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Adrenal beta-arrestin 1 inhibition in vivo attenuates post-myocardial infarction progression to heart failure and adverse remodeling via reduction of circulating aldosterone levels

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List of nonstandard abbreviations: AngII: Angiotensin II; AT_1R: AngII receptor type I; AZG: Adrenocortical Zona Glomerulosa; βarr1: beta-arrestin 1; GPCR: G-protein coupled receptor, GRK2: GPCR Kinase-2; HF: Heart failure; MI: Myocardial infarction; StAR: Steroidogenic Acute Regulatory protein**.**

ABSTRACT

OBJECTIVES: We investigated whether adrenal β–arrestin 1 (βarr1)-mediated aldosterone production plays any role in post-MI HF progression.

BACKGROUND: Heart failure (HF) represents one of the most significant health problems worldwide and new and innovative treatments are needed. Aldosterone contributes significantly to HF progression after myocardial infarction (MI) by accelerating adverse cardiac remodeling and ventricular dysfunction. It is produced by the adrenal cortex after angiotensin II (AngII) activation of AngII type 1 receptors (AT_1Rs), G protein-coupled receptors (GPCRs) that also signal independently of G proteins. G protein-independent signaling is mediated by β–arrestin (βarr) -1 and -2. We recently reported that adrenal βarr1 promotes AT_1R-dependent aldosterone production leading to elevated circulating aldosterone levels in vivo.

METHODS: Adrenal-targeted, adenoviral-mediated gene delivery in vivo in two-week post-MI rats, a time point around which circulating aldosterone significantly increases to accelerate HF progression, was performed to either increase the expression of adrenal βarr1 or inhibit its function via expression of a βarr1 C-terminal-derived peptide fragment.

RESULTS: We found that adrenal βarr1 overexpression promotes aldosterone elevation post-MI, resulting in accelerated cardiac adverse remodeling and deterioration of ventricular function. Importantly, these detrimental effects of aldosterone are prevented when adrenal βarr1 is inhibited in vivo, which markedly decreases circulating aldosterone post-MI. Finally, the prototypic AT_1R antagonist losartan appears unable to lower this adrenal βarr1-driven aldosterone elevation.

CONCLUSIONS: Adrenal βarr1 inhibition, either directly or with AT_1R "biased" antagonists that prevent receptor-βarr1 coupling, might be of therapeutic value for curbing HF-exacerbating hyperaldosteronism.

INTRODUCTION

Despite recent advances in prevention and management of heart disease, death due to chronic heart failure (HF) continues to rise and new treatments are needed (1,2). Aldosterone is one of a number of hormones with detrimental effects to the myocardium, whose circulating levels are elevated in chronic HF (3). It can contribute significantly to HF progression after myocardial infarction (MI) and to the morbidity and mortality of the disease (3-5). Its main actions on the post-MI heart include (but are not limited to) cardiac hypertrophy, fibrosis, and increased inflammation and oxidative stress, all of which result in adverse cardiac remodeling and progressive loss of cardiac function and performance (5,6). Accordingly, plasma aldosterone levels are a marker of HF severity (7,8) and aldosterone antagonists, such as spironolactone and eplerenone, have well-documented beneficial effects in HF constituting a significant segment of the chronic HF pharmacotherapeutic regimen (9,10).

Aldosterone is a mineralocorticoid produced and secreted by the cells of the zona glomerulosa of the adrenal cortex in response to either elevated serum potassium levels or to angiotensin II (AngII) acting through its type 1 receptors (AT_1Rs), endogenously expressed in the adrenocortical zona glomerulosa (AZG) cells (11). AT_1Rs belong to the superfamily of G protein coupled receptors (GPCRs), and, upon agonist activation, couple to the $G_{q/11}$ family of G proteins (12). Over the past few years, a number of GPCRs, including the AT_1Rs, have been shown to also signal through G protein-independent pathways. The protein scaffolding actions of β-arrestin-1 and -2 (βarr1 and -2, also known as arrestins -2 and -3, respectively), universal receptor adapter/scaffolding proteins originally discovered as terminators of GPCR signaling, play a central role in mediating this G protein-independent signal transduction (13,14).

We recently reported that adrenal βarr1 promotes AngII-dependent aldosterone production in vitro in human AZG cells, independently of G-proteins (15). Additionally, adrenal-specific βarr1 overexpression in vivo resulted in a marked elevation of circulating aldosterone levels in otherwise normal animals (15). In the present study, we sought to investigate whether adrenal βarr1 plays any role in regulation of circulating aldosterone levels in post-MI HF progression, as well. For this purpose, we used our previously developed methodology for adrenal-targeted gene transfer in vivo (16,17), in two-week post-MI rats, of either wild type βarr1 to induce adrenal βarr1 overexpression or of a βarr1 protein fragment comprising the βarr1 C-terminus, which inhibits βarr1 signaling activity. The two-week post-MI time point was chosen, since around this time-point circulating aldosterone levels increase dramatically to accelerate post-MI HF progression in rats (18,19).

