Src Is Required for Mechanical Stretch-Induced Cardiomyocyte Hypertrophy through Angiotensin II Type 1 Receptor-Dependent β-Arrestin2 Pathways

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Abstract

Angiotensin II (AngII) type 1 receptor (AT1-R) can be activated by mechanical stress (MS) without the involvement of AngII during the development of cardiomyocyte hypertrophy, in which G protein-independent pathways are critically involved. Although β-arrestin2-biased signaling has been speculated, little is known about how AT1-R/β-arrestin2 leads to ERK1/2 activation. Here, we present a novel mechanism by which Src kinase mediates AT1-R/β-arrestin2-dependent ERK1/2 phosphorylation in response to MS. Differing from stimulation by AngII, MS-triggered ERK1/2 phosphorylation is neither suppressed by overexpression of RGS4 (the negative regulator of the G-protein coupling signal) nor by inhibition of Gαq downstream protein kinase C (PKC) with GF109203X. The release of inositol 1,4,5-triphosphate (IP3) is increased by AngII but not by MS. These results collectively suggest that MS-induced ERK1/2 activation through AT1-R might be independent of G-protein coupling. Moreover, either knockdown of β-arrestin2 or overexpression of a dominant negative mutant of β-arrestin2 prevents MS-induced activation of ERK1/2. We further identify a relationship between Src, a non-receptor tyrosine kinase and β-arrestin2 using analyses of co-immunoprecipitation and immunofluorescence after MS stimulation. Furthermore, MS-, but not AngII-induced ERK1/2 phosphorylation is attenuated by Src inhibition, which also significantly improves pressure overload-induced cardiac hypertrophy and dysfunction in mice lacking AngII. Finally, MS-induced Src activation and hypertrophic response are abolished by candesartan but not by valsartan whereas AngII-induced responses can be abrogated by both blockers. Our results suggest that Src plays a critical role in MS-induced cardiomyocyte hypertrophy through β-arrestin2-associated angiotensin II type 1 receptor signaling.

Introduction

Angiotensin II type 1 (AT1) receptor, belonging to the G-protein-coupled receptor family (GPCRs), shares a common structure of 7-transmembrane receptor (7TMRs), and mediates the signal response of Angiotensin II (AngII), thereby regulates blood pressure, cardiac hypertrophy and heart failure [1,2]. Recent studies demonstrate that AT1-R acts as a stress-sensitive switcher which can be triggered by mechanical stress without the ligand binding [3–5]. We have previously showed that some kinds of angiotensin II receptor blockers (ARBs), such as candesartan and olmesartan, inhibit pressure overload-induced cardiac hypertrophy in angiotensinogen knockout (AGT KO) mice, while others like valsartan, exert the inhibitory effect in the presence of AngII [6]. However, the detailed molecular mechanisms of how mechanical stress-induced AT1-R activation and its inhibition are regulated by ARBs still remain elucidative.

A growing body of evidences indicated that biased agonism might selectively induce the conformational switch of GPCRs, which preferentially activated or inhibited a subset of downstream signaling [7,8]. We thus assumed there might be an unique pathway involved in mechanical stress (MS)-induced AT1-R activation and signal transduction, which was different from that induced by AngII, and resulted in the divergent effects of various ARBs. Rakesh K. previously reported that β-arrestin2 dependent pathway rather than G-protein coupling played a pivotal role in MS-induced AT1-R signaling [7]. However, little was known regarding to the downstream pathway subsequent to β-arrestin2-mediated AT1-R and ERK1/2 activation by mechanical stress. It was believed that AngII stimulated G-protein-dependent ERK1/2 activation through binding to AT1-R, but overexpression of AT1-R mutant lacking Gαq/Gqα coupling also induced ERK1/2 phosphorylation and developed severe myocardial hypertrophy both in vitro and in vivo [9,10]. We previously reported that G protein-independent calcineurin was critically involved in me-
Mechanical stretch induced myocardial hypertrophy [11], and candesartan could attenuate the hypertrophic response through regulating the tyrosine kinases cascade [3]. Therefore, we hypothesized that Src family kinase, the non-receptor tyrosine kinase, might mediate G protein-independent AT1-R signaling and cardiac hypertrophy induced by MS.

