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β -Arrestin–Biased Agonism of the Angiotensin Receptor Induced by Mechanical Stress

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Abstract

β -Arrestins, which were originally characterized as terminators of heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptor (GPCR) signaling, also act as important signal transducers. An emerging concept in GPCR signaling is β -arrestin-biased agonism, in which specific ligand-activated GPCR conformational states selectively signal through β -arrestins, rather than through G proteins. Here, we show that mechanical stretch induced β -arrestin-biased signaling downstream of angiotensin II type I receptors (AT1Rs) in the absence of ligand or G protein activation. Mechanical stretch triggered an AT1R-mediated conformational change in β -arrestin similar to that induced by a β -arrestin-biased ligand to selectively stimulate receptor signaling in the absence of detectable G protein activation. Hearts from mice lacking β -arrestin or AT1Rs failed to induce responses to mechanical stretch, as shown by blunted extra-cellular signal-regulated kinase and Akt activation, impaired transactivation of the epidermal growth factor receptor, and enhanced myocyte apoptosis. These data show that the heart responds to acute increases in mechanical stress by activating β -arrestin-mediated cell survival signals.

INTRODUCTION

Angiotensin II (AngII) type I receptors (AT1Rs) are members of the large superfamily of GPCRs [heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors] and are important in the regulation of blood pressure and hypertrophic cardiac growth in response to increased mechanical load (1). The classic paradigm for activation of AT1R signaling involves AngII stimulation of AT1Rs resulting in dissociation of G proteins into G_{α_q} and $\beta\gamma$ subunits, leading to the formation of the second messengers inositol triphosphate and diacylglycerol (DAG) by the effector enzyme phospholipase C (PLC) (1). This G protein-

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dependent signaling pathway is rapidly terminated by enzymes known as GPCR kinases (GRKs), which phosphorylate AT1Rs on their cytoplasmic tail (2) and enable the binding of the multifunctional protein β -arrestin (2,3). The binding of β -arrestins to phosphorylated receptors sterically impairs further receptor–G protein coupling in a process known as receptor desensitization (2). Although the function of β -arrestins in AT1R desensitization is now appreciated, increasing evidence supports a broader role for β -arrestins as signal transduction scaffolds that regulate a large network of signaling pathways (3). Moreover, it is also becoming apparent that some GPCR ligands may be able to selectively activate either the G protein pathway or the β -arrestin pathway, a concept known as ligand bias or biased agonism (4–6).

Biased agonism is the ability of a ligand to selectively stabilize receptor conformations that preferentially activate or inhibit a subset of downstream pathways (4–6). Inherent in the concept of ligand bias is that the receptor can exist in multiple distinct conformations in response to ligand binding (4–6). For example, in the absence of AngII, the AT1R would reside in an inactive conformation, whereas in the presence of AngII, an active conformation that couples to G proteins would predominate. In contrast, a β -arrestin–biased agonist would elicit a different conformation in the receptor, which promotes the recruitment and activation of β -arrestin without stimulation of G protein coupling (7,8).

Recent work suggests that load-induced membrane stretch on cardiomyocytes activates AT1R signaling (9) without requiring the AT1R agonist AngII (10,11). Mechanical transduction in cells can involve various mechanisms (12–16), including AT1R-mediated activation of transient receptor potential (TRP) channels that depends on G protein coupling (10,17) and the recruitment of β -arrestin to AT1Rs (10). Here, we show that mechanical activation of AT1Rs can stimulate β -arrestin–biased signaling, and investigate its pathophysiological role in the heart.

RESULTS

Mechanical stretch activates β -arrestin–mediated signaling of AT1R

We measured the effect of tensile stretch of the left ventricle (LV) on the activation of extracellular signal–regulated kinase (ERK), a kinase that can be activated by AT1Rs in a β -arrestin–dependent manner (8). A handmade balloon was placed in the LV of a Langendorff-perfused mouse heart and inflated to 30 to 40 mmHg for 10 min (Fig. 1A and fig. S1A). Increasing mechanical stretch led to a graded increase in phosphorylated ERK in the heart (Fig. 1A). To determine whether ERK signaling that is activated by mechanical stretch in the heart requires AT1Rs and β -arrestin, we applied mechanical stretch to various knockout (KO) mouse hearts. Mechanical stretch induced an increase in the abundance of phosphorylated ERK in hearts from wild-type mice, but not in hearts from either AT1R KO or β -arrestin2 KO mice (Fig. 1, B and C). Stretch-induced ERK phosphorylation occurred in hearts from β -adrenergic receptor (β AR) double KO and β -arrestin1 KO mice (fig. S1, B and C). Because β -arrestin–mediated signaling depends on the initial receptor phosphorylation by a GRK (6), we tested whether specific GRKs were needed for ERK activation in the heart by mechanical stretch. Compared to that in wild-type hearts, mechanical stretch did not increase the abundance of phosphorylated ERK in GRK5 or GRK6 KO mouse hearts (Fig. 1, D and E). In contrast, hearts lacking GRK2, like wild-type hearts, showed increased amounts of phosphorylated ERK in response to mechanical stretch (Fig. 1F). Thus, ERK activation in response to mechanical stretch requires the presence of AT1Rs, β -arrestin2, GRK5, and GRK6.

Mechanical stretch induces G protein-independent AT1R signaling in the absence of ligand stimulation

Ligand activation of AT1Rs induces a conformational change of the receptor that enables coupling to G proteins and leads to generation of the second messengers inositol triphosphate and DAG (18,19). To determine whether mechanical stretch activates G protein signaling, we used DAG reporter (DAGR), a fluorescence resonance energy transfer (FRET)-based reporter for the second messenger DAG, to measure G protein activation in real time in live cells (20). DAGR translocates from cytosol to bind DAG in the plasma membrane, where it undergoes concentration-dependent inter-molecular FRET that is proportional to G_q-coupled AT1R activation (20). Human embryonic kidney (HEK) 293 cells stably expressing AT1Rs and DAGR were stimulated with AngII or hypo-osmotic stretch. AngII treatment induced a rapid increase in DAGR FRET, which peaked within 15 s and then slowly returned to baseline (Fig. 2A). In contrast, hypo-osmotic stretch did not induce a FRET signal, indicating absence of G protein activation (Fig. 2A). To further demonstrate the absence of G protein activation with mechanical stress, we measured the amount of phosphorylated ERK in the presence of the protein kinase C (PKC) inhibitor RO318425. Although RO318425 reduced ERK phosphorylation in response to AngII, it had no effect in cells stimulated with either mechanical or osmotic stretch (Fig. 2, B and C).

