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Ezrin, Radixin, and Moesin Phosphorylation in NIH3T3 Cells Revealed Angiotensin II Type 1 Receptor Cell-Type–Dependent Biased Signaling

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Abstract. *β*-Arrestin-biased agonists are a new class of drugs with promising therapeutic effects. The molecular mechanisms of *β*-arrestin-biased agonists are still not completely identified. Here, we investigated the effect of angiotensin II (AngII) and [Sar1,Ile4,Ile8] AngII (SII), a *β*-arrestin-biased agonist, on ezrin–radixin–moesin (ERM) phosphorylation in NIH3T3 cells (a fibroblast cell line) stably expressing AngII type 1A receptor. ERM proteins are cross-linkers between the plasma membrane and the actin cytoskeleton and control a number of signaling pathways. We also investigated the role of G*α*q protein and *β*-arrestins in mediating ERM phosphorylation. We found that AngII stimulates ERM phosphorylation by acting as a *β*-arrestin-biased agonist and AngII-stimulated ERM phosphorylation is mediated by *β*-arrestin2 not *β*-arrestin1. We also found that SII inhibits ERM phosphorylation by acting as a G*α*q protein–biased agonist. We concluded that ERM phosphorylation is a unique *β*-arrestin-biased agonism signal. Both AngII and SII can activate either G*α*q protein or *β*-arrestin-mediated signaling as functional biased agonists according to the type of the cell on which they act.

Keywords: angiotensin II type 1 receptor, biased agonist, *β*-arrestin, G protein, ERM family protein

Introduction

Angiotensin II type 1 receptor (AT1R) is a G protein–coupled receptor (GPCR). There are two AT1R subtypes: AT1AR and AT1BR (1). AT1R responds to angiotensin II (AngII) and changes its conformation to couple with G*α*q protein (2). G*α*q protein activates phospholipase C-*β* (PLC*β*) that hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol triphosphate (IP_3) (3). DAG acts as a second messenger that activates protein kinase C (PKC) and IP_3 mediates elevation of cytosolic calcium levels (4). GPCR kinases (GRKs) then phosphorylate the activated AT1R mediating binding of *β*-arrestin1/2 proteins (5). *β*-Arrestin1/2 proteins desensitize and internalize the activated receptors and initiate another cascade of intracellular signaling (6). [Sar1,Ile4,Ile8] AngII (SII) was reported to be a *β*-arrestin-biased agonist that acts on AT1R. SII-induced change in the AT1R conformation is different from that of AngII stimulation (7). Previous reports suggested that SII activates *β*-arrestin signaling without G protein coupling (8). However, it was reported that SII also activates G protein signaling but in a different way from AngII, although the activation by SII is much weaker than AngII (9).

Investigation of the intracellular signaling pathways activated by AngII and SII in HEK293 cells and cardiomyocytes revealed a number of differences. Both AngII and SII can stimulate extracellular regulated kinase (ERK) phosphorylation (10). However, SII-mediated ERK phosphorylation is graded and slow with localization of phosphorylated ERK (pERK) in the cytoplasm (11). AngII activates apoptotic signals while SII activates anti-apoptotic ones (12). Great efforts have been made to identify unique signaling pathways of *β*-arrestin-biased agonists. However, the differences in cellular responses by AngII or SII-mediated signaling still remain to be determined.

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ERM proteins are key proteins inside the cell. They control and regulate many signaling pathways including migration, differentiation and survival signals (13). ERM proteins are activated by phosphorylation and the process of activation needs two sequential steps. The first step is ERM binding with PIP2 which converts ERM proteins from the closed conformation to the open one to expose phosphorylation sites (14). The second step is ERM phosphorylation by different types of kinases, e.g., GRKs, Rho A kinase (ROCK), and phosphatidylinositol 3 kinase (PI3K) (15 – 17). These kinases can be activated by G proteins and β-arrestin-dependent signals. However, G proteins, including Gαq and G$\beta\gamma$ proteins, activate PLCβ, leading to break-down of PIP2 and inhibition of the first step in ERM activation (18). Therefore, we assumed that ERM phosphorylation is an index of the unique β-arrestin-biased agonism signal.

We investigated the SII effect on ERM phosphorylation in NIH3T3 cells (a fibroblast cell line) due to the following reasons: first, published data on SII signaling pathways in fibroblast cell lines is very limited and not clearly established. Second, previous reports showed that AngII-induced responses in cardiac fibroblasts and other fibroblast cell lines are different from those in cardiomyocytes in many aspects (9, 19), which has led us to assume that SII-induced responses in fibroblast cell lines will be different from SII-induced responses in cardiomyocytes. Third, although β-arrestin-biased agonists promote cardiomyocyte protection and reduce cardiac fibrosis (20), β-arrestin signaling in different types of fibroblasts have been reported to initiate tissue fibrosis (21), and our own studies have found that β-arrestin2 mediates metoprolol-stimulated cardiac fibrosis in vivo (22). Thus, SII may activate AT1R–β-arrestin signaling in cardiac fibroblasts and initiate cardiac fibrosis in the heart that could lead to cardiac dysfunction.

Here we show that AngII stimulates ERM phosphorylation in NIH3T3 cells by acting as a β-arrestin-biased agonist and SII inhibits ERM phosphorylation by acting as a Gαq protein–biased agonist.

Materials and Methods

Constructs and cell lines

NIH3T3 cells stably over-expressing AT1AR were used in all experiments. Plasmids used in the experiments include the following: Flag-tagged AT1AR, β-arrestin1, β-arrestin2, Gq-CT (amino acids 305 – 359 of mouse Gαq), constitutively active mutant of Gαq [Gq (Q209L)], dominant negative mutant (Q209L/D277N) of Gαq (DN-Gq) cDNAs in PMX-Puro. Plasmids were stably transfected into NIH3T3 cells using retroviral infection technique. Mutations were made using the QuikChange site-directed mutagenesis according to manufacturer instructions. All constructs were verified by sequencing.

