Circulating Exosomes Induced by Cardiac Pressure Overload Contain Functional Angiotensin II Type 1 Receptors

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Background—Whether biomechanical force on the heart can induce exosome secretion to modulate cardiovascular function is not known. We investigated the secretion and activity of exosomes containing a key receptor in cardiovascular function, the angiotensin II type 1 receptor (AT1R).

Methods and Results—Exosomes containing AT1Rs were isolated from the media overlying AT1R-overexpressing cells exposed to osmotic stretch and from sera of mice undergoing cardiac pressure overload. The presence of AT1Rs in exosomes was confirmed by both electron microscopy and radioligand receptor binding assays and shown to require β-arrestin2, a multifunctional adaptor protein essential for receptor trafficking. We show that functional AT1Rs are transferred via exosomes in an in vitro model of cellular stretch. Using mice with global and cardiomyocyte conditional deletion of β-arrestin2, we show that under conditions of in vivo pressure overload the cellular source of the exocytosis of exosomes containing AT1R is the cardiomyocyte. Exogenously administered AT1R-enriched exosomes target cardiomyocytes, skeletal myocytes, and mesenteric resistance vessels and are sufficient to confer blood pressure responsiveness to angiotensin II infusion in AT1R knockout mice.

Conclusions—AT1R-enriched exosomes are released from the heart under conditions of in vivo cellular stress to likely modulate vascular responses to neurohormonal stimulation. In the context of the whole organism, the concept of G protein–coupled receptor trafficking should consider circulating exosomes as part of the reservoir of functional AT1Rs. (Circulation. 2015;131:2120-2130 DOI: 10.1161/CIRCULATIONAHA.115.015687.)

Key Words: beta-arrestin | exosomes | hypertension | receptors, angiotensin | signal transduction

Exosomes are extracellular nanovesicles 30 to 100 nm in size that are released into the extracellular space by reverse budding of multivesicular bodies containing intraluminal vesicles.1 Exosomes were initially described as cell-secreted vesicles that eliminate obsolete molecules such as the transferrin receptors in reticulocytes2 but are now known to be released by many different cell types and can be found in most bodily fluids.1,3 The biogenesis of exosomes begins when early endosomes are processed by endosomal sorting mechanisms to form intraluminal vesicles within larger multivesicular bodies.1,4 These multivesicular bodies containing intraluminal vesicles then traffic to and fuse with the plasma membrane to release their intraluminal vesicles (now called extracellular vesicles or exosomes) into the extracellular microenvironment.1,4,5

Exosomes are structurally distinct from other shed particles such as microparticles and ectosomes and vary in their abundance, size, and composition. Exosomes are enriched in molecules derived from the parent cells, including, but not limited to, adhesion and membrane trafficking molecules, signal transduction proteins, chemokines, mRNAs, noncoding RNAs and microRNAs, heat shock proteins, growth factors, and G protein–coupled receptors (GPCRs) such as the somatostatin receptor 2.1,3,5,6 Intriguingly, exosome transfer can confer new functions on target cells and represents an important mechanism for intercellular communication and signaling.3,7 This has particular relevance in cardiovascular physiology, given the central role of neurohormonal signaling that occurs through the angiotensin II (AngII) type 1 receptor (AT1R), a GPCR known to be critically involved in the maintenance of blood pressure and heart function. However, it remains unknown whether exosomes contain functional AT1Rs.

Received September 22, 2014; accepted April 10, 2015.
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The online-only Data Supplement is available with this article at http://circ.ahajournals.orglookup/suppl/doi/10.1161/CIRCULATIONAHA.115.015687-DC1.
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Circulation is available at http://circ.ahajournals.org DOI: 10.1161/CIRCULATIONAHA.115.015687
It is now appreciated that AT1Rs can be activated either by the endogenous agonist (AngII) or by biomechanical stress.\textsuperscript{8,10} Interestingly, biomechanical stress induces AT1Rs to recruit the adaptor protein β-arrestin, internalize, and activate intracellular signaling in the absence of ligand.\textsuperscript{10,11} Because it has been shown that stress conditions can lead to the cellular release of exosomes,\textsuperscript{12,13} a potentially important functional outcome of stretch-activated AT1R trafficking could be the induction and release of exosomes containing AT1Rs into the microenvironment and systemic circulation.

In this study, we tested the hypothesis that biomechanical stress induced in vitro by osmotic stretch and in vivo by cardiac pressure overload can induce secretion of AT1R-enriched exosomes. Using radioligand binding, nanoparticle tracking of purified exosomes, and mice with conditional deletion of β-arrestin2, we quantify the number of AT1Rs contained within exosomes, demonstrate the cellular source of and mechanism for their release, and show functionality by testing their capacity to restore AT1R signaling in vitro and in vivo in AT1R knockout (KO) mice.

Methods

Detailed methods are described in the online-only Data Supplement.

Exosome Isolation

Before stimulation, cells were washed with PBS and then placed in serum-free media for 30 minutes. Exosomes were isolated from conditioned media overlying \textasciitilde 4\times10^6 cells after 30 minutes of hypotonic conditions (143 mOsm/kg; osmotic stretch), 10 μmol/L AngII, or no stimulation. Exosomes were purified with previously described methods.\textsuperscript{14}

Nanoparticle Tracking Analysis

Exosome preparations were diluted in PBS to obtain a particle concentration between 2 and 20\times10^9/mL and examined with constant-flow injection as described previously\textsuperscript{15} (Figures I and II in the online-only Data Supplement).

AT1R Radioligand Binding Assay

Modification of the radioligand binding assay was used to quantify AT1R density in exosomes.\textsuperscript{16}

Experimental Animals

Eight- to 12-week-old mice of either sex of the following genotypes were used: C57/B6 wild type (WT), AT1R KO,\textsuperscript{17} global β-arrestin1 KO,\textsuperscript{18,19} global β-arrestin2 KO,\textsuperscript{20} and β-arrestin2\textsuperscript{2lox/lox}\textsuperscript{21} mice were generated by flanking exon 2 of the mouse β-arrestin2 gene (Arrb2) with LoxP sites\textsuperscript{21} and subsequent backcrossing into a C57/B6 genetic background for 7 generations. Conditional cardiomyocyte deletion of β-arrestin2 was generated by crossing β-arrestin2\textsuperscript{2lox/lox} mice with transgenic mice expressing a tamoxifen-inducible Mer-Cre-Mer recombinase under the control of the α-myosin heavy chain (αMHC) promoter\textsuperscript{22} (Jackson Laboratory, stock No. 005650) to generate β-arrestin2\textsuperscript{2lox/lox}/αMHC\textsuperscript{Cre/Cre} mice and then crossed with β-arrestin2\textsuperscript{2lox/lox} to yield the genotypes used in the study (Figure III in the online-only Data Supplement).

At 12 weeks of age, β-arrestin2\textsuperscript{2lox/lox}/αMHC\textsuperscript{Cre/Cre} and β-arrestin2\textsuperscript{2lox/lox}/αMHC\textsuperscript{Cre/Cre} mice of either sex were placed on a tamoxifen chow diet (400 mg/kg) for 7 days, followed by regular chow for an additional 21 days.