Previous in vitro studies show that activation of AT1Rs by membrane stretch occurs independently of released AngII ligand (10,11). Therefore, we tested whether mechanical stretch of cells expressing AT1Rs promoted the release of AngII. Cells stably expressing AT1Rs were stimulated with AngII or cyclic stretch, and the conditioned medium was placed on unstimulated cells stably overexpressing AT1Rs. Conditioned medium from AngII-treated cells, but not from stretched cells, triggered an increase in ERK phosphorylation in unstimulated cells (Fig. 2D).

To demonstrate that ERK activation with mechanical stretch of the heart does not occur because of released AngII ligand, we infused enalaprilat intravenously into intact mice to inhibit conversion of AngI to AngII by angiotensin-converting enzyme (Fig. 2, E and F). Enalaprilat was effective because it prevented AngI-induced increases in blood pressure (Fig. 2F). In mouse hearts that received enalaprilat pretreatment or enalaprilat in the perfusion buffer, mechanical stretch induced ERK phosphorylation to the same degree as saline-treated controls (Fig. 2E). Together, these data indicate that the activation of ERK signaling by mechanical stress occurs in the absence of release of AngII and G protein-mediated second messenger generation.

Mechanical stretch induces AT1R trafficking and β -arrestin recruitment

We used mechanical and hypo-osmotic stretch in cells stably expressing AT1Rs to examine the molecular mechanisms of stretch-induced, β -arrestin-dependent signaling. We applied static mechanical stretch to cells plated on a rubber silicone membrane with a computer-regulated vacuum to achieve expansion of cell size by 20%. Cyclic stretch was achieved by applying cycles of 10% cell expansion at 1 Hz. Hypo-osmotic stretch was used to confirm reproducibility and to allow fluorescent imaging studies. In cells transfected with small interfering RNA (siRNA) directed against β -arrestin1 and β -arrestin2 (β -arrestin1/2), stimulation with cyclic mechanical stretch or hypo-osmotic stretch significantly attenuated ERK phosphorylation compared to that in cells transfected with scrambled control siRNA (Fig. 3, A and B). In contrast, knockdown of β -arrestin1/2 followed by AngII stimulation partially attenuated increases in ERK phosphorylation, consistent with the known G protein and β -arrestin pathways for ERK activation by AngII (8). In cells stimulated with 20% static mechanical stretch, ERK phosphorylation occurred at 5 min of stimulation in a β -arrestin-dependent manner, similar to that seen in cells stimulated with cyclic or hypo-osmotic stretch

(fig. S2, A and B). Because stretch of AT1R-expressing cells led to β -arrestin-dependent ERK activation, we tested whether mechanical stretch could promote internalization and trafficking of AT1R, a process that typically occurs after ligand stimulation (21). HEK 293 cells stably expressing either hemagglutinin (HA)-tagged AT1Rs or Flag-tagged β 1ARs were stimulated with 10% cyclic stretch for 10 min, and receptor internalization was visualized by confocal microscopy. In unstimulated cells, both AT1Rs and β 1ARs showed a uniform distribution at the plasma membrane. Cyclic stretch induced the redistribution of AT1Rs into intracellular aggregates in a class B pattern (Fig. 3C), indicating a strong and long-lasting interaction with β -arrestin that is typically observed with AngII stimulation (22). Quantification of AT1R internalization was performed by measuring the percentage of cells showing intracellular aggregates in a given condition (table S1). AT1R internalization occurred in $77.0 \pm 4.6\%$ of cells after cyclic stretch and in $88.5 \pm 2.7\%$ of cells after AngII treatment compared to $2.5 \pm 0.2\%$ when unstimulated ($P < 0.001$) (table S1). In contrast, $<2\%$ of cells expressing β 1ARs showed internalization after cyclic stretch (Fig. 3C and table S1).

We next tested whether cyclic or osmotic stretch could induce AT1R-mediated β -arrestin recruitment. Cells stably expressing AT1Rs were transiently transfected with yellow fluorescent protein (YFP)-tagged β -arrestin1 or β -arrestin2 (in the presence of GRK5) and subjected to osmotic stretch (Fig. 3D). In the absence of stimulation, both β -arrestins showed a uniform distribution throughout the cell (Fig. 3D). In response to hypo-osmotic stretch, both β -arrestin1 and β -arrestin2 (in the presence of GRK5) localized to intracellular puncta, indicating the translocation of β -arrestin into endocytic vesicles (Fig. 3D). After AngII stimulation, $82.0 \pm 2.9\%$ of cells expressing β -arrestin1-YFP and $84.0 \pm 2.4\%$ of cells expressing β -arrestin2-YFP showed intracellular aggregates ($P < 0.001$ compared to unstimulated) (table S1). After hypo-osmotic stretch, intracellular aggregates were seen in $66.0 \pm 2.2\%$ of cells expressing β -arrestin1-YFP and in $66.5 \pm 4.3\%$ of cells expressing β -arrestin2-YFP ($P < 0.001$ compared to unstimulated cells) (table S1). In contrast, cells stably expressing β 1ARs did not show trafficking of β -arrestin in response to either cyclic or hypo-osmotic stretch (Fig. 3E). These data demonstrate that mechanical stress promotes the intra-cellular trafficking of β -arrestin in a class B pattern that is typical for AT1Rs after stimulation by AngII, and suggest a stable interaction between β -arrestin and internalized AT1Rs.