Cell culture

NIH3T3 cells were plated in 60-mm tissue culture dishes (30% confluency; BD Falcon, Franklin Lakes, NJ, USA) and grown in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific, Barrington, IL, USA) and penicillin and streptomycin (GIBCO). Cells were starved over night before stimulations by replacing the medium with DMEM containing 0.5% fetal bovine serum.

siRNA transfection

Chemically synthesized, double-stranded siRNAs, were purchased from Invitrogen (Carlsbad, CA, USA) in deprotected and desalted form. The siRNA sequences targeting mouse β-arrestin2 were 5′-GCCACAGAC GAUGACAUCGUCUUUG-3′. NIH3T3 cells that were 30% – 40% confluent on 60-mm dishes were transfected with 200 pmol of β-arrestin2 siRNA and control Hi siRNA (Invitrogen) 48 h before retroviral infection, using lipofectamine 2000 reagent (Invitrogen).

Retroviral infection

Retrovirus was prepared in Plat E cells. Plat E cells were plated in 60-mm tissue culture dishes (30% confluency) and grown in 5% CO_2 at 37°C in DMEM supplemented with 10% FBS, penicillin, streptomycin, blasticidin (Invitrogen), and puromycin (Sigma, St. Louis, MO USA). Blasticidin and puromycin were removed from the media 12 h before transfection. Plasmids were transfected into Plat E cells using X-treme Gene 9 (Roche, Basel, Switzerland). Forty-eight hours later, media containing retrovirus were collected and centrifuged to remove Plat E cells. Then NIH3T3 cells (plated in 60-mm tissue culture dishes, 30% – 40% confluency) were infected with 1.5 ml of supernatant. Eight hours later, 1.5 ml of fresh DMEM media supplemented with 10% FBS, penicillin, and streptomycin were added to infected NIH3T3 cells. Twenty four hours after infection, the media were removed and replaced with fresh ones. Twenty four hours later, cells were ready for experiments.

Drugs

The following drugs from the indicated sources were used: angiotensinII (SCETI Bioscience, Tokyo), [Sar1,Ile4,Ile8] AngII (was synthesized at the Cleveland Clinic, Cleveland, Lerner Research Institute, OH, USA), valsartan, and Y27632 (Wako, Osaka).

Cell lysates preparation

Cells were first washed with 1 ml 1 × phosphate-buffered saline (PBS) and then cells were lysed using 495 μl lysis buffer (50 mM Tris-Cl, 1 mM EDTA, 150 mM NaCl, 20 mM NaF, 0.5% Nonidet P-40, 10% glycerol), and 5 μl protease inhibitor (Nacalai Tesque, Kyoto). Cell lysates were transferred to 1.5-ml tubes and kept in ice for 15 min. Cell lysates were purified by centrifugation at 15,000 rpm for 15 min at 4°C. Then we added 50 μl SDS + 4 × β-mercaptoethanol (β-ME) to each 150-μl sample of cell lysate, followed by heating the samples at 95°C for 5 min. The samples were finally kept at −80°C until use.

Western blotting experiments

Cell lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, UK). After the membranes were blocked with either 3% bovine serum albumin (BSA) (Sigma) or 3% skim milk in Tris-buffered saline Tween 20 (TBS-T) (20 mM Tris PH 7.5, 137 mM NaCl, 0.2% Tween 20) at room temperature, the membranes were incubated with anti-ERM antibody (Ab) (Cell Signaling, Danvers, MA, USA), anti-pERM polyclonal Ab (Cell Signaling), anti-ERK monoclonal Ab (Cell Signaling), anti-pERK Ab (Cell Signaling), anti-β-arrestin Ab (BD Bioscience, Franklin Lakes, New Jersey, USA), anti-β-arrestin2 monoclonal Ab (Cell Signaling), or anti-GAPDH Ab (Santa Cruz, Santa Cruz, CA, USA) overnight at 4°C and then with anti-rabbit Ab (Cell Signaling) or anti-mouse Ab (Santa Cruz) for 1 h at room temperature. Specific bands were detected using chemiluminescence western blotting detection reagents. Chemiluminescent images were captured and digitized using Scion image for windows software (Scion Corporation, Meyer Instruments, Houston, TX, USA).

Statistical analyses

The results are presented as the mean ± S.E.M. from at least three independent experiments. Statistical comparisons were made with one-way analysis of variance followed by Tukey correction using Graph Pad Prism version 5 (Graph Pad Software, Inc., CA, USA) with significance imparted at P-values < 0.05.

Results

ERM phosphorylation in NIH3T3 cells is stimulated by AngII not SII

We prepared NIH3T3 cells stably over-expressing AT1AR using the retroviral infection technique. We investigated the effect of AngII (1 μM) and SII (30 μM) on ERM phosphorylation after 5-, 15-, and 30-min stimulations (Fig. 1: A, B). AngII stimulated ERM phosphorylation at each time point while SII did not (Fig. 1: A, B).