Src is a stress-sensitive kinase which plays an important role in the pathophysiological mechanisms for pressure overload-induced myocardial hypertrophy and pulmonary arterial hypertension. Src inhibition can effectively reverse the hypertensive response and hypertrophic signaling [12,13]. MS-mediated autophosphorylation of Src is highly associated with ERK1/2 activation [14]. Recent study revealed the expression and distribution of Src in the nucleus of cardiomyocytes with hypertrophy [15], and β-arrestin2 enhanced nuclear localization of ERK1/2 via GPCRs activation [16]. Mutant AT1 receptor lacking the docking site impaired Src-dependent nuclear translocation of ERK1/2 [10]. These findings prompted us to assume that Src kinase might be involved in β-arrestin2-mediated ERK1/2 activation and cardiac hypertrophy subsequent to AT1-R activation by MS. In the present study, we focused on the changes of AT1-R downstream signals, especially the role of Src kinase, in regulating β-arrestin2-dependent and AT1-R-induced signal transduction after MS.

Materials and Methods

Reagents

Anti-ERK1/2 (#9102), anti-phospho-ERK1/2 at Thr202/ Tyr204 (#9101) and anti-phospho-Src at Tyr416 (#2101) were purchased from Cell-signaling Technology; anti-Angiotensin II type 1 receptor (ab9391) were purchased from Abcam, plc; anti-HA-probe (sc-805), anti-ERK1/2 (sc-203) and anti-phospho-ERK1/2 (sc-2027) and anti-phospho-Src at Tyr416 (sc-2101) were provided by Sigma-Aldrich; anti-HA-probe (sc-805), anti-phospho-ERK1/2 at Thr 202/ Tyr204 (sc-2027) and anti-phospho-Src at Tyr416 (sc-2101) were purchased from Santa Cruz Biotechnology; and SU6656 (#572635) was purchased from Calbiochem, Merck KGaA. Mechanistic stretch-model culture plates were provided as kind gifts from Chiha University Graduate School of Medicine.

Plasmids Constructs

HA-tagged ERK2, FLAG-tagged β-arrestin1 and 2 were kindly provided by professor Issei Komuro (Tokyo University, Japan). Wild-type FLAG-tagged β-arrestin2 in vector pcDNA3 was used as the template. PCR based site-directed mutagenesis approach was performed to make a valine(54)-to-aspartic substitution in the β-arrestin2. PCR based site-directed mutagenesis approach was performed to make a valine(54)-to-aspartic substitution in the AGT KO mice were used in the present study and wide-type (WT) C57BL/6 mice were used as control littermates. All the procedures involving animals were carried out in accordance with the recommendations of the guidelines for the Care and Use of Laboratory Animals from China Council on Animal Care. The experiment was approved by the Experimental Animal Ethics Committee, Fudan University Shanghai Medical College with the permit number of 20110307-092. Pressure overload model was established by transverse aorta constriction (TAC) for 2 weeks as previously described [6,11]. To ameliorate suffering, the mice were anesthetized by intraperitoneal injection of a combination of ketamine (100 mg/kg) with xylazine (5 mg/kg), and respiration was artificially controlled with a tidal volume of 0.2 ml and a respiratory rate of 110 breaths/min. The transverse aorta was constricted with the 7-0 nylon strings by ligating the aorta together with a blunted 27-gauge needle to yield a narrowing of 0.4 mm in diameter, and the needle was pulled out later. SU6656 (1 mg/kg/day) was continuously administered by Alzet osmotic mini pumps (DURECT, Cupertino, California) and was implanted subcutaneously into the back of mice right after anesthetized with 2% inhaled isoflurane from 3 days before TAC to 2 weeks after TAC. At 2 weeks after TAC, all mice were anesthetized by inhaled anesthetic isoflurane for cardiac echocardiography and hemodynamics analysis, and then mice were quickly sacrificed before they woke up. The hearts were excised for further examination, and the bodies were recorded, collected and centralized processing.