We next tested whether mechanical stretch induces AT1R phosphorylation in a GRK5- and GRK6-dependent manner. Stimulation of HEK 293 cells transiently expressing AT1Rs with static mechanical stretch for 10 min led to robust phosphorylation of AT1Rs, to an extent comparable to that seen with AngII stimulation (fig. S2C). Moreover, cells transfected with siRNA targeting GRK5 and GRK6 showed decreased AT1R phosphorylation in response to stretch.

Mechanical stretch induces a conformational change in β -arrestin

The absence of G protein activation with mechanical stress suggests that AT1Rs adopt a conformation that promotes β -arrestin recruitment and enables activation of downstream signaling (23,24). To determine the time course of interaction between AT1R and β -arrestin, we performed FRET experiments in HEK 293 cells transiently transfected with AT1R-cyan fluorescent protein (CFP), GRK5, and β -arrestin1-YFP or β -arrestin2-YFP. We quantified the formation of the AT1R-mCFP- β -arrestin-mYFP complex by measuring the percentage of total whole-cell CFP-excited fluorescence. In response to AngII, the maximal FRET detected was $\sim 25\%$ for AT1R-CFP and β -arrestin1-YFP, and $\sim 19\%$ for AT1R-CFP and β -arrestin2-YFP (Fig. 4A). Similar to ligand stimulation, osmotic stretch induced β -arrestin recruitment to the AT1R, but to a lower extent: $\sim 5\%$ for the AT1R-CFP and β -arrestin1-YFP pair and 3% for the AT1R-CFP and β -arrestin2-YFP pair (Fig. 4B).

Biased signaling by β -arrestin presumes that the interaction of β -arrestin with the receptor causes β -arrestin to adopt a conformation that enables β -arrestin to trigger cellular signaling cascades (6,25). To assess whether mechanical stress acts as a biased ligand by inducing conformational changes in β -arrestin, we used a β -arrestin-based biosensor and measured intramolecular bioluminescence resonance energy transfer (BRET) (7,26). The β -arrestin biosensor contains the bioluminescent *Renilla* luciferase (Rluc) and the YFP fused to the N and C termini of β -arrestin2, respectively (7,26). Changes in β -arrestin conformation alter the distance or orientation of the two ends of the β -arrestin molecule (or both), leading to changes to the efficiency of BRET (7,26). Cells cotransfected with AT1Rs and the β -arrestin reporter showed an increase in BRET ratio when stimulated with AngII (Fig. 4C). In contrast, when cells were stretched under hypo-osmotic conditions, the intramolecular BRET ratio for β -arrestin2 was decreased, indicating that β -arrestin adopted a different conformation with mechanical stretch compared to that with the ligand AngII (Fig. 4C). These data are similar to the changes in intramolecular BRET ratios observed when the β -arrestin-biased AT1R ligand SII (Sar1, Ile4, Ile8-AngII) is used to activate β -arrestin-mediated ERK signaling (8,24). Together, these data demonstrate that membrane stretch stimulates AT1R to recruit β -arrestin and induces a conformation in β -arrestin that promotes receptor internalization and intracellular signaling.

Mechanical stretch stimulates AT1R-mediated EGFR transactivation and ERK signaling

β -Arrestins mediate the transactivation of epidermal growth factor receptors (EGFRs) in response to ligand stimulation of AT1R or β 1ARs through the shedding of membrane-bound EGF to promote EGFR dimerization, autophosphorylation, and subsequent EGFR internalization (27,28). We therefore tested whether mechanical stretch could induce transactivation of EGFR in a β -arrestin-dependent manner by monitoring EGFR-green fluorescent protein (GFP) internalization with confocal microscopy (28). In cells stably expressing AT1Rs transiently transfected with GFP-tagged EGFR, mechanical stretch induced EGFR internalization in $74.2 \pm 2.4\%$ of cells, indicating transactivation (Fig. 5A). Transactivation was blocked by the EGFR inhibitor AG1478 and the Src tyrosine kinase inhibitor PP2 ($8.0 \pm 3.5\%$ and $11.0 \pm 4.2\%$ cells showed EGFR internalization, respectively) (Fig. 5A). Mechanical stretch induced EGFR internalization in $77.7 \pm 5.5\%$ of the AT1R-expressing cells transfected with control siRNA, and in $15.0 \pm 0.5\%$ in AT1R cells transfected with siRNA directed against β -arrestin1/2 ($P < 0.001$, control siRNA compared to β -arrestin1/2 siRNA) (Fig. 5B and table S1). AngII treatment induced internalization in $77.6 \pm 3.5\%$ of the control siRNA-transfected cells and in $12.0 \pm 2.7\%$ of the β -arrestin1/2 siRNA-transfected cells ($P < 0.001$ compared to control siRNA) (Fig. 5B and table S1). In contrast, mechanical stretch did not induce EGFR internalization in HEK 293 cells stably expressing β 1ARs (fig. S1D). To measure EGFR phosphorylation induced by mechanical stretch, we transiently transfected HEK 293 cells stably expressing AT1Rs with EGFR-GFP, with or without siRNA targeting β -arrestin1/2, and stimulated with $1 \mu\text{M}$ AngII, EGF (10 ng/ml), osmotic stretch, or 10% cyclic stretch. Knockdown of β -arrestin1/2 significantly decreased the amount of EGFR phosphorylation in response to AngII or stretch (Fig. 5C). To determine whether mechanical stretch stimulates EGFR-mediated ERK activation in the heart, we examined whether pretreatment with the specific EGFR inhibitor erlotinib blocked mechanical stretch-induced ERK signaling. Stimulation of wild-type hearts with mechanical stretch or EGF ligand significantly increased the amount of phosphorylated ERK, an increase that was blocked by the EGFR inhibitor erlotinib (Fig. 5D), indicating that mechanical stress induces EGFR-dependent ERK signaling.

