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Mechanical activation of the angiotensin II type 1 receptor contributes to abdominal aortic aneurysm formation

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ABSTRACT

Objective: The angiotensin II type 1 receptor (AT1R) can be activated under conditions of mechanical stretch in some cellular systems. Whether this activity influences signaling within the abdominal aorta to promote to abdominal aortic aneurysm (AAA) development remains unknown. We evaluated the hypothesis that mechanical AT1R activation can occur under conditions of hypertension (HTN) and contribute to AAA formation.

Methods: BPH/2 mice, which demonstrate spontaneous neurogenic, low-renin HTN, and normotensive BPN/3 mice underwent AAA induction via the calcium chloride model, with or without an osmotic minipump delivering 30 mg/kg/d of the AT1R blocker Losartan. Systolic blood pressure (SBP) was measured at baseline and weekly via a tail cuff. The aortic diameter (AoD) was measured at baseline and terminal surgery at 21 days by digital microscopy. Aortic tissue was harvested for immunoblotting (phosphorylated extracellular signal-regulated kinase-1 and -2 [pERK1/2] to ERK1/2 ratio) and expressed as the fold-change from the BPN/3 control mice. Aortic vascular smooth muscle cells (VSMCs) underwent stretch with or without Losartan (1 μM) treatment to assess the mechanical stimulation of ERK1/2 activity. Statistical analysis of the blood pressure, AoD, and VSMC ERK1/2 activity was performed using analysis of variance. However, the data distribution was determined to be log-normal (Shapiro-Wilk test) for ERK1/2 activity. Therefore, it was logarithmically transformed before analysis of variance.

Results: At baseline, the SBP was elevated in the BPH/2 mice relative to the BPN/3 mice ($P < .05$). Losartan treatment significantly reduced the SBP in both mouse strains ($P < .05$). AAA induction did not affect the SBP. At 21 days after induction, the percentage of increase in the AoD from baseline was significantly greater in the BPH/2 mice than in the BPN/3 mice (101.28% ± 4.19% vs 75.59% ± 1.67% above baseline; $P < .05$). Losartan treatment significantly attenuated AAA growth in both BPH/2 and BPN/3 mice (33.88% ± 2.97% and 43.96% ± 3.05% above baseline, respectively; $P < .05$). ERK1/2 activity was increased approximately fivefold in the BPH/2 control mice relative to the BPN/3 control mice ($P < .05$). In the BPH/2 and BPN/3 mice with AAA, ERK1/2 activity was significantly increased relative to the respective baseline control ($P < .05$) and effectively reduced by concomitant Losartan therapy ($P < .05$). Biaxial stretch of the VSMCs in the absence of angiotensin II demonstrated increased ERK1/2 activation ($P < .05$ vs static control), which was significantly inhibited by Losartan.

Conclusions: In BPH/2 mice with spontaneous neurogenic, low-renin HTN, AAA growth was amplified compared with the normotensive control and was effectively attenuated using Losartan. ERK1/2 activity was significantly elevated in the BPH/2 mice and after AAA induction in the normotensive and hypertensive mice but was attenuated by Losartan treatment. These data suggest that AT1R activation contributes to AAA development. Therefore, further investigation into this signaling pathway could establish targets for pharmacotherapeutic engineering to slow AAA growth. (JVS—Vascular Science 2021;2:194-206.)

Clinical Relevance: Hypertension (HTN) and abdominal aortic aneurysm (AAA) have been epidemiologically linked for decades; however, a biomechanical link has not yet been identified. Using a murine model of spontaneous neurogenic HTN experimentally demonstrated to have low circulating renin, mechanical activation of the angiotensin II type 1 receptor (AT1R) was identified with elevated blood pressure and AAA induction. HTN amplified AAA growth. However, more importantly, blocking the activation of AT1R with the angiotensin receptor blocker Losartan effectively abrogated AAA development. Although inhibiting the production of angiotensin II has previously been unsuccessful in altering AAA growth, the results from the present study suggest that blocking the activation of AT1R through direct ligand binding or mechanical stimulation might alter aortic wall signaling and warrants further investigation.

Keywords: Aortic aneurysm; Angiotensin II type 1 receptor; Losartan; Vascular remodeling; Hypertension

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Despite advances in the surgical management of abdominal aortic aneurysms (AAAs), no medical therapy has been found to be effective in slowing disease progression.[1] An epidemiologic association exists between AAA development and hypertension (HTN), although a biomechanical link has not yet been established. Angiotensin II (AngII), an endogenous vasoactive peptide involved in blood pressure autoregulation, has been shown to be upregulated in human AAAs.[2] Antagonism of AngII signaling, using either angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs), has been shown to reduce AAA development in various animal models.[3–6] ARBs attenuated AAA formation in the AngII-infusion model using ApoE$^{-/-}$ mice and in the intra-aortic porcine pancreatic elastase model using C57BL/6 mice.[6] AAA development via the porcine pancreatic elastase model was also inhibited in AT1aR knockout mice, highlighting the importance of AngII signaling in this disease process.[5] Despite these promising results in animal studies, disruption of AngII signaling has been ineffective in slowing AAA progression in human trials. In the AARDVARK (aortic aneurysmal regression of dilation: value of ACE-inhibition on risk) trial, no difference in annual AAA growth rate was observed between patients receiving ACE inhibitors, β-blockers, and placebo.[7]

In vascular smooth muscle cells (VSMCs), AngII signals through the AngII type 1 receptor (AT1R) to activate cascades of intracellular events, which can alter contraction, cell growth, migration, extracellular matrix deposition, and production of inflammatory cytokines.[8] Several of these cascades subsequently converge to phosphorylate and activate members of the mitogen-activated protein kinases, in particular, the extracellular signal-regulated kinase-1 and -2 (ERK1/2), which translocates to the nucleus and phosphorylates transcription factors to affect gene expression.[8,9] Mounting evidence has shown that the AT1R can undergo mechanical activation in the absence of ligand binding. COS7 cells expressing AT1R with a mutated ligand-binding site did not respond to AngII treatment but demonstrated a conformational shift, mechanical stress-induced AT1R activation, and ERK1/2 phosphorylation that was inhibited by concomitant treatment with ARBs.[10,11] Furthermore, it was demonstrated that in cardiomyocytes isolated from angiotensinogen knockout mice, which do not express detectable levels of AngII, mechanical stretch resulted in AT1R activation and pretreatment of these cells with ARBs attenuated ERK1/2 phosphorylation.[10,11] Because mechanical activation occurs independently of ligand binding, it is unaffected by ACE inhibition, indicating that ARBs might be more effective at inhibiting this AT1R signaling trigger.

Mechanical activation of the AT1R has not been evaluated in the healthy abdominal aorta or in AAAs; however, given the epidemiologic association between HTN and AAA, mechanical AT1R activation might play a major role in these disease states. In the present study, we sought to demonstrate that mechanical activation of AT1R can occur in HTN, can contribute to murine AAA formation, and might be attenuated by AT1R blockade.

ARTICLE HIGHLIGHTS

- **Type of Research:** An original basic science research study
- **Key Findings:** Losartan decreased the systolic blood pressure in mice with spontaneous neurogenic, low-renin hypertension (HTN) and normotensive control mice. Abdominal aortic aneurysm **(**AAA) growth was augmented by HTN and abrogated by Losartan. Amplified extracellular signal-regulated kinase-1 and -2 (ERK1/2) activity observed in HTN and AAA was inhibited by Losartan therapy.
- **Take Home Message:** HTN was associated with increased AAA growth and increased ERK1/2 activity. Angiotensin II type 1 receptor blockade with Losartan antagonized both AAA growth and ERK1/2 activation, suggesting that angiotensin II type 1 receptor activation, either by direct ligand binding or mechanical stimulation, contributed to AAA growth and could be an effective pharmacotherapeutic target.

METHODS

The Medical University of South Carolina institutional animal care and use committee approved all animal care and surgical procedures (approval no. 00209).