Results

Membrane Stretch and Cardiac Pressure Overload Increase Exosome Secretion

We used nanoparticle tracking analysis\textsuperscript{15} to determine whether exosomes are released into the media in vitro and into the circulation in vivo under conditions of mechanical overload. We found \textasciitilde 50,000 exosomes/µL media overlying WT HEK293T cells and cells stably overexpressing AT1Rs (receptor density, 2.0±0.22 pmol/mg protein; Figure 1A). When stimulated by hypotonicity (osmotic stretch) or by AngII, there was an \textasciitilde 2-fold increase in the number of exosomes released from AT1R-expressing cells into the overlying media (Figure 1A). This was abrogated with the addition of 15 mmol/L dimethyl amiloride, an inhibitor of exosome release (Figure 1A). We next tested whether exosome release occurs in vivo under conditions of pressure overload in the intact animal. WT mice were subjected to either transverse aortic constriction (TAC) or sham surgery, and serum was harvested after 1 or 7 days. We found that under basal conditions, 1 µL serum contains \textasciitilde 9 million exosomes, which increased 3-fold to \textasciitilde 28 million exosomes/µL serum after 7 days of pressure overload (Figure 1A).

We characterized exosomes isolated from HEK293T cells by transmission electron microscopy and immunoblotting (Figure IA–IC in the online-only Data Supplement) and showed the presence of common exosome markers such as CD9, CD63 and Alix.\textsuperscript{13} Calnexin was not detected in the exosome fraction, demonstrating the lack of contaminating endoplasmic reticulum proteins. In addition, using immunogold labeling, we demonstrate the presence of CD9 and tagged AT1R (AT1R-HA) on exosomes from media overlying cells stably expressing AT1R-HA (Figure ID in the online-only Data Supplement). We analyzed the size distribution in exosomes isolated from cell media after osmotic stress and from sera of mice after 7 days of TAC. Exosomes ranged in size from 30 to 100 nm with a mean size \textasciitilde 60 to 65 nm (Figures IE and II in the online-only Data Supplement), confirming the efficacy of our exosome isolation procedure with little contamination from larger microvesicles.

Exosomes Contain AT1Rs

We next used saturation radioligand binding to confirm the presence of AT1Rs in exosomes released by cells after stimulation. Using G50 size exclusion columns to isolate the exosomes containing AT1R bound to a saturating concentration of \textsuperscript{[125]I}-SAR1-ILE8-AngII (Figure 1B; see Methods for Statistical Analysis

Data are expressed as median with first and third quartiles. Statistical significance was determined by the Kruskal-Wallis test. Correction for multiple comparisons was made by use of a Dunn correction. Analysis of blood pressure (online-only Data Supplement) comparing basal with AngII was performed with the Wilcoxon signed-rank test for repeated measurements within each group, whereas comparison of percent changes of hemodynamic parameters between ≥3 independent groups was assessed by the Kruskal-Wallis test. Quantitative polymerase chain reaction data were analyzed by the Mann-Whitney U test (online-only Data Supplement). A threshold value of \(P \leq 0.05\) was considered statistically significant. All analyses were performed with GraphPad Prism version 6.01.
Exosomes isolated from media overlying HEK 293T cells

Exosomes isolated from media overlying AT1R-stable cells

Exosomes isolated from serum of wild type mice

A

B

C

D

E

Figure 1. Osmotic stretch (OSM) significantly augments the secretion of angiotensin II (AngII) type I receptor (AT1R)–enriched exosomes. A, Nanotrack particle analysis was performed to determine concentration of exosomes isolated from conditioned media overlying HEK 293T cells or sera from mice. Left, Hypotonic-conditioned media (osmotic stretch) significantly increased exosome concentration compared with isotonic media (nonstimulated [NS]) of HEK 293T cells; the addition of dimethyl amiloride (DMA; an inhibitor of exosome release) prevented the shedding of particles. Middle, Conditioned media from AT1R-stable cells after stimulation with osmotic stretch or AngII showed significantly increased concentration of exosomes that was also blocked by DMA treatment. Right, Exosomes isolated from sera of mice increased after pressure overload (transverse aortic constriction [TAC]) compared with sham-operated mice.

B

C

D

E

Radioligand binding was used to measure AT1R density in exosomes (Exo). AT1R density in exosomes isolated from overlying media after osmotic stretch and AngII stimulation were significantly increased compared with the basal condition without stimulation. D, AT1R density of exosomes derived from sera of wild-type (WT) mice after TAC was significantly greater than sham serum.

E

The number of AT1Rs calculated from receptor binding data was normalized to the starting volume of overlying media of AT1R-stable cells stimulated with either osmotic stretch (open bar) or AngII (solid bar); the same normalization was performed considering the starting volume of mouse serum after 1 day (open bar) or 7 days (solid bar) of TAC. A through E, Statistical significance was determined by the Kruskal-Wallis and Dunn tests, comparing each group with the control nonstimulated or sham group. Data are presented as medians with first and third quartiles.
μ receptors per 1 to stretch in vitro or TAC in vivo, the AT1R density increased serum in WT mice. After a stress condition such as osmotic II), a process previously shown to involve both microvesicles (Figure 2A). To monitor for the presence of exosomes, we used donor cells overexpressing mCherry-tagged Alix, a commonly used exosome marker protein.\(^1\) AT1R-YFP cells transfected with Alix-mCherry were stimulated by osmotic stretch, and \(\approx 40\%\) of cells under these hypotonic conditions showed cell membrane surface budding (Figure 2B, panel II), a process previously shown to involve both microvesicles\(^2\) and exosomal secretion.\(^3\) Confocal microscopy of WT HEK 293T cells 12 hours after exposure to exosomes derived from hypotonic-stimulated conditioned media overlying these AT1R-YFP– and Alix-mCherry–expressing cells showed transfer of Alix-mCherry to the inside of cells and AT1R-YFP to the plasma membrane (Figure 2B, panel IV). To determine whether the AT1Rs transferred through exosomes were functional, we tested whether transferred AT1Rs would undergo internalization after AngII stimulation. Coculturing WT HEK 293T cells with exosomes isolated from osmotic stretch or AngII-conditioned media of AT1R-YFP cells resulted in the transfer of AT1Rs to recipient cells that were localized to the plasma membrane at basal conditions (Figure 2C, panels 4 and 5). Exposure to 15 minutes of AngII resulted in internalization of AT1Rs, thus demonstrating that the exosome-mediated transfer of receptors retained their ability to traffic inside the cell after agonist stimulation (Figure 2C, panels 9 and 10, white arrows).

To test whether exosome-transferred AT1Rs can activate agonist dependent signaling, we measured ERK1/2 phosphorylation in WT HEK 293T cells 12 hours after the addition of AT1R-HA–containing exosomes (Figure 3A). AngII stimulation of exosome-treated WT 293T cells resulted in a 2- to 3-fold increase in ERK phosphorylation relative to T-ERK.

