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An adrenal beta-arrestin 1-mediated signaling pathway underlies angiotensin II-induced aldosterone production in vitro and in vivo.

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Abstract

Aldosterone produces a multitude of effects in vivo, including promotion of post-myocardial infarction adverse cardiac remodeling and heart failure progression. It is produced and secreted by the adrenocortical zona glomerulosa (AZG) cells after angiotensin II (AngII) activation of AngII type 1 receptors (AT_1Rs). Up until now, the general consensus for AngII signaling to aldosterone production has been that it proceeds via activation of $G_{q/11}$-proteins, to which the AT_1R normally couples. Here, we describe a novel signaling pathway underlying this AT_1R-dependent aldosterone production mediated by β–arrestin (βarr) -1, a universal heptahelical receptor adapter/scaffolding protein. This pathway results in sustained extracellular signal-regulated kinase (ERK) activation and subsequent upregulation of StAR, a steroid transport protein regulating aldosterone biosynthesis in AZG cells. In addition, this βarr1-mediated pathway appears capable of promoting aldosterone turnover independently of G-protein activation, since treatment of AZG cells with SII, an AngII analog that induces βarr, but not G-protein coupling to the AT_1R, recapitulates the effects of AngII on aldosterone production and secretion. In vivo, increased adrenal βarr1 activity, by means of adrenal-targeted adenoviral-mediated gene delivery of a βarr1 transgene, resulted in a marked elevation of circulating aldosterone levels in otherwise normal animals, suggesting that this adrenocortical βarr1-mediated signaling pathway is operative, and promotes aldosterone production and secretion in vivo, as well. Thus, inhibition of adrenal βarr1 activity on AT_1Rs might be of therapeutic value in pathological conditions characterized and aggravated by hyperaldosteronism.

\body

Aldosterone is one of a number of hormones that can be detrimental to myocardium, and whose circulating levels are elevated in chronic heart failure (HF). It contributes significantly to HF progression after myocardial infarction (MI) and to the morbidity and mortality of the disease (1-3). Aldosterone`s 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 (2-4).

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 1A receptors ($AT_{1A}Rs$), which are endogenously expressed in the adrenocortical zona glomerulosa (AZG) cells (5,6). AT_1Rs belong to the superfamily of 7-transmembrane spanning G-protein coupled receptors (GPCRs), and, upon agonist activation, couple to the $G_{q/11}$ family of G-proteins (6). Over the past few years however, a number of GPCRs, including the AT_1R, 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), originally discovered as terminators of GPCR signaling following phosphorylation of these receptors by the GPCR kinases (GRKs), play a central role in mediating G protein-independent signal transduction by these receptors (7,8).

We recently reported that adrenal GRK2, the major co-factor of βarr action toward receptors, is upregulated in HF leading, through its concerted action with βarr1, to increased desensitization/down-regulation of α_2-adrenoceptors, and this mediates the increased adrenal catecholamine output seen in HF (9). Since adrenal aldosterone production stimulated by AngII is increased in HF (1-3), and βarr1 also regulates AT_1R signaling (7,8), we hypothesized that

adrenal βarr1 might mediate the signaling of AT_1R to aldosterone production and secretion. In order to test this in vitro, we used the human AZG cell line H295R, which endogenously expresses the AT_1R, but not the AT_2R (the other AngII receptor type). Importantly, these cells produce and secrete aldosterone in response to AngII stimulation (10,11). To examine whether adrenal βarr1 influences aldosterone turnover in vivo, we used our previously developed methodology for adrenal-targeted, adenoviral-mediated gene transfer (9,12) of wild-type full-length βarr1 in normal rats. We have uncovered a novel signaling pathway mediated by βarr1 that leads to aldosterone production by the AT_1R in AZG cells in vitro, which, importantly, is also operative in vivo, since adrenal βarr1 overexpression was found to be capable of increasing circulating levels of aldosterone in vivo.

Results

βarr1-mediated AngII-induced aldosterone production in vitro. Since AngII is known to promote aldosterone production in AZG cells, we set out to explore a potential role for βarrs in this effect. In H295R cells, treatment with 10 nM AngII leads to a significant induction of aldosterone secretion, as expected (Fig. 1*A*). Western blotting with an antibody against both βarr isoforms in native extracts from these cells revealed that only βarr1 is expressed endogenously in significant amounts (Fig. 1*B*). Consistent with this, human adrenal glands express βarr1 robustly (SI Fig. 1), and importantly, βarr1 co-localizes with the known adrenocortical protein, steroidogenic acute regulatory protein (StAR), in human adrenocortical sections (Fig. 1*C*).

Aldosterone synthesis in AZG cells is initiated by the mitochondrial uptake of cholesterol, the precursor of all adrenal steroids (10). Mitochondrial cholesterol uptake is the rate-limiting step of this procedure and is catalyzed by the steroid transport protein StAR, whose levels are upregulated in response to AngII stimulation (10,13). Consistent with this, we observed a large StAR upregulation in H295R cells 6 hrs post-AngII stimulation (Fig. 1*B*).

In order to test whether endogenous βarr1 plays a role in AngII-induced aldosterone production/secretion, we transfected H295R cells with the V53D dominant negative βarr1 mutant (DN βarr1), which prevents βarr1 from interacting with its various intracellular non-receptor binding partners (14,15). As shown in Fig. 1*D*, DN βarr1 overexpression led to marked inhibition of AngII-induced aldosterone secretion, compared to control empty vector (EV)-transfected cells. In addition, the AngII-induced StAR upregulation normally observed in EV-transfected cells was also absent in DN βarr1-transfected cells (Fig. 1*E*). Conversely,

transfection of H295R cells with an adenovirus encoding for wild-type βarr1 (Adβarr1) led to significantly enhanced AngII-induced aldosterone secretion compared to control AdGFP (Green Fluorescent Protein)-transfected cells (Fig. 1*F*), which was also accompanied by a marked enhancement of AngII-induced StAR upregulation (Fig. 1*G*). Taken together, these results show that βarr1 is necessary for AngII-induced StAR upregulation and subsequent aldosterone production in AZG cells in vitro.

βarr1-mediated AT_1R signaling to aldosterone production involves DAG and sustained ERK activation.