Cell Culture and transfection

In vitro cardiomyocytes were cultured as previously reported [34]. In brief, neonatal (one-day-old) rats were sacrificed under ether anesthesia, and then ventricular tissues were surgically isolated from the anesthetized rats, all operations were made to minimize suffering. The isolated tissues were minced and placed in culture medium containing the buffers of 5.4 mmol/L KCl, 0.44 mmol/L NaH2PO4, 137 mmol/L NaCl, 4.2 mmol/L NaHCO3 and 5 mmol/L glucose at pH 7.4. Cells were then dissociated at 37°C by a combination of mechanical agitation and enzymatic digestion with 0.1 mg/mL DNase II (Sigma) and 0.125% pancreatin trypsin (Calbiochem). Cells were pre-plated for 2 h in 100 mm dishes with Dulbecco’s modified Eagle’s medium (DMEM) with 10% defined bovine calf serum (FBS, HyClone), penicillin (100 units/mL) and streptomycin (100 mg/mL) (Sigma), then the unattached cardiomyocytes were collected and plated at a field density of 1×105 cells/cm2 on silicone rubber culture dishes. Stretching of cardiomyocytes by 10% was conducted as described previously [3]. HEK-293-AT1 cells lines were kindly provided by professor Issei Komuro (Tokyo University, Japan), and were cultured in Dulbecco’s modified Eagles medium (DMEM) with 10% FBS and 1% penicillin/streptomycin as described previously [3]. Transient transfection of AT1 plasmids into cells were performed by using Gene Transfection System (Invitrogen) according to the manufacturer’s instructions. The stable selection of transfection cells were achieved by adding Aminoglycoside G418 (200 mg/mL) to cells 3 days after transfection. All cell cultures were transferred to serum-free media 24 h before experiment.

Experimental Animal Model

Angiotensinogen gene knockout (AGT KO) mice were provided as kind gifts from professor Issei Komuro. Aged 8~10 weeks of AGT KO mice were used in the present study and wide-type (WT) C57BL/6 mice were used as control littermates. All the procedures involving animals were carried out in accordance with the recommendations of the guidelines for the Care and Use of Laboratory Animals from China Council on Animal Care. The experiment was approved by the Experimental Animal Ethics Committee, Fudan University Shanghai Medical College with the permit number of 20110307-092. Pressure overload model was established by transverse aorta constriction (TAC) for 2 weeks as previously described [6,11]. To ameliorate suffering, the mice were anesthetized by intraperitoneal injection of a combination of ketamine (100 mg/kg) with xylazine (5 mg/kg), and respiration was artificially controlled with a tidal volume of 0.2 ml and a respiratory rate of 110 breaths/min. The transverse aorta was constricted with the 7-0 nylon strings by ligating the aorta together with a blunted 27-gauge needle to yield a narrowing of 0.4 mm in diameter, and the needle was pulled out later. SU6656 (1 mg/kg/day) was continuously administered by Alzet osmotic mini pumps (DURECT, Cupertino, California) and was implanted subcutaneously into the back of mice right after anesthetized with 2% inhaled isoflurane from 3 days before TAC to 2 weeks after TAC. At 2 weeks after TAC, all mice were anesthetized by inhaled anesthetic isoflurane for cardiac echocardiography and hemodynamics analysis, and then mice were quickly sacrificed before they woke up. The hearts were excised for further examination, and the bodies were recorded, collected and centralized processing.

ERK2 kinase activity Assay

HA-tagged ERK2 plasmid was transient transfected into cardiomyocytes 24 h after plating the cells on stretch-model culture plates. ERK2 kinase activity was determined as we described previously [17]. In brief, RGS4 plasmid DNA (7.5 μg) was co-transfected into each dish of HEK-293-AT1 cells with or without HA tagged-ERK2 (2.5 μg), then the transfected cells were lysed, and the lysates were incubated with anti-HA antibody for 1 h at 4°C. Then, the immunocomplex was precipitated using A/ G Plus-agarose beads, washed, resuspended in 25 ml kinase buffer with 2 m Ci of [γ-32P] ATP, and incubated with 25 mg of MBP as the substrate at 25°C for 10 min. After incubation, the reaction was terminated by adding Laemmli sample buffer (0.002% bromophenol blue, 0.01M sodium phosphate buffer, pH 7.0, 10%
Mechanical Stretch Induced Src-Dependent AT1 Receptor Activation

RGS4 failed to inhibit mechanical stretch-induced ERK1/2 phosphorylation

Increasing evidence implicated that AT1-R could function through a heterotrimeric G-protein coupling or a G-protein-independent mode [18–20]. AngII, as the receptor agonist, activated AT1-R via a traditional GTP analog sensitive pathway. Ligand binding to G-protein-coupled receptors (GPCRs) induced GTP binding to the Gz subunit and dissociation from the βγ subunits. Here, we tested the possibility of Gz subunit activation during the process of mechanical stretch-induced AT1-R activation. It was reported that RGS4 negatively regulated Gzq signaling though promoting the hydrolysis of GTP [19,20]. Thus, the effect of Gzq coupling was attenuated by overexpressing RGS4 in cardiomyocytes. As shown in Figure 1A, Gzq-dependent phosphorylation of ERK1/2 was significantly reduced in AngII

Statistical Analysis

All data were expressed as means ± s.e.m. The between-group comparisons of means were done by one-way ANOVA followed by Tukey-Kramer test. P-values smaller than 0.05 were considered statistical significance.