Murine hypertension model. The commercially available BPH/2 mouse strain (Jackson Laboratories, Bar Harbor, Me) was used because it develops spontaneous neurogenic HTN, which begins at 5 weeks of age and peaks at 21 weeks. The normotensive BPN/3 mouse strain, also commercially available from Jackson Laboratories, was used as a control.

Several investigations have demonstrated that the HTN in BPH/2 mice is mediated by overactivity of the sympathetic nervous system owing to upregulated signaling in the medial amygdala, a known cardiovascular regulatory region.[12,13] The contribution of the renin-angiotensin system (RAS) has remained controversial. Although central RAS signaling has been effectively eliminated as a major component,[14] the influence of the peripheral RAS continues to be considered owing to reports of significant blood pressure decreases in BPH/2 mice treated with ACE inhibitors.[15] However, the investigators could not conclusively state that the peripheral RAS had caused the HTN in BPH/2 mice because they had not accounted for the effect of bradykinin, a potent vasodilator known

to show increased activity as a result of ACE inhibitor treatment and to lead to decreased systolic blood pressure.[16–18]

Previous studies from our laboratory have demonstrated differences in regional gene expression and protease production between mice with AngII-induced HTN and the spontaneous neurogenic HTN in the BPH/2 strain, further supporting the fundamental differences in the RAS contribution to HTN in this mouse strain.[19] Moreover, low circulating and tissue concentrations of angiotensin I in BPH/2 mice have been reported.[17,18] Considering these conflicting descriptions of the factors influencing HTN in the BPH/2 mouse strain and how they might influence comparisons with the BPN/3 normotensive control mice, we analyzed our mouse colony regarding AT1R abundance in the infrarenal abdominal aorta, plasma renin level, and plasma bradykinin level. The method has been described in detail in the Supplementary Methods, and the data have been included in Supplementary Figs 1 to 3.

In brief, the abundance of AT1R was not significantly different between the BPH/2 and BPN/3 mice (P = NS, t test; Supplementary Fig 1). The baseline aortic diameters also did not differ between the BPH/2 (n = 28; 404 ± 7 μm) and BPN/3 (n = 32; 415 ± 5 μm) mice (P = NS, t test; Supplementary Fig 2). Significantly lower plasma renin levels were found in the BPH/2 mice (7308.4 ± 561.6 pg/mL vs 15,537.2 ± 2104.6 pg/mL; $P < .05$, t test; Supplementary Fig 3). The bradykinin levels were equivalent (220.7 ± 31.7 pg/mL in the BPH/2 mice vs 225.1 ± 19.5 pg/mL in the BPN/3 mice; P = NS, t test). Therefore, the BPH/2 mice can be characterized as having spontaneous, neurogenic low-renin HTN, permitting study of AT1R activation in a physiologic system with negligible circulating ligand, which can be extrapolated to mechanical stimulation.

AAA induction. After induction of anesthesia with 3% isoflurane and subcutaneous injection of 0.05 mg/kg buprenorphine, BPN/3 and BPH/2 mice aged 12 to 20 weeks underwent midline laparotomy. Intraoperatively, the heart rate was continuously monitored. The abdominal aorta was exposed from the left renal vein to the aortic bifurcation, and the baseline aortic diameter (AoD) was measured using calibrated digital microscopy. A sponge soaked in 0.5 M calcium chloride ($CaCl_2$) was placed directly against the aortic adventitia for 10 minutes. The sponge was then removed, and the abdomen was closed. The mice were allowed to recover in a heated and oxygenated chamber before being returned to their home cage. In addition, sham-induced BPN/3 and BPH/2 mice were treated with a sponge soaked in NaCl in a similar fashion.

Osmotic minipump implantation. Losartan potassium was dissolved in sterile saline and loaded into Alzet osmotic minipumps (model 1004; Durect Corp, Cupertino, Calif) to achieve an infusion rate of 30 mg/kg/d.[20] After induction of anesthesia using 3% isoflurane, the BPH/2 and BPN/3 mice underwent subcutaneous placement of the Alzet osmotic minipump via a left flank incision. In the mice undergoing both AAA induction and Losartan infusion, the osmotic minipump was placed 24 hours after the AAA induction procedure.

Blood pressure measurement. The mouse blood pressure was measured before and weekly after induction of AAA and pump implantation using the CODA8 tail-cuff method (Kent Scientific, Torrington, Conn), as described previously.[19] All systolic blood pressure (SBP) measurements were conducted between 9 AM and 12 PM for the duration of the study. The mice were placed in a restraint corridor and allowed ≥10 minutes of acclimation. The area was warmed with a heating pad, and a dark, quiet environment was maintained to facilitate accurate measurements. Each mouse had three cuff cycling events at each measurement point, and an average of the validated values was recorded as the SBP for that mouse on that day.

Tissue harvest and processing. Terminal procedures were performed at 21 days. After the induction of anesthesia, laparotomy was performed. The AoD was measured using digital microscopy, and the abdominal aorta was then harvested from the left renal vein to the level of the aortic bifurcation. The endothelium was denuded, and the aorta was snap-frozen in liquid nitrogen. The aortic samples were homogenized in extraction buffer using the Bullet Blender Tissue Homogenizer (Next Advance, Troy, NY), and the protein concentration was determined using the Pierce BCA total protein assay (ThermoFisher Scientific, Waltham, Mass).

Immunoblot analysis. The relative abundance of phosphorylated ERK1/2 (pERK1/2), total ERK1/2, and β-actin was determined by immunoblotting. In brief, 15 μg of aortic tissue homogenate was loaded into each well of a 4% to 12% Bis-Tris gradient gel (Invitrogen Corp, Carlsbad, Calif) and fractionated by electrophoresis. The fractionated proteins were transferred to nitrocellulose membranes (0.45 mm; Bio-Rad, Hercules, Calif) and blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline for 1 hour at room temperature (RT). The membranes were incubated in antiserum specific for pERK1/2 (1:5000 in 5% BSA), ERK1/2 (1:5000 in 5% BSA), and β-actin (1:500 in 5% BSA) for an additional 75 minutes at RT. After incubation with the primary antibody, the membranes were repeatedly washed to reduce nonspecific antibody interactions. Secondary peroxidase-conjugated antibodies were then applied (1:10,000; 5% BSA) and allowed to incubate for 1 hour at RT. The membrane was again washed before imaging. Immunoreactive signals were detected using a chemiluminescent substrate (Western Lightning

Chemiluminescence Reagent Plus; Perkin Elmer, Akron, Ohio) and imaged using the Amersham Imager 600 series (GE Healthcare, Chicago, Ill). Between immunoblotting for each protein of interest, the membranes were stripped using the Blot Restore Membrane Rejuvenation Kit (EMD Millipore, Burlington, Mass). The images were converted to TIFF format, and the band intensity was quantified using Gel-Pro Analyzer, version 3.1.14 (Media Cybernetics, Silver Spring, Md).

FlexCell analysis of VSMCs. Primary VSMC lines from murine aortic biopsies were established using an accepted outgrowth technique, as previously described.[21] The isolated VSMCs were maintained in smooth muscle cell–specific growth media with an added supplement pack (SMC Growth Medium 2; C-22062; PromoCell, Heidelberg, Germany) at 37°C in 5% carbon dioxide. Confluent VSMCs from culture passages 2 to 10 were seeded at a density of 5000 cells/cm^2 into amino acid–coated BioFlex-6 well plates (BF-3001A; FlexCell International Corp, Burlington NC) and allowed to adhere overnight. Complete VSMC media were then replaced with serum-free media (no AngII). After 6 hours of serum starving, the media were replaced with fresh serum-free media with and without Losartan (1 μM). The plates were held under static conditions or subjected to 12% biaxial cyclic tension for 3 hours using a FlexCell culture system (FlexCell International Corp). The cell lysate was harvested to quantify the ratio of pERK1/2 to ERK1/2.