To further dissect the signaling pathway of AngII-induced aldosterone production mediated by βarr1 in AZG cells, we focused on βarr1-promoted ERK1/2 activation. ERK1/2 play a central role in StAR upregulation by means of inducing StAR gene transcription in response to AngII stimulation in AZG cells (13). βarrs have been shown to mediate AT_1R signaling to ERKs in various heterologous cell systems in vitro (7,8). Following AngII stimulation for various times, we found that βarr1 overexpression does lead to sustained AngII-induced ERK1/2 activation in H295R cells, lasting at least 6 hrs and contrary to a more transient ERK1/2 activation by AngII in control AdGFP-transfected cells (Figs. 2*A* & 2*B*). Conversely, inhibition of endogenous βarr1 by DN βarr1 completely abrogates AngII-induced ERK1/2 activation in H295R cells compared to EV-transfected cells (Figs. 2*A* & 2*B*). These data indicate that βarr1 promotes a sustained AngII-induced ERK1/2 activation, which could underlie the observed βarr1-promoted StAR upregulation and aldosterone production in response to AngII in AZG cells.

Recently, βarr1 was shown to recruit the diacylglycerol (DAG) kinases (DGKs) to activated M_1 muscarinic cholinergic receptors, which also couple to Gq-proteins like the AT_1Rs,

thereby catalyzing the conversion of the Gq-dependent second messenger DAG to phosphatidic acid (PA) at the cell membrane (16). PA is a potent ERK cascade activator by means of bringing together Ras and Raf1 kinase at the level of the plasma membrane to interact with each other (17). Therefore, we hypothesized that this βarr1-mediated mechanism could be at play in AngII-induced sustained ERK1/2 activation in AZG cells, as well. To test this, we pretreated transfected H295R cells with the phospholipase C (PLC) inhibitor U73122 (18) to suppress all DAG production before AngII stimulation. In the presence of PLC inhibition, βarr1 overexpression is unable to induce StAR upregulation or ERK activation in response to AngII stimulation, which are also absent in control AdGFP-transfected cells, as expected (Figs. 2*D* & 2*C*, respectively, and quantitation in Figs. 2*F* & 2*E*, respectively). Adding the cell-permeable DAG analog dioctanoylglycerol (DiC8-DAG) however (19), which circumvents PLC inhibition and is a DGK substrate, immediately before applying AngII to the PLC inhibitor-treated cells, rescues the ability of βarr1 to mediate StAR upregulation and ERK activation in response to AngII, both in βarr1-overexpressing and in control AdGFP-transfected cells (Figs. 2*D* & 2*C*, respectively, and quantitation in Figs. 2*F* & 2*E*, respectively). Importantly, in the presence of PLC inhibition, βarr1 is also incapable of promoting AngII-induced aldosterone production in H295R cells (Fig. 2*G*), and this capability is again rescued by the addition of DiC8-DAG (Fig. 2*H*). Finally, application of the MEK1 inhibitor PD98059 which abolishes ERK1/2 activation led to a complete abrogation of AngII-induced ERK activation and StAR upregulation (Figs. 2*D* & 2*C*, respectively, and quantitation in Figs. 2*F* & 2*E*, respectively), as well as of AngII-induced aldosterone production (Fig. 2*G*) both in βarr1-overexpressing and in control AdGFP-transfected cells, thus confirming the vital role of ERK1/2 in mediation of AngII-induced aldosterone production in AZG cells (13). Taken together, these results indicate that DAG is necessary for

βarr1-mediated ERK1/2 activation, StAR upregulation and aldosterone production in AZG cells induced by AngII, probably via βarr1-recruited DGK-catalyzed conversion to PA.

The βarr1-mediated signaling pathway operates independently of G-protein activation. Next, we examined whether this βarr1-mediated signaling pathway of AngII-dependent aldosterone production can proceed without the activation of the cognate AT_1R G-protein pathway. To this end, we took advantage of the well characterized AngII analog [Sar^1,Ile^4,Ile^8]-AngII (SII), which is a biased AT_1R agonist, in that it does not induce the coupling of AT_1R to G-proteins, but instead induces receptor interaction with βarrs and downstream βarr-mediated signaling (20). As shown in Fig. 3*A*, SII, at the relatively high concentration of 10μM, is also able to induce aldosterone secretion from H295R cells, and this is enhanced in cells overexpressing βarr1 (Fig. 3*A*). Conversely, transfection with DN βarr1 abolishes SII-induced aldosterone secretion (Fig. 3*A*). Of note, 1 μM SII treatment could stimulate aldosterone secretion only in the presence of βarr1 overexpression (data not shown), consistent with far less potency of this compound at stimulating βarrs compared to AngII (21). In addition, 10μM SII treatment results also in ERK1/2 activation (Fig. 3*B*) and StAR upregulation (Fig. 3*C*), which are again enhanced by βarr1 overexpression and abrogated by DN βarr1 (Figs. 3*B* & 3*C*). Taken together, these results indicate that βarr1 is able to mediate AT_1R signaling to aldosterone production in AZG cells in its own right, i.e. even without concomitant activation of G-proteins by the AT_1R.

βarr1 mediates aldosterone production in vivo. Next, we examined whether adrenal βarr1 can affect aldosterone production in vivo, as well. Adrenal gland-specific overexpression of βarr1 in normal rats via infection with Adβarr1 in vivo led to a significant increase in plasma aldosterone

levels compared to control AdGFP rats (536±50 pg/ml vs. 235±40 pg/ml, respectively, n=5, $p<0.01$) (Fig. 4*A*) at seven days post-in vivo gene delivery. βarr1 was markedly overexpressed in the adrenals of Adβarr1 rats (Fig. 4*B*).

Since GRK2 is a co-factor of βarr1 activity towards receptors, we also delivered an adenovirus carrying GRK2 (AdGRK2) to normal rat adrenal glands. As shown in Fig. 4*A*, GRK2 overexpression resulted in a small but significant increase in plasma aldosterone at 7 days post-gene delivery compared to control AdGFP-treated rats (322±20 pg/ml, n=5, $p<0.05$ vs. AdGFP), indicating that increased activity/expression of GRK2 in the adrenal gland increases aldosterone production, as well. This is consistent with induced βarr1 acting at the plasma membrane. Fig. 4*B* shows the overexpression of the respective transgenes in the adrenals of normal rats. Of note, all transgenes delivered in vivo displayed adrenal-specific overexpression with no ectopic expression in any other tissue tested (Ref. 12, and data not shown). In addition, plasma aldosterone values in saline-treated rats were similar to AdGFP-treated rats (Fig. 4*A*), indicating the absence of any non-specific effects of the adenoviral infection on plasma aldosterone values.