Mechanical stress in the heart activates AT1R- and β -arrestin-dependent prosurvival signaling

Because mechanical stretch can induce cardiomyocyte and smooth muscle cell apoptosis (12, 16), and β -arrestins have been implicated in pathways that promote cell survival (29), we tested

whether mechanical stretch of the heart stimulates prosurvival pathways in a β -arrestin-dependent manner. Wild-type, AT1R KO, and β -arrestin KO mouse hearts were subjected to 30 min of balloon stretch to increase diastolic wall stress and induce apoptosis. Mechanical stretch of wild-type hearts led to Akt phosphorylation, which was absent in hearts from AT1R KO and β -arrestin2 KO mice (Fig. 6A). Moreover, the rate of apoptotic cell death as measured by TUNEL [terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling] staining was significantly increased in hearts from β -arrestin2 KO or AT1R KO mice compared to those from wild-type or β -arrestin1 KO mice (Fig. 6B).

The angiotensin receptor blocker losartan blocks stretch-induced AT1R signaling

To test whether stretch induces AT1Rs to adopt a signaling conformation, we measured ERK phosphorylation in the presence of an angiotensin receptor blocker (ARB), which stabilizes the receptor in an inactive conformation (30). Stimulation of HEK 293 cells stably expressing AT1R with AngII or stretch increased phosphorylated and activated ERK (Fig. 7A). In contrast, pretreatment with losartan abolished both the AngII- and the stretch-mediated activation of ERK. To block AT1Rs in vivo, we pretreated mice with losartan and losartan was added to the perfusion buffer during ex vivo stretch experiments. Pretreatment with losartan prevented the increase in blood pressure caused by AngII infusion (Fig. 7B, lower panel). Administration of losartan prevents mechanical stretch-induced ERK activation in hearts (Fig. 7B), consistent with the concept that an ARB locks the AT1R in an inactive conformation and prevents its activation by mechanical stretch. We further investigated whether treatment with losartan enhanced the rate of apoptosis in hearts subjected to ex vivo mechanical stress. We found that mechanical stretch of wild-type hearts treated with losartan significantly increased the rate of apoptotic cell death as measured by TUNEL staining compared to untreated wild-type hearts (Fig. 7C).

Because TUNEL staining cannot distinguish between apoptotic cells and cells undergoing DNA repair (31), additional assays for apoptosis were performed. In hearts subjected to mechanical stretch, losartan treatment increased DNA fragmentation, caspase 3/7 activity, and the ratio of Bax to Bcl2 protein compared to untreated stretched hearts (fig. S3).

DISCUSSION

An emerging concept in GPCR signaling is β -arrestin-biased agonism, the notion that distinct ligand-activated conformational states selectively stimulate GPCRs to signal through β -arrestin. Our studies provide evidence that membrane stretch activates AT1R-mediated β -arrestin signaling that is equivalent to a pure biased ligand. We show that (i) membrane stretch induces β -arrestin-biased signaling of AT1Rs in the absence of ligand or G protein activation; (ii) membrane stretch triggers an AT1R-mediated conformational change in β -arrestin that is identical to that found with a pure biased ligand; and (iii) in response to mechanical stretch, hearts lacking β -arrestin2 or AT1Rs fail to induce β -arrestin-biased AT1R signaling and show blunted ERK activation, impaired EGFR transactivation, and enhanced apoptosis.

Transduction of mechanical force on cells can involve various mechanisms, including GPCRs, cell cytoskeleton, integrins, and the TRP family of ion channels (12–16). Indeed, membrane stretch can lead to AT1R-mediated activation of TRP channels in a G protein- and PLC-dependent manner (10). Although mechanically stretched cultured cardiomyocytes show hypertrophic responses such as activation of EGFR (32) and ERK (9,11), the precise molecular mechanism by which this occurs is not well understood. Here, we demonstrate that mechanical stretch can lead to β -arrestin-biased signaling through the AT1R that requires β -arrestin2, GRK5, and GRK6.

The concept of β -arrestin-biased signaling refers to the ability of a ligand to selectively stabilize a receptor conformation such that it promotes β -arrestin recruitment and β -arrestin-dependent signaling independently of G proteins (6). Stimulation of AT1R by AngII induces G_q -mediated activation of PLC and downstream second messenger signaling, which are important for the development of cardiac hypertrophy in response to pressure overload (33). Studies with the biased ligand SII show that β -arrestin activation of ERK signaling can occur in the absence of G protein coupling and second messenger generation (8,24). It is becoming apparent that β -arrestin-biased signaling requires receptor phosphorylation by a subset of GRKs, specifically GRK5 or GRK6 (28,34–36), which appears to induce a receptor- β -arrestin conformational state that activates signaling (7). Our data demonstrate that mechanical stress functions as a biased ligand by inducing a ligand-independent conformation of AT1R that is favorable for GRK5- and GRK6-mediated phosphorylation, β -arrestin translocation, and induction of cellular pathways that promote cell survival in the heart.

β -Arrestin1 and β -arrestin2 show a ubiquitous distribution and function in the desensitization of most seven-transmembrane receptors (29,37). Although the amino acid sequences of the two β -arrestin isoforms (β -arrestin1 and β -arrestin2) are 78% identical, their activities do not appear to be redundant (38,39). Although either β -arrestin isoform can mediate desensitization and signaling through AT1R, our results show that only β -arrestin2 appears to mediate stretch-induced biased signaling of AT1R in the heart, whereas our cellular studies show that stretch can mediate translocation of either β -arrestin1 or β -arrestin2 (in the presence of GRK5). In our cellular studies, we found that the abundance of endogenous GRK5 in HEK 293 cells was low, and therefore, β -arrestin2 recruitment with stretch could only be observed if exogenous GRK5 was transfected. Moreover, our ex vivo heart studies demonstrate selectivity for β -arrestin2, GRK5, and GRK6. These data are also consistent with data showing a role for only β -arrestin2 in mediating EGFR transactivation in vascular smooth muscle cells with endogenous amounts of AT1Rs (27).

