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Intravital Förster resonance energy transfer imaging reveals elevated $[Ca^{2+}]_i$ and enhanced sympathetic tone in femoral arteries of angiotensin II-infused hypertensive biosensor mice

Youhua Wang[1], Ling Chen[1,2], W. Gil Wier[1] and Jin Zhang[1]

Departments of [1]Physiology and [2]Medicine, University of Maryland School of Medicine, Baltimore, MD, USA

Key points

- It is desirable to study altered artery function in hypertension in living animals, where factors influencing artery function are intact.
- We infused 'biosensor' mice chronically with angiotensin II to produce hypertension, and used intravital Förster resonance energy transfer microscopy to measure, simultaneously, $[Ca^{2+}]_i$ and artery diameter *in vivo*.
- Femoral arteries in hypertensive mice had increased basal (resting) $[Ca^{2+}]_i$ and were more constricted than femoral arteries in saline-infused mice. These differences were abolished by blocking (1) all peripheral sympathetic nerve activity (SNA), or (2) vascular α_1-adrenoceptors, but not by blocking vascular angiotensin II or arginine vasopressin receptors.
- Neither contractility nor structural changes were found in femoral arteries of angiotensin II-infused mice.
- The results support previous suggestions that angiotensin II infusion raises blood pressure by acting on the CNS to increase SNA, which increases smooth muscle $[Ca^{2+}]_i$. Infused angiotensin II does not act directly on arterial angiotensin II receptors.

Abstract Artery narrowing in hypertension can only result from structural remodelling of the artery, or increased smooth muscle contraction. The latter may occur with, or without, increases in $[Ca^{2+}]_i$. Here, we sought to measure, in living hypertensive mice, possible changes in artery dimensions and/or $[Ca^{2+}]_i$, and to determine some of the mechanisms involved. Ca^{2+}/calmodulin biosensor (Förster resonance energy transfer-based) mice were made hypertensive by S.C. infusion of angiotensin II (Ang II, 400 ng kg^{-1} min^{-1}, 2–3 weeks). Intravital fluorescence microscopy was used to determine $[Ca^{2+}]_i$ and outer diameter of surgically exposed, intact femoral artery (FA) of anaesthetized mice. Active contractile FA 'tone' was calculated from the basal-state diameter and the passive (i.e. Ca^{2+}-free) diameter (PD). Compared to saline control, FAs of Ang II-infused mice had (1) ~21% higher active tone and (2) ~78 nM higher smooth muscle $[Ca^{2+}]_i$, but (3) the same PDs. The local Ang II receptor (AT_1R) blocker losartan had negligible effect on tone or $[Ca^{2+}]_i$ in control FAs, but reduced the basal tone by ~9% in Ang II FAs. Both I.V. hexamethonium and locally applied prazosin abolished the difference in FA tone and $[Ca^{2+}]_i$, suggesting a dominant role of sympathetic nerve activity (SNA). Changes in diameter and $[Ca^{2+}]_i$ in response to locally applied phenylephrine, Ang II, arginine vasopressin, elevated $[K^+]_o$ and acetylcholine were not altered. In summary, FAs of living Ang II hypertensive mice have higher $[Ca^{2+}]_i$, and are more

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constricted, due, primarily, to elevated SNA and some increased arterial AT_1R activation. Evidence of altered artery reactivity or remodeling was not found.

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Corresponding author J. Zhang: Department of Physiology, University of Maryland School of Medicine, 655 W. Baltimore Street, Baltimore, MD 21201, USA. Email: jzhan002@umaryland.edu

Abbreviations ACh, acetylcholine; Ang II, angiotensin II; AT_1R, angiotensin II type 1 receptor; AVP, arginine vasopressin; BP, blood pressure; $[Ca^{2+}]_i$, intracellular $[Ca^{2+}]$; FRET, Förster resonance energy transfer; (ex)MLCK, (exogenous) myosin light chain kinase; FA, femoral artery; NPY, neuropeptide Y; PD, passive diameter; PE, phenylephrine; PSS, physiological salt solution; SNA, sympathetic nerve activity.

Introduction

Determination of the mechanisms of vascular pathology in hypertension, such as increased artery resistance and stiffness, has been challenging. Although it is certain that increased arterial vascular resistance, as opposed to increased cardiac output, underlies the elevated arterial blood pressure (BP) in primary hypertension, the mechanisms and sites of decreased (narrowed) artery internal diameter that cause the increased resistance remain largely unknown. In general, artery narrowing in hypertension can only result from structural remodelling of the artery (Korsgaard *et al.*, 1993; Martinez-Lemus *et al.* 2009; Mulvany, 2011), or increased contraction, via increased smooth muscle intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) (Linde *et al.* 2012) and/or increased 'Ca^{2+}' sensitivity (Hilgers *et al.* 2007). The problem of determination of arterial smooth muscle $[Ca^{2+}]_i$ is particularly challenging because the $[Ca^{2+}]_i$ that is activating contraction will certainly change immediately upon removal of an artery from the animal for study. In contrast to structural remodelling, the dynamic factors of membrane potential, transmural pressure, endothelial influences, local substances in the blood and wall of the artery, and autonomic nervous system activity (sympathetic and non-adrenergic/non-cholinergic) will be different or completely absent in an artery studied *ex vivo* (Knot & Nelson, 1998). Thus, determining whether $[Ca^{2+}]_i$ is altered in arteries in hypertension almost mandates an *in vivo* approach (Zhang *et al.* 2010; Bagher & Segal, 2011). Similarly, the mechanisms of some changes may exist only in the hypertensive animal, such as altered sympathetic nerve activity (SNA; Esler *et al.* 2010) or altered plasma levels of vasoactive substances (Blasutein *et al.* 2012). In the present study we have used our previously developed method (Zhang *et al.* 2010) to measure $[Ca^{2+}]_i$ in femoral artery (FA) of optical biosensor mice (Isotani *et al.* 2004; Wier *et al.* 2008) *in vivo* to attempt to determine whether $[Ca^{2+}]_i$ is altered in arteries of hypertensive mice. This method also allows a determination of passive diameter (PD) *in vivo*, which indicates structural remodelling (Mulvany, 2011), as PD is determined in the absence of any contractile activation by Ca^{2+}. Hypertension was induced in mice by chronic, s.c. infusion with angiotensin II (Ang II). This type of experimental hypertension is believed to be relevant to human essential hypertension, and to act by altering mechanisms in the CNS that control vascular resistance, arterial baroreceptors and renal function (Huang *et al.* 2010; Blaustein *et al.* 2012). Although FA is not a 'resistance' artery, it is exposed to circulating substances and nerve activity that may be altered in hypertension. Thus, $[Ca^{2+}]_i$ in FA may reflect some of the systemic factors involved in altering diameter of the true resistance vessels, the small arteries and arterioles. Indeed, in human essential hypertension, conduit arteries such as FA are stiffer than normal, and this contributes to the pathology of hypertension, causing increased arterial pulse wave velocity and increased cardiac work (Sparks *et al.* 2011; Sudano *et al.* 2011).