Discussion

Over the past few years, a novel role for βarr1 and -2, molecules initially discovered as terminators of G protein signaling by GPCRs, has emerged, i.e. that these two proteins, after uncoupling the activated receptor from its cognate G-protein, actually serve as signal transducers for the receptor in their own right (7,8). However, this novel role of βarrs has thus far been demonstrated almost exclusively in heterologous cell systems in vitro. The present study delineates a novel signaling pathway mediated by βarr1, which operates in vitro and in vivo, in a specialized cell type/tissue (ZG cells of the adrenal cortex), and which leads to an important physiological effect (AngII-induced aldosterone production). Moreover, this increased aldosterone production may then precipitate diseases that are characterized and aggravated by enhanced circulating levels of this hormone, such as post-MI HF progression (3,4).

Additionally, our data strongly suggest that blocking adrenal βarr1 actions on AT_1R might serve as a novel therapeutic strategy for lowering aldosterone levels in pathological conditions characterized and precipitated by elevated aldosterone levels, one of the most important of which is post-MI progression to HF. Testing the effects of adrenal βarr1 inhibition in vivo in post-MI HF in order to validate this important receptor regulatory protein as a novel therapeutic target in HF will be the focus of our next study.

Suppression of aldosterone production at its various sources, the most important of which physiologically is the adrenal cortex, is of particular importance, since aldosterone has been shown to exert some of its actions (its so-called "non-genomic" actions) by binding other molecular targets than the mineralocorticoid receptor (MR), the molecular target that normally mediates its cellular actions (2,3). These MR-independent actions are of course unaffected by the currently available MR antagonist drugs, such as eplerenone and spironolactone, used in the

treatment of HF. 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 its actions at its receptor level and we are planning to investigate this in an in vivo experimental model of HF.

The pathway of βarr1-dependent AT_1R signaling to aldosterone production appears to be initiated by the recruitment of βarr1 to the activated AT_1R which scaffolds DGK(s) to the activated receptor. This, in turn, leads to conversion of DAG to PA, a membrane phospholipid that can directly activate the ERK cascade. The resulting sustained ERK activation leads to activation of StAR gene transcription, thereby causing up-regulation of this cholesterol-transporting protein. The StAR-facilitated mitochondrial uptake of cholesterol subsequently initiates aldosterone synthesis in AZG cells. This signaling pathway is schematically depicted in Fig. 5. Of note, StAR is the major regulator of the biosynthesis of all adrenal steroids throughout the adrenal cortex (10), not only of aldosterone, therefore βarr1 is very likely to be involved in regulation of the synthesis of glucocorticoids and androgens (the other two categories of adrenal steroids) by the adrenal cortex, as well.

Although AT_1R has been shown to result in sustained ERK activation via βarrs, it is the βarr2 isoform that has actually been shown to mediate this effect, whereas βarr1 has actually been shown to act in the opposite direction, i.e. rather inhibiting AT_1R-induced ERK activation (22). Recently, it was shown in transfected HEK293 cells that βarr1 only inhibits AT_1R signaling to ERK by classically desensitizing the receptor (i.e. uncoupling it from the G protein), and βarr2 instead promotes the G protein-independent signaling of AT_1R to ERKs, but this βarr2-mediated ERK activation produces no transcriptional effects (23). Our present findings seem to be in discordance with these studies. However, it should be emphasized here that these studies were

done in transfected heterologous systems, with overexpressed receptors at supra-physiological levels, and also with recombinant (not natural) AT_1Rs engineered in such a way that they cannot couple to any G proteins. Given that signaling from a given GPCR to ERKs can vary widely depending on the relative concentrations of receptor, G-proteins, GRKs, and βarrs, as well as on the cellular context in general (i.e. cell type and cellular signaling machinery) (8,23), this apparent discrepancy can be easily explained. Additionally, the H295R cells used in the present study do not express significant amounts of βarr2 endogenously, so βarr2 is unlikely to be involved in AngII-induced aldosterone production, at least in this system (see Figure 1*B*). Nevertheless, it is entirely plausible that βarr2 might have different or even opposite effects on the signaling pathway leading from AT_1R activation to aldosterone production in other AZG cell lines or in vivo, and this is going to be the focus of future studies.

βarr-activated ERKs have been shown to be largely retained in the cytosol due to their association with receptor-arrestin complexes, and thus not to be able to translocate to the nucleus to induce transcriptional effects (22,23). However, βarr1 localizes in the cytoplasm, as well as in the nucleus by virtue of possessing a nuclear localization sequence, whereas βarr2 is excluded from the nucleus due to a nuclear export sequence present in its molecule (24). In fact, βarr1 translocates into the nucleus in response to stimulation of the μ-opioid receptor, a $G_{i/o}$-coupled receptor, wherein it interacts with the p27 and c-Fos promoters and stimulates transcription by recruiting histone acetyltransferase p300 and enhancing local histone H4 acetylation (25,26). On the other hand, ERK1/2 not only target nuclear transcription factors, but also numerous other plasma membrane, cytoplasmic, and cytoskeletal substrates (27), some of which mediate the reportedly non-transcriptional effects of βarr-activated ERKs, such as chemotactic T- and B-cell migration (28,29). Some other ERK substrates though, such as the Rsk and Mnk protein kinases,

can translocate to the nucleus and activate transcription factors, thereby producing the transcriptional effects of activated ERK1/2 indirectly (27). Indeed, the cardiac-specific overexpression of a G protein-uncoupled mutant AT_1R has been reported, which induces ERK1/2 activation that promotes a histologically distinct form of cardiac hypertrophy from that caused by the wild type receptor, with greater cardiomyocyte hypertrophy and less cardiac fibrosis (30). This suggests that ERK1/2 activated independently of G proteins can produce transcriptional effects from AT_1R activation in vivo, albeit different from the transcriptional effects of G-protein-activated ERKs. In the same vein, βarr1 was very recently shown in 3T3-L1 adipocytes to mediate ERK activation from the endogenous TNFα receptor (a cytokine receptor) through $G_{q/11}$-proteins, and this βarr1-mediated ERK activation coupled TNFα receptor activation to lipolysis, phosphatidylinositol 3-kinase activation and inflammatory gene expression (31). Taken together, all these studies indicate that βarr1-activated ERK1/2 can lead to transcriptional effects, which is in complete agreement with our present findings, i.e. that βarr1-activated ERK1/2 increases StAR expression and aldosterone synthesis in AZG cells. Indeed, βarr1-activated ERK1/2 appears to increase StAR expression in H295R cells transcriptionally, via suppression of the early intermediate gene DAX-1 (13), a transcriptional repressor of the StAR gene (data not shown).