METHODS

In vivo adrenal gene delivery in post-MI rats. All animal procedures and experiments were performed in accordance with the guidelines of the IACUC committees of Thomas Jefferson and Nova Southeastern Universities. MI was performed using a cryo-infarct method we have previously described (16). Adrenal-specific in vivo gene delivery was done essentially as described (17), via direct delivery of adenovirus in the adrenal gland. Drug treatments were performed with 50 mg/kg/day of losartan potassium (in drinking water) and 100 (or 5) mg/kg/day eplerenone (both drugs from Sigma-Aldrich, USA).

Construction and purification of adenoviruses. Recombinant adenoviruses that encode full length wild type βarr1 (Adβarr1) or a rat βarr1 C-terminal fragment (aa. 369-418, Adβarr1ct, see Supplemental Fig. 1A), were constructed as described previously (15,16). Briefly, transgenes were cloned into shuttle vector pAdTrack-CMV, which harbors a CMV-driven green fluorescent protein (GFP), to form the viral constructs by using standard cloning protocols. As control adenovirus, empty vector which expressed only GFP (AdGFP) was used. The resultant adenoviruses were purified, as described previously, using two sequential rounds of CsCl density gradient ultracentrifugation (15,16).

Plasma aldosterone measurements. Rat plasma aldosterone levels were determined by EIA (Aldosterone EIA kit, ALPCO Diagnostics, Salem, NH, USA), as described (15,20).

Echocardiographic and hemodynamic measurements. Two-dimensional guided M-mode and Doppler echocardiography using a 14-MHz transducer (Vevo 770 Echograph, VisualSonic Inc., Toronto, Canada), and closed chest cardiac catheterization were performed in rats, as described previously (16,21). Three independent echocardiographic measurements were taken in both modes.

Western blotting. Western blots to assess protein levels of StAR (sc-25806), cardiac levels of PAI-1 (sc-8979), TGF-β1 (sc-1460), βarr1 transgenes (A1CT antibody, a generous gift from Dr. R.J. Lefkowitz, Duke University Medical Center, Durham, NC, USA), and GAPDH (MAB374; Chemicon, Temecula, CA, USA) were done using protein extracts from rat adrenal glands or hearts, as described previously (15,16). Visualization of western blot signals was performed with Alexa Fluor 680– (Molecular Probes) or IRDye 800CW–coupled (Rockland Inc.) secondary antibodies on a LI-COR infrared imager (Odyssey).

Real-time PCR. Total heart RNA isolation, reverse transcription and real-time RT-PCR were carried out as previously described (16,21). The following primer pairs were used: 5`-GTCCACGAGGTGACAAAGGT-3` and 5`-CATCTTTTCCAGGAGGTCCA-3` for Col3α1, 5`-CACCCCTTCTGCGTTGTATT-3` and 5`-TTGACCCTAACCAAGGATGC-3` for Col1α1, 5`-TGCCTGCACCTTTGTGATATCG-3` and 5`-CATGGCAGGACAATCGAACC-3` for BNP (NPR-B), 5`-CATCCTGGACAACCTGC-3` and 5`-TAGGTCCGAACCTTGCC-3` for ANP (NPR-A), and, finally, 5′-TCAAGAACGAAAGTCGGAGG-3′ and 5′-

6

GGACATCTAAGGGCATCAC-3′ for 18S rRNA. Real time RT-PCR was performed using SYBR® Green Supermix (Bio-Rad). Normalization was done with 18S rRNA levels. No bands were seen in the absence of reverse transcriptase.

Masson-Trichrome staining. Masson-trichrome staining was performed as described (22).

Statistical analyses. Data are generally expressed as mean ± SEM. Unpaired 2-tailed Student's *t* test and one- or two-way ANOVA with Bonferroni test were generally performed for statistical comparisons, unless otherwise indicated. For most 3-group statistical comparisons Dunnett's 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

Adrenal βarr1 and post-MI aldosterone levels. In the present study, we set out to investigate the potential role played by adrenal βarr1 in modulation of in vivo post-MI HF aldosterone levels. To this end, we overexpressed, specifically in the adrenal glands of two-week post-MI rats, wild-type βarr1 or a βarr1 C-terminal fragment (βarr1ct), which is unable to bind receptor substrates, thus acting as an inhibitor of βarr1 scaffolding/signaling activity (Supplemental Fig. 1A). To confirm the inhibitory effects of βarr1ct on βarr1 activity in vitro, we performed an extensive molecular characterization of its effects on AngII-induced signaling to aldosterone production in the human AZG cell line H295R (Supplemental Fig. 1B). βarr1ct was indeed found to