Results

RGS4 failed to inhibit mechanical stretch-induced ERK1/2 phosphorylation

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Mechanical stretch-induced ERK1/2 phosphorylation did not depend on Gz coupling pathway

Previous in vivo studies showed that mechanical stretch-induced ERK1/2 activation was significantly attenuated in hearts from β-arrestin KO mice [7,18]. To explore whether mechanical stretch induced ERK1/2 response via β-arrestin-dependent pathway or Gz-dependent signaling, we first determined the release of inositol 1,4,5-triphosphate (IP3) in stretch-induced cardiomyocytes, since CAMP-dependent IP3 bioactivity was the major event of GPCRs activation through Gz protein coupling. As shown in Figure 2A, IP3 release was significantly attenuated in stretching-induced cardiomyocytes compared with that in AngII (10⁻⁷ mol/L)-induced cardiomyocytes. Both AngII and mechanical stretch mediated IP3 release were blocked by candesartan (10⁻⁶ mol/L), suggesting that AT1 receptor was essential for Gz protein coupling. In addition, inhibition of PKC with GF109203X, a specific PKC inhibitor, effectively attenuated AngII (10⁻⁷M) induced ERK1/2 phosphorylation, but the phosphorylation level of ERK1/2 stimulated by stretching was not affected (Fig. 2B). These data suggested that mechanical stretch mediated ERK1/2 signal might not through activating the “classical” G-protein-dependent pathway.

Next, we examined the role of β-arrestin in ERK1/2 activation induced by mechanical stretch. Results showed that both the mRNA and protein levels of β-arrestin1/2 remained unchanged in response to mechanical stretch (Fig. 2C). However, knocking down endogenous β-arrestin1/2 caused a significant decrease in the phosphorylation level of ERK1/2 in response to mechanical stretch (Fig. 2D). We further found that a robust increase of phosphorylated ERK1/2 at 10 min after stretching, which was induced by β-arrestin2 but not by β-arrestin1 in β-arrestin1 or 2 single transfected HEK-293-AT1 cells after knocking down the endogenous β-arrestin1/2 with specific siRNA (Fig. 2E).
β-arrestin2 regulated ERK1/2 phosphorylation through interacting with the tyrosine kinase Src during mechanical stretch

Above data indicated that β-arrestin2 was critically involved in mechanical stretch-induced ERK1/2 phosphorylation, however, both mRNA and protein levels of β-arrestin2 were not affected by stretching, but the phosphorylation level of Src was suppressed by knocking down of β-arrestin2 (Fig. 3A). Therefore, we assumed that β-arrestin2 might be the scaffold protein that mediated AT1-R-dependent tyrosine kinase phosphorylation. In the Co-IP analysis, we revealed that Src was immunoprecipitated by anti-β-arrestin2 antibody in a time dependent manner (Fig. 3B), suggesting that Src was recruited to the cell membrane and interact with AT1-R during stretching. Our previous observation indicated that one kind of Src family tyrosine kinase was critically involved in mechanical stretch-evoked AT1-R signaling [3], but a direct interaction between Src and β-arrestin2-V54D might not be demonstrated in the model of mechanical stretch. Hence, we cotransfected FLAG-tagged β-arrestin2 and HA-tagged Src into HEK-293-AT1 cells. Afterwards, coimmunoprecipitation assays were conducted in stretched or unstretched HEK-293-AT1 cells. The proteins were collected after stretching for 10 min, and followed by immunoprecipitation of β-arrestin2 with anti-FLAG antibody. We probed the immunoprecipitated sample by Western blotting using anti-HA antibody. The results showed that β-arrestin2 interacted with Src only in stretched cells (Fig. 3C), suggesting that AT1-R mediated recruitment of β-arrestin2 might be important for Src binding and activation. But Src kinase was markedly reduced in sample from cells transfected with dominant negative β-arrestin2-V54D (Fig. 3C). Previous studies reported that β-arrestin, which functioned as a scaffold protein, could recognize and interact with the catalytic domain of Src, by which regulated its kinase activity. Therefore, the attenuated interaction between Src and β-arrestin2-V54D might be due to the lack of binding site for SH2 domain kinase docking [25]. Cell immunofluorescence further confirmed that Src kinase was phosphorylated and recruited to membrane by accumulated β-arrestin2 after mechanical stretch, but this effect was abolished by mutating the Src binding site in β-arrestin2-V54D transfected cardiomyocytes (Fig. 3D). Taken together, these data suggested that mechanical stretch-induced translocation of β-arrestin2 was essential for Src docking and signal transduction. To further investigate the effect of β-arrestin2/Src interaction after stretching on cardiomyocyte hypertrophy, we examined stretch-induced ERK1/2 phosphorylation in both β-arrestin2 and β-arrestin2-V54D transfected cardiomyocytes. The results showed that ERK1/2 phosphorylation was significantly attenuated in cells transfected with β-arrestin2-V54D compared with that in β-arrestin2 transfected cells (Fig. 3E). In addition, mechanical stretch-induced ERK1/2 activation was also inhibited by pretreating cardiomyocytes with SU6656 (10 μmol/L), a selective Src family kinase inhibitor. Of note, the inhibitory effect of SU6656 on ERK1/2 phosphorylation was not observed in cardiomyocytes stimulated by AngII (10^{-7} mol/L) (Fig. 3F). Collectively, these data confirmed that β-arrestin2/Src interaction was uniquely involved in mechanical stretch-mediated ERK1/2 activation.