Methods

Ethical approval

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. Mice that express exogenous myosin light chain kinase (exMLCK) biosensor with a C57BL/6 genetic background (Zhang *et al.* 2010) were used. All mice were maintained on a 12:12 h light/dark schedule at 22–25°C and 45–65% humidity and fed ad libitum on a standard rodent diet and tap water.

Osmotic Ang II pump implantation

Mice (12–26 weeks) were anaesthetized with 1.5% inhalational isoflurane (IsoFlo, Abbott Animal Health, Abbott Park, IL, USA), weighed and then implanted s.c. with an Alzet osmotic minipump (Model 1004, Durect Corp., Cupertino, CA, USA) filled with either Ang II ($n = 29$) or saline (vehicle control, $n = 24$). Ang II was delivered at 400 ng kg^{-1} min^{-1} for 2–3 weeks.

Intra-carotid arterial BP measurement and i.v. injection setup

Mice were anaesthetized with 2% isoflurane (IsoFlo); isoflurane was maintained at 2% in O_2 during

surgery and then reduced to 1.5% for the subsequent experiments. Core temperature was maintained at 37.5–38°C. The left carotid artery was isolated and cannulated with a 1.4 French Mikro-tip pressure catheter (Millar Instruments, Houston, TX, USA) for BP recording. The left jugular vein was cannulated with a PE10 catheter (Braintree Scientific, Inc., Braintree, MA, USA) for I.V. injection of drugs. Following 20 min stabilization, BP was recorded for at least 30 min and calculated off-line (BioPac System, Santa Barbara, CA, USA). At the end of the experiment, the mouse was killed with an overdose of isoflurane followed by cervical dislocation.

Data collection was performed blinded: the individual measuring BP did not know whether the animal had been infused with Ang II or saline. Animal code numbers were matched with experimental group and BP after the data on all the mice had been collected.

Preparation of FA for simultaneous diameter and fluorescence recording

The method used here was that of Zhang *et al.* (2010). Briefly, FA of isoflurane anaesthetized mice was dissected lightly of connective tissue via a cutaneous incision in the upper thigh. The artery was then exposed to an Olympus MVX10 MacroView fluorescence microscope (Olympus America, Center Valley, PA, USA) and superfused with physiological salt solution (PSS) to allow local application of drugs.

For fluorescence recording, the MVX10 microscope was equipped with an objective lens of 0.5 NA, 2× magnification, 20 mm working distance, and further variable 'zoom' magnification of 0.63–6.3× (Zhang *et al.* 2010). Fluorescence illumination was provided by a xenon arc lamp (Lambda LS, Sutter Instrument Co., Novato, CA, USA). Illumination at 436 ± 10 nm was gated and adjusted in intensity through the use of a programmable shutter (Smart Shutter, Sutter Instrument Co.). The microscope was fitted with an image splitting device (DualView, Photometrics, Tucson, AZ, USA) equipped with a dichroic beam splitter centred at 505 nm, and two emission filters, 470/30 and 535/30 nm, providing detection of cyan fluorescence protein (CFP) and yellow fluorescence protein (YFP) fluorescence emission, respectively. A sensitive CCD camera (ORCA ER, Hamamatsu Corp., Bridgewater, NJ, USA) in conjunction with the image splitting device was used. The camera was controlled and images were acquired using HCImage (Hamamatsu). Image processing was performed with custom software, written using IDL 8.1 (ITT Visual Information Solutions, Boulder, CO, USA).

Calculation of $[Ca^{2+}]_i$

To obtain a calibration curve relating exMLCK Förster resonance energy transfer (FRET) CFP/YFP ratio to $[Ca^{2+}]_i$, isolated mesenteric small arteries mounted on a wire myograph were put on the stage of the microscope equipped with the optics for exMLCK FRET measurements. Arteries were first permeabilized by exposure to 1000 U ml^{-1} α-toxin (*Staphylococcus aureus*) for 1 h in a 'relaxing' solution {pCa (–log $[Ca^{2+}]$) >9.0}. The permeabilized vessels were then bathed in a series of calibration solutions that had free pCa of 9.0, 7.0, 6.5, 6.0, 5.5, 5.0 and 4.5, obtained by mixing Ca-EGTA stock solutions of pCa 9.0 and pCa 4.5, in the appropriate ratios, while force was recorded simultaneously at 37°C, pH 7.1 (Fig. 1*A*). Ionic strength was adjusted with potassium methanesulfonate to 150 mM.

The pCa–FRET ratio relationship (Fig. 1*B*) was then obtained using measured FRET ratios in the solutions of different pCa. R_{min} and R_{max} denote FRET ratios in pCa 9.0 and 4.5 solutions and correspond to Ca^{2+}/calmodulin-free and Ca^{2+}/calmodulin-saturated exMLCK, respectively. The exMLCK was half-maximally activated at pCa 6.05, with a Hill coefficient of 1.4. For each individual artery *in vivo*, R_{min} and R_{max} were determined in $[Ca^{2+}]_o$-free (0 Ca) and high (117 mM) $[K^+]_o$ (117 K) superfusion PSS (Fig. 1*C*). Figure 1*C* shows a representative recording of CFP/YFP ratio and calculated $[Ca^{2+}]_i$ using the calibration curve in Fig. 1*B* under basal condition and in response to 0 Ca and 117 K solution in an exMLCK FA *in vivo*. As was the case with local application of receptor agonists and antagonists, local application of the calibrating solutions had no effect on BP or heart rate (Zhang *et al.* 2010).

Reagents and solutions

Artery superfusion solution (PSS, in mM) comprised: NaCl, 112; $NaHCO_3$, 25.7; KCl, 4.9; $CaCl_2$, 2.5; $MgSO_4.7H_2O$, 1.2; KH_2PO_4, 1.2; glucose, 11.5; Hepes, 10 (pH 7.4, equilibrated with gas of 5% O_2, 5% CO_2, 90% N_2 at 37°C). 117 K solution was made by replacing NaCl with equimolar KCl of normal PSS. 0 Ca solution was made by omitting Ca^{2+} and adding 0.5 mM EGTA in normal PSS.