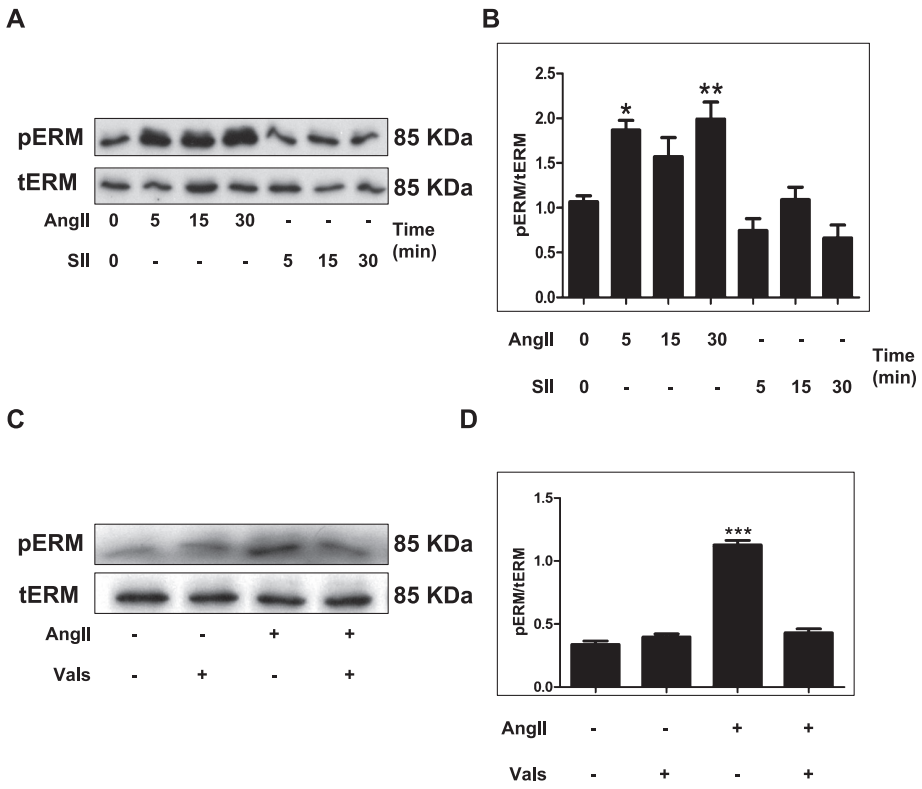

Fig. 1. ERM phosphorylation in NIH3T3 cells is stimulated with AngII not SII. A, B) NIH3T3 cells stably over-expressing AT1AR using retroviral infection technique were fasted overnight and then treated with AngII (1 μM) or SII (30 μM) for 5, 15, and 30 min. Then cells were lysed and samples were subjected to SDS-PAGE analysis and western-blotted using polyclonal pERM and total ERM (tERM) antibodies. *P < 0.05; **P < 0.01, compared with 0-min stimulation group, n = 3 independent experiments. C, D) NIH3T3 cells stably over-expressing AT1AR using the retroviral infection technique were fasted overnight, treated with (10 μM) valsartan (Vals) for 30 min, and then stimulated with AngII (1 μM) for 30 min. Then cells were lysed and samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) analysis and western-blotted using polyclonal pERM and total ERM (tERM) antibodies. ***P < 0.001, compared with 0-min stimulation group, n = 3 – 5 independent experiments. All P-values were analyzed by one-way analysis of variance (ANOVA) with the Tukey correction. Data are shown as means ± S.E.M.

AngII-stimulated ERM phosphorylation was blocked by treatment with an AT1R blocker, valsartan, for 30 min before stimulation with AngII (Fig. 1: C, D). We also observed that the AngII- and SII-stimulated ERK phosphorylation pattern is different from that reported in HEK293 cells (data not shown). AngII stimulated ERK phosphorylation in a graded manner with maximum response occurring after 30 min. The SII effect on ERK phosphorylation was rapid, sharp, and brief with maximum response after 5 min. It was reported that AngII-stimulated ERK phosphorylation is biphasic in HEK293 cells. The first phase is rapid, sharp, brief, and Gαq-dependent and it lasts from 2 to 5 min. The second phase is graded, slow, and β-arrestin-dependent and lasts from 10 to 60 min (10). SII-stimulated ERK phosphorylation on the other hand is monophasic, graded, slow, and β-arrestin-dependent (10). These results suggest that SII signaling in NIH3T3 cells is different from that in HEK293 cells or cardiomyocytes.

AngII-stimulated ERM phosphorylation in NIH3T3 cells is independent of Gαq-protein

We investigated the mechanism by which AngII stimulates ERM phosphorylation. We examined the role of Gαq protein in AngII-stimulated ERM phosphorylation by using 3 interventions: 1) Over-expression of Gαq carboxy terminal peptide (Gq-CT) (Fig. 2: A, B), which inhibits Gαq coupling with AT1AR (23); 2) Over-expression of Gαq constitutive active mutant [Gq (Q209L)] (Fig. 2: C, D), which desensitizes AT1AR and down-regulates Gαq downstream signals (24); and 3) Over-expression of Gαq dominant negative mutant (Q209L/D277N) (DN-Gq) (Fig. 2: E, F), which down regulates Gαq downstream signals (25).

Gq-CT did not affect AngII-stimulated ERM phosphorylation (Fig. 2: A, B), Gq (Q209L) increased both basal and AngII-stimulated ERM phosphorylation (Fig. 2: C, D), and DN-Gq did not affect AngII-stimulated ERM phosphorylation (Fig. 2: E, F). These results indicate that, as we initially assumed, Gαq protein signaling inhibits ERM phosphorylation. These results also indicate that AngII-stimulated Gαq protein signaling in NIH3T3 cells expressing AT1AR is too weak to inhibit ERM phosphorylation.

AngII-stimulated ERM phosphorylation in NIH3T3 cells is dependent on β-arrestin2

We investigated the role of β-arrestin1, β-arrestin2 (Fig. 3: A – D), and ROCK (Fig. 3: G, H) in AngII-stimulated ERM phosphorylation. Over-expression of β-arrestin1 (Fig. 3E) significantly reduced ERM phosphorylation (Fig. 3: A, B), while over-expression of β-arrestin2 (Fig. 3E) significantly increased both basal and AngII-stimulated ERM phosphorylation (Fig. 3: A, B). The role of β-arrestin2 in AngII-stimulated ERM phosphorylation was confirmed with knockdown experiments. β-Arrestin2 siRNA knockdown (Fig. 3F) caused 51% reduction in AngII-stimulated ERM phosphorylation (Fig. 3: C, D). These results indicate that AngII-stimulated ERM phosphorylation is dependent on β-arrestin2. Inhibition of ROCK signaling by Y27632 significantly increased both basal and AngII-stimulated ERM phosphorylation (Fig. 3: G, H). This is the first time that ROCK has been reported to inhibit ERM phosphorylation. These results also suggest that β-arrestin2 signaling is more powerful than both Gαq protein and β-arrestin1 signaling. Thus, AngII acts as a β-arrestin-biased agonist in NIH3T3 cells.