Figure 2. Mechanical stretch preferentially activated a heterotrimeric G protein-independent AT1 signaling pathway. (A) In vitro cultured cardiomyocytes were seeded on 24 well plates (1.5 × 10^5 cells) and labeled by myo-[3H] inositol (1.0 μCi/ml) at 37°C for 24 h. Inositol phosphates (IPx) release was determined at the different indicated time points triggered by AngII (10^{-7} mol/L) or stretch respectively, and including the treatment of candesartan. (B) The effect of GF109203X on ERK1/2 phosphorylation stimulated by stretch or AngII (10^{-7} mol/L). (C) The endogenous mRNA and protein levels of β-arrestin1 and β-arrestin2 in cardiomyocytes were determined before and after stretch for 10 min. (D) Time-dependent ERK1/2 phosphorylation was determined after treatment with β-arrestin1/2 siRNA or scrambled siRNA. (E) Mechanical stretch-induced expression of phosphorylated ERK1/2 was determined in a single plasmid (β-arrestin1 or β-arrestin2) transfected HEK-293-AT1 cells after knock-down of endogenous β-arrestin1/2. * P<0.05 vs. AngII-induced group (n = 3 separated experiments). doi:10.1371/journal.pone.0092926.g002
Src inhibition attenuated pressure overload-induced myocardial hypertrophy

We then investigated the in vivo consequence of the inhibition of β-arrestin2/Src signaling on pressure overload-induced myocardial hypertrophy in AGT KO mice. At 2 weeks after TAC, echocardiography assessment showed a significant increase of left ventricular anterior wall at end-diastolic (LVAWd) and posterior wall at end-diastolic (LVPWd) thickness in both C57BL/6 mice and AGT KO mice, accompanied by higher left ventricular ejection fraction (LVEF) and left ventricular end-systolic pressure (LVESP). However, Efficacy of attenuating myocardial hypertro-
phy by pretreatment with SU6656 was more pronounced in AGT KO mice than in C57BL/6 mice (Fig. 4A). Cardiac reprogramming of specific genes expressions were activated during pressure overload, we therefore determined the immediate-early response genes and fetal genes in the heart after TAC. Pressure overload enhanced the transcriptional levels of ANP, BNP and α-skeleton in AGT KO mice and C57BL/6 littersmates, but expression of these re-enhanced genes was significantly lower in AGT KO mice than in C57BL/6 mice after SU6656 treatment, except for sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) (Fig. 4B).

**Effects of different ARBs on AngII and TAC-induced cardiac hypertrophic response in AGT KO mice**

We further compared the effects of candesartan and valsartan on myocardial hypertrophic response induced by TAC and AngII stimulation in AGT KO mice. Results showed that both candesartan and valsartan exerted inhibitory impacts on AngII-induced cardiac hypertrophy, but only candesartan effectively reversed TAC-induced hypertrophic response (Fig. 5A–B). Furthermore, Src was weakly upregulated by AngII but robustly enhanced by TAC for 2 weeks, and AngII-induced upregulation of Src was inhibited by both candesartan and valsartan, while TAC-induced Src activation was inhibited only by candesartan (Fig. 5C).