The final important finding of the present study is that SII can completely recapitulate the AngII effects on aldosterone production, albeit at significantly lower concentrations, consistent with its lower potency at AT_1R compared to the physiological full agonist AngII. This finding has enormous pharmacological and therapeutic ramifications, since it strongly argues for the existence of at least two different active conformations of the AT_1R, one of which would lead only to βarr1 but not G-protein activation, but which both result in aldosterone production in

AZG cells. Therefore, complete blockade of both of these conformations would be warranted in order to achieve the most effective suppression of AngII-dependent aldosterone production. To our knowledge, the relative efficacy of the currently available AT_1R antagonist drugs (the sartans) at inhibiting these two signaling pathways emanating from AT_1R (i.e. the G protein- and the βarr-mediated) has never been tested and is completely unknown and thus will also be the focus of our future studies. In fact, there have been several reports of limited efficacy of some AT_1R antagonists at suppressing aldosterone in HF (32-34), despite their more or less equal capability to inhibit G-protein activation by the AT_1R. It would thus be interesting to examine whether variations in the efficacy of these agents at inhibiting AT_1R–βarr coupling could account for their reduced efficacy at suppressing aldosterone. Based on the results of the present study however, the most effective AT_1R antagonist at inhibiting AngII-dependent aldosterone production should be an agent that would inhibit both AT_1R-G protein and AT_1R-βarr1 coupling equally well.

In conclusion, the present study reports a novel, G protein-independent signaling pathway mediated by βarr1 in adrenocortical zona glomerulosa cells that underlies aldosterone production in response to AngII in vitro and in vivo. Activity of this pathway appears to regulate adrenal aldosterone production, and hence circulating levels of this mineralocorticoid in vivo. Thus, adrenal βarr1 activity towards the AT_1R might represent a novel therapeutic target for reducing plasma aldosterone levels in pathological conditions where this is desirable, including several endocrinological disorders characterized by hyperaldosteronism and cardiovascular disease.

Methods

Materials. [Sar[1],Ile[4],Ile[8]]-AngII (SII) was a generous gift from Dr. P. Cordopatis (University of Patras School of Pharmacy, Patras, Greece). U73122 was from Biomol (Plymouth Meeting, PA, USA) and DiC8-DAG from Sigma (Sigma-Aldrich, St Louis, MO, USA).

In vivo adrenal gene delivery in normal rats. All animal procedures and experiments were performed in accordance with the guidelines of the IACUC of Thomas Jefferson University. Adrenal-specific in vivo gene delivery was done essentially as described (12), via direct delivery of adenovirus in the adrenal glands.

Construction and purification of adenoviruses. Recombinant adenoviruses that encode GRK2 (AdGRK2) or rat wild type, full length β-arrestin1 (Adβarr1) were constructed as described previously (9). 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 (9).

H295R cell culture and transfection. H295R cells were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured as previously described (35). Transfection was performed either with Adβarr1 or AdGFP or with pcDNA3.1 plasmid encoding either for the V53D dominant negative βarr1 mutant (14) (DN βarr1, a generous gift of Dr. Lefkowitz, Duke University Medical Center, Durham, NC, USA) or just empty pcDNA3.1 vector (EV). Plasmid transfections were performed using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

Plasma and in vitro aldosterone secretion measurements. Rat plasma aldosterone levels and in vitro aldosterone secretion in the culture medium of H295R cells were determined by EIA (Aldosterone EIA kit, ALPCO Diagnostics, Salem, NH, USA), as described (34).

Western blotting. Western blots to assess protein levels of StAR (sc-25806), GRK2 (sc-562; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-ERK1/2 (#9106), total ERK1/2 (#4696), and total ERK2 (#9108; Cell Signaling Technology, Danvers, MA), βarr1 (A1CT antibody, Ref. 16, a generous gift of Dr. 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 in H295R cell extracts, as described previously (9). 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).

Co-immunofluorescence. Immunofluorescence imaging of human adrenal cross-sections was carried out as described previously (9). Briefly, human adrenal cross-sections were fixed, permeabilized and labeled with rabbit polyclonal anti-StAR (sc-25806) and goat anti-βarr1 (sc-9182; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies, followed by the corresponding Alexa Fluor 594 anti-goat (red) and Alexa Fluor 568 anti-rabbit (green) secondary antibodies (Molecular Probes, Eugene, OR, USA). Confocal images were obtained using a ×40 objective on a Leica Microsystems TCS SP laser scanning confocal microscope.

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 all tests, a p value of <0.05 was generally considered to be significant.

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Figure Legends

Fig. 1. Involvement of βarr1 in AngII-induced aldosterone production and secretion in H295R cells. (*A*) Aldosterone secretion in response to 10 nM AngII treatment (AngII) or vehicle (Control) for 6 hrs in H295R cells. *, $p<0.05$, n=4 independent experiments. (*B*) Top: Representative immunoblots for endogenous βarrs and StAR in protein extracts from vehicle- (Cont.) and AngII-treated (AngII) H295R cells, including blots for GAPDH as loading control. A lane run with extract from HEK293 cells (HEK), as a positive control for both βarr isoforms, is also shown. Bottom: Densitometric analysis of StAR protein expression normalized to GAPDH levels. *, $p<0.05$, n=4 independent experiments. (*C*) Co-immunofluorescence in sections of human adrenal glands using antibodies specific for StAR (green) and βarr1 (red), showing co-localization of the two fluorescent signals (yellow), which indicates endogenous expression of βarr1 in human adrenocortical cells. Scale bar: 100 μm. (*D*) Aldosterone secretion in H295R cells transfected with empty vector (EV) or with a plasmid encoding for the V53D dominant negative βarr1 (DN βarr1), and stimulated with 10 nM AngII or vehicle for 6 hrs. *, $p<0.05$, n=5 independent experiments. (*E*) Western blotting for StAR in these cells at the end of the indicated treatments. Blots for βarr1 to confirm DN βarr1 overexpression are also shown, along with GAPDH as loading control. On top: representative blots, on bottom: densitometric quantification of 5 independent experiments. *, $p<0.05$, n=5. (*F*) Aldosterone secretion in H295R cells transfected with Adβarr1 or AdGFP, and stimulated with 10 nM AngII or vehicle for 6 hrs. *, $p<0.05$, n=5 independent experiments. (*G*) Western blotting for StAR. Representative blots confirming the overexpression of βarr1, along with GAPDH as loading control, are shown on top, and the densitometric quantification of 5 independent experiments on bottom. *, $p<0.01$, n=5.