Reagents and sources were: acetylcholine (ACh) α-toxin from *staphylococcus aureus*, Ang II, arginine vasopressin (AVP), guanethidine, hexamethonium, losartan, phentolamine, phenylephrine (PE), prazosin, SR49059, suramin, BIBO 3304, neuropeptide Y (NPY) and α,β-methylene ATP (Sigma-Aldrich, St Louis, MO, USA); other reagents were reagent grade or the highest grade available. Reagents were dissolved in deionized water or DMSO as stock solutions. Appropriate dilution in experimental superfusing solution was made for local application, whereas sterile saline was used to dissolve hexamethonium for I.V. injection.

Data analysis and statistics

Basal vascular tone is defined as the difference between absolute diameter under steady state resting conditions and the maximal diameter in 0 Ca solution as a percentage of maximal diameter (Fig. 2*A*). Vasoconstrictor responses are expressed as: (maximal diameter – absolute diameter for each dose)/maximal diameter × 100. Vasodilator responses are expressed as change from baseline for each dose/(maximal diameter – baseline) × 100.

The data are expressed as mean ± SEM; n denotes the number of arteries studied. Comparisons of data were made using Student's paired or unpaired t test, as appropriate; a two-way ANOVA was used where indicated. Differences were considered significant at $P < 0.05$.

Results

Chronic Ang II infusion elevates arterial BP

BP in mice receiving a chronic infusion of Ang II begins to rise within 1–2 days, then increases gradually over the first 2 weeks and remains stably high for ∼2 weeks (Gonzalez-Villalobos *et al.* 2009; Kirabo *et al.* 2011). In the present study, mean arterial BP, measured during week 3, was 105 ± 3 mmHg ($n = 11$) in Ang II-infused exMLCK biosensor mice, significantly higher than that (97 ± 4 mmHg, $n = 7$, $P < 0.05$) in control (saline-infused) exMLCK biosensor mice.

Chronic Ang II infusion results in enhanced basal vascular tone in FA *in vivo*

Figure 2*A* shows representative recordings of diameter and calculated $[Ca^{2+}]_i$ under basal condition and in response to 0 Ca and 117 K solution in an artery of saline-infused exMLCK biosensor mouse and an artery of Ang II-infused biosensor mouse. FAs of saline-infused mice in the basal state were ∼275 μm in outer diameter when superfused with normal PSS, and dilated to ∼416 μm when superfused with 0 Ca solution (PD, Fig. 2*A*, top, and *Ba*). Thus, FAs *in vivo* exhibited a relatively large amount of basal vascular tone, 33 ± 2% of PD ($n = 20$, Fig. 2*Bb*). In the Ang II-infused mice, basal state diameter of FAs was less, ∼236 μm, and dilated to PD, ∼396 μm. PD of Ang II arteries was not significantly different from that in the saline group. Basal vascular tone, however, in arteries of the Ang II-infused mice, 40 ± 1% of PD ($n = 21$), was significantly higher, ∼21%, than that in arteries of saline-infused mice (Fig. 2*Bb*).

$[Ca^{2+}]_i$, measured by FRET fluorescence in FA walls, ∼314 nM in arteries of Ang II-infused mice, was significantly higher than that (∼236 nM) in arteries of saline-infused mice (Fig. 2*A*, bottom, and *Bc*). The increased (∼78 nM) $[Ca^{2+}]_i$ (Fig. 2*Bc*) was consistent with the augmented basal tone (Fig. 2*Bb*). Mechanisms that possibly contribute to the increased $[Ca^{2+}]_i$ and basal tone during Ang II infusion were then examined.

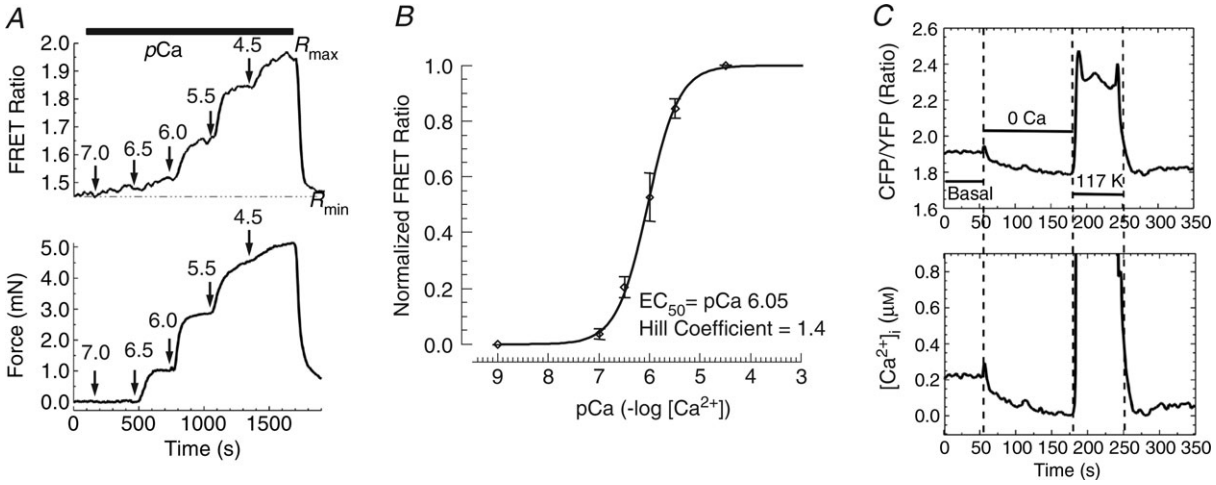

Figure 1. Calibration of exMLCK FRET ratio for determination of $[Ca^{2+}]_i$ in α-toxin permeabilized arteries
A, representative recordings of FRET (CFP/YFP) ratio (top) and force (bottom) changes during incubation with a series of calibration solutions (pCa = 9.0–4.5) in an isolated, α-toxin permeabilized mouse mesenteric artery mounted on a confocal wire myograph. Data were obtained at 37°C, pH 7.1. *B*, pCa–FRET ratio relationship. This curve was determined using FRET ratios in various $[Ca^{2+}]_o$ solutions obtained in *A*. Normalized FRET ratio = $(R - R_{min})/(R_{max} - R_{min})$, where R represents the measured FRET ratio. EC_{50} = pCa 6.05; Hill coefficient = 1.4. Data were normalized to the maximum (1.0 at pCa = 4.5) and minimum (0 at pCa = 9) FRET ratios; $n = 6$ arteries. *C*, representative recordings of FRET ratio (top) and calculated $[Ca^{2+}]_i$ (bottom) in response to $[Ca^{2+}]_o$-free (0 Ca) and high $[K^+]_o$ (117 K) superfusion solutions in an exMLCK femoral artery *in vivo*. Calculated $[Ca^{2+}]_i$ was obtained using the calibration curve in *B*. Time of exposure to 0 Ca and 117 K solutions is indicated by horizontal bars. 0 Ca and 117 K solutions were applied routinely in each experiment to determine R_{min} and R_{max}, respectively.

