5).

Supplementary References


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**Table S1: In vivo cardiac functional parameters of βarr1KO and WT mice.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham/WT</th>
<th>Sham/KO</th>
<th>post-MI/WT</th>
<th>post-MI/KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVIDs (mm)</td>
<td>2.31±0.08</td>
<td>2.35±0.11</td>
<td>4.36±0.12#</td>
<td>3.21±0.21#*</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.9±0.15</td>
<td>3.9±0.08</td>
<td>5.4±0.05#</td>
<td>4.6±0.03#*</td>
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<tr>
<td>FS (%)</td>
<td>41.5±0.14</td>
<td>40.8±2.39</td>
<td>10.7±1.13#</td>
<td>22.1±0.35#*</td>
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<tr>
<td>EF (%)</td>
<td>72.5±0.97</td>
<td>71.1±3.02</td>
<td>21.9±2.65#</td>
<td>41.0±2.97#*</td>
</tr>
<tr>
<td>PWTd (mm)</td>
<td>0.96±0.05</td>
<td>1.01±0.02</td>
<td>1.26±0.01#</td>
<td>1.10±0.04#*</td>
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</table>

**Basal Hemodynamic Measurements**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham/WT</th>
<th>Sham/KO</th>
<th>post-MI/WT</th>
<th>post-MI/KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (min–1)</td>
<td>465.2±2.9</td>
<td>474.6±11.5</td>
<td>459.6±12.5</td>
<td>457.3±8.7</td>
</tr>
<tr>
<td>+dP/dt&lt;sub&gt;max&lt;/sub&gt; (mmHg/s)</td>
<td>5,410±149</td>
<td>5,632±179</td>
<td>3,152±93#</td>
<td>4,148±75#*</td>
</tr>
<tr>
<td>–dP/dt&lt;sub&gt;min&lt;/sub&gt; (mmHg/s)</td>
<td>-5,119±206</td>
<td>-5,051±101</td>
<td>-3,379±104#</td>
<td>-3,998±157#*</td>
</tr>
<tr>
<td>LVESP (mmHg)</td>
<td>97.4±1.1</td>
<td>97.8±1.86</td>
<td>82.4±4.4#</td>
<td>85.1±1.8#</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>3.7±0.4</td>
<td>4.1±0.4</td>
<td>11.5±0.8#</td>
<td>10.9±1.4#</td>
</tr>
</tbody>
</table>
### Hemodynamic Measurements after maximal Isoproterenol (333 ng/kg BW)

<table>
<thead>
<tr>
<th></th>
<th>Sham/WT</th>
<th>Post-MI/WT</th>
<th>Post-MI/βarr1KO</th>
<th>Post-MI/WT/βarr1KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (min⁻¹)</td>
<td>579±17.2</td>
<td>583±7.2</td>
<td>575±13.3</td>
<td>580±7.1</td>
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<tr>
<td>+dP/dt max (mmHg/s)</td>
<td>8,917±740</td>
<td>10,651±330</td>
<td>4,789±128</td>
<td>5,673±289</td>
</tr>
<tr>
<td>–dP/dt min (mmHg/s)</td>
<td>-8,446±438</td>
<td>-9,380±255</td>
<td>-4,457±192</td>
<td>-5,100±218</td>
</tr>
<tr>
<td>LVESP (mmHg)</td>
<td>102±2.3</td>
<td>103±1.8</td>
<td>85±3.1</td>
<td>87±3.4</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>4.9±1.4</td>
<td>4.7±0.6</td>
<td>12.1±0.9</td>
<td>12.0±0.5</td>
</tr>
</tbody>
</table>

Echocardiographic and hemodynamic analysis data from 3-month-old sham-operated (Sham) or post-MI WT and βarr1KO mice on day 28 post-MI. +dP/dt max, maximal first derivative of LV pressure rise; –dP/dt min, minimal first derivative of LV pressure fall; HR, heart rate; LVESP, LV end systolic pressure; LVEDP, LV end diastolic pressure; LVIDd, LV inner diameter during diastole; LVIDs, LV inner diameter during systole; FS, fractional shortening; EF, ejection fraction; PWTd, posterior wall thickness in diastole; LV: Left ventricular; BW: Body weight; *, p<0.05, vs. post-MI/WT; †, p<0.05, vs. Sham/WT; n=7 mice/group for echo, 5 mice/group for hemodynamic measurements. One-way ANOVA with Bonferroni test were performed among all groups. Data are presented as mean ± SEM.