Assay to quantify ERK1/2 activation. Immediately after completion of FlexCell treatment with or without Losartan, the cells were washed with phosphate-buffered saline and lysed. Collected cell lysates were plated on InstantOne enzyme-linked immunosorbent assay 96 well plates (ThermoFisher Scientific). Two wells per sample were treated with pERK1/2 antiserum and the remaining two wells with ERK1/2 antiserum. After 1 hour of incubation shaking at RT, the wells were washed with a washing buffer. The TMB (3,3′,5,5′-tetramethylbenzidine) detection reagent was added to each sample and underwent react shaking at RT in the dark for 30 minutes. At exactly 30 minutes, the reaction was stopped, and the plates were read three times at 450 nm using a SpectraMax M3 plate reader (Molecular Devices LLC, San Jose, Calif). Duplicate wells from each sample were averaged and analyzed for the ratio of pERK1/2 to ERK1/2.

Statistical analysis. The SBP was compared among treatment groups at each measurement point using repeated measures analysis of variance (ANOVA). The proportion of increase in AoD was calculated as previously described [(terminal AoD − baseline AoD)/baseline AoD × 100] for each individual mouse, with the baseline control AoD represented as 100% and differences between groups compared using ANOVA.[22] The data are presented as the mean ± standard error of the mean. The pERK1/2 to ERK1/2 ratio was calculated for each sample and compared among the groups. The normality of the data was determined using the Shapiro-Wilk test, and non-normally distributed results were logarithmically transformed before ANOVA. Summary statistics were performed on the transformed data and reported as the geometric mean ^{x}/geometric standard error of the mean.[23] Statistical tests were performed using STATA, version 15 (StataCorp, College Station, Tex). A power analysis was conducted using the preliminary data from our laboratory regarding the extent of aortic dilation with periadventitial $CaCl_2$ application. A sample size of five mice per group was determined to provide ≥80% power ($\alpha = .05$) to detect global differences.

RESULTS

Losartan decreased SBP in BPN/3 and BPH/2 mice. The baseline (day 0) SBP measurements were significantly elevated in all BPH/2 mice relative to the BPN/3 controls ($P < .05$; Fig 1, *A*). In the absence of intervention, the blood pressure of the BPH/2 and BPN/3 mice remained stable during the 21-day treatment period (Fig 1, *B-D*). In the BPH/2 + Losartan group, the SBP was significantly decreased on days 7, 14, and 21 ($P < .05$ vs BPH/2) but remained elevated compared with that of the normotensive BPN/3 controls (Fig 1, *B-D*). Thus, although Losartan produced a decrease in SBP in the hypertensive BPH/2 mice, these mice continued to demonstrate significantly elevated SBP compared with the normotensive controls. In the BPH/2 + AAA group, the SBP was not altered by the development of AAA at any time point (Fig 1, *B-D*). Finally, in the BPH/2 + AAA + Losartan group, the SBP was significantly decreased on days 7, 14, and 21 ($P < .05$; Fig 1, *B-D*). The SBP in the latter group had decreased and was consistent with that of the BPN/3 controls at 7 and 14 days (Fig 1, *B* and *C*) but had rebounded to be significantly elevated by 21 days ($P < .05$; Fig 1, *D*).

The BPN/3 mice responded to Losartan treatment in a similar fashion. The BPN/3 + Losartan treatment group had had a significant decrease in SBP on days 7, 14, and 21 ($P < .05$; Fig 1, *B-D*). Similarly, in the BPN/3 + AAA + Losartan group, the SBP was significantly decreased at all time points after AAA induction ($P < .05$ vs BPN/3; Fig 1, *B-D*). AAA induction alone did not significantly affect the SBP.

$CaCl_2$-induced AAA formation was augmented in BPH/2 mice and attenuated by Losartan. All changes in AoD are presented as the percentage of change from the baseline measurement for each individual mouse (Fig 2). The overall baseline AoD for the BPN/3 and BPH/2 mice was not significantly different (P = NS; Supplementary Fig 3). In the sham procedures, application of the NaCl-soaked sponge did not produce a

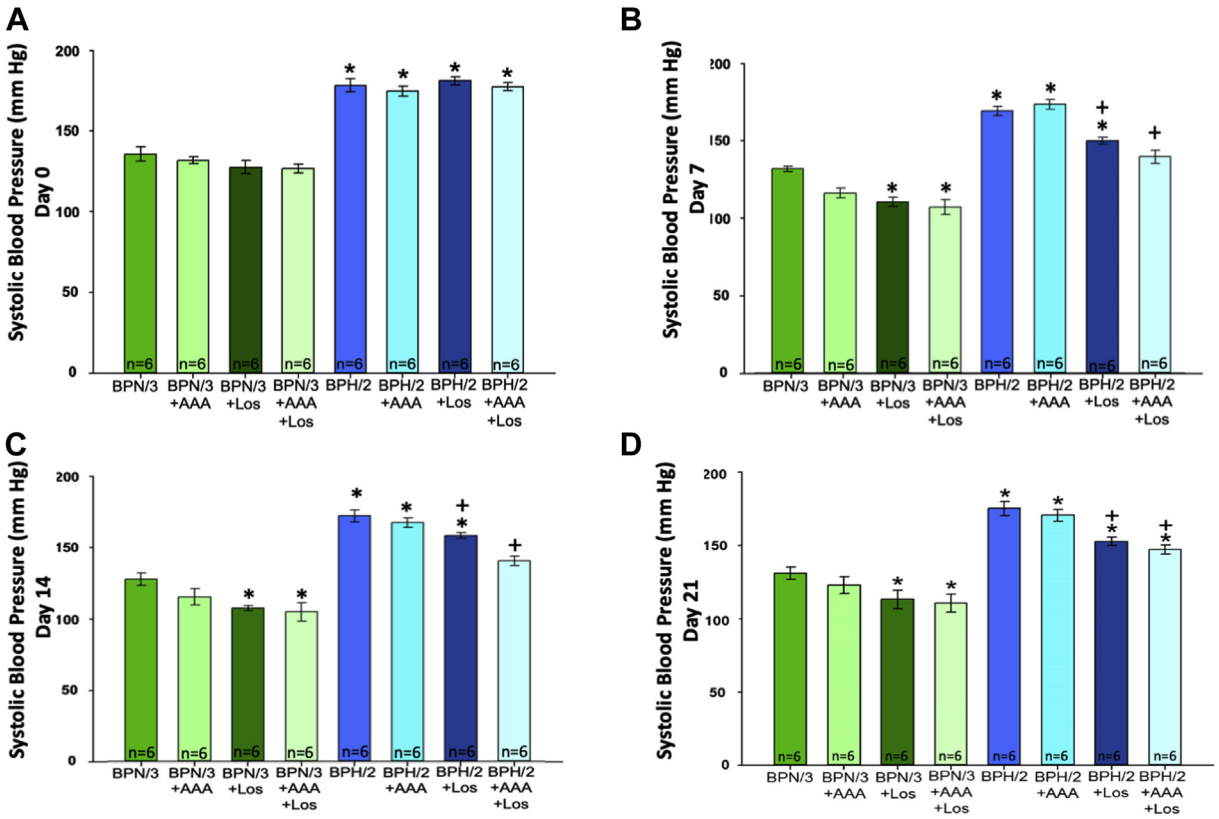

Fig 1. Systolic blood pressure (SBP ±standard error) readings via Coda tail cuff system at days 0 **(A)**, 7 **(B)**, 14 **(C)**, and 21 **(D)**. *$P < .05$ vs BPN3 control; +$P < .05$ vs BPH2 control; analysis of variance (ANOVA) for both. *AAA,* Abdominal aortic aneurysm; *Los,* Losartan.

significant increase in the AoD in either the BPN/3 or BPH/2 mice. For the BPN/3 + AAA mice, an AoD increase of 75.59% ± 1.67% above baseline had occurred ($P < .05$). In the BPN/3 + AAA + Losartan group, the AoD had increased compared with baseline ($P < .05$) but was significantly attenuated relative to the BPN/3 + AAA group, measuring only 43.96% ± 3.05% above baseline ($P < .05$). In the BPH/2 + AAA mice, the increase in the AoD was 101.28% ± 4.19% above baseline, significantly greater than that observed in the BPN/3 + AAA group ($P < .05$), suggesting that AAA formation was enhanced by concurrent spontaneous neurogenic, low-renin HTN. AAA development was attenuated in the BPH/2 + AAA + Losartan group (33.88% ± 2.97% above baseline) compared with the BPH/2 + AAA group ($P < .05$). Thus, Losartan abrogated AAA growth in the normotensive BPN/3 mice and was also effective in the hypertensive BPH/2 mice, a novel finding that suggests that the AT1R pathway contributes to degenerative remodeling.