Ligand activation of AT1Rs results in a conformational change of the receptor that enables coupling to G proteins and leads to generation of the second messengers inositol triphosphate and DAG (18,19). Although mechanical stretch-activated signaling has been previously suggested to require G proteins (10,11), we show that G protein activation is not required for activation of β -arrestin-dependent ERK signaling in cells expressing AT1Rs or in hearts. Our data support a model in which mechanical stretch under some conditions mediates the activation of β -arrestin signals in a manner that does not seem to require G_q or the generation of second messengers. Transactivation of EGFRs after ligand stimulation of AT1Rs and β 1ARs requires β -arrestins (27,28), as well as GRK5, GRK6, Src, matrix metalloproteinases, and shedding of heparin-binding EGF-like growth factor (27,28). Consistent with our findings that mechanical stress activates β -arrestin-biased signaling, we show that stretch-induced AT1R-mediated EGFR transactivation requires the presence of β -arrestin and is sensitive to inhibitors of Src and EGFR.

A potential role for EGFR transactivation in the heart is to promote cell survival signaling (28). Indeed, ligand activation of both AT1Rs and β ARs can activate antiapoptotic β -arrestin-dependent signaling in vascular smooth muscle cells and the heart (28,40). It is possible that the high diastolic pressures we imposed on the hearts ex vivo may have induced other stresses, such as endocardial ischemia and hypoxic stress, which could have independently led to EGFR activation. Nonetheless, blood pressure in the mouse is similar to that in humans (41,42), and previous studies have documented increased LV end-diastolic pressures in patients with coronary artery disease during exercise (43) or with severe aortic stenosis (44–46).

We therefore propose that mechanical stretch of the heart uses AT1Rs to stimulate β -arrestin-dependent prosurvival pathways under conditions of increased wall stress, such as that which

occurs with hypertension and heart failure with preserved systolic function, conditions that are frequently treated with angiotensin-converting enzyme inhibitors and ARBs.

Controversy exists whether dual AT1R inhibition with a combination of an angiotensin-converting enzyme inhibitor and an ARB is favorable for patients with heart failure (47,48). Indeed, the ARB candesartan can inhibit mechanical stress-induced ERK activation by AT1R (30). In our study, losartan blocked stretch-mediated β -arrestin-biased AT1R signaling and promoted apoptosis in hearts exposed to ex vivo diastolic stretch. We postulate that treatment of patients with an ARB may block stretch-mediated biased AT1R signaling, which potentially could inhibit beneficial β -arrestin signaling in the heart.

In summary, we demonstrate that mechanical stress in cells and in the heart activates AT1R-induced ERK signaling in a β -arrestin-dependent manner that does not require G proteins, second messenger generation, or AngII ligand release. Mechanical stress activates GRK5 and GRK6, which promotes AT1R internalization and β -arrestin recruitment, and subsequently triggers β -arrestin to adopt a signaling conformation that is functionally equivalent to that seen with a biased ligand. The formation of an AT1R- β -arrestin signalosome by mechanical stress induces EGFR transactivation and subsequent ERK signaling. The activation of β -arrestin-biased signaling in the heart in response to increases in diastolic mechanical stretch induces pro-survival pathways, which function to limit cardiomyocyte injury. These findings have important implications for patients who present to the hospital with acute diastolic heart failure, a common disease that is associated with high mortality (49).

MATERIALS AND METHODS

Experimental animals

Eight- to 12-week-old β -arrestin1 and β -arrestin2 KO [β arr1 KO (50) and β arr2 KO (51)], AT1R KO (52), double β AR KO (double β 1AR and β 2AR KO) (53), GRK2 KO, GRK5 KO, GRK6 KO (54), and control C57/B6 wild-type (WT) mice were used for this study.

Cell culture and transfections

HEK 293 cells were maintained as previously described (28). HEK 293 cells stably expressing AT1_AR were cultured in zeocin (100 μ g/ml; Invitrogen) (24). β -Arrestin1 and β -arrestin2 transfections were carried out with Fugene 6 (Roche) (55). Briefly, 2 μ g of β -arrestin1 or β -arrestin2 plasmid was used for transfection. Cells were incubated for 24 to 48 hours and then replated in 35-mm confocal dishes or six-well plates containing a silicone membrane bottom. Cells were serum-starved for 12 hours before stimulation. Fugene 6 was also used to transfect EGFR into HEK 293 cells (24,56).

Isolated perfused heart preparation

Mice were heparinized [500 U/kg intraperitoneally (ip)] and anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg). Before dissection, the size of the LV was measured by echocardiography and the approximate LV volume was computed. Hearts were removed from animals on a heated operation board under a dissecting microscope (Nissho Optical T-240; Labtek) by median thoracotomy. Hearts were cannulated and retrogradely perfused at 37°C and 80 to 100 mmHg with Krebs-Henseleit buffer [118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, 0.5 mM Na-EDTA, and 11 mM glucose, saturated with 95% O₂–5% CO₂ (pH 7.4)] through the aorta in a noncirculating Langendorff apparatus (Hugo Sachs Harvard Apparatus). Buffer was initially passed through a 5.0- μ m filter on preparation.

Intraventricular balloon and myocardial stretch

The LV balloon was made by stretching a polypropylene membrane (57) into the shape of the LV cavity, inserted into the LV through the mitral valve, and inflated with water to yield an LV end-diastolic pressure of 30 to 50 mmHg. The water-filled balloon was secured to a polyethylene-50 tube and connected to a Statham P23Db pressure transducer (Gould Instruments). LV pressure was continuously recorded with a pressure-recording system (Mac Lab, Millar Instruments). Hearts of age- and sex-matched mice that were perfused without inflation of the balloon for identical periods of time served as the perfusion control (PC). Mice with neither perfusion nor inflation served as an excision control (EC). Balloon inflation pressure was held constant with minor adjustments of volume during the experiment. After 10 min of balloon inflation, experiments were terminated and hearts were snap-frozen for analysis. Hearts were excluded from analysis if they failed to beat spontaneously when perfused, or if balloon pressure was <25 or >50 mmHg for 10 min after inflation.