**Figure 2.** HEK 293T cells respond to osmotic stretch and angiotensin II (AngII) by secreting AT1R–yellow fluorescent protein (YFP) and Alix-mCherry under basal conditions (I). After 30 minutes of osmotic stress, there is budding on the surface of cells, and Alix– (mCherry) and AT1R (YFP)–enriched vesicles appear on the surface (II). Wild-type (WT) HEK 293T cells 12 hours after being exposed to exosomes derived from nonstimulated media show low transfer of Alix-Cherry protein (III), whereas hypotonic-stimulated conditioned media (IV) shows transfer of Alix-mCherry to the inside of cells and AT1R-YFP to the plasma membrane. C, Exosomes collected from overlying media of cells expressing AT1R-YFP were transferred to recipient cells and tested for their ability to internalize after agonist stimulation. Confocal microscopy analysis shows AT1R-YFP localized to the plasma membrane in cells stably expressing AT1R-YFP (2) and in recipient HEK293 cells after incubation with exosomes derived from osmotic stretch and AngII stimulation (4 and 5). No green fluorescent protein (GFP) fluorescence was observed in recipient cells when incubated with exosomes isolated from unstimulated AT1R-YFP–stable cells (3). Stimulation of the transferred receptors with 10 µmol/L AngII for 15 minutes induced robust AT1R internalization (9 and 10, white arrows). B and C, Scale bar, 15 µm; n=4 independent experiments; representative images shown. The cell confluence (\(\approx 70\%\)) for all confocal experiments was monitored under bright field with a lower-magnification microscope.
Figure 3. Exosome-derived angiotensin II (AngII) type I receptors (AT1Rs) are able to stimulate cellular signaling. A, HEK 293T cells preincubated with exosomes (Exo) derived from conditioned media of AT1R-HA–stable cells after osmotic stretch or AngII stimulation show a significant increase in phosphorylated (p)-ERK levels after AngII stimulation but not when cells were preincubated with exosomes derived from nonstimulated AT1R-HA–stable cells. The AngII-induced p-ERK response was blocked by the AT1R receptor blocker telmisartan (Telm). B, In a separate experiment, AT1R-HA–stable cells were pretreated with the inhibitor of exosome release dimethyl amiloride (DMA;15 nmol/L), and exosomes were isolated from conditioned media of cells after stimulation with osmotic stretch or AngII. HEK 293T cells harvested with exosomes derived from AT1R-HA–stable cells treated with DMA failed to respond to AngII stimulation. A and B, Statistical significance was determined by 5 independent Kruskal-Wallis tests with a post hoc Dunn test comparing the p-ERK/total (T) ERK levels of AngII and telmisartan+AngII with the p-ERK/T-ERK levels of control nonstimulated (NS) in each group of recipient cells. HEK 293T cells preincubated with cycloheximide (0.05 mol/L) and exosomes derived from conditioned media of AT1R-stable cells after osmotic stretch or AngII stimulation show a significant increase in p-ERK levels after AngII stimulation. Inhibiting protein synthesis with cycloheximide did not result in a reduction in AngII-induced p-ERK levels in recipient cells treated with exosomes. Statistical significance was determined by 6 independent Kruskal-Wallis tests with a post hoc Dunn test comparing the p-ERK/T-ERK levels of AngII and telmisartan+AngII with the p-ERK/T-ERK levels of control nonstimulated (NS) in each group of recipient cells. Additional comparison of the p-ERK/T-ERK levels of preselected pairs of AngII columns with or without cycloheximide was performed in each of the 3 types of recipient cells (AT1R-HA–stable cells, no exosomes; HEK 293T cells+exosomes with osmotic stretch; HEK 293T cells+exosomes with AngII). Data are presented as medians with first and third quartile.
that was completely blocked by pretreatment with the AT1R receptor blocker telmisartan (Figure 3A). To test whether the transfer of functionally competent AT1Rs occurred via an exosome-dependent mechanism, we pretreated donor cells with the exosome release blocker dimethyl amiloride to block the secretion of exosomes. Recipient cells cocultured with overlying media from dimethyl amiloride–treated cells failed to respond to AngII stimulation (Figure 3B), indicating that, in overlying media of stimulated cells, the mechanism for AT1R responsiveness is most likely attributable to the transfer of AT1R contained in exosomes.

To exclude the possibility that transfer of AT1Rs resulted from new synthesis of transferred mRNAs, we incubated recipient cells with cycloheximide (0.05 mol/L) for 12 hours before AngII stimulation. Although the level of ERK phosphorylation was slightly lower in the cells stably expressing AT1Rs treated with cycloheximide, no significant effect on ERK phosphorylation was observed in recipient cells (Figure 3C), suggesting that exosomes deliver functional AT1R protein and not mRNA.

Exosomes Containing AT1R Can Modulate Blood Pressure Responses In Vivo

Previous studies have shown that exosomes contain functional molecules such as microRNAs, mRNAs, and proteins and when injected into animal models can lead to important physiological effects on atherosclerosis formation or angiogenesis during peripartum cardiomyopathy. To determine whether AT1Rs transferred by exosomes could modulate a physiological response in vivo, we injected AT1R–enriched exosomes into the tail vein of AT1R KO mice and 24 hours later measured the blood pressure response to AngII infusion (2 µg·kg⁻¹·min⁻¹; Figure 4A). AT1R KO mice injected with PBS or exosomes isolated from media overlying nonstimulated cells showed a minimal increase in blood pressure, likely as a result of the infusion of volume (Figure 4B and Table I in the online-only Data Supplement). However, when AT1R KO mice received exosomes isolated from overlying media of hypertonic-stimulated cells or from sera of WT mice 7 days after TAC, systolic blood pressure increased by ≈30% (Figure 4B and Table I in the online-only Data Supplement). We also found that heart lysates from AT1R KO that received exosomes containing AT1R showed elevated levels of phosphorylated ERK compared with either non–exosome-treated or AT1R-deficient exosome control hearts (Figure 4C). Using radioligand binding, we quantified AT1R expression in the heart, skeletal muscle, lung, and kidney tissue of WT and AT1R KO mice that received exogenous exosomes. We detected significant AT1R expression in both heart and skeletal muscle of exosome-injected AT1R KO mice (~9 fmol/mg), which was approximately half the receptor density (~19 fmol/mg protein) found in the heart and skeletal muscle of WT mice (Table II in the online-only Data Supplement). Interestingly, AT1Rs were not detected in the kidney or lung of AT1R KO mice after intravenous exosome injection.

Figure 4. Exosomes can serve as a means of receptor transfer in vivo. A, Diagram of the in vivo study performed by injecting exosomes in tail vein of angiotensin II (AngII) type I receptor (AT1R) knockout (KO) mice. The day after injection of exosomes into mice, responsiveness to AngII stimulation was assessed by invasive hemodynamic monitoring of blood pressure. B, In response to AngII infusion, systolic blood pressure in WT mice increased, whereas no increase was detected in AT1R KO mice when injected with an exosome-free saline solution. Administration of exosomes derived from osmotic stretch–treated AT1R-stable cells or from sera of wild-type (WT) mice subjected to transverse aortic constriction (TAC) restored the AngII-dependent increase in systolic blood pressure. C, After AngII infusion, hearts were removed to assess AT1R-mediated signaling. AngII–induced phosphorylated (p)–ERK signaling in the heart was restored in mice injected with AT1R–enriched exosomes. B and C, Data are presented as medians with first and third quartiles. Statistical significance was determined by the Kruskal-Wallis with a Dunn test for multiple comparisons.

AT1Rs Within Circulating Exosomes Traffic to Cardiac and Skeletal Myocytes and Resistance Vessels

To investigate which cells in the heart and skeletal muscle uptake AT1Rs from circulating exosomes, we injected WT mice with exosomes derived from media overlying AT1R–YFP cells with or without osmotic stretch (Figure 5A). Confocal microscopy of heart cryosections shows the presence of fluorescently labeled AT1Rs only within troponin-positive...
cardiomyocytes, not within platelet-derived growth factor receptor–positive fibroblasts (Figure 5B, left). A similar pattern was observed in skeletal muscle (Figure 5B, right). These findings are supported by separate experiments of freshly isolated cardiomyocytes and fibroblasts from WT mouse heart 24 hours after the intravenous injection of AT1R-YFP–containing exosomes. Immunostaining for YFP, cardiac troponin I, and platelet-derived growth factor receptor-α showed incorporation of AT1R-YFP only in cardiomyocytes, not in fibroblasts (Figure IV in the online-only Data Supplement).

Because injection of exosomes containing AT1R resulted in the reconstitution of an AngII-stimulated blood pressure...
response in AT1R KO mice (Figure 4A), we investigated whether the uptake of AT1Rs also occurred within cells of mesenteric resistance vessels. Consistent with their role in regulating blood pressure, we found the presence of AT1R-YFP within endothelial and smooth muscle cells of mesenteric vessels (Figure 5C).