Local vascular Ang II receptor activation mediates a small component of the enhanced vascular tone during Ang II infusion

We first sought to determine whether any of the increased FA tone in Ang II-infused mice was due to the elevated circulating Ang II in the plasma acting directly on the vascular type-1 Ang II receptors (AT_1Rs) in FA, causing increased vasoconstriction directly. FA constricted and $[Ca^{2+}]_i$ was elevated in response to acute, exogenous, local application of Ang II (100 nM, Fig. 3*A*), indicating the presence of AT_1Rs. Moreover, both the acute vasoconstriction and $[Ca^{2+}]_i$ elevation to locally applied exogenous Ang II were significantly blunted in arteries of chronic Ang II-infused mice compared with those in arteries of saline-infused mice (Fig. 3*A* and *C*), suggesting that the augmented basal tone observed in Ang II-infused mouse FA was not due to over-reactivity of AT_1Rs, nor to an up-regulated expression of AT_1Rs. Nevertheless, acute, locally applied Ang II-induced $[Ca^{2+}]_i$ elevation and vasoconstriction were significantly reduced by the specific AT_1R blocker losartan (100 nM), in both saline- and Ang II-infused arteries (Fig. 3*B* and *C*). Losartan (100 nM) alone, applied locally to the exposed artery, had negligible effect on basal tone (reduced by ~1%) or $[Ca^{2+}]_i$ (reduced by ~32 nM) in FAs of saline-infused mice ($n = 12$, Fig. 3*B* and *D*). In FAs of Ang II-infused mice, in contrast, losartan reduced basal tone to ~37% of PD ($n = 10$, $P < 0.05$) and lowered $[Ca^{2+}]_i$ to ~270 nM ($P < 0.01$). Therefore, in chronic Ang II-infused mice, ~9% of basal FA tone was attributable to the activation of AT_1Rs by plasma circulating Ang II on the FA itself.

Elevated FA tone in Ang II mice is not attributable to vascular AVP receptor activation

Plasma Ang II acts in the brain to modify hormonal output, such as AVP, as well as sympathetic drive, to the periphery, thus influencing the function of blood vessels (Zimmerman *et al.* 2004; Marvar *et al.* 2010; Young *et al.* 2012; Lob *et al.* 2013). Therefore, we examined the effects of activating and blocking the V_{1A} receptor, the primary vascular vasopressinergic receptor (Fig. 4). AVP dose-dependently caused vasoconstriction with an EC_{50} of 2.9 nM ($n = 4$). At 10 nM, AVP caused a remarkable increase of basal tone and significant elevation of $[Ca^{2+}]_i$ in both saline and Ang II groups ($n = 9$ per group, Fig. 4*A* and *C*). At 10 nM, SR49059, a specific V_{1A} receptor antagonist, significantly blocked the 10 nM AVP-induced vasoconstriction (Fig. 4*B* and *C*). At this concentration, in the absence of exogenous AVP, SR49059 reduced basal

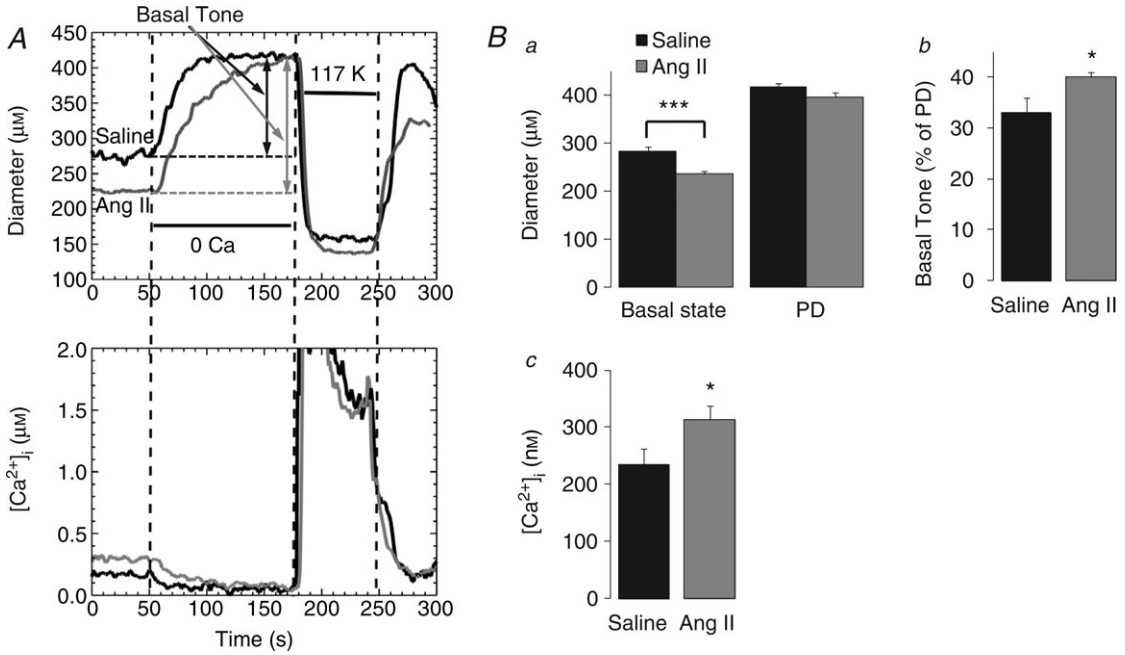

Figure 2. Chronic angiotensin II (Ang II) infusion results in elevated $[Ca^{2+}]_i$ and basal vascular tone in exMLCK mouse FAs *in vivo*

A, representative recordings of diameter (top) and calculated $[Ca^{2+}]_i$ (bottom) in response to 0 Ca and 117 K solutions in FAs of saline- (control, black line) and Ang II (grey line)-infused mice. Basal tone is defined as the difference between the absolute diameter under basal condition and outside passive diameter (PD) in 0 Ca solution. *B*, summary of basal diameter and PD (*a*), basal vascular tone (*b*) and $[Ca^{2+}]_i$ under basal state (*c*) in FAs of saline- (filled bars) and Ang II-infused (Ang II, shaded bars) exMLCK mice; $n = 20$ (saline) and 21 (Ang II) arteries. PD, in this and following experiments, was determined by the outer diameter in 0 Ca solution. $^*P < 0.05$; $^{***}P < 0.001$ *vs.* saline controls.

FA tone by ∼4% in arteries from both saline- and Ang II-infused mice ($n = 6$ per group, Fig. 4*B* and *D*). These results suggest that V_{1A} receptor activation by endogenous AVP had a negligible effect on the *increased* FA tone of Ang II-infused mice.