**Discussion**

Cardiac hypertrophy is one of the independent risk factors responsible for myocardial injury and remodeling, and such compensatory response may transit to heart failure. Plenty of previous studies have demonstrated that AT1-R is one of the most important receptors mediating cardiac hypertrophic response through multiple signaling pathways [2,3,21,22]. However, the diversity of AT1-R-mediated signaling in response to hypertrophic stimuli in the heart is still not fully understood. We have previously revealed that mechanical stretch can activate AT1-R without the involvement of AngII [3]. In this study, we further confirm a critical role of Src in regulating AT1-R-mediated intercellular signaling transduction stimulated by mechanical stretch.

Immerging evidences indicated that activation model of GPCRs might undergo different phases due to different stimuli. It was generally believed the first phase of AT1-R activation was G-protein coupling dependent, and this process was hypersensitive to ligand binding. G-protein signaling subtype 4 (RGS4), an

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**Figure 4. In vivo analyses of the cardiac function by echocardiography and hemodynamic measurements.** Both AGT KO mice and the C57BL/6 WT littersmates were pretreated with or without Src kinase inhibitor (SU6656), followed by TAC for 2 weeks. (A) Quantifications of LVAWd, LVPWd, LVEF and LVESP by representative M-mode tracing and hemodynamic recording from five mice. (B) Quantifications of cardiac immediate-early response genes in C57BL/6 mice and AGT KO mice with or without pretreatment of SU6656 (n = 5 separated experiments). * P<0.05 vs. saline-treated TAC-operated AGT KO mice.

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inhibitory regulator of GPCR signaling through accelerating GTPase activity, was mainly expressed on cardiomyocytes [23,24]. In this study, intervention of G-protein coupling by overexpressing RGS4 in cardiomyocytes effectively blocked AngII-stimulated ERK1/2 phosphorylation, the indicator of hypertrophic response, but this effect was almost invisible in mechanical stretched cardiomyocytes. These results suggested that stretch-mediated AT1-R activation in the absence of the ligand binding might be sensitive to a second phase of G-protein signaling transduction which was independent of G-protein coupling.

Previous studies illustrated that PKC and IP3, two important molecules were required for AngII-mediated modulation of myofilament calcium sensitivity and cardiac excitation-contraction coupling [17,25]. However, IP3 was not fully activated in cardiomyocytes by short time effect of mechanical stretch. Interestingly, MS-induced ERK1/2 phosphorylation was not, suppressed by blocking PKC, suggesting a G-protein coupling independent signaling pathway was involved in stretching-induced cardiac hypertrophy. Of note, SU6656, a Src kinase inhibitor, effectively suppressed stretching-induced ERK1/2 activation, indicating a crucial role of Src but not PKC was required for mechanical stretch-mediated AT1 activation and intracellular signaling.

Rakesh K. previously reported that mechanical stretch evoked an abundant increase of ERK1/2 phosphorylation in hearts from WT mice, but not from AT1-R KO or arrestin2 KO mice [7]. It was believed that distinct conformational states of AT1-R might selectively stimulate signaling via G-protein dependent or independent pathway, while β-arrestin–biased agonism might serve as the central mechanism for mechanical stretch-mediated GPCR signaling. In line with this finding, our data suggested that β-arrestin2 not β-arrestin1 was required for stretch-induced ERK1/2 phosphorylation. However, both mRNA and protein levels of β-arrestin2 remained unchanged after mechanical stretch. Instead, stretch triggered β-arrestin2 translocation to the membrane of cardiomyocyte. Previous reports indicated the divergent roles of β-arrestin1 and β-arrestin2 in regulating intercellular signal via translocation to nucleus and cell surface [16,18,26,27]. In this study, we revealed that β-arrestin2 was highly expressed on cell membrane suggesting that mechanical stretch promoted the membrane recruitment of β-arrestin2 and binding with AT1-R. Plenty of studies demonstrated that GPCRs-mediated β-arrestin2 recruitment was essential not only for receptor internalization, but also for signal transduction [18,26]. It was also proved that tyrosine kinases-dependent signal cascade was crucial for GPCRs mediated ERK1/2 phosphorylation through transactivating...
EGFR [7,27,28]. However, the underlying mechanism was not yet fully clear. Our data demonstrated that AT1-R-mediated accumulation of Src kinase in response to mechanical stretch was β-arrestin2 dependent, dominant negative β-arrestin2 greatly attenuated Src docking and accumulation after stretching. Recruitment of Src to activated GPCRs had multiple functions, such as MAP kinase activation, receptor internalization, and granule release [29,30]. In our study, Src activation was essential for β-arrestin2-mediated ERK1/2 phosphorylation. Transfection with mutant β-arrestin2 V54D or inhibition of Src by SU6656 in cardiomyocytes effectively suppressed MS-induced ERK1/2 activation. Src-dependent Ras-ERK1/2 pathway was also reported in the activation of AT1a-2m receptor, which lacking the binding site for G-protein [9,10]. Therefore, we proposed that β-arrestin2-mediated Src activation might play an important role in G-protein-independent AT1-R activation and subsequent hypertrophic response.