**β-Arrestin Is Necessary for the Packaging of AT1R Cargo Into Exosomes**

Because GPCR internalization requires the multifunctional adapter protein β-arrestin for efficient trafficking,26 we tested whether β-arrestin is necessary for the packaging of AT1R cargo into exosomes. Consistent with previous data for internalization and signaling,10,27 silencing β-arrestin1 and β-arrestin2 in donor cells stably expressing AT1R-YFP impaired both osmotic stretch and AngII-induced AT1R internalization (Figure 6A). Importantly, when exosomes were isolated from media overlying osmotic-stretch-overexpressing cells, silencing of β-arrestin1/2 blocked the exosomal transfer of AT1R-YFP to WT HEK 293 recipient cells (Figure 6A). β-Arrestin1/2 knockdown did not affect the exosome concentration released by either osmotic or AngII.

**Cardiomyocytes Are the Principal Source of AT1R-Enriched Exosomes Secreted During Pressure Overload**

On the basis of our observation that packaging of AT1Rs into exosomes requires β-arrestin, we next tested whether this is also true for exosomes released in vivo into the circulation. In experiments using β-arrestin1 and β-arrestin2 global KO mice, we found that after pressure overload the total number of exosomes released into the circulation was the same as in WT for both β-arrestin1 and β-arrestin2 KO mice (Figure 6B and Figure VA in the online-only Data Supplement). However, only the β-arrestin2 KO mice showed a markedly reduced number of AT1Rs in the serum after TAC (Figure 6C and Figure VB–VD in the online-only Data Supplement), indicating that β-arrestin2 is needed for the in vivo packaging of AT1Rs into exosomes and is consistent with the known role of β-arrestin2, not β-arrestin1, in mechanical stretch–induced AT1R signaling.10

We next sought to identify the cellular source of AT1R-enriched exosomes after cardiac pressure overload. We crossed β-arrestin2flox/flox mice with αMHCMerCreMer transgenic driver mice and after tamoxifen treatment achieved an ≈80% knockdown.

Figure 6. Exosomes containing angiotensin II (AngII) type 1 receptors (AT1Rs) require β-arrestin for packaging and in vivo are secreted into the circulation by cardiomyocytes. A, siRNA targeting β-arrestin1/2 prevents AT1R internalization after osmotic stretch or AngII stimulation in AT1R–yellow fluorescent protein (YFP) donor cells (top). Although siRNA targeting β-arrestin1/2 has no effect on the number of exosomes secreted, it prevented the enrichment of AT1Rs within exosomes, leading to the inability to transfer AT1R-YFP to recipient wild-type (WT) HEK 293T recipient cells (bottom). Representative Western blot showing silencing of β-arrestin1/2.

B, Pressure overload induced a similar ≈3-fold increase in circulating exosomes as measured by nanoparticle tracking analysis in WT, β-arrestin2 knockout (KO), β-arrestin2flox/flox, and β-arrestin2flox/+;α-MHCcreMer transgenic driver mice (top). Statistical significance was determined by the Kruskal-Wallis with a Dunn post hoc test comparing different genotypes at 7 days of TAC. P=N.S., β-arrestin2flox/flox/α-MHCcreMer vs β-arrestin2 KO. Both the β-arrestin2flox/flox and β-arrestin2flox/+;α-MHCcreMer mice were treated with tamoxifen for 1 week, followed by a 3-week washout period.

C, Marked reduction in the number of AT1Rs in exosomes from sera of β-arrestin2 KO and β-arrestin2flox/flox mice compared with WT and β-arrestin2flox/+;α-MHCcreMer, mice, suggesting that the principal cellular source for AT1R-enriched exosome in the serum after transverse aortic constriction (TAC) are cardiomyocytes. Data are presented as medians with first and third quartiles.
reduction in β-arrestin2 mRNA in cardiomyocytes (Figure III in the online-only Data Supplement). After 1 week of pressure overload, we observed a marked reduction in circulating AT1Rs in the serum of β-arrestin2fl/fl/αMHC-CreCrem compared with tamoxifen-treated β-arrestin2fl/flox lacking the αMHCCreCrem transgene (Figure 6C and Figure VD in the online-only Data Supplement). These data suggest that the cellular source of AT1Rs released into the circulation under conditions of pressure overload is principally cardiomyocytes.

Discussion

In this study, we show that mechanical stress induces the release of exosomes containing AT1R in vitro under hypotonic conditions and in vivo with pressure overload. Using confocal microscopy and radioligand binding, we show that exocytosis of exosomes by AT1R-expressing cells after osmotic stretch or AngII infusion contain functional AT1Rs. We demonstrate that the in vivo injection of AT1R-containing exosomes reconstitutes AngII-induced blood pressure response and cardiac ERK signaling in AT1R KO animals and that cardiac and skeletal myocytes and mesenteric resistance vessels are the principal targets for exosomal delivery of AT1Rs. Most strikingly, we show that the trafficking and sorting of AT1Rs into exosomes require the multifunctional adaptor protein β-arrestin and that the cellular source for AT1R-enriched exosomes released into the circulation after pressure overload is predominantly the cardiomyocyte. Our study supports the concept that exosomes contain functional AT1Rs and provide a means of intercellular communication both at the local tissue level and at a distance to maintain cardiovascular homeostasis.

It is now appreciated that cells continuously release exosomes containing proteins and RNAs as an important mechanism for cell-to-cell communication in multicellular organisms. A wide variety of cell types have been reported to release exosomes under normal or pathological conditions, including astrocytes, microglial cells, tumor cells, endothelial cells, and cardiomyocytes. In this study, we show that cardiomyocytes are the principal cellular source of AT1R-containing exosomes released into the circulation after TAC because cardiomyocyte-specific deletion of β-arrestin2 in mice markedly impaired the packaging of AT1Rs into secreted exosomes (Figure 6C). Interestingly, the total number of exosomes released into the circulation was not reduced (Figure 6B), suggesting that β-arrestin is not required for intracellular maturation and exocytosis of exosomes but is required for the sorting and packaging of GPCRs into the exosome. These data suggest a new role for the multifunctional adaptor protein β-arrestin2, which not only regulates the intracellular trafficking and signaling of an agonist-stimulated GPCR but also is an important mechanism regulating the intracellular pathways that lead to packaging of AT1Rs into exosomes in response to a biomechanical stress.

Although virtually all cells release microvesicles and nanovesicles (exosomes), the main distinction is particle size, with nanovesicles ranging between 30 and 100 nm and microvesicles ranging between 100 and 1000 nm in diameter. Therefore, distinguishing exosomes from the other types of microparticles secreted by cells can be technically challenging if rigorous methods are not used. In this study, we used a number of methods to purify and quantify high-quality exosomes such as nanoparticle tracking, electron microscopy, differential centrifugation, size exclusion chromatography, and ultracentrifugation. Together with our modification of the radioligand binding assay, we show that, although only a few receptors can be detected per 100 exosomes, the number of exosomes released into the circulation increases 3-fold with hemodynamic overload, leading to the striking assessment that a microliter of serum contains ~10 to 20 million AT1Rs. Furthermore, the number of circulating exosomes in our mouse studies (≈2×10⁹ per 1 mL) is consistent with data in humans showing ~10¹⁰ exosomes per 1 mL plasma.