Application of mechanical strain and osmotic stress

Flexible silicone elastomer membrane bottom culture plates (BF-300-1U untreated BioFlex culture plate; Flexcell International) were coated with 0.002% collagen (Roche) for adherence. After cells were plated at a confluence of 70 to 80%, they were allowed to incubate for 24 hours and then serum-starved for 12 hours before mechanical stimulation. Mechanical stretch was applied with a computer-regulated vacuum strain apparatus (Flexcell Strain Unit FX-4000 Tension plus, Flexcell International). Alternate cycles of strain and relaxation were applied at 1 Hz (0.5 s of strain, 0.5 s of relaxation) with regulated vacuum pressure sufficient to generate 10% stretch of culture well diameter. Control samples were maintained at static conditions with no application of strain. Hypo-osmotic stretch was induced by adding double-distilled H₂O at a ratio of 1:1. As a result, the osmolality of phosphate-buffered saline (PBS) changed from 285 to 147 mosm/kg, and the osmolality of imaging buffer (for FRET analysis) changed from 255 to 137 mosm/kg.

Knockdown of β -arrestin1/2 with siRNA

β -Arrestin-targeting siRNA is a 21-nucleotide siRNA that has been previously described (8, 58). Cells were plated at 30 to 40% confluence in 10-cm dishes. siRNA (3.5 μ g) was used with GeneSilencer Transfection reagent (Gene Therapy Systems) (35). All experiments were performed 60 to 72 hours after siRNA transfection. Cells were serum-starved for 12 hours before stimulation (28).

Immunoblotting

LV tissue was homogenized in NP-40 lysis buffer containing 20 mM tris (pH 7.4), 137 mM NaCl, 1% NP-40, 20% glycerol, 10 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 10 mM NaF, aprotinin (2.5 μ g/ml), and leupeptin (2.5 μ g/ml). Protein concentrations were assayed with Bio-Rad protein assay reagent, and 100 μ g of protein was denatured by heating at 95°C for 5 min before resolving by SDS-polyacrylamide gel electrophoresis. Immunoblotting for myocardial total ERK and phosphorylated ERK was performed as previously described (59). The following dilutions of primary antibody were used: total ERK (Upstate), 1:3000; ERK1/2 (Cell Signaling), 1:1200; total Akt (Cell Signaling), 1:1000; phosphorylated Akt (Cell Signaling), 1:1000; phosphorylated EGFR (Tyr⁸⁴⁵), 1:500; total EGFR (Cell Signaling), 1:500; Bax (Cell Signaling), 1:500; and Bcl2 (Cell Signaling), 1:500. Detection was carried out by ECL (Amersham Biosciences). Densitometric analysis was performed with Bio-Rad Fluoro-S Multi-Image software.

Confocal imaging

HEK 293 cells stably expressing HA-AT1Rs or Flag- β 1ARs and transiently transfected with β arr1-YFP, β arr2-YFP, or EGFR-GFP were visualized by confocal imaging (56).

FRET assay

HEK 293 cells were transiently transfected with AT1R-CFP and β arr1-YFP or β arr2-YFP in 35-mm dishes. Imaging buffer [125 mM NaCl, 5 mM KCl, 1.5 mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose, 0.2% bovine serum albumin (BSA), 10 mM Hepes (pH 7.4)] was added to the dish on a 37°C heated stage. Images were acquired on a Zeiss Axiovert 200M microscope (Carl Zeiss Microimaging Inc.) (60). Calculation of percent FRET was performed as previously described (61).

DAGR assay

The DAGR assay was performed on cells stably expressing AT1R and DAGR (20). Cells were split and plated on a 35-mm dish coated with collagen. Twenty-four hours later, cells were washed with PBS and placed in imaging buffer [125 mM NaCl, 5 mM KCl, 1.5 mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose, 0.2% BSA, 10 mM Hepes (pH 7.4)]. After basal activity was recorded for 1 min, cells were stimulated with AngII or hypo-osmotic stretch and the DAGR ratio was recorded for 8 min.

BRET assay

BRET assays were performed as described (26). Briefly, HEK 293 cells were transiently transfected with Flag-AT1R and Rluc- β arr2-YFP with Eugene 6 reagent. After 48 hours of transfection, cells were plated into a 96-well microplate at ~50,000 per well. Luciferase substrate coelenterazine H at a final concentration of 5 μ M was added 10 min before stimulation. Cells were then stimulated with AngII (1 μ M) or H₂O (1:1), and readings were collected at 10 min after stimulation. BRET signal was calculated as the ratio of light emitted by the acceptor (YFP) and light emitted by the donor (Rluc). The values for BRET signal were corrected by subtracting the background BRET signal detected when Rluc- β arr2 was expressed alone. The change in BRET signal was calculated as the difference between BRET signal after stimulation and BRET signal before stimulation.

Treatment protocol for enalaprilat or losartan infusion in mice

Enalaprilat (1 μ g/g, Sigma), the active metabolite of the angiotensin-converting enzyme inhibitor enalapril, was infused into WT mice for 10 min to block the formation of AngII. Angiotensin-converting enzyme inhibition was confirmed by monitoring the blood pressure in response to infusion of AngI (10 pmol/kg) for 3 min with and without pretreatment with enalaprilat. In similar experiments, the AT1R antagonist losartan (1 μ g/g, Sigma) was administered to mice by intravenous infusion for 10 min. The efficacy of AT1R blockade was monitored by the infusion of AngII (10 pmol/kg) for 3 min in mice pretreated with losartan. In separate experiments, hearts from enalaprilat- and losartan-treated mice underwent balloon mechanical stretch as described above. Enalaprilat (1 μ M) or losartan (100 μ M) was also added to the Krebs-Henseleit buffer. After 10 min of ex vivo mechanical stretch, hearts were removed and assayed for phosphorylated ERK.

EGF or erlotinib treatment protocol for mice

WT mice were pretreated for 1 hour with erlotinib (20 mg/kg) or 10% dimethyl sulfoxide (DMSO) ip, followed by infusion of 10% DMSO or EGF (100 μ g/kg). Five minutes after drug administration, hearts were excised and flash-frozen in liquid nitrogen for biochemical assays as described previously (56).