Systemic ganglionic blockade abolishes Ang II-induced $[Ca^{2+}]_i$ elevation and tone augmentation

Acute blockade of autonomic ganglionic transmission causes a larger fall in mean arterial BP, a 'depressor' response, in Ang II-infused animals compared to controls (Kuroki *et al.* 2012). Thus, increased arterial pressure after Ang II treatment involves increased SNA, or increased efficacy of SNA, as might be the result of increased 'vascular reactivity' (Anning *et al.* 2005; Touyz *et al.* 2005; Ding *et al.* 2007; Hercule *et al.* 2007; Kirabo *et al.* 2011; Kharade *et al.* 2013). Because increased SNA may be region specific (Yoshimoto *et al.* 2010), it was unknown whether Ang II treatment increased SNA or SNA efficacy in FA. Hexamethonium, a ganglionic blocker, administered (I.V.) at 30 mg kg^{-1} over periods of 30 s resulted in dilation of FA and a decrease of $[Ca^{2+}]_i$ in both saline- and Ang II-infused mice (Fig. 5*A*). The reduction of $[Ca^{2+}]_i$ and basal tone caused by hexamethonium was significantly larger in Ang II-infused arteries, i.e. the difference of $[Ca^{2+}]_i$ and basal tone between saline and Ang II groups was abolished by hexamethonium (Fig. 5*B*). Thus, the elevated $[Ca^{2+}]_i$ and additional vascular tone of FA in Ang II-infused mice were largely due to increased SNA or to increased SNA efficacy.

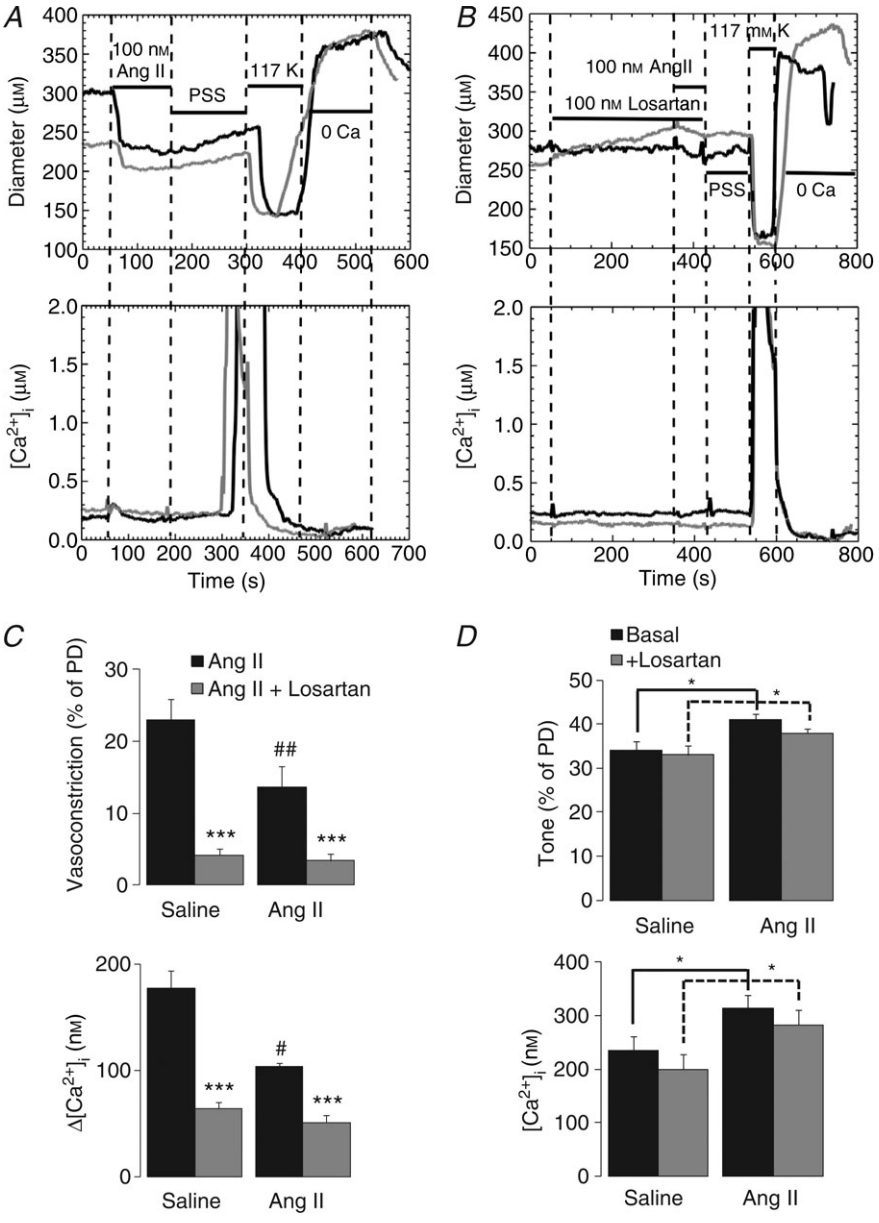

Figure 3. Local vascular Ang II receptor activation mediates a small component of the enhanced vascular tone during Ang II infusion
A, representative experiments showing the diameter (top) and $[Ca^{2+}]_i$ (bottom) changes in response to acute, local application of Ang II (100 nM) in arteries of saline- (black line) and Ang II- (grey line) infused mice. Time of exposure to Ang II, physiological salt solution (PSS), and 117 K and 0 Ca solutions are indicated by horizontal bars. *B*, representative experiments showing the effects of local application of losartan (100 nM) on diameter (top) and $[Ca^{2+}]_i$ (bottom) under basal condition and during acute Ang II (100 nM) application in arteries of saline- (black line) and Ang II- (grey line) infused mice. *C*, summary of local application of Ang II-induced vasoconstriction (top, filled bars) and $[Ca^{2+}]_i$ elevation (bottom, filled bars) that was blocked by local pretreatment of losartan (100 nM, shaded bars) in FAs of saline- and Ang II-infused mice. $^{***}P < 0.001$with *vs.* without losartan; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* saline group; $n = 6$–8 arteries. *D*, top: summary of basal tone in the absence (filled bars) and presence (shaded bars) of 100 nM losartan in arteries of saline- and Ang II-infused mice; $n = 12$ per group. bottom: comparison of losartan-induced $[Ca^{2+}]_i$ changes in FAs of saline- and Ang II-infused mice. $^{*}P < 0.05$.