The activation of cellular oncogenes such as c-myc and c-Src were critically involved in the reprogram progress of gene expression during pressure overload-induced myocardial hypertrophy [31]. Here, we also found enhanced that cardiac reprogramming genes were enhanced in the pressure overload model of AGT KO mice, which indicated that AngII binding signal was not essential for regulation of fetal genes reprogramming and hypertrophy induced by mechanical stretch. Instead, inhibition of Src effectively suppressed the transcripts of ANP, BNP and α-skeleton, reversed the ventricular remodeling and hypertrophic response. In this study, we also found that mechanical stress-induced ERK1/2 response could only be suppressed by candeasartan, one kind of inverse agonism, but not by valsartan. Of note, candesartan inhibited the AT1-R activation through binding two domains of Glu257 in TM6 and Thr207 in TM7, both located at the carboxyl of the receptor, thereby stabilized AT1-R in the inactive state [4,32]. Previous studies suggested that the carboxyl-terminal residues of AT1-R were required for G-protein independent signal transduction and transactivation of EGFR [28,33]. Mutation of the conserved YIPP motif in the C terminus of AT1-R, resulted in diminished EGFR transactivation and cardiac hypertrophy in Tg-Y193P mice [33]. Because β-arrestin2-dependent Src recruitment was critically involved in GPCRs-mediated EGFR transactivation, the data presented here further supported the notion that the special effect of candeasartan on mechanical stretch-induced ERK1/2 phosphorylation was achieved via inhibition of G-protein independent but β-arrestin2 dependent Src recruitment.

In summary, our study presented the divergent mechanisms for mechanical stretch- and AngII-mediated β-arrestin2-dependent and G-protein coupling-dependent signaling pathway in cardiac hypertrophy. Mechanical stretch-induced conformational switch of AT1-R might be different from that stimulated by AngII, thus resulted in different signal transduction (Fig. 6). The uncovering of β-arrestin2/Src-mediated ERK1/2 phosphorylation in response to mechanical stretch might be crucial for the establishment of pressure overload-induced cardiac hypertrophy, and clarification of this novel mechanism might be helpful for the development of more potent inverse agonist for AT1-R.

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Author Contributions
Conceived and designed the experiments: YZ SW HG. Performed the experiments: SW GJ YY JW. Analyzed the data: SW JY. Contributed reagents/materials/analysis tools: GZ IK JG. Wrote the paper: SW YZ. Helped to complete the response work and gave suggestions for study: AS.