Previous studies have shown that the release of exosomes containing growth factor receptors into the tumor microenvironment may be a mechanism to promote metastatic spread of tumor cells. A proteomic study of plasma from healthy donors showed that microvesicles contain proteins mostly associated with the complement and coagulation signal-transduction cascade and the cytoskeletal and integrin complex. Indeed, exosomes secreted from platelets confer proadhesive properties to endothelial cells or tumor cells by the transfer of adhesion molecules such as CD41. Although it has been shown that AT1R agonist stimulation can increase secretion of functionally active microparticles to promote reactive oxygen species production and proinflammatory responses, whether AngII stimulation increased secretion of exosomes enriched with AT1Rs was not investigated. Here, we show that cardiomyocytes release AT1Rs containing exosomes in response to pressure overload and likely play an important role in cardiovascular homeostasis.

Our findings extend the physiological relevance of exosomal transfer of proteins to include the cardiovascular system. Previous in vitro studies have shown that adenosine 2A receptors and dopamine 2 receptors are released within microvesicles and can be transferred to acceptor cells, retaining their ability to respond to agonist stimulation. Because hypotonic osmotic stretch in vitro is not comparable to the complex physiology of in vivo pressure overload, we tested whether exosomes secreted in vivo contain functional GPCRs and could facilitate intercellular signaling. We speculate that, under pathological conditions of increased circulating AngII levels or elevated cardiac filling pressures resulting from cardiac dysfunction or hypertension, enhanced vesicle secretion from cardiomyocytes and transfer of AT1Rs to distant cellular sites may represent a mechanism to offset the physiological downregulation after agonist stimulation. Importantly, this may impair cardiac function during blood pressure overload, promoting reactive oxygen species production or proinflammatory response in an AT1R-dependent manner, and lead to aggravation of cardiac remodeling during pressure overload.

The identification of cardiomyocytes as the principal cellular source for exocytosing AT1R-enriched exosomes into the circulation with pressure overload, with subsequent uptake by skeletal muscle and resistance vessels, suggests that the current concept of GPCR trafficking may need to be expanded when the whole organism is considered. The accepted model for GPCR trafficking is a multistep process
that begins with the processing and folding of newly synthesized receptors within the endoplasmic reticulum, followed by transit through the Golgi into vesicles that become targeted to the plasma membrane.\textsuperscript{42} Once on the cell surface, ligand exposure promotes the endocytosis of receptors and involves a number of dynamic protein-protein interactions that are initiated by the recruitment of β-arrestin. β-Arrestin facilitates the formation of clathrin-coated vesicles, and subsequent interaction with multiple trafficking proteins leads to the formation of intracellular endosome-containing receptor.\textsuperscript{26,42} Internalized GPCRs within endosomes may experience several fates: They are recycled back to the plasma membrane to once again be available for stimulation by ligand; they activate signaling pathways as signalosomes; or they are targeted for lysosomal degradation.\textsuperscript{42} The data from our study suggest that an additional fate should be considered, particularly under conditions of biomechanical stress: the trafficking of GPCRs into endosomes for release into the circulation. Thus, we propose the concept that the trafficking of AT1R should not be limited to the cell but expanded to include the whole organism in which the total reservoir of functional AT1R includes both those in the cell and those circulating as exosomes. For the AT1R, this includes the β-arrestin2-dependent extrusion of functional receptors into endosomes from cardiomyocytes, which then circulate, are incorporated into endothelial and smooth muscle cells of resistance vessels and skeletal myocytes, and are available to respond to neurohumoral stimulation.

The precise mechanism of how exosomes in the circulation cross the capillary endothelium to release their cargo into underlying cells is not clearly defined. Studies using fluorescently labeled ovalbumin within vesicles have suggested that an equilibrium between endocytosis and subsequent exocytosis occurs in the capillary endothelium. This process has been called transcytosis, whereby endothelial cells can endocytose proteins across the endothelium.\textsuperscript{43} Additionally, other mechanisms of intercellular communication have been described such as the formation of short membranous nanotubes that allow the transfer of B-cell antigen receptor to bystander B cells, thereby increasing the pool of antigen-presenting cells.\textsuperscript{44} Nonetheless, our data demonstrate the remote transfer of AT1Rs by means of exosomes from cardiomyocytes to endothelial and smooth muscle cells of mesenteric vessels.

Acknowledgments
We sincerely thank Dr Thomas M. Coffman for kindly providing AT1R KO mice and Dr Sudha K. Shenoy for her gift of the YFP-tagged AT1R-stable cells. We thank Dr Julia Walker and Barbara Theriot for maintaining, backcrossing, and providing the β-arrestin2Flox/Flox mice. We thank Dr Robrt J. Lefkowitz for his considerable support in the development of the β-arrestin2Flox/Flox mice. Requests for these mice should be addressed to Dr Lefkowitz or Dr W. Chen.

Sources of Funding
This work was supported by National Institutes of Health grants HL56687 and HL75443 to Dr Rockman; the institutional grant T32 HL007101 to Dr Watson; Clinical Oncology Research Center Development grant 5K12-CA100639-08 to Dr M. Chen; and R01-CA172570 to Dr W. Chen.

Disclosures
Dr Rockman is a scientific cofounder for Trevena Inc, a company that is developing G protein–coupled receptor–targeted drugs. The other authors report no conflicts.

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Circulating Exosomes Induced by Cardiac Pressure Overload Contain Functional Angiotensin II Type 1 Receptors

Circulation. 2015;131:2120-2130; originally published online May 20, 2015;
doi: 10.1161/CIRCULATIONAHA.115.015687

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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Cell culture and transfection

HEK293T cells (American Type Culture Collection, USA) were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM-glutamine, 100 units/ml penicillin/streptomycin, and 10 % (v/v) heat-inactivated fetal bovine serum (FBS) at 37 °C and in an atmosphere of 5% CO₂. Cells stably expressing the HA tagged AT1R (2 ± 0.22 pmol/mg protein) were used as previously described¹ and YFP tagged AT1R (1 ± 0.31 pmol/mg protein) were a kind gift from Dr. Shenoy. In separate co-localization experiments, AT1R-YFP stable cells were transiently transfected with (2 µg DNA) of the plasmid containing Alix-mCherry (Addgene, plasmid 21504).

Exosomes isolation

Briefly, differential centrifugation at 4 °C was performed starting with a centrifugation at 1000 x g (15 min) followed by centrifugations at 12,000 x g (20 min) and 18,000–20,000 x g (20 min) to obtain a supernatant free of free floating cells and cellular debris. The supernatant was filtered by vacuum or gravity (Figure S1, Figure S2 A, B) through a 0.22 µm filter to remove cellular debris, apoptotic bodies and microvesicles. Purified exosomes were then collected by ultracentrifugation of the filtered solution at 100,000 x g (70 min) using a Ti70 rotor in a Beckman ultracentrifuge and resuspended in 100 µl of
1X PBS. No difference were observed for the size and quantity of exosomes isolated from serum compared to citrated plasma (Figure S2 C,D).

**Nanoparticle Tracking Analysis (NTA)**

NTA is based on the principle that the rate of Brownian movement of nanoparticles in solution is related to their size, and by tracking the movement of individual nanoparticles over time the particle diameter can be calculated.\(^2\) Briefly, samples from the 100,000 x g centrifugation step were used for NTA, approximately 0.3 ml supernatant was loaded into the sample chamber of an LM10 unit (Nanosight, Ltd) and three videos of either 30 or 60 seconds were recorded of each sample.