abrogate βarr1- and G protein-mediated signaling from AT_1R to ERK activation and Steroidogenic Acute Regulatory (StAR) protein up-regulation, both of which signaling events are absolutely necessary for AngII-driven aldosterone production and secretion from these adrenocortical cells (15, see Supplemental Fig. 1B). Thus, after confirming that βarr1ct acts as an inhibitor of adrenal βarr1-mediated aldosterone production in vitro, we overexpressed either the full length βarr1 (to increase adrenal βarr1 levels/activity) or the βarr1ct (to inhibit adrenal βarr1 activity in vivo) specifically in the adrenals of the post-MI rats. Experimental animals were randomized to three different groups: one group receiving adrenal gene transfer of AdGFP (control group), one receiving full length wild type βarr1 (Adβarr1), and one receiving the βarr1ct (Adβarr1ct). One day before adrenal gene transfer, all groups were analyzed by echocardiography to confirm presence of similar levels of LV dysfunction and HF prior to gene delivery. All groups were then studied over the course of the following 7 days (i.e. up to 3 weeks post-MI).

In vivo expression of the respective transgenes in the adrenal glands of the animals at 7 days post-gene delivery was confirmed by Western blotting (Supplemental Fig. 2). Of note, the adrenal-targeted gene transfer methodology employed results in no ectopic transgene expression (17 & data not shown). As expected, plasma circulating aldosterone levels at 7 days post-gene delivery were found markedly elevated in control AdGFP-treated post-MI rats (470 ± 20 pg/ml, ~2-fold of the aldosterone levels of normal AdGFP-treated rats) (15), compared to normal (i.e. sham-operated) AdGFP-treated rats, indicating marked MI-induced aldosterone elevation. Importantly, adrenal βarr1 overexpression resulted in an even more pronounced aldosterone elevation post-MI, on top of that normally present due to the occurrence of MI (845 ± 150 pg/ml in Adβarr1-

treated vs. 470±20 pg/ml in control AdGFP-treated post-MI rats, n=6, p<0.05) (Fig. 1). In contrast, levels in Adβarr1ct-treated rats (350±30 pg/ml, n=6, p<0.05 vs. AdGFP) were significantly lower than in control AdGFP-treated post-MI rats (Fig. 1). Aldosterone levels in post-MI AdGFP rats were similar to saline-treated post-MI rats (data not shown), indicating no non-specific effects of the adenoviruses used on plasma aldosterone values.

Consistent with the above findings, βarr1 overexpression led to significant up-regulation of adrenal StAR protein, the most critical enzyme in adrenocortical biosynthesis of aldosterone (as well as of the other adrenal steroids) (15), compared to control AdGFP-treated post-MI rats, indicating enhanced aldosterone synthesis in vivo, whereas overexpression of βarr1ct reduced adrenal StAR levels below the levels of the control rats (Supplemental Fig. 2). Taken together, these results indicate that adrenal βarr1 promotes post-MI-associated hyperaldosteronism, and inhibition of its activity reduces aldosterone production and plasma circulating aldosterone levels post-MI in vivo.

In vivo cardiac function and dimensions at 7 days post-gene delivery. Next, we examined the impact of this adrenal βarr1-mediated hyperaldosteronism on the post-MI myocardium. Indeed, we found that ejection fraction (EF) was markedly reduced in Adβarr1-treated post-MI rats at 7 days post-gene delivery, compared to control AdGFP-treated post-MI rats (41.4±1.2 % vs. 48.7±1.1 %, respectively, n=7, p<0.05) (Fig. 2A). EF in both groups was similar before gene delivery, and EF of AdGFP-treated rats at 7 days post-gene delivery was slightly but significantly reduced compared to pre-gene

9

delivery, as expected, given that cardiac function deteriorates over time after MI, although at 3 weeks post-MI (when post-gene delivery measurements were taken) there is limited dysfunction with this model (Fig. 2A). Indeed, previous studies by us have shown that this model in the rat does not lead to significant cardiac dysfunction before ~10 weeks post-MI (21). Furthermore, LV end diastolic diameter (LVEDD), a marker of cardiac dimensions, was significantly increased in Adβarr1-treated rats at 3 weeks post-MI compared to control AdGFP post-MI rats, in which heart enlargement was less pronounced at 3 weeks post-MI (Fig. 2B). This indicates that adrenal βarr1 overexpression significantly accelerates the progression of cardiac hypertrophy by promoting aldosterone elevation post-MI. Of note, EF and LVEDD of saline-treated 3-week post-MI rats were similar to those of control AdGFP-treated post-MI rats at 7 days post-gene delivery, indicating no non-specific effects of the adenoviral gene delivery on cardiac function (data not shown).

Importantly, these adrenal βarr1-induced cardiac alterations are aldosterone-mediated, i.e. due to the elevated aldosterone levels caused by adrenal βarr1 overexpression in vivo, since EF reduction and LVEDD increase are prevented (i.e. are similar to control AdGFP-treated rats) by treatment of post-MI Adβarr1 rats with the aldosterone antagonist eplerenone (Figs. 2C&D), although this drug, as expected, has no effect on the plasma aldosterone increase caused by the Adβarr1 treatment of the adrenals of these post-MI animals (Supplemental Fig. 3). Of note, eplerenone prevented the effects of adrenal βarr1 overexpression at two completely different doses (a high one, 100 mg/kg/d, Figs. 2C&D, and a low one, 5 mg/kg/d, data not shown). Thus, the cardiac

10

effects observed upon adrenal βarr1 overexpression are indeed mediated by circulating aldosterone.