ERK1/2 activity was increased in BPH/2 mice and by AAA induction and antagonized by Losartan treatment. ERK1/2 activity, as quantified by the pERK1/2 to ERK1/2 ratio, was calculated and compared among the treatment groups after logarithmic transformation. The BPN/3 + Losartan group demonstrated a paradoxical increase in ERK1/2 activity relative to the BPN/3 control group (1.66 ^{x}/1.26 vs 0.42 ^{x}/1.27; $P < .05$; Fig 3, *A*). In the BPN/3 + AAA group, ERK1/2 activity was increased eightfold relative to the BPN/3 control (3.35 ^{x}/1.54 vs 0.42 ^{x}/1.27; $P < .05$; Fig 3, *A*). However, in the BPN/3 + AAA + Losartan group, ERK1/2 activity showed a trend toward returning to baseline ($P = .08$; Fig 3, *A*). A representative image of the immunoblot is shown in Fig 3, *B*.

BPH/2 mouse ERK1/2 activity was increased approximately fivefold relative to the BPN/3 control mice (2.20 ^{x}/1.20 vs 0.42 ^{x}/1.27; $P < .05$; Fig 3, *A*), suggesting that AT1R activation was elevated in the abdominal aorta in the presence of neurogenic, low-renin HTN. Alternatively, ERK1/2 activity in the BPH/2 + Losartan group was significantly reduced ($P < .05$ vs BPH/2) and was not different from the normotensive BPN/3 control mice ($P =$ NS). For the BPH/2 + AAA mice, the ERK1/2 activity was amplified relative to the BPN/3 mice (3.09 ^{x}/1.27 vs 0.42 ^{x}/1.27; $P < .05$). However, the variance among the samples prohibited the establishment of a significant difference compared with the BPH/2 controls (Fig 3, *A*). In the BPH/2 + AAA + Losartan group, the ERK1/2 activity was significantly decreased relative to the BPH/2 + AAA group ($P < .05$; Fig 3) and consistent with that of the

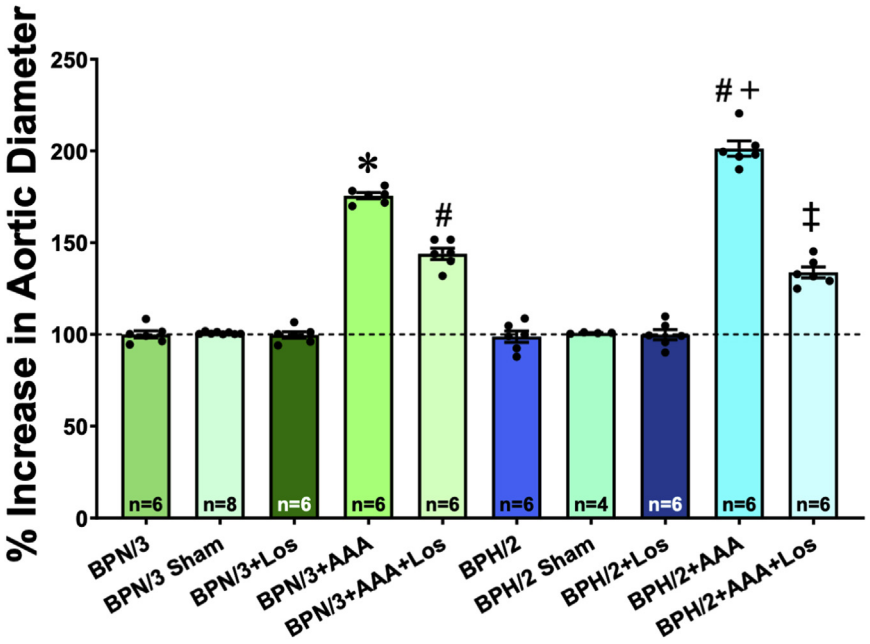

Fig 2. Percentage of increase of aortic diameter (AoD) above baseline (100%) for BPN3 mice **(A)** and BPH2 mice **(B)**. Individual data represented by *dots; bars* indicate mean ± standard error of the mean. *$P < .05$ vs BPN3 control; #$P < .05$ vs BPN3 + abdominal aortic aneurysm (*AAA*); +$P < .05$ vs BPH2 control; ‡$P < .05$ vs BPH2 + AAA; analysis of variance (ANOVA) for all. *Los,* Losartan.

BPN/3 control mice (P = NS; Fig 3, *A*). We found physiologic symmetry such that AT1R blockade with Losartan decreased ERK1/2 activity and inhibited AAA development, supporting activation of the AT1R with downstream ERK1/2 phosphorylation as an integral signaling pathway in the development of AAAs.

Mechanical activation of AT1R in aortic VSMCs. Primary aortic VSMCs were maintained in serum-free media to minimize activation of confounding signaling pathways, including, but not limited to, ligand-specific binding of AngII, and assessing stretch as a mechanical stimulant for ERK1/2 activation (Fig 4). Under Static conditions, treatment with Losartan had no effect on ERK1/2 activation (P = NS). However, 3 hours of cyclic Stretch induced a significant increase compared with the Static control state (1.81 ± 0.22-fold change; $P < .05$), indicating that ERK1/2 phosphorylation can be mechanically stimulated in aortic VSMCs. Blocking AT1R with Losartan concurrent with the Stretch treatment significantly reduced this response (1.38 ± 0.20-fold change in the Stretch + Losartan group vs 1.81 ± 0.22-fold change in the Stretch group; $P < .05$) and brought ERK1/2 activation back to equivalence with the Static control (P = NS). This in vitro assessment of ERK1/2 phosphorylation in the presence and absence of Stretch and the abrogation of the response during treatment with Losartan supports mechanical activation of AT1R as a major trigger for ERK1/2 activation in aortic VSMCs.

DISCUSSION

Experimental evidence has suggested that AT1R can be activated by mechanical stretch. However, this has not been previously examined in AAAs. Using a validated periadventitial $CaCl_2$ model of murine AAAs in a mouse strain that develops spontaneous neurogenic HTN with low circulating renin (evidence indicating that the HTN is not primarily dependent on AngII signaling), we have demonstrated that HTN augments AAA development, that AT1R inhibition with Losartan abrogates aortic degenerative remodeling, and that ERK1/2 signaling is amplified in the presence of HTN and AAA but is effectively attenuated by AT1R inhibition. Mechanical activation of AT1R to increase ERK1/2 activity was further confirmed in aortic VSMCs. Collectively, these findings suggest that mechanical activation of AT1R can contribute to AAA development, supporting further investigation into the potential therapeutic benefit of ARB therapy in patients with AAAs.