SII inhibits ERM phosphorylation in NIH3T3 cells by acting as a Gαq protein–biased agonist

We investigated the effect of β-arrestin2 (Fig. 4: A, B) and Gαq protein (Fig. 4: C – H) on SII-ERM signaling. Over-expression of β-arrestin2 significantly increased basal levels of phosphorylated ERM (pERM) proteins. However, β-arrestin2 over-expression had no effect on SII-ERM signaling (Fig. 4: A, B). In contrast to the over-expression of β-arrestin2, over-expression of Gq-CT significantly increased SII-stimulated ERM phosphorylation (Fig. 4: C, D), but decreased SII-stimulated ERK phosphorylation (data not shown). Over-expression of Gq (Q209L) significantly increased both basal and SII-stimulated ERM phosphorylation (Fig. 4: E, F), but decreased both basal and SII-stimulated ERK phosphorylation (data not shown). Over-expression of DN-Gq increased SII-stimulated ERM phosphorylation (Fig. 4: G, H). It also significantly decreased SII-stimulated ERK phosphorylation (data not shown). These results indicate that SII stimulates AT1AR coupling with Gαq protein. These results also show that SII mediated Gαq protein signaling is more powerful than β-arrestin signaling. Thus, SII acts as a Gαq protein–biased agonist in NIH3T3 cells.

Discussion

Here we demonstrated that AngII but not SII stimulates ERM phosphorylation in NIH3T3 cells stably over-expressing AT1AR. AngII-stimulated ERM phosphorylation in NIH3T3 cells is independent of Gαq protein and β-arrestin1 and dependent on β-arrestin2. Gαq protein and β-arrestin1 work together to antagonize β-arrestin2-mediated ERM phosphorylation. AngII weakly stimulates Gαq protein but powerfully stimulates β-arrestin2 signaling in NIH3T3 cells to phosphorylate ERM proteins (Fig. 5). SII stimulates Gαq protein signal-

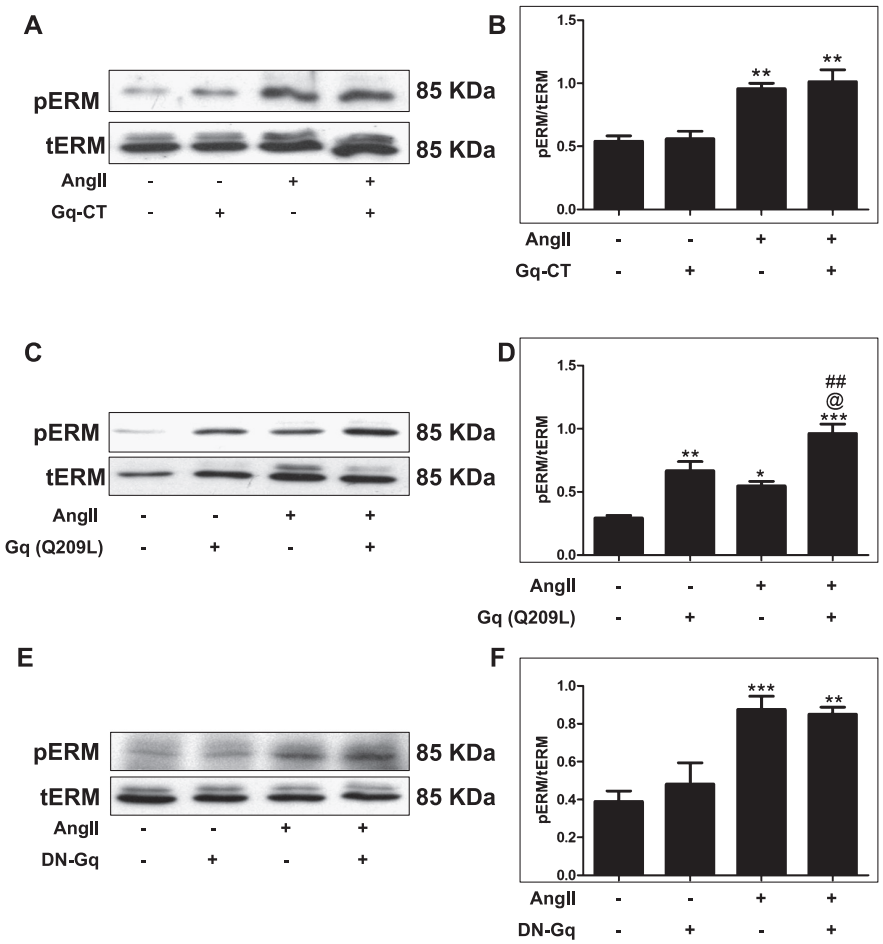

Fig. 2. AngII-stimulated ERM phosphorylation in NIH3T3 cells is independent of Gαq protein. A, B) NIH3T3 cells stably over-expressing AT1AR and Gq-CT (inhibits Gαq coupling to AT1AR) using the retroviral infection technique were fasted overnight and then stimulated with AngII (1 μM) for 5 min. Then cells were lysed and samples were subjected to SDS-PAGE and western-blotted using polyclonal pERM and tERM antibodies. $^{**}P < 0.01$, compared with the AngII control group, n = 3 independent experiments. C, D) NIH3T3 cells stably over-expressing AT1AR and Gq (Q209L) (desensitizes AT1AR and down regulates Gαq down stream signals) using the retroviral infection technique were fasted overnight and then stimulated with AngII (1 μM) for 5 min. Then cells were lysed and samples were subjected to SDS-PAGE and western-blotted using polyclonal pERM and tERM antibodies. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the AngII control group; $^{@}P < 0.05$, compared with the Gq (Q209L) control group; $^{\#\#}P < 0.01$, compared with the AngII 5-min stimulation group, n = 3 – 4 independent experiments. E, F) NIH3T3 cells stably over-expressing AT1AR and DN-Gq (down regulates Gαq activity) using the retroviral infection technique were fasted overnight and then stimulated with AngII (1 μM) for 5 min. Then cells were lysed and samples were subjected to SDS-PAGE and western-blotted using polyclonal pERM and tERM antibodies. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the AngII control group. n = 3 – 5 independent experiments. All *P*-values were analyzed by ANOVA with the Tukey correction. Data are shown as means ± S.E.M.