Finally, hemodynamic analysis revealed that Adβarr1-treated post-MI rats exhibited significantly reduced basal and maximal dose of isoproterenol-induced cardiac contraction and relaxation indices, compared to control AdGFP-treated rats (Figs. 2E&F). At this early post-MI time-point, when cardiac dysfunction has not yet manifested as HF, echocardiographic and hemodynamic parameters of Adβarr1ct-treated post-MI rats did not display statistically significant differences from those of control AdGFP-treated post-MI rats, as one might expect, although there was some trend towards functional improvement in the Adβarr1ct group (see Table 1 for complete in vivo cardiac functional parameters in all three post-MI groups at one week after gene delivery). These results show that the adrenal βarr1-mediated hyperaldosteronism indeed results in significantly accelerated deterioration of function of the post-MI rat heart.

Cardiac remodeling and functional biomarkers at 7 days post-gene delivery. We also performed molecular and structural evaluation of the post-MI rat hearts at 7 days post-gene delivery. Consistent with the in vivo functional data, real time PCR in total mRNA isolated from these hearts showed a marked upregulation of collagen types 1α1 and 3α1, markers of cardiac fibrosis, and of ANP (Atrial Natriuretic Peptide) and BNP (B-type Natriuretic Peptide), markers of cardiac hypertrophy, in the post-MI hearts of Adβarr1-treated rats, compared to control AdGFP-treated animals (Figs. 3A-D). Conversely, upregulation of all these markers was prevented in Adβarr1ct-treated rats (Figs. 3A-D), despite the fact this group did not show significant improvement in cardiac function,

which is not surprising given the early post-MI time-point these measurements were taken at. Thus, lowering of circulating aldosterone levels by adrenal βarr1 inhibition in vivo causes a marked reduction in the expression of adverse remodeling-related genes, which might help halt the post-MI cardiac decline at later time-points. Additionally, heart weight-to-body weight ratio measurements also confirmed the accelerated cardiac hypertrophy (i.e. enhanced at one week post-adrenal gene delivery, compared to control AdGFP-treated) displayed by Adβarr1-treated post-MI rats (Table 1, see also above, Fig. 2B).

Cardiac fibrosis at 7 days post-gene delivery. Masson-trichrome staining for cardiac fibrosis at 3 weeks post-MI (7 days post-gene delivery) showed markedly increased fibrosis in Adβarr1-adrenal treated rat hearts compared to control AdGFP-treated rat hearts, whereas fibrosis was almost completely absent in Adβarr1ct-adrenal treated rat hearts (Figs. 4A&5B). As expected, no fibrosis was detectable in sham-operated rat hearts (Fig. 4A). In addition, eplerenone treatment markedly reduced fibrosis in Adβarr1-adrenal treated rat hearts (Supplemental Fig. 4), thus providing another indication that the cardiac effects of βarr1 are aldosterone-dependent.

Cardiac mediators of aldosterone at 7 days post-gene delivery. Immunoblotting in cardiac protein extracts revealed a marked upregulation of cardiac Plasminogen Activator Inhibitor (PAI)-1 and Transforming Growth Factor-β (TGF-β), two of the most important molecular mediators of aldosterone`s cardiac fibrotic and adverse remodeling actions (5), in the post-MI hearts of Adβarr1-treated rats compared to control AdGFP-treated rats

12

(Figs. 4C&D). In contrast, in the hearts of Adβarr1ct-treated rats, not only was upregulation of PAI-1 and TGF-β prevented, but the levels of these proteins were actually lowered below the levels of control AdGFP-treated rats (Figs. 4C&D). Taken together, these results indicate that adrenal βarr1-mediated hyperaldosteronism accelerates cardiac adverse remodeling and progression to HF after MI, and that these effects can be reciprocally mitigated by adrenal βarr1 inhibition, which significantly reduces circulating aldosterone levels.

Angiotensin antagonism and βarr1-mediated aldosterone levels post-MI. Finally, we examined whether adrenal βarr1 can affect the efficacy of AT_1R antagonism at curbing AngII-induced aldosterone production. For this purpose, we treated post-MI rats with the prototypic AT_1R antagonist losartan (23,24) for the entire 7-day post-gene delivery period at a dose of 50 mg/kg/day. As expected, in control AdGFP-treated post-MI rats, losartan produced a small but significant plasma aldosterone reduction (from 470 ± 20 in saline-treated to 402 ± 10 pg/ml in losartan-treated rats, $p<0.05$, n=6) (Fig. 5). In Adβarr1-treated post-MI rats however, losartan is virtually unable to lower aldosterone levels (845 ± 150 in saline-treated vs. 880 ± 88 pg/ml in losartan-treated rats, Not Significant at $p<0.05$, n=6) (Fig. 5). In the Adβarr1ct-treated group, no significant aldosterone reduction by losartan was observed, probably because plasma aldosterone levels were already reduced below the levels of AdGFP-treated rats by Adβarr1ct alone. Consistent with this, losartan seems also incapable of reducing the cardiac fibrosis induced by adrenal βarr1-mediated hyperaldosteronism (Supplemental Fig. 4). However, levels in both the saline- and losartan-treated Adβarr1ct rats were significantly lower than