Epidemiologically, the association between HTN and the incidence of AAA has been known for many years. However, a recent retrospective review found that HTN was also a significant predictor of AAA expansion.[24] Looking to animal models to confirm this phenomenon, spontaneous hypertensive rats with AAA induction via elastase infusion resulted in a nearly twofold increase in the aortic growth rate relative to Wistar-Kyoto normotensive controls and significantly larger overall AAA diameter.[25] In a separate study using Wistar-Kyoto rats with

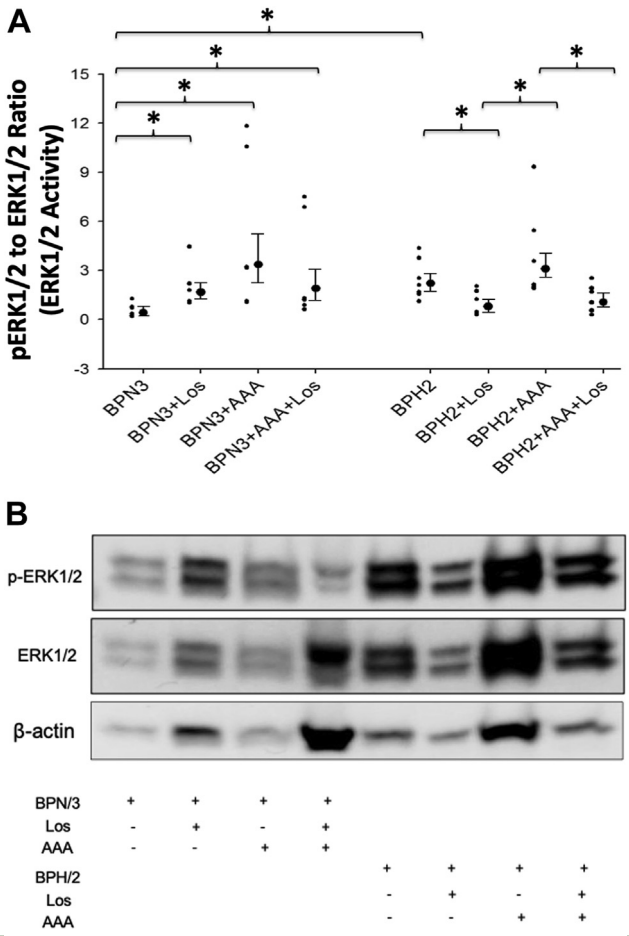

Fig 3. Extracellular signal-regulated kinase-1 and -2 (ERK1/2) activity logarithmically transformed and compared using analysis of variance (ANOVA) is represented as the geometric mean ˣ/geometric standard error in BPN3 and BPH2 mice treated with Losartan (*Los*) or abdominal aortic aneurysm (*AAA*) induction, or both. **A,** $^{*}P < .05$ with bracket indicating comparative groups. **B,** Representative immunoblot. *Los,* Losartan; *pERK1/2,* phosphorylated ERK1/2.

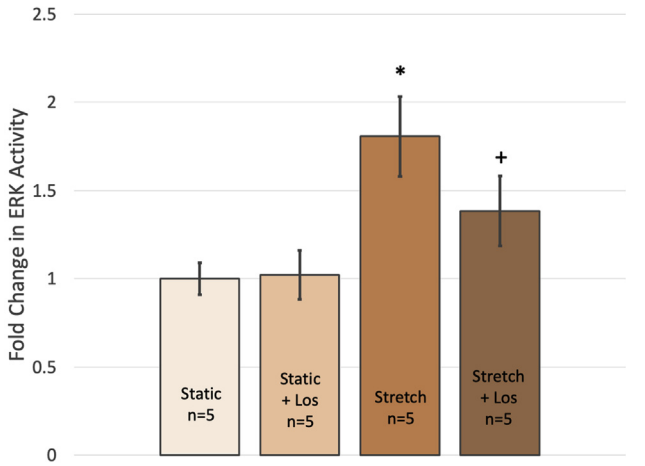

Fig 4. The fold change in extracellular signal-regulated kinase-1 and -2 (*ERK1/2*) activity in aortic vascular smooth muscle cells (VSMCs) subjected to cyclic Stretch with or without Losartan (*Los*) normalized to VSMCs held at Static. $^{*}P < .05$ vs Static; $+P < .05$ vs Stretch; analysis of variance (ANOVA) for both.

HTN by nephrectomy, it was again demonstrated that AAA induction by elastase infusion in the hypertensive cohort resulted in significantly larger AAAs compared with that of normotensive rats.[26] Using a mouse strain with spontaneous, neurogenic, low-renin HTN, the present study has likewise provided support for augmented AAA development with concurrent HTN, and we have begun to explore integral pathways for mechanical signaling.

Mechanical activation of the AT1R has been well-described in cardiomyocytes but has not been fully explored in aortic VSMCs. Recent experimental evidence has suggested that peripheral arteriolar VSMC mechanical sensitivity can be mediated by AT1R activation.[27-30] The myogenic reflex refers to the phenomenon by which arteriolar vasoconstriction or dilatation occurs in response to changes in intraluminal pressure to maintain consistent blood flow.[29] In rat skeletal muscle arterioles, mechanical activation of the AT1R was demonstrated to occur in the absence of AngII and resulted in polymerization and reorganization of the actin cytoskeleton to produce myogenic vasoconstriction.[30] Additionally, using pressure myography in isolated rat cremasteric arterioles, ARB treatment inhibited pressure-induced vasoconstriction in a concentration-dependent manner.[30] Further targeting mechanical AT1R signaling, myogenic reactivity of renal and mesenteric VSMCs was abolished in AT1R knockout mice ($Agtr1a^{-/-}$).[27,28] Although the present investigation included a normotensive mouse strain with an intact RAS axis and confirmed that blockade of AT1R with Losartan therapy attenuated AAA expansion as demonstrated in previous studies,[3-6] we also incorporated a mouse model with neurogenic, low-renin HTN. In these BPH2 mice, AAA expansion was similarly attenuated with concomitant ARB therapy, suggesting that AT1R activation occurred despite minimal circulating ligand, was inhibited by Losartan, and significantly affected intracellular signaling pathways that promoted degenerative aortic remodeling.

Evolving research on the molecular interaction of AT1R, ligand binding, non—ligand-dependent activation, and ARBs has demonstrated that these drugs function as inverse agonists.[10] Inverse agonists are defined as compounds that bind to a given receptor and induce the opposite pharmacologic response induced by agonist binding, in contrast to competitive antagonists, which compete for the ligand binding site and, thereby, prevent agonist binding. A recent receptor mutagenesis study further supported the potent inverse agonist activity of ARBs at the AT1R.[31] Losartan has demonstrated weak inverse agonist activity; however, its major metabolite

EXP3174 was much stronger in previous mutagenesis studies and investigations of myogenic reactivity.[32,33] It is this inverse agonist activity that enables ARBs to inhibit mechanical and ligand-specific activation of AT1R and might extend their therapeutic capabilities beyond that of AngII-driven vascular pathology to modulate tension-derived cell signaling throughout the vasculature and, specifically, within the abdominal aorta.

To investigate this signaling phenomenon in the murine abdominal aorta, ERK1/2 activity was used as a reliable marker of AT1R activation. A G-protein–coupled receptor such as AT1R uses ERK1/2 as a major effector of intracellular signaling and conformational changes at the receptor level can lead to ERK1/2 phosphorylation through the G-protein–derived pathways (eg, via Ras, protein kinase C, or tyrosine kinases) or the β-arrestin pathway.[8,34] The apparent convergence of several AT1R-driven signaling pathways on ERK1/2 emphasizes the importance of measuring the activity of this kinase as evidence grows to demonstrate that ligand-dependent and ligand-independent (mechanical) activation of AT1R engage different intracellular cascades.[35] More specifically, mechanical AT1R activation has been repeatedly associated with the β-arrestin pathway and effectively quantified by ERK1/2 phosphorylation.[36-38] Additionally, ARBs have shown efficacy in the inhibition of mechanical AT1R activation, as quantified by a decrease in ERK1/2 phosphorylation.[10] Because the present investigation used one mouse strain with a normal RAS and one in which this system has minimal circulating ligand, using ERK1/2 phosphorylation as an indicator of AT1R activation allowed the capture of both stimuli and the opportunity to effectively block both stimuli with Losartan. The ERK1/2 activity inhibited by Losartan in a mouse strain characterized as neurogenic, low-renin HTN can then be largely extrapolated to mechanical activation at AT1R. This contention has been further supported by the in vitro VSMC mechanical activation of ERK1/2 that was significantly and nearly completely abrogated by Losartan.

In the present investigation exploring the activation of AT1R in AAA growth under hypertensive (BPH/2) and normotensive (BPN/3) conditions, the BPH/2 mice had increased ERK1/2 activity relative to BPN/3 mice, even at baseline. Furthermore, with AAA induction, ERK1/2 activity was elevated in both mouse strains and effectively attenuated by Losartan treatment, supporting this pathway as a major contributor to AAA growth. To be more specific, activation of the AT1R and increased downstream ERK1/2 activity were observed in the spontaneous neurogenic, low-renin hypertensive BPH/2 mice at baseline and after AAA induction, leading to the interpretation that ligand-directed signaling has little effect on AT1R activation and supporting the mechanical activation of AT1R under conditions of elevated aortic wall tension (HTN).