ing more strongly than β-arrestin2 signaling in NIH3T3 cells to inhibit ERM phosphorylation (Fig. 5). Our study showed for the first time that both AngII and SII can stimulate either Gαq protein– or β-arrestin-biased signaling, depending on the type of the cell on which they act.

Biased agonism is the ability of an agonist to selectively stimulate one signaling pathway more strongly than another (26). β-Arrestin-biased agonists are a new class of drugs with promising therapeutic effects, especially in the treatment of heart failure (27). TRV120027 is the first β-arrestin-biased agonist designed to treat acute heart failure patients and is currently under phase 2a trial (28). β-Arrestin-biased agonists have been found to improve cardiomyocyte function and protect them against apoptosis (29). β-Arrestin-biased agonists have been also found to reduce cardiac fibrosis (20). However, we recently reported that chronic treatment with the β-

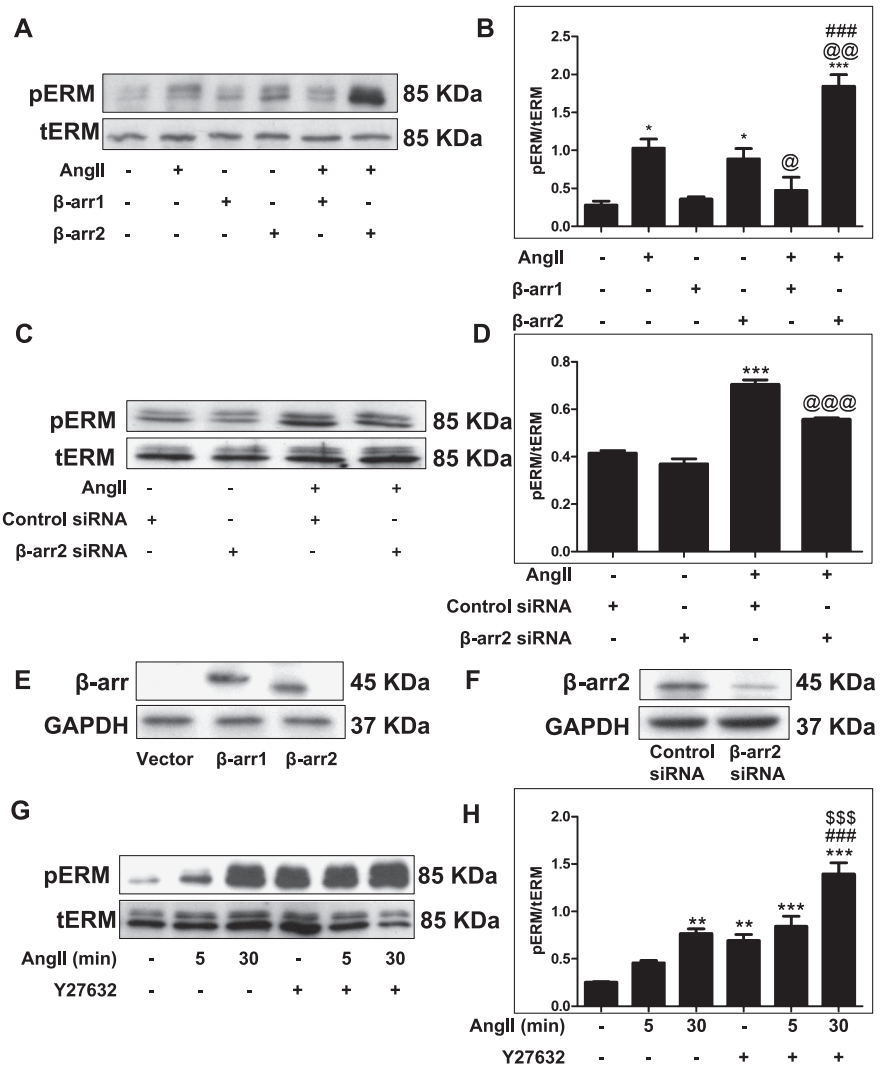

Fig. 3. AngII-stimulated ERM phosphorylation in NIH3T3 cells is dependent on β-arrestin2. A, B) NIH3T3 cells stably over-expressing AT1AR and β-arrestin1 (β-arr1) or β-arrestin2 (β-arr2) using the retroviral infection technique were fasted overnight and then stimulated with AngII (1 μM) for 30 min. Then cells were lysed and samples were subjected to SDS-PAGE and western-blotted using polyclonal pERM and tERM antibodies. *$P < 0.05$, ***$P < 0.001$, compared with the AngII control group; $^{@}P < 0.05$, $^{@@}P < 0.01$, compared with the AngII 30-min stimulation group; $^{\#\#\#}P < 0.001$, compared with the AngII 30 min + β-arr1 group, n = 3 – 4 independent experiments. C, D) NIH3T3 cells stably over-expressing AT1AR using the retroviral infection technique and treated with β-arr2 siRNA to knockdown it were fasted overnight and then stimulated with AngII (1 μM) for 30 min. Then cells were lysed and samples were subjected to SDS-PAGE and western-blotted using polyclonal pERM and tERM antibodies. ***$P < 0.001$, compared with the AngII control group; $^{@@@}P < 0.001$, compared with the AngII 30-min group, n = 4 independent experiments. E) Over expression of β-arr1 and β-arr2. F) β-arr2 knockdown. G, H) NIH3T3 cells stably over-expressing AT1AR using the retroviral infection technique were fasted overnight, treated with (10 μM) Y27632 (ROCK inhibitor) for 30 min, and then stimulated with AngII (1 μM) for 30 min. Then cells were lysed and samples were subjected to SDS-PAGE and western-blotted using polyclonal pERM and tERM antibodies (E, F). **$P < 0.01$, ***$P < 0.001$, compared with the AngII control group; $^{\$\$\$}P < 0.001$, compared with the AngII 30 min group; $^{\#\#\#}P < 0.001$, compared with the Y27632 control group, n = 3 – 4 independent experiments. All P-values were analyzed by ANOVA with the Tukey correction. Data are shown as means ± S.E.M.