**AT1R radioligand binding assay**

Briefly 50 µl of exosome suspension was incubated with a saturating concentration of radiolabeled AT1R antagonist \([^{125}\text{I}]-\text{SAR1-ILE8-ANGIOTENSIN II}\) (2 nmol/L, Perkin Elmer NEX 248) in a buffer containing 50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 12.5 mmol/L Mg Cl\(^2\), 0.2% BSA. Specific binding was determined by subtracting non-specific binding in the presence of 10 µmol/L Telmisartan from total binding measured by the addition of assay buffer. After 90 minutes of incubation at RT, the binding assay was loaded onto chromatography columns (BioRad) containing 10 ml of Sephadex G50 medium resin (Sigma-Aldrich) and the exosomes were eluted by gravity with 5 ml of elution buffer (50 mmol/L Tris pH 7.4). Previous calibration experiments with Blue Dextran (2000 kDa) and Orange G (500 Da) showed that 5 ml was sufficient to completely elute the void volume containing the exosomes while retaining the free
radioligand (Figure 1B). Bound radioactivity was quantified using a Packard Cobra Gamma Counter. Specific radioactivity was converted to femtomole (fmol) of AT1R and normalized to $10^2$ exosomes, then expressed as the number of receptors using the Avogadro constant: $\text{N° of AT1Rs in } 1 \text{ fmol} = \frac{6.23 \times 10^{23}}{10^{-15}}$.

**Transmission Electron Microscopy and Immunogold labeling**

The main marker of exosomes is their morphology, including oval-biconcave shape and size of 30 to 100 nm. To define their purity and quality, we examined exosome morphology using Transmission Electron Microscopy (T.E.M.). 5 μL of purified exosomes was loaded on a Formvar-coated copper grid (200 mesh) for 5 min and excess exosome suspension was blotted with filter paper. The grid was placed on a drop of 1% uranyl acetate solution in milli-Q H2O for 30 sec, and excess stain was removed. Exosomes were examined using a Philips/FEI T.E.M. CM12 transmission electron microscope at 80 kV.

For the immunogold-labeling the exosomes isolated by differential centrifugation were incubated overnight with a primary antibody 100 μl 1:5 dilution (Rabbit anti HA, Clontech and Mouse CD9, Millipore). Exosomes were then washed in PBS and centrifuged at 100 000 x g for 70 min in order to eliminate the excess of unbound antibody. The antibody labeled exosomes were loaded on Formvar-coated grid and fixed with 2% paraformaldehyde in PBS for 5 min. The exosome loaded grids were washed three times in PBS and incubated with Goat Blocking solution for 10 min and then with secondary antibody gold labeled Goat anti Rabbit (10 nm beads size) and
Goat anti Mouse (25 nm beads size) for 30 min. After 3 washes in PBS the grid was placed on a drop of 1% uranyl acetate solution in milli-Q H2O for 30 seconds, and excess stain was removed.

**Exosomes transfer by confocal microscopy**

Confocal microscopy was carried out as previously described using a Zeiss 780 inverted laser scanning confocal microscope. HEK 293T cells stably transfected with the plasmids containing cDNAs encoding the AT1R-YFP and Alix-mCherry (ADDgene, Plasmid 21504) were used as positive control while not transfected HEK 293 served as the negative control. Cells were plated onto glass-bottom dishes, pre-coated with Collagen I (0.001 %), before observation on the confocal microscope. HEK 293 cells were treated overnight with exosomes derived from AT1R-YFP conditioned media of three different conditions: Osmotic Stretch (OSM), Angiotensin II (Ang II) 10 µmol/L and No Stimulation (NS). In order to assess if AT1R was active once transferred to recipient cells, live cells were treated with Ang II (10 µmol/L) and images were collected 15 minutes after stimulation. Samples were visualized using single sequential line excitation filter at 514 and 568 nm and emission filter at 505–550 nm for YFP and mCherry detection respectively.

**Pressure overload**

Animal studies were carried out according to approved protocols and animal welfare regulations of Duke University Medical Center’s Institutional Review Boards. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg). Chronic
pressure overload was induced by transverse aortic constriction (TAC) in WT mice for 1 week while in Sham operated mice the suture was passed around the aorta but not ligated as previously described.

**In vivo exosomes administration**

In separate experiments, AT1R-KO mice were injected through tail vein with a range between 1.0-2.3 x 10^{11} exosomes isolated from AT1R stable expressing cells, and between 4.6-5.6 x 10^{13} exosomes isolated from the serum of wild type mice. Hemodynamic assessment was performed 24 hours after injection on mice at baseline and during AngII infusion (2 μg/kg/min).

An additional set of injections were performed on WT mice using ~5 x 10^{13} exosomes isolated from AT1R-YFP stable cells, then heart, skeletal muscle and mesenteric vessels were removed after 24 hours for histological analysis.

**Hemodynamic study (blood pressure response)**

After bilateral vagotomy, a polyethylene-50 (PE-50) catheter was inserted into the left axillary artery and connected to a Statham P23 Db pressure transducer (Gould Statham Instruments, Bayamon, Puerto Rico) for pressure monitoring. Blood pressure was recorded continuously with a pressure-recording system (MacLab, Millar Instruments, Houston, TX). Intravenous drug infusion was performed via the right jugular vein. Continuous infusion of AngII (2 μg/kg/min) was administered via the right jugular vein. Mean arterial pressure was recorded for 3 minutes before infusion (Basal).
and for 5 min after AngII infusion.

**Histology**

Wild type male mice (N=20) were injected through tail vein with a range between 4 to 5 x 10\(^{13}\) exosomes isolated from AT1R-YFP stable expressing cells. The day after the mice were sacrificed and tissues, after fixation in PFA (Paraformaldehyde) 4%, embedded in OCT compound and snap frozen prior to cryostat sectioning. For ex vivo confocal microscopy analysis, cryosections (7\(\mu\)m) of mice tissues, after ice cold fixation with PFA 4%, were immunostained for different markers using the following dilution of primary antibodies: Anti GFP (Invitrogen, A10262) 1:200, Anti Cardiac Troponin I 1:100 (AbCam ab19615), Anti PDGFRα (R&D systems AF1062) 20 \(\mu\)g/ml, Anti \(\alpha\)-actin SMC (AbCam 5694) 1:50, Anti CD31 (BD Pharmigen 553370), 1:50.

Secondary antibodies dilution were used as follows : Anti chicken FITC (Invitrogen, A16131) 1:500, Anti mouse Alexa Fluor 568 (Invitrogen, A10037) 1:200, Anti goat Alexa Fluor 633 (Invitrogen, A21082) 1:200, Anti rat Alexa Fluor 633 (Invitrogen, A21094) 1:200.

**Myocyte and Fibroblast Isolation**

Adult myocytes were isolated as described previously.\(^5,6\) Following anesthesia, the heart was excised and the aorta was cannulated with a 20-gauge needle then mounted on the perfusion apparatus. The perfusion solution was composed of Joklik’s minimum essential medium containing (in mmol/L) 113 NaCl, 4.7 KCL, 0.6 KH2PO2, 0.6 Na2PO4, 1.2 MgSO4, 0.5 MgCl2, 10 HEPES, 20 D-glucose, 30 taurine, 2.0 carnitine,
2.0 creatine, and 20 mM Ca2+ at pH 7.4. The aorta was perfused for 2–3 min, then 150 units/ml of type-II collagenase (Worthington) was added and perfused for 15 min. The temperature of perfusate was maintained at 34 °C and all solutions were continuously bubbled with 95% O2, 5% CO2. LV tissue was separated from the great vessels, atria and right ventricle, minced, and allowed to digest in perfusate for 15 min. The digested heart was filtered through 200 mm nylon mesh, placed in a conical tube, and spun at 250 x g to allow viable myocytes to settle. The supernatant was centrifuged at 250 x g in order to pellet down the cardiac fibroblast. Cardiomyocytes and fibroblast were placed in confocal dishes for immunostaining experiments.