13

in vehicle-administered control AdGFP post-MI rats (Fig. 5). These results strongly suggest that losartan`s post-MI aldosterone lowering effects are antagonized by adrenal βarr1, therefore, adrenal βarr1 inhibition can potentiate the hypoaldosteronic actions of this drug in post-MI HF. Effects of losartan in AdGFP-treated and saline-treated post-MI rats were similar (data not shown).

DISCUSSION

We recently reported that adrenal βarr1 promotes AngII-dependent aldosterone production in vitro in human AZG cells, independently of G-proteins (15). Additionally, adrenal-specific βarr1 overexpression in vivo resulted in marked elevation of circulating aldosterone levels in otherwise normal animals (15). In the present study, we sought to investigate whether adrenal βarr1 plays any role in regulation of circulating aldosterone levels in post-MI HF progression. We found that adrenal βarr1 is indeed a crucial regulator of circulating aldosterone levels in vivo during post-MI HF progression, in that increased adrenal βarr1 levels/activity promotes aldosterone elevation post-MI, resulting in accelerated cardiac adverse remodeling and deterioration of function, whereas blockade of its activity in vivo lowers post-MI aldosterone levels, attenuating or even preventing these detrimental effects of aldosterone on the failing heart.

These findings strongly suggest that blockade of adrenal βarr1 action on AT_1R might serve as a novel therapeutic strategy for lowering aldosterone levels post-MI and in HF. This is particularly important, since aldosterone has been shown to exert some of its actions (its so-called "non-genomic" actions) independently of the mineralocorticoid receptor (MR), its molecular target that normally mediates its cellular actions (4,5). These

14

MR-independent actions are unaffected by the currently available MR antagonists, such as eplerenone and spironolactone, used in the treatment of HF (9,10). Therefore, curbing aldosterone production at its major source, i.e. the adrenal cortex, by inhibiting βarr1 actions, could presumably be more effective therapeutically than inhibiting aldosterone`s actions at its receptor level.

In addition, since adrenal βarr1 appears necessary for upregulation of StAR, the enzyme that regulates synthesis of all adrenal steroids, its inhibition presumably leads to suppression of the production of the other adrenocortical steroids as well, i.e. of glucocorticoids and corticosterone (15). Of note, glucocorticoids have been reported to actually occupy the cardiac mineralocorticoid receptors under normal conditions instead of aldosterone (25). Therefore, adrenal βarr1 inhibition, by suppressing production of glucocorticoids and mineralocorticoids alike, has the unique potential of keeping cardiac MRs completely at bay. For this very same reason, adrenal βarr1 emerges as a much superior target for post-MI cardiac remodeling and heart failure treatment than MR inhibition (e.g. with eplerenone) or aldosterone synthase inhibition, given that the latter strategies cannot counter all the adverse effects of all adrenal steroids post-MI, as suppression of all adrenal steroid production via adrenal βarr1 inhibition is projected to do.

Another important ramification of the present study is that pathological situations that cause elevation of adrenal βarr1 activity towards receptors can lead to abnormally high AngII-induced aldosterone production and hyperaldosteronism. Indeed, we recently reported that in chronic HF, adrenal GRK2, a protein kinase that induces receptor-βarr coupling, is dramatically upregulated resulting in chronically enhanced catecholamine

production by the adrenal medulla (16). Thus, it is entirely plausible that, driven by the enhanced GRK2 activity, adrenal βarr1 activity towards receptors, including the AT_1Rs, is also increased in chronic HF or during progression from MI to HF, which could mediate (at least in part) the chronically elevated circulating levels of aldosterone that precipitate this disease. Importantly, we have previously shown that GRK2 can desensitize AngII receptors in the heart in vivo (26), and that overexpression of GRK2 in rat adrenal glands also causes elevation of plasma aldosterone (15). Both of these findings argue in favor of the aforementioned scenario.

Furthermore, it is now well established that, in addition to the circulatory renin-angiotensin-aldosterone system (RAAS), there are also several other local RAAS`s in peripheral tissues, including the heart (intracardiac RAAS) and the kidneys (intrarenal RAAS), and these systems also hyperfunction in HF contributing to the HF-associated hyperaldosteronism (27,28). Therefore, it would be worth investigating whether βarr1 is involved in aldosterone production by these local RAAS`s, and whether it contributes to their increased aldosterone output during HF as well. In fact, specifically for the intracardiac RAAS, this possibility is very likely, given the elevated cardiac GRK2 levels in HF (29).