Histological analysis

Freshly harvested cardiac samples were placed in sucrose-PBS solution at 4°C for 2 to 4 hours, placed in cross section in optimum cutting temperature compound (Miles Pharmaceuticals), and snap-frozen in liquid nitrogen. DNA fragmentation was detected in situ by TUNEL (28). In brief, DNA fragments were labeled with fluorescein-conjugated dUTP using terminal deoxynucleotidyl transferase (Roche Diagnostics Corp.). The total number of nuclei was determined by manual counting of propidium iodide fluorescence-stained nuclei in six random fields per section (magnification, ×200). All TUNEL-positive nuclei were counted in each section.

Angiotensin receptor phosphorylation

Flag-tagged mutant AT1R in which the PKC phosphorylation sites have been substituted for alanine (34) were transfected into HEK 293 cells. Metabolic labeling was conducted for 1 hour in medium containing 200 µCi of [³²P]P_i (inorganic phosphate)/ml (34,56). After stimulation with 1 µM AngII or mechanical stretch for 10 min, Flag-AT1Rs were immunoprecipitated from each sample and their phosphorylation was analyzed by autoradiography with a PhosphorImager (Amersham Biosciences).

DNA laddering

DNA laddering was detected with the enhanced apoptotic DNA ladder detection kit (Biovision). Mouse hearts were subjected to the indicated treatments and 50 mg of heart tissue was cut into fine pieces, solubilized in tris-EDTA lysis buffer, and treated with ribonuclease-free deoxyribonuclease (Qiagen) for 1 hour at 37°C. Enzyme B was added to the sample and incubated at 50°C for 30 min. Samples were then treated with ammonium acetate and isopropanol and incubated at −20°C for 10 min. DNA was washed with 70% ethanol and loaded on a 1.8% agarose gel with ethidium bromide. DNA ladder was visualized by illuminating with ultraviolet light.

Caspase 3/7 activity assay

Caspase 3/7 activity in heart samples was measured with the Caspase-Glo 3/7 Assay kit (Promega) (62). Briefly, 10 µg of protein in a 50-µl total volume was mixed with 50 µl of caspase-Glo 3/7 reagent and incubated for 1 hour at room temperature. Luminescence was measured with a TD-20/20 luminometer (Turner Designs), with each heart sample run in triplicate. Results are expressed as relative units of luminescence.

Statistics

Data are expressed as mean ± SEM. Statistical significance was determined with a one-way analysis of variance (ANOVA) (with Bonferroni correction or Tukey's test for multiple comparisons) with GraphPad Prism software. A *P* value of <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Mechanical stretch activates ERK in an AT1R-, β -arrestin2-, and GRK5/6-dependent manner. Diastolic stress in Langendorff-perfused mouse hearts was acutely increased with a left ventricular balloon that was inflated for 10 min. (A) Representative immunoblot of five experiments showing ERK phosphorylation and pressure tracings from LVs after graded amounts of mechanical stretch. Acute increases in diastolic stretch resulted in increased left ventricular diastolic pressure and ERK phosphorylation. (B to E) Mechanical stretch induced ERK phosphorylation in hearts of wild-type (WT) mice, but not in hearts from AT1R KO, β -arrestin2 KO, GRK5 KO, or GRK6 KO mice. (F) Mechanical stretch induced similar amounts of phosphorylated ERK in WT mice and GRK2 KO mice. Summary data in (B) to (F) represent the mean $\pm$ SEM of eight to nine KO and WT hearts for stretch and four KO and WT littermate hearts for EC (excision control) and PC (perfusion control) conditions. Str (stretch), inflation of LV balloon for 10 min. * $P < 0.0001$, compared to EC or PC in the same group.

Fig. 2.

Mechanical stretch induces ligand- and G protein-independent signaling. **(A)** Real-time DAGR assay was performed on cells stably expressing AT1Rs, which showed increased DAGR fluorescence ratio with AngII treatment, whereas no DAGR accumulation occurred after hypo-osmotic stretch. **(B and C)** In HEK 293 cells stably expressing AT1Rs, pretreatment with the PKC inhibitor RO318425 (1 μ M) significantly reduced ERK phosphorylation in response to 1 μ M AngII, but not when cells were also exposed to mechanical (Mch) or hypo-osmotic stretch (Osm). * $P < 0.001$, No pretreatment NS compared to No pretreatment AngII or No pretreatment Str; † $P < 0.001$, RO318425 NS compared to RO318425 AngII or RO318425 Str; ‡ $P < 0.001$, No pretreatment AngII compared to RO318425 AngII. $n = 3$ independent experiments for each condition with 2×10^6 to 3×10^6 cells per experiment. **(D)** Cells stably expressing AT1Rs were stimulated with AngII or cyclic stretch, and conditioned medium was used to treat a new set of cells. Cells treated with stretch-conditioned medium did not show ERK activation in response to AngII. $n = 5$ independent experiments for each condition with 2×10^6 to 3×10^6 cells per experiment. **(E)** Pretreatment of mice with enalaprilat (Enal) or perfusion of mice with buffer containing enalaprilat to block AngII production did not affect stretch-induced ERK phosphorylation in the heart. * $P < 0.0001$, compared to EC or PC in the same group. EC, $n = 4$ hearts; PC, $n = 4$ hearts; stretch, $n = 8$ hearts. IV, intravenous. **(F)** Enalaprilat blocked the AngI-mediated increase in blood pressure, confirming that enalaprilat inhibited angiotensin-converting enzyme and AngII production.

Fig. 3.