**Western Blotting**

Western blot analysis was performed as previously described. Total cell lysate and exosome lysate were performed using NP-40 lysis buffer containing 20 mmol/L Tris (pH 7.4), 137 mmol/L NaCl, 1% NP-40, 20% glycerol, 10 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na3VO4, 10 mmol/L NaF, aprotinin (2.5 mg/ml), and leupeptin (2.5 mg/ml). Protein concentrations were assayed with Bio-Rad protein assay reagent, and 50 mg of protein was denatured by heating at 95°C for 5 min before resolving by SDS–polyacrylamide gel electrophoresis. Immunoblotting for ERK and phosphorylated ERK in HEK 293 cells after AngII (1μmol/L) stimulation was performed as previously described. The following dilutions of primary antibody were used: total ERK (Millipore), 1:3000; ERK1/2 (Cell Signaling), 1:1200; CD9 (Millipore) 1:1000; Calnexin (AbCam) 1:1000; CD63 (Santa Cruz) 1:1000; GAPDH (Santa Cruz) 1:1000; β-arrestin 2 (Lefkowitz laboratory) 1:1000. Detection was carried out by ECL (Amersham
Biosciences). Densitometric analysis was performed with Bio-Rad Fluoro-S Multi-Image software.

**β-arrestin 2 siRNA silencing in AT1R-YFP cells**

β-arrestin-targeting siRNA 21-nucleotide sequence was customized and bought from Quiagen as follows: β-Arrestin1 siRNA: 5'AAAGCCTTCTGCGCGGAGAAT-3' ; β-Arrestin2 siRNA: 5'AAGGACCGAAAGTGGTTTG-3', β-Arrestin1&2 siRNA: 5'AAACCTGCGCTTCCGCTATG-3' and Control siRNA: 5'-AATTCTCCGAACGTGTCACGT-3'. Cells were plated at 30 to 40% confluence in 10-cm dishes. siRNA (20 μg) was used with GeneSilencer Transfection reagent (Gene Therapy Systems). All experiments were performed 60 to 72 hours after siRNA transfection. Cells were serum-starved for 1 hour before stimulation.

**Exosomes isolation from Citrate plasma or serum of wild type mice**

Citrate plasma and serum were kept on ice upon blood collection to minimize platelets activation then stored at -80° C after centrifugation. Exosomes were purified, modifying previous methods⁹,¹⁰, by differential centrifugation at 4 °C, starting with a centrifugation at 1000 x g (15 min) and followed by centrifugations at 12,000 x g (20 min), 18,000–20,000 x g (20 min). The resulting supernatant was filtered through a 0.22 μm filter to obtain a solution free of cell debris, apoptotic bodies and microvesicles. Purified exosomes were then collected by ultracentrifugation at 100,000 x g (70 min) using a Ti70 rotor in a Beckman ultracentrifuge and resuspended in 100 μl of 1 X PBS.
Statistical analysis

Data are expressed as median with 1\textsuperscript{st} and 3\textsuperscript{rd} quartile. Statistical significance was determined by Kruskal-Wallis. Correction for multiple comparisons was made using a Dunn’s correction. Analysis of blood pressure (Supplemental Material) comparing Basal vs Ang II was performed using Wilcoxon signed rank test for repeated measurements within each group, while comparison of % changes of hemodynamic parameters between 3 or more independent groups was assessed by Kruskal Wallis. QT-PCR data were analyzed by Mann-Whitney t test (Supplemental Material). A threshold value of p<0.05 was considered statistically significant. All analyses were performed with GraphPad Prism version 6.01.
Supplemental References


Supplemental Figures and Tables:
Figure S1

A Anti CD9 (25 nm beads) Exosomes isolated from HEK 293 T cells after Osmotic Stretch 100 nm Alix Exo Calnexin CD 9 Cell lysate

B Exo Cell

KDa

95

67

42

25

37

Alix Calnexin CD 63 CD 9 GAPDH

C Exosomes isolated from HEK 293 T cells after Osmotic Stretch

D Exosomes isolated from AT1R-HA stable cells after:

No Stimulation Osmotic Stretch

E Analysis of size distribution in exosomes with different conditions

- Osmotic Stretch media; Mean size = 64.2 ± 0.7 (nm) (N=6)
- Serum of mice after TAC 7 days; Mean size = 60.7 ± 0.7 (nm) (N=6)
Figure S2

A  Exosomes isolated from media overlying AT1R stable cells

B  Gravity vs Vacuum after Osmotic Stretch

C  Exosomes isolated from Serum or citrated plasma

D  Serum vs Plasma after TAC
Figure S4

<table>
<thead>
<tr>
<th>Exosomes derived from overlying media of AT1R-YFP cells after</th>
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<th>Cardiac Fibroblast</th>
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Cardiomyocytes

Cardiac Fibroblast
Figure S5

A) Exosomes isolated from serum of mice

B) Number of AT1R per microliter of serum

C) Exosomes isolated from serum of mice

D) Exosomes isolated from serum of mice

Exosomes isolated from serum of mice

Number of AT1R per microliter of serum

Exosomes isolated from serum of mice

Exosomes isolated from serum of mice
Supplemental Figure Legends

Figure S1: Characterization of exosomes isolated from cell media and mice serum.

A) Isolated exosomes show CD9 on the vesicle surface following immune-gold labeling and visualization with T.E.M. (scale bar is 100 nm). B) Biochemical characterization of exosome and cell lysate blotted for the typical exosome markers Alix, CD63, CD9 and the cellular marker as Calnexin. C) Exosomes derived from conditioned HEK 293T cells showed the typical cup shape and ranged in size between 30 and 100 nm (scale bare = 100 nm). D) T.E.M. was used to identify CD9 and AT1Rs on the surface exosomes derived from conditioned media overlying HEK 293T AT1R-HA stable transfected cells before and after osmotic stretch. Exosomes derived from HEK 293T AT1R-HA stable transfected cells after osmotic stretch exhibited both CD9 (25 nm) and AT1R-HA (10 nm) on their surface. E) Size distribution analysis of exosomes isolated from media of cells after Osmotic stretch and from serum of mice after TAC 7 days. Exosomes isolated from different conditions showed a similar size distribution with a peak at around 60 nm. Data expressed as mean ± SEM.

Figure S2: Different methods of isolation do not affect exosomes concentration and size distribution.

A) Exosomes isolated from AT1R-HA stable expressing cells using Gravity or Vacuum filtration step showed similar concentration in no stimulation (~30,000 exosomes/μl) and after osmotic stretch condition (~150,000 exosomes/μl). B) Exosomes size distribution analysis showed a similar range size distribution of exosomes isolated with gravity
filtration step and exosomes isolated with vacuum filtration step. C) Exosomes isolate from citrate plasma showed a concentration similar to exosomes isolated from serum without citrate in SHAM and TAC condition. D) Circulating exosomes isolated from citrate plasma or serum showed a similar size distribution.