One of the major physiological effects of aldosterone is an increase in blood pressure via salt and water retention (4,5). Thus, alterations in mean arterial pressure by the elevated aldosterone levels caused by adrenal βarr1 overactivity might very well have contributed to the observed cardiac phenotype of adrenal βarr1-overexpressing post-MI rats. It should be noted here however that βarr1 knockout mice do not show any changes in blood pressure compared to wild type age-matched control mice (30). Additionally, the

16

direct effects of aldosterone on cardiac tissue are bound to have played the most important role in the observed cardiac phenotype of the post-MI animals, given the relatively small time-period (only 7 days) between genetic manipulation of adrenal βarr1 levels which raises aldosterone levels (i.e. gene delivery) and the day of cardiac measurements/examination, which is rather insufficient for blood pressure to affect cardiac function and remodeling that dramatically. Besides, whether changes in blood pressure play any role in the cardiac effects of aldosterone is still an open question in its own right, since there are several reports in the literature showing aldosterone to affect cardiac function and fibrosis in post-MI rats independently of changes in mean blood pressure (31,32). Indeed, no differences in systemic mean arterial pressure among the three post-MI treatment groups of the present study (i.e. AdGFP, Adβarr1, Adβarr1ct) were observed at one week post-gene delivery (data not shown), further supporting the notion that blood pressure did not play any major role in the observed cardiac effects of βarr1-dependent aldosterone at this early post-MI time-point (3 weeks).

The last finding of the present study is that the aldosterone-lowering actions of losartan, the prototypic drug of the class of $AT_{1A}R$ antagonists (sartans) (23,24), are countered by adrenal βarr1. Although at normal βarr1 levels (control AdGFP-treated post-MI rats) it is capable of producing a small but significant plasma aldosterone lowering as expected, when adrenal βarr1 is overactive (Adβarr1-treated post-MI rats), losartan does not decrease plasma aldosterone at all. This finding implies that inhibition of adrenal βarr1 in vivo can facilitate the inhibitory effects of losartan (and possibly also of the other sartans) on AngII-induced aldosterone production. Of note, limited efficacy of losartan and other sartans at lowering aldosterone levels in HF patients and in

experimental animals, the so-called "aldosterone escape", has been reported (20,33,34). Therefore, the finding that losartan`s effects on aldosterone production can be antagonized by adrenal βarr1-AT$_1$R coupling might explain (at least in part) this reported limited efficacy of losartan and related drugs at curbing aldosterone levels. On the other hand, increased activity of the βarr1 co-factor GRK2 on cardiac AT$_1$Rs also attenuates the pro-contractile signaling of these receptors (26). Therefore, the development of novel, functionally selective (or "biased") AT$_1$R ligands (35,36), which would inhibit AT$_1$R-induced GRK2/βarr1 activation, at least as effectively as AT$_1$R-induced G-protein activation, might prove extremely beneficial in the treatment of HF-related hyperaldosteronism and decreased cardiac function.

Clinical implications. We have found that circulating aldosterone levels are reciprocally regulated by adrenal βarr1 activity in vivo, in that they are directly proportional to βarr1 activity toward AngII receptors in the adrenal glands. Therefore, inhibiting adrenal βarr1 action markedly decreases circulating aldosterone and attenuates its detrimental effects on the post-MI heart, such as fibrosis, hypertrophy, and dilatation, thereby preventing or even reversing adverse remodeling post-MI and maintaining cardiac function in the face of post-MI-driven cardiac decline. Additionally, losartan, a classical AngII receptor antagonist drug used in the treatment of hypertension, appears unable to counter this adrenal βarr1-promoted hyperaldosteronism post-MI. Taken together, the present findings suggest adrenal βarr1 as a major driving force behind post-MI aldosterone elevation, whose inhibition in vivo, either via gene therapy or pharmacologically, could potentially be of enormous therapeutic value in the management of post-MI HF patients. Finally, from the pharmacotherapeutic standpoint,

18

an evaluation of the whole class of AT_1R antagonists (sartans) in terms of their efficacy at antagonizing βarr1-driven hyperaldosteronism is highly warranted, as it could help explain some well-known existing differences in therapeutic efficacy, and also identify the most efficacious agents at lowering post-MI aldosterone, within this very important cardiovascular drug class.

In summary, the present study reports that adrenal βarr1 promotes the well-documented post-MI-associated elevation of circulating aldosterone, and thus, direct inhibition of its activity via adrenal-targeted gene therapy or via development of novel AT_1R "biased" or "functionally selective" ligands that can prevent/reduce GRK2/βarr1 activation by the AT_1R might be of therapeutic value in post-MI ensuing HF, as well as in already established chronic HF, both of which are precipitated by the cardiotoxic actions of elevated aldosterone.

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FIGURE LEGENDS

Figure 1. Regulation of plasma aldosterone levels by adrenal βarr1. Plasma aldosterone levels in AdGFP-, Adβarr1-, or Adβarr1ct-treated two-week post-MI rats, at 7 days post-in vivo gene delivery. *****, $p < 0.05$, vs. AdGFP, ******, $p < 0.05$ vs. Adβarr1, n=6 rats/group.