Mechanical stretch induces AT1R trafficking and β -arrestin recruitment. (A and B) HEK 293 cells stably expressing AT1R were transfected with scrambled siRNA (Ctrl siRNA) or β -arrestin1/2 siRNA and stimulated with 1 μ M AngII, mechanical stretch (A), or hypo-osmotic stretch (B) for 10 min. $n = 5$ independent experiments with 2×10^6 to 3×10^6 cells per experiment. * $P < 0.001$, Ctrl siRNA NS compared to Ctrl siRNA AngII or Ctrl siRNA Str; † $P < 0.05$, Ctrl siRNA AngII or Ctrl siRNA Str compared to β arr1/2 siRNA AngII or β arr1/2 siRNA Str; ‡ $P < 0.01$, β arr1/2 siRNA NS compared to β arr1/2 siRNA AngII. (C) HEK 293 cells stably expressing HA-tagged AT1R were stimulated with AngII (1 μ M) or 10% cyclic mechanical stretch for 10 min. AT1R redistributed from the cell membrane to internalized vesicles. In cells expressing Flag-tagged β 1ARs, isoproterenol stimulation (1 μ M), but not mechanical stretch, resulted in receptor internalization. $n = 4$ independent experiments for each condition. Arrowheads show internalized AT1Rs. (D) Hypo-osmotic stretch induced redistribution of β -arrestin1-YFP and β -arrestin2-YFP into endocytic vesicles in a class B pattern similar to that induced by treatment with 1 μ M AngII in HEK 293 cells stably expressing AT1R. $n = 4$ independent experiments for each treatment. Arrowheads, β -arrestin-YFP within endocytic vesicles. (E) Treatment with isoproterenol (Iso) (1 μ M), but not cyclic (Cyc) or osmotic stretch (Osm Str), resulted in redistribution of β -arrestin1-YFP and β -arrestin2-YFP from the cytosol to the plasma membrane in a class A pattern, indicating a transient and weak interaction of β -arrestin with the receptor in HEK 293 cells stably expressing β 1AR. Scale bar, 10 μ m.

Fig. 4.

Specific β -arrestin conformation induced by mechanical stretch. **(A and B)** After agonist stimulation with AngII (1 μ M) or hypo-osmotic stretch and GRK5 transfection, β -arrestin1 and β -arrestin2 (with GRK5 transfection) were recruited to AT1Rs, as detected by increased FRET in HEK 293 cells transiently transfected with AT1R-CFP and β -arrestin1-YFP or β -arrestin2-YFP. $n = 4$ to 6 independent experiments with 1×10^6 to 2×10^6 cells per experiment. **(C)** Different conformations of β -arrestin2 were detected with the intramolecular BRET biosensor (Luc- β arr-YFP) when AT1R-expressing cells were stimulated with AngII (1 μ M) or hypo-osmotic stretch. $n = 5$ independent experiments with 1×10^6 to 2×10^5 cells per experiment. $*P < 0.0001$, AngII AT1R compared to AngII Mock transfection; $\dagger P < 0.0001$, Osm Str AT1R compared to AngII AT1R or Osm Str Mock transfection.

Fig. 5.

Mechanical stress induced AT1R-mediated EGFR transactivation. **(A)** EGFR-GFP was internalized in HEK 293 cells stably expressing AT1R that were stimulated with 1 μ M AngII, EGF (10 ng/ml), or 10% cyclic stretch. This internalization was blocked by pretreatment with the Src inhibitor PP2 (5 μ M) or the EGFR inhibitor AG1478 (1 μ M). Arrowheads, internalized EGFRs. **(B)** Knockdown of β -arrestin1/2 by siRNA blocked AngII- and mechanical stretch-induced EGFR transactivation in HEK 293 cells stably expressing AT1Rs ($n = 4$ independent experiments with 50 to 80 cells per experiment). **(C)** Knockdown of β -arrestin1/2 in HEK 293 cells stably expressing AT1Rs prevented EGFR phosphorylation in response to 1 μ M AngII, osmotic stretch, or 10% cyclic stretch, but not to EGF ligand (10 ng/ml). * $P < 0.001$, control siRNA no stimulation (NS) compared to control siRNA treated with AngII and Str; † $P < 0.001$, control siRNA Ang or Str compared to β -arrestin1/2 siRNA AngII or Str. $n = 3$ independent experiments with 2×10^6 to 3×10^6 cells per experiment. **(D)** Mechanical stretch in WT hearts stimulated activation of ERK, which was sensitive to the EGFR inhibitor erlotinib (Erl) ($n = 4$ hearts). * $P < 0.0001$, Str no treatment (open bar) compared to Str treated with Erl (solid bar); * $P < 0.0001$, EC EGF treated (solid bar) compared to EC no treatment (open bar). Scale bar, 10 μ m.

Fig. 6.

Mechanical stress in the heart activates prosurvival antiapoptotic signaling in an AT1R- and β -arrestin2-dependent manner. **(A)** Diastolic stretch applied for 30 min stimulated Akt phosphorylation in WT hearts, but not in hearts from AT1R KO or β -arrestin2 KO mice. **(B)** Mechanical stretch-induced apoptosis was enhanced in hearts from AT1R KO and β -arrestin2 KO mice as measured by TUNEL staining compared to hearts from WT or β -arrestin1 KO mice. Str, balloon stretch for 30 min. $n = 4$ hearts for each group. * $P < 0.0001$, compared to PC in the same group; $^{\dagger}P < 0.01$, compared to stretch in WT or β arr1 KO mice. Scale bar, 500 μ m.

Fig. 7.

Blockade of stretch-induced signaling by an angiotensin receptor antagonist. **(A)** AngII and stretch increased phosphorylation of ERK. Pretreatment of cells with losartan abolished AngII- and stretch-mediated ERK phosphorylation ($n = 4$ independent experiments). **(B)** Mice pretreated with losartan failed to activate stretch-induced ERK phosphorylation (left). Losartan blocked the AngII-induced increase in blood pressure, confirming that losartan inhibited AT1Rs (right) ($n = 3$ to 5 hearts for each condition). **(C)** Mechanical stretch-induced apoptosis was higher in losartan-treated WT mice as measured by TUNEL staining compared to untreated WT mice. Str, balloon stretch for 30 min. $n = 4$ hearts for each group. Arrowheads show TUNEL-positive nuclei. * $P < 0.05$, untreated PC compared to untreated Str hearts; * $P < 0.001$, losartan PC compared to losartan Str hearts; † $P < 0.05$, losartan Str hearts compared to untreated Str. Scale bar, 500 μ m.