Extrinsic hemodynamic and intrinsic biomechanical forces might contribute to stress within the vascular system. Therefore, simply treating HTN might not be adequate to quench mechanotransduction within the aortic wall. Previous studies have reported a positive association between AAA growth and biomechanical markers of aortic wall stress, suggesting that intrinsic wall tension itself might contribute to greater aortic expansion.[39] In the BPN3 normotensive mouse strain used in the present investigation, the efficacy of Losartan in attenuating AAA growth might have been attributable to ligand blocking. However, based on the theory of increased intrinsic aortic wall tension when AAA develops (the law of LaPlace), this medical therapy could also have been blocking mechanical AT1R activation. With the present evidence that activation of AT1R contributes to AAA development and can be effectively abrogated with ARBs, further mechanistic and prospective studies are warranted to fully characterize this pharmacotherapeutic opportunity.

Study limitations. As briefly discussed, activation of AT1R might stimulate multiple signaling pathways and future investigation of the breadth of the effects of AT1R activation are necessary to fully characterize the contribution to aortic remodeling. In the present project, activation of AT1R was measured by phosphorylation of ERK1/2, a known major downstream kinase; however, this is not an exclusive association. ERK1/2 participates in multiple signaling cascades; therefore, it is possible that alternative compensatory phosphorylation of this kinase might have occurred when AT1R was blocked. Additionally, it is possible that the decrease in SBP noted in the BPN/3 + Losartan mice altered the signaling through another mechanosensitive pathway, thereby inhibiting aortic dilation but without a direct association with the blockade of AT1R. The influence of these alternative signaling cascades will be further explored because the mechanistic aspects of tension-induced aortic remodeling continues to be a focus of experimentation in our laboratory.

ERK1/2 has been shown to be an effector pathway for both ligand-dependent and ligand-independent AT1R activation and a marker of effective inhibition by ARBs; therefore, the present project could not definitively quantify the proportion of ERK1/2 phosphorylation attributable to mechanical AT1R activation. Similarly, the variation in total ERK1/2 abundance was not compared between these treatment groups, because our primary interest was identifying increases in the pERK1/2 to ERK1/2 ratio as an indicator of AT1R activation. Also, our assessment was conducted in each individual replicate; however, the relative abundance of total ERK1/2 could be included in future investigations of this signaling cascade.

The present project used a single time point for aortic harvest and assessment of AT1R activation. Therefore, future work with interval assessments might demonstrate a trend in activity that would allow for more effective drug dosing. Moreover, additional dosing regimens can be explored to optimize the effect of Losartan inhibiting AAA growth in this model. Given the lack of aortic growth observed with the sham surgical procedure and the goal of characterizing how HTN and AAA induction alters cellular signaling to initiate and promote degenerative remodeling, all molecular assessments in the treatment groups were compared with the nonoperated control mice. Future investigations considering time point analysis might incorporate sham surgical mice in each treatment group.

Finally, the characterization of the BPH/2 mice as low-renin HTN was made using unoperated control mice. The potential changes in plasma renin and bradykinin levels due to AAA induction or Losartan therapy were not further explored as contributors to aortic dilation, blood pressure decreases, or ERK1/2 activity. As our laboratory prepares to further investigate the relationship between the RAS, the BPH/2 mouse model, and aortic remodeling, we recognize that integration of ACE inhibitor therapy, the AT1R knockout mouse (Agtr1$^{-/-}$), and serial plasma protein assessments will be paramount.

CONCLUSIONS

Using a spontaneous neurogenic, low-renin mouse model of HTN, the present study has demonstrated that AT1R activation occurs under conditions of elevated blood pressure and augmented AAA formation, thereby supporting epidemiologic reports that HTN can be an important contributor to AAA development and expansion. Blockade of AT1R signaling with Losartan effectively abrogated AAA growth in normotensive and hypertensive mice, indicating a vital role for this signaling pathway in pathologic aortic remodeling. Understanding the key mediators in this cascade will be vital to confirming AT1R as a viable pharmacotherapeutic target to abrogate AAA growth and warrants further investigation.

AUTHOR CONTRIBUTIONS

Conception and design: NW, JJ, JR
Analysis and interpretation: SH, NW, YX, RM, JJ, JR
Data collection: SH, NW, RP, AAJ, HL, RG, CC, YX
Writing the article: SH, NW, RP, AAJ, HL, RG, CC, YX, JR
Critical revision of the article: SH, RM, JJ, JR
Final approval of the article: SH, NW, RP, AAJ, HL, RG, CC, YX, RM, JJ, JR
Statistical analysis: SH, YX, RM, JJ, JR
Obtained funding: JJ, JR
Overall responsibility: JR

REFERENCES

1. Baxter BT, Terrin MC, Dalman RL. Medical management of small abdominal aortic aneurysms. Circulation 2008;117:1883-9.
2. Tsunemi K, Takai S, Nishimoto M, Yuda A, Hasegawa S, Sawada Y, et al. Possible roles of angiotensin II-forming enzymes, angiotensin converting enzyme and chymase-like enzyme, in the human aneurysmal aorta. Hypertens Res 2002;25:817-22.
3. Xiong F, Zhao J, Zeng G, Huang B, Yuan D, Yang Y. Inhibition of AAA in a rat model by treatment with ACEI perindopril. J Surg Res 2014;189:166-73.
4. Inoue N, Muramatsu M, Jin D, Takai S, Hayashi T, Katayama H, et al. Involvement of vascular angiotensin II-forming enzymes in the progression of aortic abdominal aneurysms in angiotensin II-infused ApoE-deficient mice. J Atheroscler Thromb 2009;16:164-71.
5. Xuan H, Xu B, Wang W, Tanaka H, Fujimura N, Miyata M, et al. Inhibition or deletion of angiotensin II type 1 receptor suppresses elastase-induced experimental abdominal aortic aneurysms. J Vasc Surg 2018;67:573-84.e2.
6. Iida Y, Xu B, Schultz GM, Chow V, White JJ, Sulaimon S, et al. Efficacy and mechanism of angiotensin II receptor blocker treatment in experimental abdominal aortic aneurysms. PLoS One 2012;7:e49642.
7. Bicknell CD, Kiru G, Falaschetti E, Powell JT, Poulter NR; AARDVARK Collaborators. An evaluation of the effect of an angiotensin-converting enzyme inhibitor on the growth rate of small abdominal aortic aneurysms: a randomized placebo-controlled trial (AARDVARK). Eur Heart J 2016;37:3213-21.
8. Touyz RM, Schiffrin EL. Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. Pharmacol Rev 2000;52:639-72.
9. Treisman R. Regulation of transcription by MAP kinase cascades. Curr Opin Cell Biol 1996;8:2-5-15.
10. Zou Y, Akazawa H, Qin Y, Sano M, Takano H, Minamino T, et al. Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II. Nat Cell Biol 2004;6:499-506.
11. Jiang G, Gong H, Niu Y, Yang C, Wang S, Chen Z, et al. Identification of amino acid residues in angiotensin II type 1 receptor sensing mechanical stretch and function in cardiomyocyte hypertrophy. Cell Physiol Biochem 2015;37:105-16.
12. Davern PJ, Nguyen-Huu T-P, La Greca L, Abdelkader A, Head GA. Role of the sympathetic nervous system in Schlager genetically hypertensive mice. Hypertension 2009;54:852-9.
13. Jackson KL, Palma-Rigo K, Nguyen-Huu T-P, Davern PJ, Head GA. Major contribution of the medial amygdala to hypertension in BPH/2J genetically hypertensive mice. Hypertension 2014;63:811-8.
14. Palma-Rigo K, Jackson KL, Davern PJ, Nguyen-Huu T-P, Elghozi J-L, Head GA. Renin—angiotensin and sympathetic nervous system contribution to high blood pressure in Schlager mice. J Hypertens 2011;29:2156-66.
15. Leckie BJ. The action of salt and captopril on blood pressure in mice with genetic hypertension. J Hypertens 2001;19:1607-13.
16. Taddei S, Bortolotto L. Unraveling the pivotal role of bradykinin in ACE inhibitor activity. Am J Cardiovasc Drugs 2016;16:309-21.
17. Schlager G, Sides J. Characterization of hypertensive and hypotensive inbred strains of mice. Lab Anim Sci 1997;47:288-92.
18. Uddin M, Harris-Nelson N. Renin activity and angiotensin I concentration in genetically selective inbred line of hypertensive mice. Biochem Biophys Res Commun 2004;316:842-4.
19. Ruddy JM, Akerman AW, Kimbrough D, Nadeau EK, Stroud RE, Mukherjee R, et al. Differential hypertensive protease expression in the thoracic versus abdominal aorta. J Vasc Surg 2017;66:1543-52.
20. Tada Y, Wada K, Shimada K, Makino H, Liang EI, Murakami S, et al. Roles of hypertension in the rupture of intracranial aneurysms. Stroke 2014;45:579-86.
21. Ray JL, Leach R, Herbert J-M, Benson M. Isolation of vascular smooth muscle cells from a single murine aorta. Methods Cell Sci 2001;23:185-8.
22. Ikonomidis JS, Gibson WC, Gardner J, Sweterlitsch S, Thompson RP, Mukherjee R, et al. A murine model of thoracic aortic aneurysms. J Surg Res 2003;115:157-63.
23. Motulsky H. Intuitive Biostatistics: A Nonmathematical Guide to Statistical Thinking. 4th ed. New York: Oxford University Press; 2010.
24. Baba T, Ohki T, Kanaoka Y, Maeda K, Ito E, Shukuzawa K, et al. Risk factor analyses of abdominal aortic aneurysms growth in Japanese patients. Ann Vasc Surg 2019;55:196-202.
25. Gadowski GR, Ricci MA, Hendley ED, Pilcher DB. Hypertension accelerates the growth of experimental aortic aneurysms. J Surg Res 1993;54:431-6.