Figure 2. Effect of adrenal βarr1-mediated hyperaldosteronism on cardiac function, dimensions and contractility. **(A)** Ejection fraction (EF%) of Adβarr1- and control AdGFP-treated post-MI rats pre- and post-gene delivery (see also Table 1). *, $p < 0.05$, vs. AdGFP-post-gene delivery or Adβarr1-pre-gene delivery, **, $p < 0.05$, vs. AdGFP-pre-gene delivery, n=7 rats/group. **(B)** Left Ventricular End Diastolic Diameter (LVEDD) of these rats. *, $p < 0.05$, vs. AdGFP-post-gene delivery or Adβarr1-pre-gene delivery, n=7 rats/group. **(C)** Ejection fraction (EF%) and **(D)** Left Ventricular End Diastolic Diameter (LVEDD) of Adβarr1-treated post-MI rats administered either with saline (vehicle) or with eplerenone (Adβarr1-Eplerenone) for 7 days, at 1 week post-gene delivery (3 weeks post-MI). AdGFP post-MI rats (treated with vehicle) are also shown at 1 week post-gene delivery (3 weeks post-MI) for comparisons. *, $p < 0.05$, vs. either AdGFP or Adβarr1-Eplerenone, no significant difference between AdGFP and Adβarr1-Eplerenone was observed at $p=0.05$, n=5 rats/group. **(E, F)** Basal and maximal dose of isoproterenol (Max. Iso)-stimulated +dP/dt$_{max}$ **(E)** and –dP/dt$_{min}$ **(F)** responses of Adβarr1- and control AdGFP-treated post-MI rats at 7 days post-adrenal gene delivery (see also Table 1). *, $p < 0.05$, vs. AdGFP, n=7 rats/group.

Figure 3. Effect of aldosterone levels on cardiac remodeling markers. Heart mRNA levels of (**A**) collagen I (Col1a1); (**B**) collagen III (Col3a1); (**C**) atrial natriuretic peptide (ANP); (**D**) brain natriuretic peptide (BNP) in all experimental groups at 7 days post-gene delivery (3 weeks post-MI). All values were standardized to amplified 18S rRNA. Data are presented as mean ± SEM and plotted as fold of AdGFP values. *****, p<0.05, vs. AdGFP or Adβarr1ct, ******, p<0.05 vs. AdGFP, n=5 rat hearts/group.

Figure 4. Impact of aldosterone levels on cardiac fibrosis and adverse remodeling mediators. (**A**) Trichrome-Masson`s staining in myocardial cross-sections from AdGFP-, Adβarr1-, or Adβarr1ct-treated post-MI rats at 7 days post-adrenal gene delivery. Blue denotes collagen fibers, red denotes muscle fibers, and black represents cell nuclei. Representative images are shown from 5-6 rat hearts stained per group, along with staining in sham rat hearts, in which no blue staining was detectable. (**B**) Quantification of the % fibrotic area visualized upon Trichrome-Masson`s staining. *, p<0.05, vs. AdGFP, ******, p<0.05 vs. Adβarr1, n=5-6 rat hearts/group. (**C**) Western blotting for cardiac PAI-1 and TGF-β1 in AdGFP-, Adβarr1-, or Adβarr1ct-treated post-MI rats, at 7 days post-gene delivery, including GAPDH as loading control. (**D**) Densitometric analysis of 5 heart samples tested per group. *, p<0.05, vs. AdGFP, ******, p<0.05 vs. Adβarr1, n=5 rat hearts/group.

Figure 5. Adrenal βarr1-dependent aldosterone levels and losartan. Plasma aldosterone levels 7 days post-adrenal gene delivery of post-MI rats after concomitant vehicle (-Los)

or losartan (+Los) treatment. *****, $p < 0.05$, vs. AdGFP/-Los or Adβarr1/+Los, n=5 rats/group/treatment.
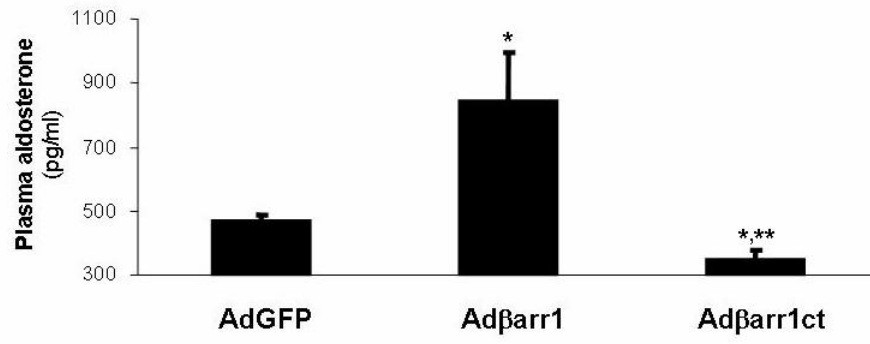
Fig. 1