blocker metoprolol induces cardiac fibrosis through β-arrestin2 (22). Another report also mentioned that β-arrestins mediate fibroblast invasion and the development of pulmonary fibrosis (21). These results suggest that β-arrestins can mediate anti-fibrotic and profibrotic pathways depending on stimulants, receptor, and cell types.

Previous reports suggested that AngII-induced responses in cardiac fibroblasts and other fibroblast cell

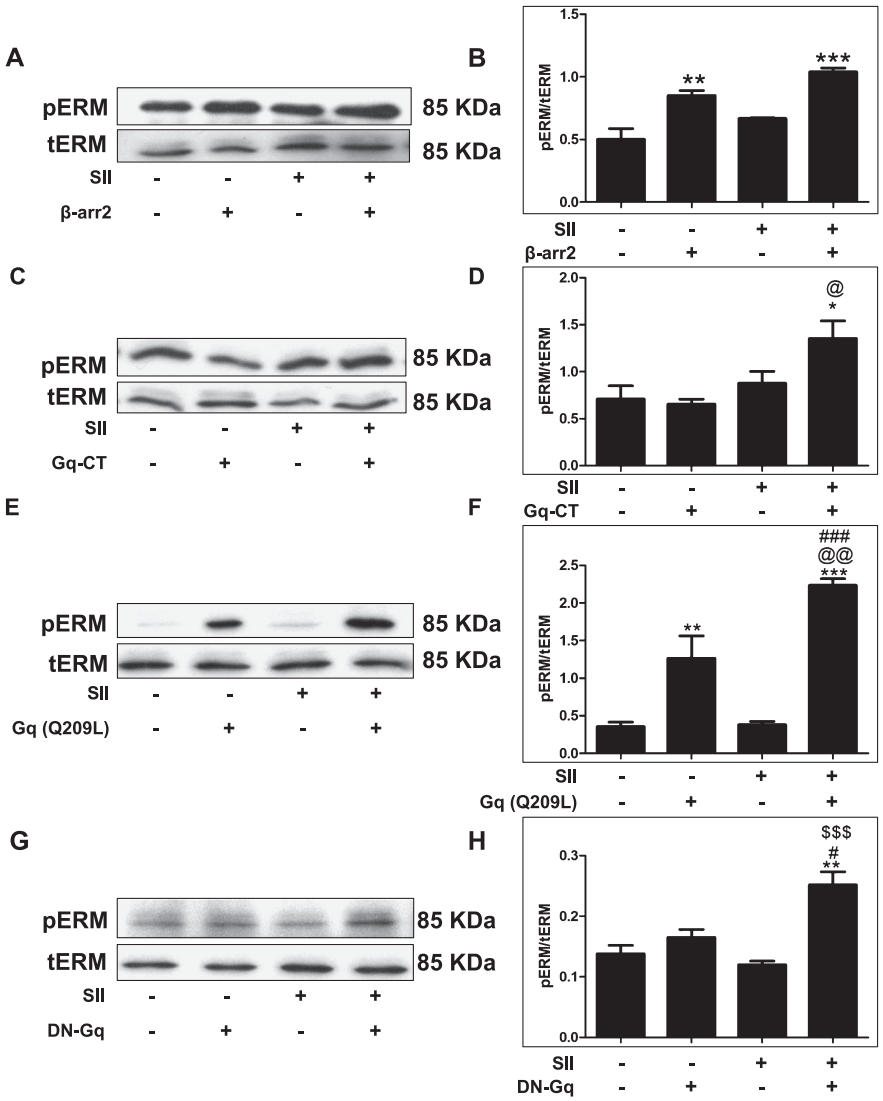

Fig. 4. SII inhibits ERM phosphorylation in NIH3T3 cells by acting as a Gαq protein–biased agonist. A, B) NIH3T3 cells stably over-expressing AT1AR and β-arr2 using the retroviral infection technique were fasted over night and then stimulated with SII (30 μM) for 30 min. Then cells were lysed and samples were subjected to SDS-PAGE and western-blotted using polyclonal pERM and tERM antibodies. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the SII control group, n = 3 independent experiments. C, D) NIH3T3 cells stably over-expressing AT1AR and Gq-CT using the retroviral infection technique were stimulated with SII (30 μM) for 5 min. Then cells were lysed and samples were subjected to SDS-PAGE and western-blotted using polyclonal pERM and tERM antibodies. $^{*}P < 0.05$, compared with the SII control group; $^{@}P < 0.05$, compared with SII 5-min stimulation group, n = 3 – 5 independent experiments. E, F) NIH3T3 cells stably over-expressing AT1AR and Gq (Q209L) using retroviral infection were stimulated with SII (30 μM) for 5 min. Then cells were lysed and samples were subjected to SDS-PAGE and western-blotted using polyclonal pERM and tERM antibodies. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the SII control group; $^{@@}P < 0.01$, compared with the Gq (Q209L) group; $^{###}P < 0.001$, compared with the SII 5-min stimulation group, n = 3 – 5 independent experiments. G, H) NIH3T3 cells stably over-expressing AT1AR and DN-Gq using retroviral infection were stimulated with SII (30 μM) for 5 min. Then cells were lysed and samples were subjected to SDS-PAGE and western-blotted using polyclonal pERM and tERM antibodies. $^{**}P < 0.01$, compared with the SII control group; $^{#}P < 0.05$, compared with the DN-Gq group; $^{\$\$\$}P < 0.001$, compared with the SII 5-min stimulation group, n = 3 – 5 independent experiments. All *P*-values were analyzed by ANOVA with the Tukey correction. Data are shown as means ± S.E.M.