Fig. 2. βarr1-mediated AngII signaling to aldosterone production in H295R cells. (*A*) Western blotting for phospho-ERK1/2 and for total ERK2 in extracts from transfected H295R cells after 10 nM AngII stimulation for the indicated times. Representative blots of 3 independent experiments are shown, including blots for βarr1 to confirm the overexpression of the transfected proteins. (*B*) Densitometric quantification of the 3 independent experiments performed in (*A*). *, $p<0.05$, vs. AdGFP, **, $p<0.05$, vs. DN βarr1. (*C,D*) Western blotting in control AdGFP- or in Adβarr1-transfected H295R cells treated with vehicle or 10 nM AngII for 6 hrs following pretreatment with 10 μM U73122, 10 μM U73122 plus 10 μM DiC8-DAG, or 50 μM PD98059. Representative blots of 3 independent experiments for each cell line are shown, including blots for endogenous βarr1 and for total ERK1/2 and GAPDH as loading controls. (*E,F*) AngII-induced ERK phosphorylation and StAR upregulation as densitometrically quantitated in the 3 independent experiments performed in (*C*) and (*D*), respectively. Values are expressed as % of the AngII response of cells not pretreated with any agent (No Inhibitor). *, $p<0.05$, vs. No Inhibitor, n=3. (*G*) Aldosterone secretion in Adβarr1- or AdGFP-transfected cells pretreated with 10 μM U73122 or μM PD98059, followed by 10 nM AngII or vehicle stimulation for 6 hrs. No significant differences at $p=0.05$, n=3 independent experiments. (*H*) Aldosterone secretion in Adβarr1- or AdGFP-transfected cells pretreated with 10 μM U73122 plus 10 μM DiC8-DAG, followed by 10 nM AngII or vehicle stimulation for 6 hrs. *, $p<0.05$, vs. –AngII, n=3 independent experiments.

Fig. 3. SII-induced aldosterone production and secretion in H295R cells. (*A*) Aldosterone secretion in transfected H295R cells stimulated with 10 μM SII or vehicle for 6 hrs. Data are

shown as the % induction over vehicle (basal) levels of aldosterone secretion. ***,** $p<0.05$, vs. AdGFP or EV, n=5 independent determinations/treatment. (*B*) Western blotting for phospho-ERK1/2 and for total ERK2 after 10 μM SII or vehicle. Representative blots of 3 independent experiments are shown on top and the % SII-induced ERK activation (over basal), as derived by densitometric quantification, is shown on bottom. *****, $p<0.05$, n=3 independent experiments. (*C*) Western blotting for StAR after 10 μM SII or vehicle. Representative blots of 3 independent experiments are shown on top, including blots for βarr1 to confirm the overexpression of the respective constructs and for GAPDH as loading control, and the % SII-induced StAR induction (over basal), as derived by densitometric quantification, is shown on bottom. ***,** $p<0.05$, n=3 independent experiments.

Fig. 4. In vivo adrenal-targeted βarr1 overexpression and aldosterone production in normal rats. (*A*) Plasma aldosterone levels in AdGFP-, AdGRK2-, or Adβarr1-treated, plus in saline-treated (Saline), normal rats at 7 days post-in vivo gene transfer. *, $p<0.05$, **, $p<0.01$, vs. AdGFP or Saline, n=5 rats/group. (*B*) Representative western blots in protein extracts from adrenal glands from these rats, confirming the overexpression of the respective transgenes. GAPDH is also shown as loading control.

Fig. 5. Schematic representation of the signaling pathway of AngII-induced aldosterone production mediated by βarr1. See text for details. ZG: Zona Glomerulosa, AngII: Angiotensin II, AT_1R: AngII receptor type I, βarr1: beta-arrestin 1 (arrestin-2), Gq: Gq protein, PLC: Phospholipase C, PIP_2: Phosphatidylinositol 4`,5`-bisphosphate, DAG: Diacylglycerol, IP_3: Inositol 1`,4`,5`- trisphosphate, DGK: Diacylglycerol kinase, PA: Phosphatidic acid, pERK:

phospho-Extracellular Signal-Regulated Kinase, MEK1: MAPK-ERK Kinase-1, StAR: Steroidogenic Acute Regulatory protein**.**