**Figure S3: β-arrestin2^{floxflox} mice characterization:**

A) PCR strategy to detect allele recombination after Tamoxifen treatment of β-arrestin 2^{floxflox} /αMHC^{Mer-Cre-Mer}. The recombination primers were used as follows: βarr2-1: 5'-GCTCCCTAACCAGGCAAGAG-3', βarr2-2: 5'-TGGGAAAGGACCCCAGGTAA-3', βarr2-3: 5'-ACGGAGGGTGGTACACATA-3'. Floxed gene band is 554 bp and null gene band is 409 bp. C) Representative recombination PCR of cardiomyocytes (CM) and Fibroblast (Fb) showing the presence of null allele only in CM of β-arrestin 2^{floxflox} /αMHC^{Mer-Cre-Mer} but not β-arrestin 2^{floxflox} after Tamoxifen treatment. C) Cardiomyocytes specific knock out of β-arrestin 2^{floxflox} /αMHC^{Mer-Cre-Mer} mice was confirmed by Real time PCR using Taqman probes showing ~80% reduction of β-arrestin2. D) Real Time PCR shows upregulation of β-arrestin2 in cardiac fibroblasts of β-arrestin 2^{floxflox} /αMHC^{Mer-Cre-Mer}. Data expressed as median with 1st and 3rd quartile. Statistical significance was determined by Mann-Whitney t-Test.

**Figure S4: Circulating AT1Rs deposit in cardiomyocytes rather than fibroblast**

Cardiomyocytes and cardiac fibroblasts were isolated from wild type hearts 24 hours after the intravenous injection of exosomes and immunostained for different cellular markers as indicated. AT1R green fluorescence was detected using GFP antibody
recognizing YFP. Cardiomyocytes were detected using cardiac troponin I (left), while fibroblast were positively stained for PDGFR-α (right). Consistent with the histology data, only cardiomyocytes were positively stained for YFP-AT1R. Cardiomyocytes were fixed and processed for the staining the day after of cell isolation. Cardiac fibroblasts were fixed and stained after 4 days in culture in order to obtain a sufficient cell confluence to perform confocal microscopy. Scale bar = 15 μm; N= 4.

**Figure S5: β-arrestin1 deletion has no effect on circulating exosomes containing AT1Rs secretion after TAC**

**A)** Exosomes concentration of circulating exosomes in serum of β-arrestin1 KO was similar to exosomes isolated from serum of WT mice. **B)** The amount of circulating AT1Rs within exosome secreted after TAC in serum was similar between β-arrestin1 KO and WT mice suggesting that the isoform 1 is not important for extracellular trafficking of AT1R. **C)** AT1R density in exosomes of β-arrestin 1 KO after TAC was similar to AT1R density in WT exosomes after TAC. **D)** Exosomes isolated from β-arrestin2 KO, global and cardiac specific, showed a blunted AT1R density after 1 week TAC suggesting that the absence of β-arrestin 2 impaired the AT1R packaging in exosomes during mechanical stress and circulating AT1Rs are secreted mostly by cardiomyocytes. Data expressed as median with 1st and 3rd quartile. Statistical significance was determined by Kruskal-Wallis with Dunn’s post hoc test. p = NS β-arrestin2<sup>flox/flox</sup>/αMHC<sup>Mer-Cre-Mer</sup> vs. β-arrestin2 KO
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Table S1: Hemodynamic parameter changes before and after Ang II infusion.

Hemodynamic parameters of WT mice and AT1R-KO mice used in the in vivo exosome injection experiments. Peripheral blood pressure was recorded for 3 minutes before AngII infusion (Basal) and for 5 minutes after AngII infusion. Data expressed as median with 1st and 3rd quartile. * p<0.05 Basal vs. AngII determined by Wilcoxon signed rank test for repeated measurements in each independent group. ** p<0.05 vs control group AT1R-KO (No Exo), † p<0.05 AT1R-KO (NS) vs. AT1R-KO (Osmotic Stretch), †† p<0.05 AT1R-KO (SHAM) vs. AT1R-KO (TAC), statistical significance determined by Kruskal-Wallis with Dunn’s test for multi comparison.

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<th>% increase of HR</th>
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<tr>
<td>13.5 (4.7-6.3)</td>
<td>403.5</td>
<td>403.5</td>
<td>1.4 (-1.7-2.6)</td>
</tr>
<tr>
<td>5.0 (12.1-14.4)**</td>
<td>381.5</td>
<td>389.7</td>
<td>0.4 (-0.2-0.8)</td>
</tr>
<tr>
<td>13.1 (12.1-14.4)**</td>
<td></td>
<td></td>
<td>2.4 (1.3-2.8)</td>
</tr>
</tbody>
</table>

% increase of MBP:** **p<0.05 vs. control group AT1R-KO (No Exo), † p<0.05 AT1R-KO (NS) vs. AT1R-KO (Osmotic Stretch), †† p<0.05 AT1R-KO (SHAM) vs. AT1R-KO (TAC) determined by Kruskal-Wallis with Dunn’s test for multi comparison.
Table S2. AT1R density in tissues after exosome injection

<table>
<thead>
<tr>
<th>Genotype</th>
<th>AT1R-KO</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exosomes Donors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Exosomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exosomes isolated from AT1R stable cells after:</td>
<td>Exosomes isolated from WT mice serum after:</td>
<td></td>
</tr>
<tr>
<td>NS</td>
<td>Osmotic Stretch</td>
<td>Sham</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart (fmol/mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N.D.</td>
<td>N.D.</td>
<td>8.3±2.0</td>
</tr>
<tr>
<td>Skeletal Muscle (fmol/mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N.D.</td>
<td>N.D.</td>
<td>10.8±2.4</td>
</tr>
<tr>
<td>Kidney (fmol/mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Lung (fmol/mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D.= Not Detectable
Table S2: Distribution of AT1R in membrane fractions of AT1R-KO mice derived from tissue biopsy: Heart, kidney, lung and skeletal muscle were removed following injection of exosomes and AT1R density was determined by radioligand binding. AT1Rs were detected in the heart and skeletal muscle, but not the lung or kidney of AT1R KO mice. Mean ± SEM.
심장에 압력 과부하를 주면 안지오텐신 II Type 1 수용체를 갖는 엑소좀이 혈액 내에서 증가한다

이 상연 교수 서울대학교병원 순환기내과

초록

배경
심장에 생물학적 혹은 물리적 과부하를 주면 심혈관 기능을 조절할 수 있는 엑소좀(exosome)의 분비가 증가할 수 있는지는 알려져 있지 않다. 본 연구는 심혈관 기능을 조절하는 혈 심 수용체인 안지오텐신 II type I 수용체(angiotensin II type I receptor, AT1R)를 갖는 엑소좀의 분비와 기능을 조사하였다.

방법 및 결과
압력 과부하 자극을 받은 AT1R 과발현 세포의 배양액이나 압력 과부하 생쥐 모델의 혈청에서 AT1R을 갖는 엑소좀을 분리하여 그 증가를 확인하였다. 엑소좀에서 AT1R이 발현하는지는 전자현미경과 radioligand receptor binding assay를 통해 조사하였다. 엑소좀에서 AT1R이 발현되기 위해서는 수용체의 이동에 해isOk적인 역할을 하는 다기능 조절 단백인 β-arrestin2가 필요하다. 압력 과부하를 받은 세포에서 발현되는 기능성 AT1R이 엑소좀을 통해 다른 세포로 전달되었다. β-arrestin2가 전체 조직 또는 심근세포에서만 결핍된 생쥐 모델을 이용하여 압력 과부하 상태에서 AT1R을 갖는 엑소좀은 심근세포에서 발현되다는 것을 발견하였다. AT1R knock-out 생쥐 모델에 AT1R을 갖는 엑소좀을 주입하면 심근세포, 근육세포, 그리고 장간막의 저항성 혈관에서 충분한 압력을 부가하여 안지오텐신 II에 대한 반응도 회복하였다.

결론
AT1R을 갖는 엑소좀은 과부하 상태의 심장에서 발현되어 혈관의 신경호르몬 자극에 대한 반응을 조절할 수 있다. 이는 혈액 내 엑소좀이 기능성 AT1R의 중요한 소스가 될 수 있을음을 보여 준다.

Basic Research