Neurogenic tone in FA is dominated by α_1-adrenoceptors

The hypothesis that increased vascular tone depended on SNA was tested by locally blocking some of the vascular receptors involved in sympathetic neuromuscular transmission. When applied locally, phentolamine (1.0 μM), a non-specific α-adrenoceptor blocker, and prazosin, a specific α_1-adrenoceptor blocker, significantly reduced

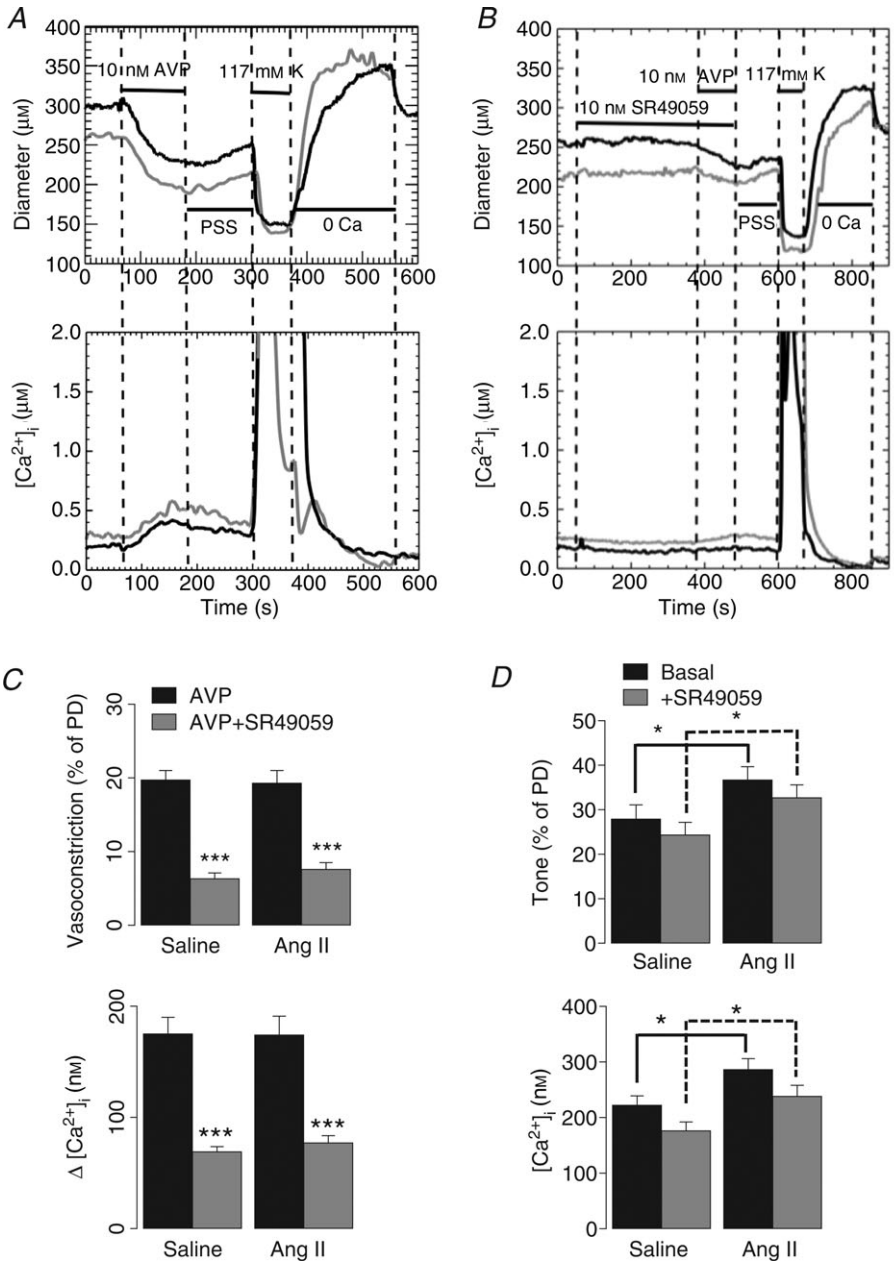

Figure 4. Elevated FA tone in Ang II mice is not attributable to vascular AVP receptor activation
A, representative experiments showing the diameter (top) and $[Ca^{2+}]_i$ (bottom) changes in response to acute, local application of AVP (10 nM) in an artery of saline-infused (black line) mice and an artery of Ang II-infused (grey line) mice. *B*, representative experiments showing the effects of local application of a specific V_{1A} receptor antagonist, SR49059 (10 nM), on diameter (top) and $[Ca^{2+}]_i$ (bottom) under basal conditions and during acute application of AVP (10 nM). *C*, summary of AVP-induced vasoconstriction (top, filled bars) and $[Ca^{2+}]_i$ elevation (bottom, filled bars) that were blocked by local application of SR49059 (10 nM, shaded bars) in FAs of saline- and Ang II-infused mice. $^{***}P < 0.001$ with *vs.* without SR49059; $n = 9$ arteries per group. *D*, top: summary of basal tone in the absence (filled bars) and presence (shaded bars) of 10 nM SR49059 in arteries of saline- and Ang II-infused mice. Bottom: comparison of SR49059-induced $[Ca^{2+}]_i$ changes in FAs from saline- and Ang II-infused mice. $n = 6$ per group. $^{*}P < 0.05$.

FA vascular tone (Fig. 6*A*, left) and $[Ca^{2+}]_i$ (Fig. 6*A*, right) in control exMLCK biosensor mice. In comparison, suramin, an ATP receptor blocker, and BIBO 3304, an NPY receptor blocker, had negligible effect on tone or $[Ca^{2+}]_i$ (Fig. 6*A*). At the same concentrations, these specific inhibitors, prazosin, suramin and BIBO 3304, significantly abolished the vasoconstriction and $[Ca^{2+}]_i$ elevation induced by PE (Fig. 6*B* and *C*), α,β-methylene ATP (Fig. 6*D*) and NPY (Fig. 6*E*), respectively. These results suggested that the neurogenic tone observed in FA was dominated by α_1-adrenoceptors.

The hypothesis that increased vascular tone depended on SNA was tested further by local application of post-ganglionic catecholamine release blockers. Guanethidine (3.0 μM, 20 min), a selective inhibitor of neurotransmitter release from adrenergic nerves, applied locally caused a significant reduction of basal vascular tone and $[Ca^{2+}]_i$. In the presence of guanethidine, the effects of prazosin and phentolamine were abolished (data not shown).

Local application of α_1-adrenoceptor blocker causes larger reduction of FA vascular tone and $[Ca^{2+}]_i$ in Ang II-infused mice than in saline-infused mice

After the dominant role of α_1-adrenergic receptor on FA tone was determined, the effect of local application of prazosin was then compared in FAs of saline- and Ang II-infused mice (Fig. 7). Prazosin dose-dependently reduced basal tone (Fig. 7*A* and *B*, top) and $[Ca^{2+}]_i$ (Fig. 7*A* and *B*, bottom) in both groups. The reduction in both tone (Fig. 7*C*, top) and $[Ca^{2+}]_i$ (Fig. 7*C*, bottom), however, was significantly larger in arteries of Ang II-infused mice.