26. Shiraya S, Miwa K, Aoki M, Miyake T, Oishi M, Kataoka K, et al. Hypertension accelerated experimental abdominal aortic aneurysm through upregulation of nuclear factor κB and Ets. Hypertension 2006;48:628-36.
27. Blodow S, Schneider H, Storch U, Wizemann R, Forst A-L, Gudermann T, et al. Novel role of mechanosensitive AT1B receptors in myogenic vasoconstriction. Pflügers Arch 2014;466:1343-53.
28. Schleifenbaum J, Kassmann M, Szijártó IA, Hercule HC, Tano J-Y, Weinert S, et al. Stretch—activation of angiotensin II type 1a receptors contributes to the myogenic response of mouse mesenteric and renal arteries. Circ Res 2014;115:263-72.
29. Hahn C, Schwartz MA. Mechanotransduction in vascular physiology and atherogenesis. Nat Rev Mol Cell Biol 2009;10:53-62.
30. Hong K, Zhao G, Hong Z, Sun Z, Yang Y, Clifford PS, et al. Mechanical activation of angiotensin II type 1 receptors causes actin remodelling and myogenic responsiveness in skeletal muscle arterioles. J Physiol 2016;594:7027-47.
31. Miura S, Fujino M, Hanzawa H, Kiya Y, Imaizumi S, Matsuo Y, et al. Molecular mechanism underlying inverse agonist of angiotensin II type 1 receptor. J Biol Chem 2006;281:19288-95.
32. Miura S, Saku K, Karnik SS. Molecular analysis of the structure and function of the angiotensin II type 1 receptor. Hypertens Res 2003;26:937-43.
33. Le MT, Vanderheyden PML, Szaszák M, Hunyady L, Kersemans V, Vauquelin G. Peptide and nonpeptide antagonist interaction with constitutively active human AT1 receptors. Biochem Pharmacol 2003;65:1329-38.
34. Eishingdrelo H, Kongsamut S. Minireview: targeting GPCR activated ERK pathways for drug discovery. Curr Chem Genom Transl Med 2013;7:9-15.
35. Storch U, Mederos y Schnitzler M, Gudermann T. G protein-mediated stretch reception. Am J Physiol Heart Circ Physiol 2012;302:H1241-9.
36. Rakesh K, Yoo B, Kim I-M, Salazar N, Kim K-S, Rockman HA. Beta-arrestin-biased agonism of the angiotensin receptor induced by mechanical stress. Sci Signal 2010;3:ra46.
37. Tang W, Strachan RT, Lefkowitz RJ, Rockman HA. Allosteric modulation of β-arrestin-biased angiotensin II type 1 receptor signaling by membrane stretch. J Biol Chem 2014;289:28271-83.
38. Wang J, Hanada K, Gareri C, Rockman HA. Mechanoactivation of the angiotensin II type 1 receptor induces β-arrestin-biased signaling through $G\alpha_i$ coupling. J Cell Biochem 2018;119:3586-97.
39. Indrakusuma R, Jalalzadeh H, Planken RN, Marquering HA, Legemate DA, Koelemay MJW, et al. Biomechanical imaging markers as predictors of abdominal aortic aneurysm growth or rupture: a systematic review. Eur J Vasc Endovasc Surg 2016;52:475-86.

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SUPPLEMENTARY METHODS.

Immunoblot analysis of AT1R in murine aorta

The relative abundance of angiotensin II type 1 receptor (AT1R) and α-tubulin from four BPH/2 aged 12 to 20 weeks and four BPN/3 mice aged 16 to 20 weeks was determined using immunoblotting (SAB2100073; Sigma Aldrich, St Louis, Mo). Aortic tissue homogenate (30 μg) was loaded into each well of a 10% acrylamide gel (Bio-Rad, Hercules, Calif) and fractionated by electrophoresis. The fractionated proteins were transferred to 0.45-mm nitrocellulose membranes (Bio-Rad). The membranes were subsequently blocked with 5% milk in 1% Tween 20 in Tris-buffered saline (1% TBST) for 1 hour at room temperature (RT). The membranes were incubated in antiserum specific for AT1R (1:1000 in 1% TBST) and α-tubulin (1:1000 in 1% TBST) for an additional 2 hours at RT. After incubation with the primary antibody, the membranes were repeatedly washed (5 × 5 minutes) with 10 mL of 1% TBST to reduce the nonspecific antibody interactions. Secondary peroxidase-conjugated antibodies were then applied (1:5000 in 1% TBST) and allowed to incubate for 1 hour at RT. The membranes were again washed extensively (5 × 5 minutes) with 10 mL of 1% TBST before imaging. Immunoreactive signals were detected using a chemiluminescent substrate (Western ECL Substrate; Bio-Rad) and then imaged using Amersham Imager 600 series (GE Healthcare, Chicago, Ill). Between immunoblotting for each protein of interest, the membranes were stripped using Restore Stripping Buffer (Thermo Fisher Scientific, Waltham, Mass). The images were transferred to a computer and converted to TIFF format. The band intensity was quantified using Gel-Pro Analyzer, version 3.1.14 (Media Cybernetics, Silver Spring, Md). The abundance of AT1R was not significantly different between the BPH/2 and BPN/3 mice used in our study (Supplementary Fig 1; P = NS, t test). The baseline aortic diameters also did not differ between the BPH/2 (n = 28; 404 ± 7 μm) and BPN/3 (n = 32; 415 ± 5 μm) mice (Supplementary Fig 2; P = NS, t test).

Serum analysis of BPH/2 and BPN/3 mice

Serum was collected from four BPH/2 mice aged 12 to 20 weeks and four BPN/3 mice aged 16 to 20 weeks. After the induction of anesthesia, medial thoracotomy was performed and the heart was visualized. A 1-mL, 25-gauge syringe was conditioned with ethylenediaminetetraacetic acid and punctured into the beating left ventricle. Approximately 1 mL of blood was collected per mouse. The blood was immediately spun at 2000g for 15 minutes at 4°C. Supernatant serum was collected, separated into aliquots, and stored at −20°C.