lines are different from those in cardiomyocytes and HEK293 cells (9, 19). In cardiac fibroblasts, AngII activates ERKs through a pathway including the G$\beta\gamma$ subunits of G_i protein and tyrosine kinases, whereas Gαq and protein kinase C are important for ERK activation in cardiac myocytes (30). SII (previously classified as a β-arrestin-biased agonist) stimulates Gαq protein–independent / β-arrestin-dependent ERK phosphorylation in

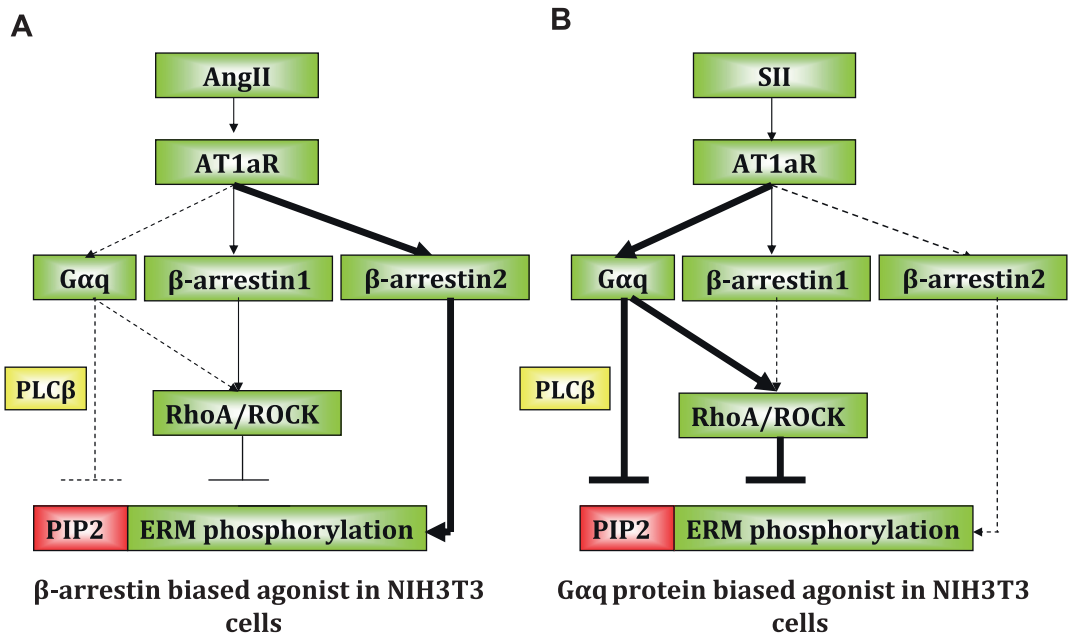

Fig. 5. AngII and SII signaling pathways in NIH3T3 cells.

HEK293 cells and cardiomyocytes (31, 27). The ability of SII to stimulate Gαq protein signaling has been reported recently (9). This recent report demonstrated that SII can stimulate Gαq protein signaling in a different way from that of AngII, although the degree of activation was much weaker than AngII. Published data on SII signaling pathways in cardiac fibroblasts and fibroblast cell lines is very limited and not clearly established. Therefore, we focused on the responses mediated by AngII and SII in NIH3T3 cells as a fibroblast cell line. We showed that AngII phosphorylates ERM proteins in NIH3T3 cells by acting as a *β*-arrestin-biased agonist. We also showed that SII inhibits ERM phosphorylation by acting as a Gαq protein–biased agonist in NIH3T3 cells.

This data would suggest that AngII induces heart failure and cardiac fibrosis by acting as a Gαq protein–biased agonist in cardiomyocytes and a *β*-arrestin-biased agonist in cardiac fibroblasts. The ability of SII to induce cardioprotection and reduce cardiac fibrosis may be due to stimulation of biased *β*-arrestin signaling in cardiomyocytes and biased Gαq protein signaling in cardiac fibroblasts.

Our findings explained the contradictory points regarding the effects of *β*-arrestins on the heart. It also showed for the first time a new behavior of receptor agonists. We demonstrated that AT1AR agonists can induce their responses through G proteins or *β*-arrestins depending on the cell type. The exact mechanism by which AngII and SII induce cell-type dependent responses is unknown. We think this is a particular point of interest and needs intensive investigation. Our study also introduced a new simple method for pharmacological screening of AT1R-biased agonists. Moreover, NIH3T3 cells provided us with another face of AT1R-biased agonism that can facilitate our understanding of drug–receptor–cell interaction in more detail.

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References

1 Lorell BH. Role of angiotensin AT1, and AT2 receptors in cardiac hypertrophy and disease. Am J Cardiol. 1999;83:48–52.
2 Ushio-Fukai M, Griendling KK, Akers M, Lyons PR, Alexander RW. Temporal dispersion of activation of phospholipase C-beta1 and -gamma isoforms by angiotensin II in vascular smooth muscle cells. Role of alphaq/11, alpha12, and beta gamma G protein subunits. J Biol Chem. 1998;273:19772–19777.
3 Ardaillou R. Angiotensin II receptors. J Am Soc Nephrol.