Figure 1

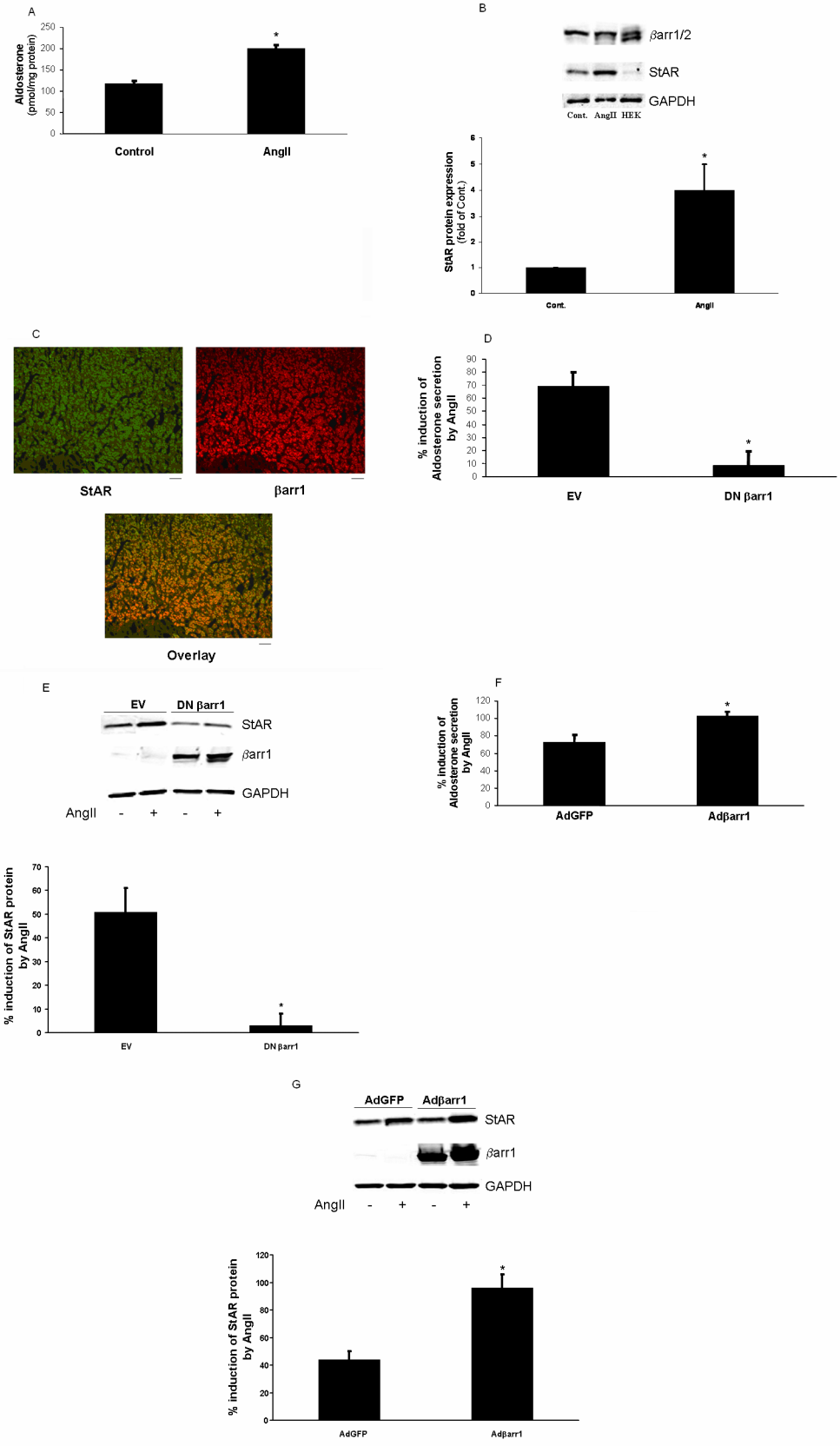

A
Aldosterone (pmol/mg protein)
250
200
150
100
50
0
*
Control
AngII
B
βarr1/2
StAR
GAPDH
Cont. AngII HEK
StAR protein expression (fold of Cont.)
6
5
4
3
2
1
0
*
Cont.
AngII
C
StAR
βarr1
Overlay
D
% induction of Aldosterone secretion by AngII
90
80
70
60
50
40
30
20
10
0
*
EV
DN βarr1
E
EV
DN βarr1
StAR
βarr1
GAPDH
AngII - + - +
% induction of StAR protein by AngII
70
60
50
40
30
20
10
0
*
EV
DN βarr1
F
% induction of Aldosterone secretion by AngII
120
100
80
60
40
20
0
*
AdGFP
Adβarr1
G
AdGFP
Adβarr1
StAR
βarr1
GAPDH
AngII - + - +
% induction of StAR protein by AngII
120
100
80
60
40
20
0
*
AdGFP
Adβarr1