Arterial contractility is not affected by chronic systemic Ang II infusion

As Ang II can promote both structural vascular remodelling (Porteri *et al.* 2003; Zhou *et al.* 2005) and changes in vascular reactivity (Ding *et al.* 2007; Hercule *et al.* 2007; Kirabo *et al.* 2011; Kharade *et al.* 2013), the increased FA tone and $[Ca^{2+}]_i$ could be explained by an increased responsiveness to neurally released noradrenaline rather than, or in addition to, increased release of noradrenaline as a result of increased SNA. To determine whether increased vascular reactivity had occurred, the responsiveness of FA *in vivo* to locally applied PE, a commonly used analogue of noradrenaline, was examined (Fig. 8). PE, at concentrations of 0.01–100 μM, in the solution superfusing the artery segment induced dose-dependent vasoconstriction (Fig. 8*A* and *B*, top) and increase in exMLCK FRET ratio (i.e. $[Ca^{2+}]_i$) (Fig. 8*A* and *B*, bottom). Neither $[Ca^{2+}]_i$ nor arterial contractility to PE was sensitized by chronic Ang II infusion (Fig. 8*B*). Vasoconstriction induced by 117 K in FAs was also similar between saline- and Ang II-infused groups. These results suggest that the increased sympathetic tone was not due to an increased vascular reactivity to neurotransmitters.

Endothelium-dependent vasorelaxation is not affected by systemic Ang II infusion

Finally, hypertension usually involves endothelial dysfunction (Huang *et al.* 1995; Anning *et al.* 2005). Therefore, endothelium-dependent vasodilation and accompanying $[Ca^{2+}]_i$ changes were also measured by locally applied ACh (0.01–10.0 μM) (Fig. 9). The EC_{50}

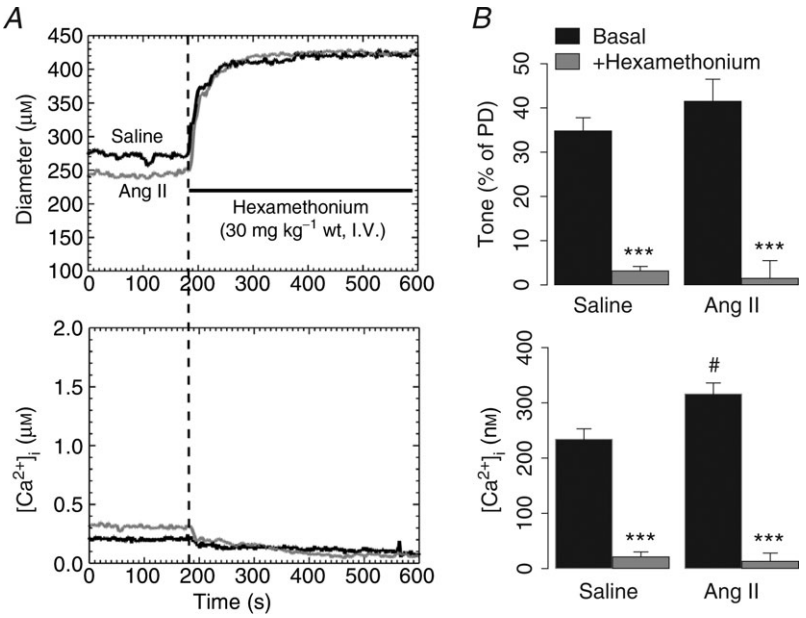

Figure 5. Systemic (I.V.) application of hexamethonium abolished the difference in basal tone and $[Ca^{2+}]_i$ between arteries of saline- and Ang II-infused exMLCK mice
A, representative recordings of diameter (top) and $[Ca^{2+}]_i$ (bottom) changes during systemic application of hexamethonium (30 mg kg^{-1}), a sympathetic ganglionic blocker, in arteries from saline- (black line) or Ang II- (grey line) infused mice. *B*, summary of basal vascular tone (top) and $[Ca^{2+}]_i$ (bottom) changes in response to hexamethonium (30 mg kg^{-1}) in arteries from saline- and Ang II-infused mice; $n = 6$ per group. $^{***}P < 0.001$ *vs.* basal; $^{\#}P < 0.05$ *vs.* saline.

of ACh was not significantly different between saline and Ang II groups (Fig. 9*B*), reflecting an absence of endothelial dysfunction that otherwise might also contribute to the greater tone in FAs of Ang II-infused mice.

Discussion

FA diameter

We observed that outer diameters of FAs in anaesthetized Ang II-infused mice were significantly less than those of saline-infused (control) mice, an effect we attribute primarily to increased SNA in the Ang II-infused mice, which increases $[Ca^{2+}]_i$, through activation of the α_1-adrenergic receptor. Although the difference in outer diameter (under tonic steady state) was small (~39 μm, Fig. 2*Ba*), it was readily detectable because of the bright fluorescence in the smooth muscle cells (see Zhang *et al.* 2010, fig. 2), and because of the stability and low variance of the diameter measurement from the video data (e.g. Fig. 2). Nevertheless, we were not able to determine inner diameter with this method. It seems likely that inner diameter decreased relatively more than outer diameter, as