1999;10:30–39.
4 Mishra S, Ling H, Grimm M, Zhang T, Bers DM, Brown JH. Cardiac hypertrophy and heart failure development through Gq and CaM kinase II signaling. J Cardiovasc Pharmacol. 2010;56: 598–603.
5 Lefkowitz RJ. G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. J Biol Chem. 1998;273:18677–18680.
6 Shenoy SK, Lefkowitz RJ. β-Arrestin-mediated receptor trafficking and signal transduction. Trends Pharmacol Sci. 2011;32: 521–533.
7 Violin JD, Lefkowitz RJ. Beta-arrestin-biased ligands at seven-transmembrane receptors. Trends Pharmacol Sci. 2007;28:416–422.
8 Tilley DG. Functional relevance of biased signaling at the angiotensin II type 1 receptor. Endocr Metab Immune Disord Drug Targets. 2011;11:99–111.
9 Saulière A, Bellot M, Paris H, Denis C, Finana F, Hansen JT, et al. Deciphering biased-agonism complexity reveals a new active AT_1 receptor entity. Nat Chem Biol. 2012;8:622–630.
10 Ahn S, Shenoy SK, Wei H, Lefkowitz RJ. Differential kinetic and spatial patterns of β-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. J Biol Chem. 2004; 279:35518–35525.
11 Tohgo A, Pierce KL, Choy EW, Lefkowitz RJ, Luttrell LM. β-arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. J Biol Chem. 2002;277: 9429–9436.
12 Ahn S, Kim J, Hara MR, Ren XR, Lefkowitz RJ. β-Arrestin-2 mediates anti-apoptotic signaling through regulation of BAD phosphorylation. J Biol Chem. 2009;284:8855–8865.
13 Neisch AL, Fehon RG. Ezrin, Radixin and Moesin: key regulators of membrane-cortex interactions and signaling. Curr Opin Cell Biol. 2011;23:377–382.
14 Fievet BT, Gautreau A, Roy C, Del Maestro L, Mangeat P, Louvard D, et al. Phosphoinositide binding and phosphorylation act sequentially in the activation mechanism of ezrin. J Cell Biol. 2004;164:653–659.
15 Cant SH, Pitcher JA. G protein-coupled receptor kinase 2-mediated phosphorylation of ezrin is required for G protein-coupled receptor-dependent reorganization of the actin cytoskeleton. Mol Biol Cell. 2005;16:3088–3099.
16 Hébert M, Potin S, Sebbagh M, Bertoglio J, Bréard J, Hamelin J. Rho-ROCK-dependent ezrin-radixin-moesin phosphorylation regulates Fas-mediated apoptosis in Jurkat cells. J Immunol. 2008;181:5963–5973.
17 Jeon S, Park JK, Bae CD, Park J. NGF-induced moesin phosphorylation is mediated by the PI3K, Rac1 and Akt and required for neurite formation in PC12 cells. Neurochem Int. 2010;56: 810–818.
18 Debell KE, Rellahan BL, Shaw S. Phospholipase C-mediated hydrolysis of PIP2 releases ERM proteins from lymphocyte membrane. J Cell Biol. 2009;184:451–462.
19 Kim S, Iwao H. Molecular and cellular mechanisms of angiotensin II mediated cardiovascular and renal diseases. Pharmacol Rev. 2000;52:11–34.
20 Tilley DG. G protein-dependent and G protein-independent signaling pathways and their impact on cardiac function. Circ Res. 2011;109:217–230.
21 Lovgren AK, Kovacs JJ, Xie T, Potts EN, Li Y, Foster WM, et al. β-Arrestin deficiency protects against pulmonary fibrosis in mice and prevents fibroblast invasion of extracellular matrix. Sci Transl Med. 2011;3:74ra23.
22 Nakaya M, Chikura S, Watari K, Mizuno N, Mochinaga K, Mangmool S, et al. Induction of cardiac fibrosis by β-blocker in G protein-independent and GRK5/β-arrestin2-dependent signaling pathways. J Biol Chem. 2012;287:35669–35677.
23 Esposito G, Prasad SV, Rapacciuolo A, Mao L, Koch WJ, Rockman HA. Cardiac overexpression of a G(q) inhibitor blocks induction of extracellular signal-regulated kinase and c-Jun NH(2)-terminal kinase activity in vivo pressure overload. Circulation. 2001;103:1453–1458.
24 Liu F, Usui I, Evans L, Austin DA, Mellon PL, Olefsky JM, et al. Involvement of both G(q/11) and G(s) proteins in gonadotropin-releasing hormone receptor-mediated signaling in L beta T2 cells. J Biol Chem. 2002;277:32099–32108.
25 Kumar V, Jong YJ, O'Malley KL. Activated nuclear metabotropic glutamate receptor mGlu5 couples to nuclear Gq/11 proteins to generate inositol 1,4,5-trisphosphate-mediated nuclear Ca2+ release. J Biol Chem. 2008;283:14072–14083.
26 Ibrahim AAE-H, Kurose H. β-Arrestin-mediated signaling improves the efficacy of therapeutics. J Pharmacol Sci. 2012;118: 408–412.
27 Rajagopal K, Whalen EJ, Violin JD, Stiber JA, Rosenberg PB, Premont RT, et al. Beta-arrestin2-mediated inotropic effects of the angiotensin II type 1A receptor in isolated cardiac myocytes. Proc Natl Acad Sci U S A. 2006;103:16284–16289.
28 Violin JD, DeWire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K, et al. Selectively engaging β-arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. J Pharmacol Exp Ther. 2010;335:572–579.
29 Rakesh K, Yoo B, Kim IM, Salazar N, Kim KS, Rockman HA. beta-Arrestin-biased agonism of the angiotensin receptor induced by mechanical stress. Sci Signal. 2010;3:125ra46.
30 Zou Y, Komuro I, Yamazaki T, Kudoh S, Aikawa R, Zhu W, et al. Cell Type–specific Angiotensin II–evoked signal transduction pathways: critical roles of $G_{\beta\gamma}$ subunit, Src family, and Ras in cardiac fibroblasts. Circ Res. 1998;82:337–345.
31 Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, et al. Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. Proc Natl Acad Sci U S A. 2003;100: 10782–10787.