Figure 2

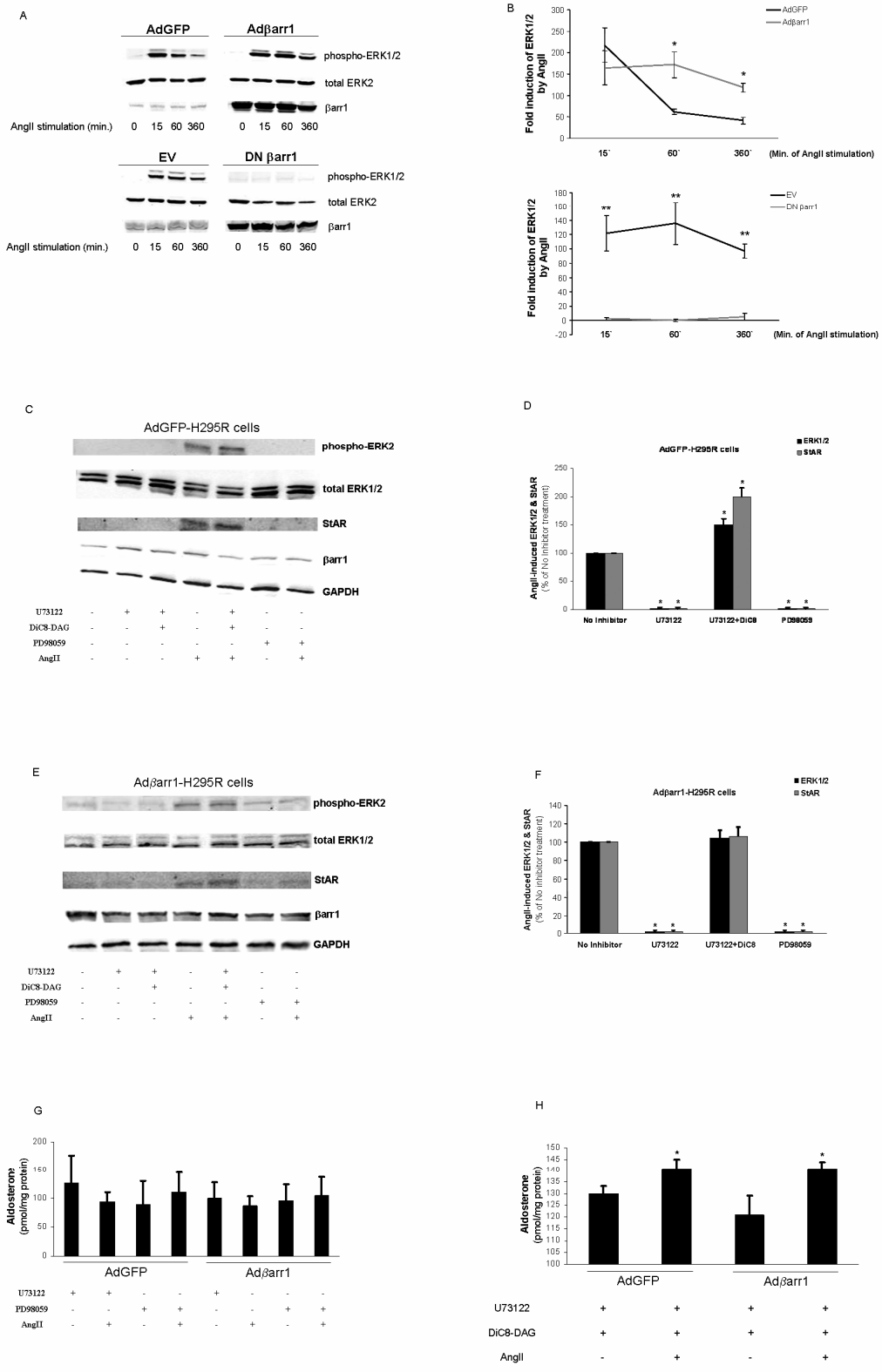

A
AdGFP
Adβarr1
phospho-ERK1/2
total ERK2
βarr1
AngII stimulation (min.) 0 15 60 360 0 15 60 360
EV
DN βarr1
phospho-ERK1/2
total ERK2
βarr1
AngII stimulation (min.) 0 15 60 360 0 15 60 360
B
AdGFP
Adβarr1
Fold induction of ERK1/2 by AngII
15' 60' 360' (Min. of AngII stimulation)
EV
DN βarr1
Fold induction of ERK1/2 by AngII
15' 60' 360' (Min. of AngII stimulation)
C
AdGFP-H295R cells
phospho-ERK2
total ERK1/2
StAR
βarr1
GAPDH
U73122 - + + - + - -
DiC8-DAG - - + - + - -
PD98059 - - - - - + +
AngII - - - + + - +
D
AdGFP-H295R cells
ERK1/2
StAR
AngII-induced ERK1/2 & StAR (% of No inhibitor treatment)
No Inhibitor U73122 U73122+DiC8 PD98059
E
Adβarr1-H295R cells
phospho-ERK2
total ERK1/2
StAR
βarr1
GAPDH
U73122 - + + - + - -
DiC8-DAG - - + - + - -
PD98059 - - - - - + +
AngII - - - + + - +
F
Adβarr1-H295R cells
ERK1/2
StAR
AngII-induced ERK1/2 & StAR (% of No inhibitor treatment)
No Inhibitor U73122 U73122+DiC8 PD98059
G
Aldosterone (pmol/mg protein)
AdGFP
Adβarr1
U73122 + + - - + - - -
PD98059 - - + + - - + +
AngII - + - + - + - +
H
Aldosterone (pmol/mg protein)
AdGFP
Adβarr1
U73122 + + + +
DiC8-DAG + + + +
AngII - + - +

Figure 3

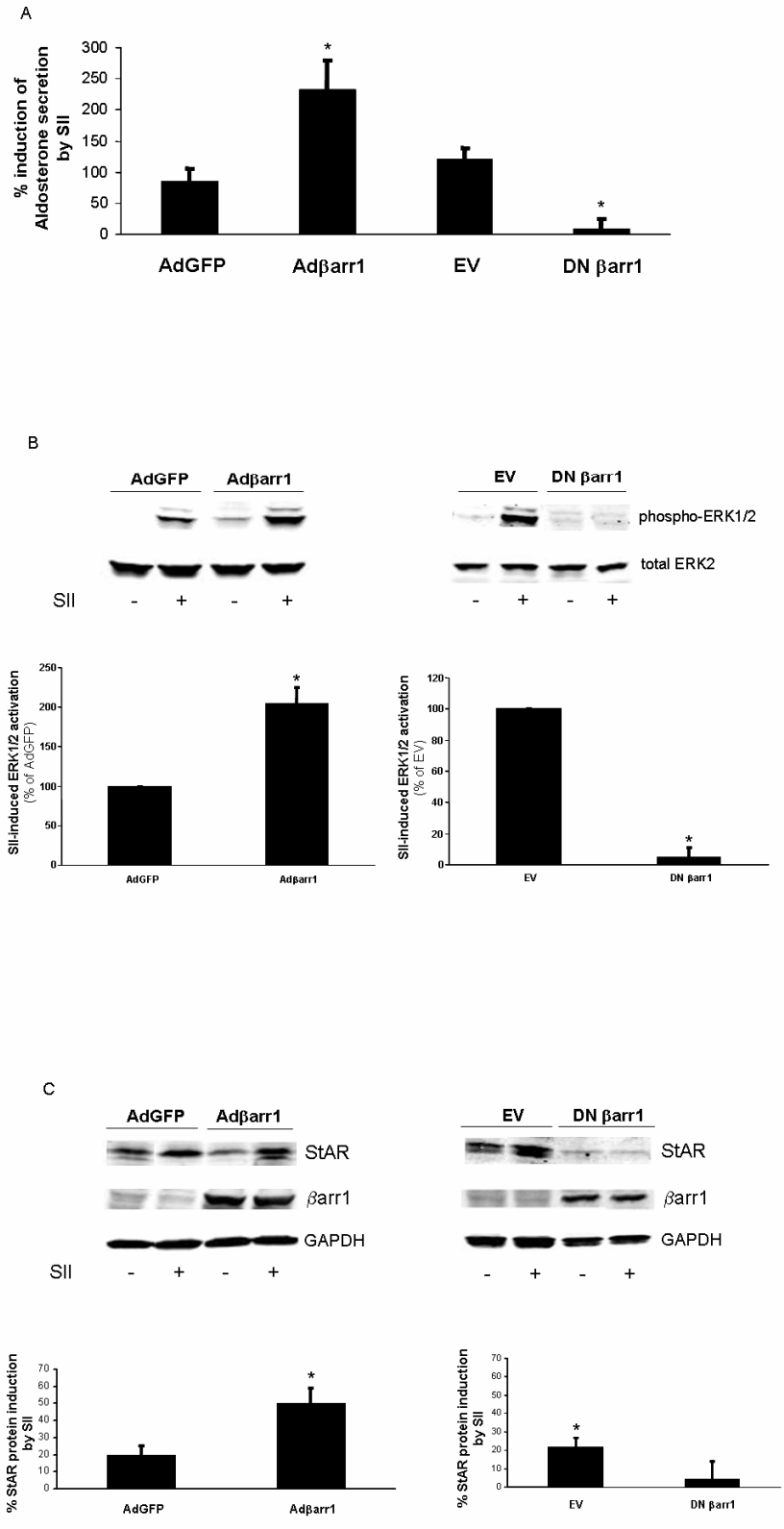

A
% induction of Aldosterone secretion by SII
300
250
200
150
100
50
0
*
*
AdGFP
Adβarr1
EV
DN βarr1
B
AdGFP
Adβarr1
EV
DN βarr1
phospho-ERK1/2
total ERK2
SII
- + - +
- + - +
SII-induced ERK1/2 activation (% of AdGFP)
SII-induced ERK1/2 activation (% of EV)
AdGFP
Adβarr1
EV
DN βarr1
C
AdGFP
Adβarr1
EV
DN βarr1
StAR
βarr1
GAPDH
SII
- + - +
- + - +
% StAR protein induction by SII
AdGFP
Adβarr1
EV
DN βarr1