References
1. Murphy TJ, Alexander RW, Griendling KK, Runge MS, Bernstein KE (1991) Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. Nature 351: 231-236
2. Lorell BH (1999) Role of angiotensin AT1, and AT2 receptors in cardiac hypertrophy and disease. Am J Cardiol 83: 48H-52H.
3. Zou Y, Akazawa H, Qin Y, Sano M, Takano H, et al. (2004) Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II. Nat Cell Biol 6: 499-506.
4. Yasuda N, Akazawa H, Qin Y, Zou Y, Komuro I (2008) A novel mechanism of mechanical stress-induced angiotensin II type 1-receptor activation without the involvement of angiotensin II. Naunyn Schmiedebergs Arch Pharmacol 377: 383-393.
5. Akazawa H, Yasuda N, Komuro I (2009) Mechanisms and functions of agonist-independent activation in the angiotensin II type 1 receptor. Mol Cell Endocrinol 302: 140-147.
6. Li L, Zhou N, Gong H, Wu J, Lin L, et al. (2010) Comparison of angiotensin II type 1-receptor blockers to regress pressure overload-induced cardiac hypertrophy in mice. Hypertens Res 33: 1209-1297.
7. Rakesh K, Yoo B, Kim IM, Salazar N, Kim KS, et al. (2010) beta-Arrestin-biased agonism of the angiotensin receptor induced by mechanical stress. Sci Signal 3: ra46.
8. Godin CM, Ferguson SS (2012) Biased agonism of the angiotensin II type 1 receptor. Mini Rev Med Chem 12: 812-816.
9. Zhai P, Yamamoto M, Galeotti J, Liu J, Masurekar M, et al. (2005) Cardiac-specific overexpression of AT1 receptor mutant lacking G alpha q/G alpha i coupling causes hypertrophy and bradycardia in transgenic mice. J Clin Invest 115: 3045-3056.
10. Seta K, Nanamori M, Modragl JJ, Neubig RR, Sadoshima J (2002) AT1 receptor mutant lacking heterogeneous G protein coupling activates the Src-Ras-ERK pathway without nuclear translocation of ERKs. J Biol Chem 277: 9267-9277.
11. Zhou N, Li L, Wu J, Gong H, Niu Y, et al. (2010) Mechanical stress-evoked but AngII-mediated β-arrestin2/Src-mediated ERK1/2 phosphorylation in response to mechanical stretch was critical for the establishment of pressure overload-induced cardiac hypertrophy. Biochem Biophys Res Commun 397: 263-269.
12. Pullamsetti SS, Berghausen EM, Dhabal S, Tretyn A, Butrous E, et al. (2012) Role of Src tyrosine kinases in experimental pulmonary hypertension. Arterioscler Thromb Vasc Biol 32: 1354-1365.
13. Reineke EL, York B, Stashi E, Chen X, Tsimelzon A, et al. (2012) SRC-2 coactivator deficiency decreases functional reserve in response to pressure overload of mouse heart. PLoS One 7: e53395.

14. Boutahar N, Guignandon A, Vico L, Lafage-Proust MH (2004) Mechanical strain on osteoblasts activates autophosphorylation of focal adhesion kinase and proline-rich tyrosine kinase 2 tyrosine sites involved in ERK activation. J Biol Chem 279: 30588–30599.

15. Chen P, Li F, Xu Z, Li Z, Yi XP (2013) Expression and distribution of Src in the nucleus of myocytes in cardiac hypertrophy. Int J Mol Med 32: 165–173.

16. Kobayashi H, Narita Y, Nishida M, Kurose H (2005) Beta-arrestin2 enhances beta2-adrenergic receptor-mediated nuclear translocation of ERK. Cell Signal 17: 1248–1253.

17. Zou Y, Komuro I, Yamazaki T, Aikawa R, Kudoh S, et al. (1996) Protein kinase C, but not tyrosine kinases or Ras, plays a critical role in angiotensin II-induced activation of Ral1 kinase and extracellular signal-regulated protein kinases in cardiac myocytes. J Biol Chem 271: 33392–33397.

18. Rajagopal K, Whalen EJ, Violin JD, Schwenke DO, et al. (2006) Beta-arrestin2-mediated inotropic effects of the angiotensin II type 1A receptor in isolated cardiac myocytes. Proc Natl Acad Sci U S A 103: 16284–16289.

19. Yan Y, Chi PP, Bourne HR (1997) RGS4 inhibits Gq-mediated activation of mitogen-activated protein kinase and phosphoinositide synthesis. J Biol Chem 272: 11924–11927.

20. Mukhopadhyay S, Ross EM (1999) Rapid GTP binding and hydrolysis by Gq) promoted by receptor and GTPase-activating proteins. Proc Natl Acad Sci U S A 96: 9339–9344.

21. Dostal DE, Baker KM (1992) Angiotensin II stimulation of left ventricular hypertrophy in adult rat heart. Mediation by the AT1 receptor. Am J Hypertens 5:276–280.

22. Sadoshima J, Izumo S (1993) Molecular characterization of angiotensin II–induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT1 receptor subtype. Circ Res 73:413–423.

23. Tokudome T, Kishimoto I, Horio T, Arai Y, Schwenke DO, et al. (2000) Regulator of G-protein signaling subtype 4 mediates anti hypertrophic effect of locally secreted natriuretic peptides in the heart. Circulation 117: 2329–2339.