Fig. 2

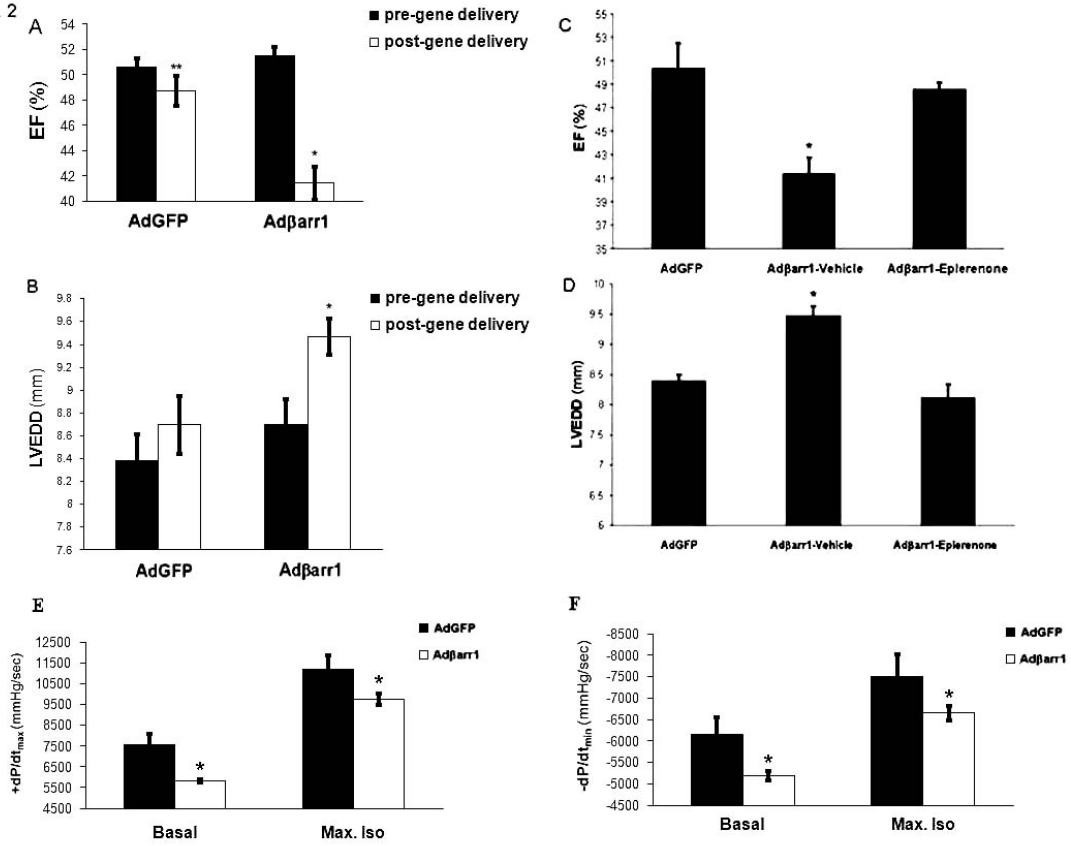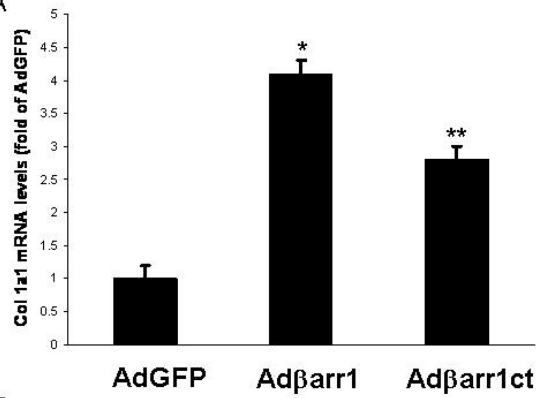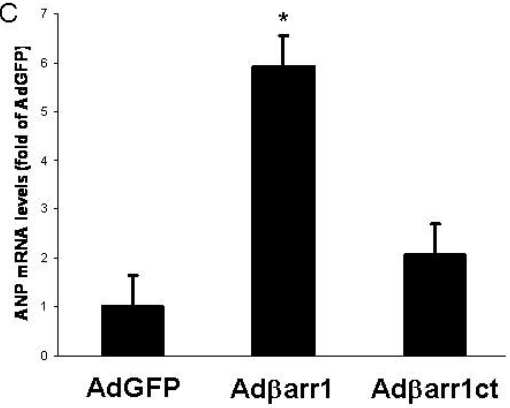Fig. 3

Fig. 4

A

AdGFP Adβarr1 Adβarr1ct

sham

C

50 kDa —— PAI-1
12 kDa —— TGF-β1
37 kDa —— GAPDH

AdGFP Adβarr1 Adβarr1ct

B

D

■ TGF-β1
□ PAI-1

30

Fig. 5