Figure 4

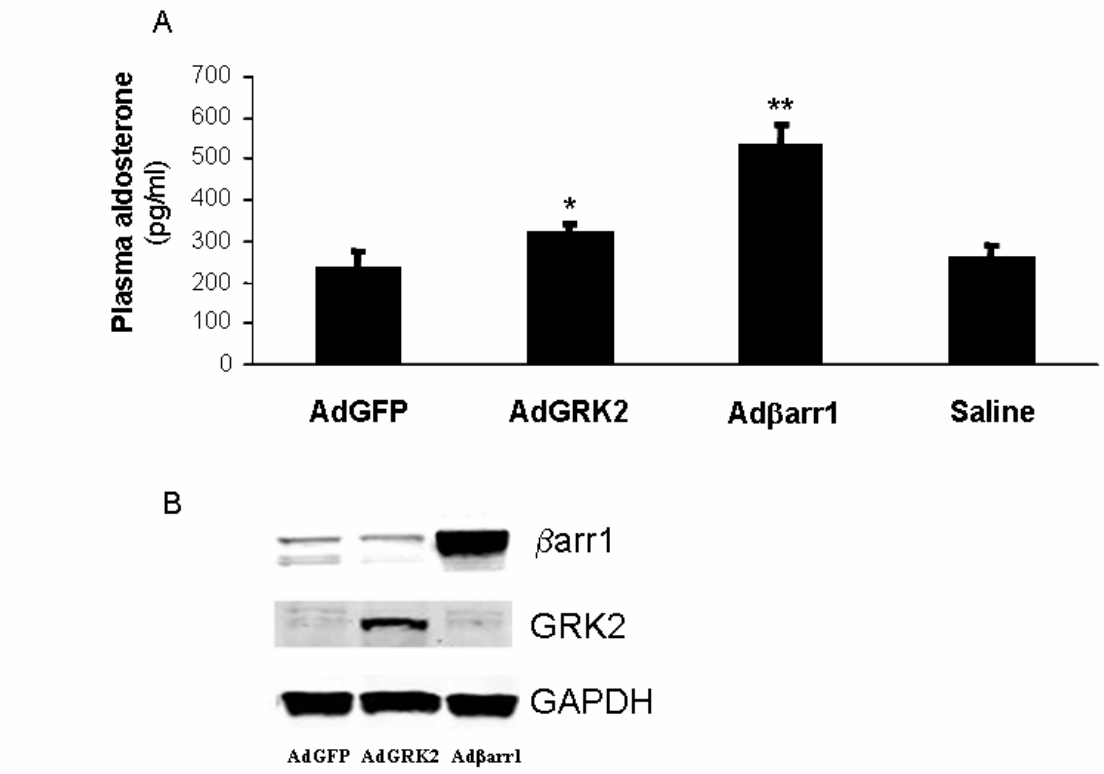
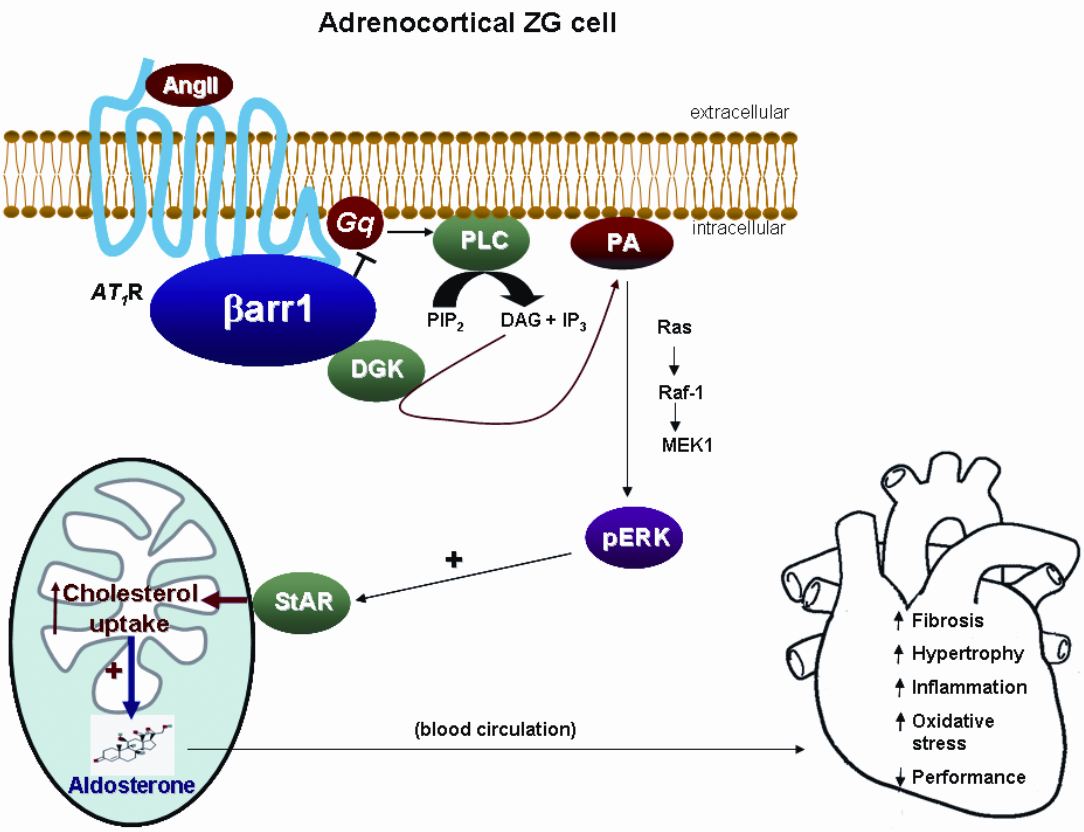

A
Plasma aldosterone (pg/ml)
700
600
500
400
300
200
100
0
*
**
AdGFP
AdGRK2
Adβarr1
Saline
B
βarr1
GRK2
GAPDH
AdGFP AdGRK2 Adβarr1

Figure 5


Adrenocortical ZG cell
AngII
extracellular
intracellular
Gq
PLC
PA
AT1R
βarr1
PIP2
DAG + IP3
DGK
Ras
Raf-1
MEK1
pERK
+
Cholesterol uptake
StAR
+
Aldosterone
(blood circulation)
Fibrosis
Hypertrophy
Inflammation
Oxidative stress
Performance