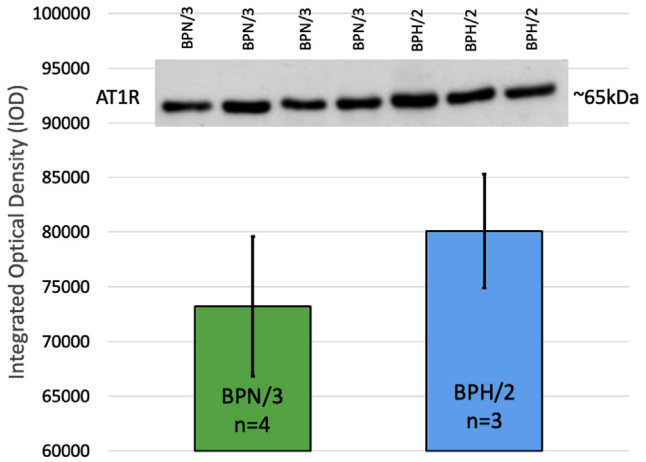
Renin quantification

To quantify the renin content in the serum samples, a Renin1 Mouse (Thermo Fisher Scientific) enzyme-linked immunosorbent assay was used. A total of 100 μL of diluted serum per sample (1:10 in Assay Diluent A; Thermo Fisher Scientific) was plated in quadruplicate on a Mouse Renin1 Antibody Coated 96 well plate (Thermo Fisher Scientific) with serial dilutions of a standard concentration. The samples were incubated for 2.5 hours at RT. After incubation, the samples were washed (4 × 300 μL 1X Wash Buffer), bound by 100 μL of biotin conjugate, and allowed 1 hour of incubation at RT. The samples were similarly washed and then stained by streptavidin-horseradish peroxidase solution, and incubated for 45 minutes at RT. After washing, the samples were treated with 100 μL of TMB (3,3′,5,5′-tetramethylbenzidine) substrate and incubated 30 minutes at RT in the dark. The reactions were stopped and immediately read at 3 × 450 nm on SpectraMax M3 plate reader (Molecular Devices LLC, San Jose, Calif). The replicate reading values were averaged, and a standard curve was calculated in accordance with the provided standards. Quadruplicate wells were averaged and compared with the standard curve for quantification of renin content.

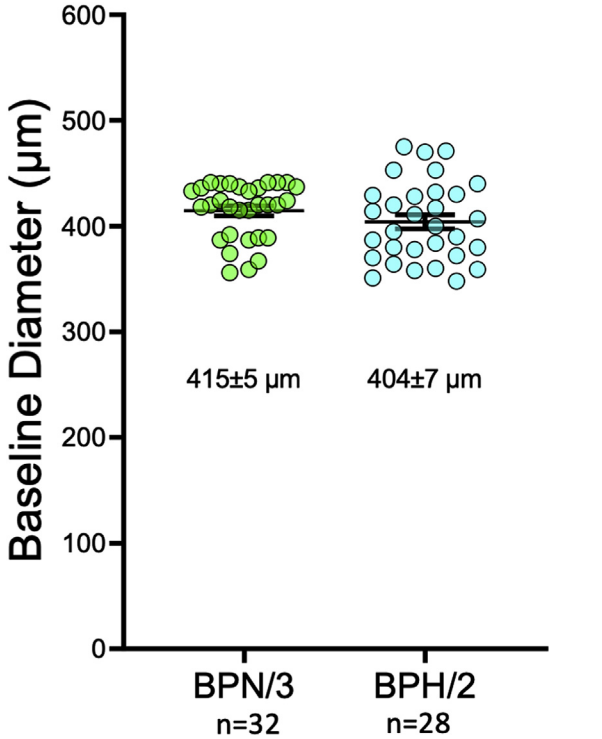
Bradykinin quantification

The bradykinin content in the serum samples was quantified using an enzyme-linked immunosorbent assay for bradykinin (MyBioSource, San Diego, Calif). The samples were diluted 1:100 and loaded onto a precoated 96 well plate (MyBioSource) in 50-μL quadruplicate wells with serial dilutions of a provided standard concentration. Next, 50 μL of Detection Reagent A (MyBioSource) was added immediately to each sample, and the plate was incubated for 1 hour at 37°C. The samples were washed 3 × 300 μL and immediately treated with 100 μL of Detection Reagent B (MyBioSource) for 30 minutes at 37°C. Similarly, the samples were washed and 90μL of TMB substrate solution was added to each well. After 20 minutes of incubation at 37°C, the reaction was stopped and immediately read on a SpectraMax M3 plate reader (Molecular Devices LLC). The replicate reading values were averaged, and a standard curve was calculated from the provided standards. Quadruplicate wells were averaged and compared with the standard curve for quantification of bradykinin content.

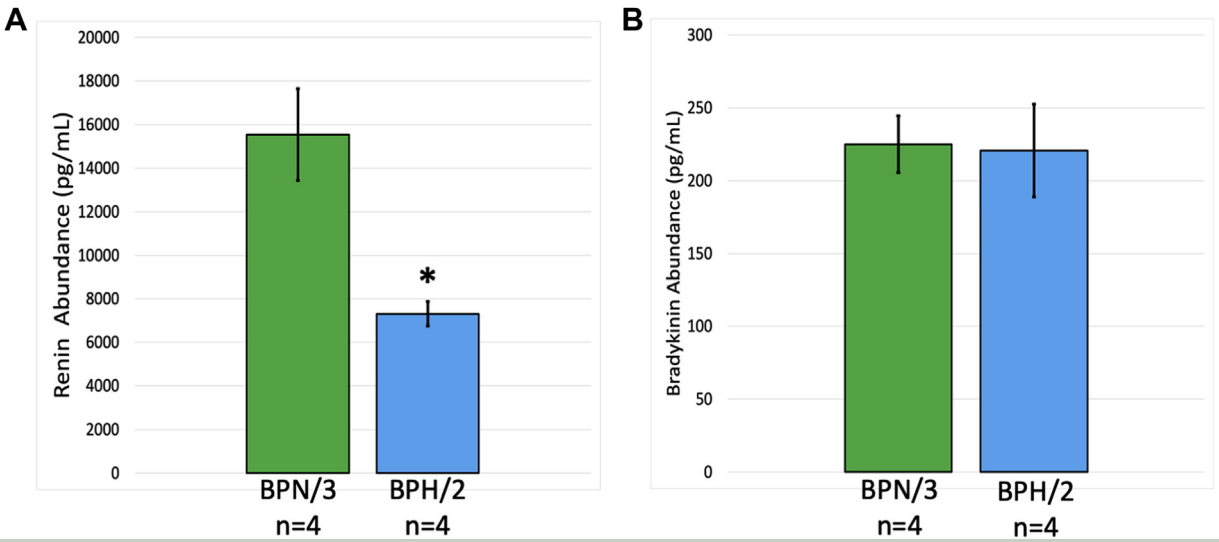
As demonstrated in Supplementary Fig 3, the experiment demonstrated significantly lower plasma renin levels in the BPH/2 mice (7308.4 ± 561.6 pg/mL vs 15,537.2 ± 2104.6 pg/mL; $P < .05$, t test). The bradykinin levels were equivalent (220.7 ± 31.7 pg/mL in the BPH/2 mice vs 225.1 ± 19.5 pg/mL in the BPN/3 mice; P = NS, t test). Therefore, the BPH/2 mice can be characterized as having spontaneous, neurogenic low-renin hypertension permitting study of AT1R activation in a physiologic system with negligible circulating ligand and can, therefore, be extrapolated to mechanical stimulation.



Supplementary Fig 1. Abundance of angiotensin II type 1 receptor (AT1R) in BPN/3 and BPH/2 infrarenal aorta. *P* = NS; *t* test.



Supplementary Fig 2. Baseline abdominal aortic diameters of BPN/3 (n = 32) and BPH/2 (n = 28) mice. *P* = NS; *t* test.



Supplementary Fig 3. Plasma renin **(A)** and bradykinin **(B)** levels in BPN/3 and BPH/2 mice. *$P < .05$; *t* test.