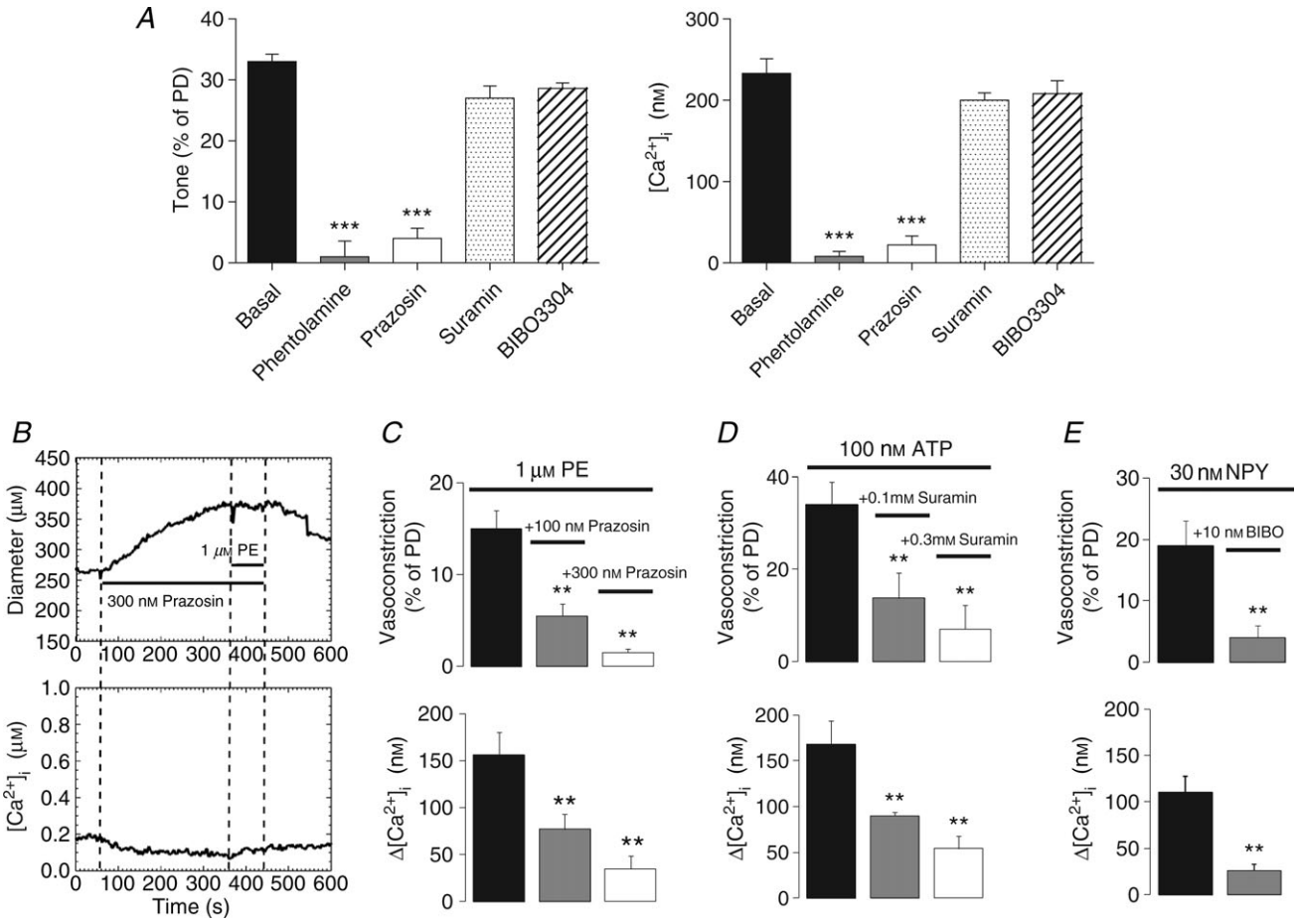

Figure 6. Local application of neurotransmitter receptor blockers reveals a dominant role of α_1-adrenergic receptor in controlling basal vascular tone in exMLCK mouse FAs
A, summary of basal tone (left) and $[Ca^{2+}]_i$ (right) in the presence of a non-specific α-adrenoceptor blocker, phentolamine (1 μM, $n = 5$), an α_1-adrenoceptor blocker, prazosin (1 μM, $n = 12$), a purinergic receptor blocker, suramin (0.3 mM, $n = 5$), and a neural peptide Y (NPY) receptor blocker, BIBO3304 (10 nM, $n = 4$) in exMLCK mouse FAs. $^{***}P < 0.001$ compared to basal tone. *B*, representative recordings of diameter (top) and $[Ca^{2+}]_i$ (bottom) showing the effect of prazosin (300 nM) on basal tone and phenylephrine- (PE, 1 μM) induced vasoconstriction. *C*, summary of vasoconstriction (top) and $[Ca^{2+}]_i$ elevation (bottom) induced by PE, an adrenergic receptor agonist, in the absence (filled bars) and presence (shaded and open bars) of prazosin (100 and 300 nM); $n = 7$ arteries. *D*, summary of vasoconstriction (top) and $[Ca^{2+}]_i$ elevation (bottom) induced by activation of purinergic receptor by α,β-methylene ATP, in the absence (filled bars) and presence (shaded and open bars) of suramin (0.1 and 0.3 mM); $n = 5$ arteries. *E*, summary of vasoconstriction (top) and $[Ca^{2+}]_i$ elevation (bottom) induced by activation of neural peptide Y (NPY) Y_1 receptor by NPY, in the absence (filled bars) and presence (shaded bars) of BIBO 3304 (10 nM); $n = 4$ arteries. $^{**}P < 0.01$, compared to respective controls.

a result of wall thickening due to contraction of the smooth muscle. Thus, our data would suggest that the vascular resistance of FA in Ang-II infused mice is significantly higher than that of control mice. Nevertheless, due to the very large diameter of FA compared to the true resistance arteries, this is unlikely to affect total peripheral resistance significantly. Because of increased contractile activation (the measured increased $[Ca^{2+}]_i$), arteries of Ang II-infused mice would be expected to be stiffer than normal.

We concluded from our measurements of PD that significant structural remodelling was unlikely to account for the decreased outer diameter in Ang II-infused mice. Previous studies have shown large decreases in PD (i.e. diameter in 0 Ca solution) of *ex vivo* arteries from human hypertensives, clearly indicating structural remodelling (Korsgaard *et al.*, 1993; Mulvany, 2011). Some uncertainty is introduced into our measurement of PD, however, because BP of the Ang II-infused mice was higher than control, and thus the PDs were actually measured at different pressures in the two groups of animals *in vivo*. Thus, a small decrease in PD, due to structural changes, could have been obscured by increased distension due to the higher pressure. We cannot rule out conclusively therefore that a small amount of structural remodelling might have occurred.

Ang II has been reported to promote cardiovascular remodelling (Porteri *et al.* 2003; Zhou *et al.* 2005), possibly involving increased reactive oxygen species, and decreased nitric oxide bioavailability. Ang II-induced inward eutrophic arterial remodelling, manifested by smaller PDs with larger media/lumen ratio, differs across vascular territories, although it has been reported in both conduit and subcutaneous small resistance arteries from human hypertension and experimental models of hypertension with chronic infusion of adrenoceptor agonists (Porteri *et al.* 2003). In our case, PDs of mouse FA were not significantly different between saline- and Ang II-infused arteries, suggesting a lack of structural alterations. This could be due to the relatively low dose of Ang II that we used and the relatively mild increase in BP. The change in mean arterial BP that we observed (∼8 mmHg) was smaller than that (25–30 mmHg) observed in other similar studies (Gonzalez-Villalobos *et al.* 2009; Feng *et al.* 2010; Kirabo *et al.* 2011). However, the dose of Ang II that we used (400 ng kg^{-1} min^{-1}) was significantly lower than that used by others (600–1000 ng kg^{-1} min^{-1}), and BP was measured under isoflurane anaesthesia in our experiments, rather than in conscious mice. Isoflurane does produce a dose-dependent decrease in arterial BP, and a depression of SNA, even at the accepted anaesthetic concentration (1.5%) (Seagard *et al.* 1984).

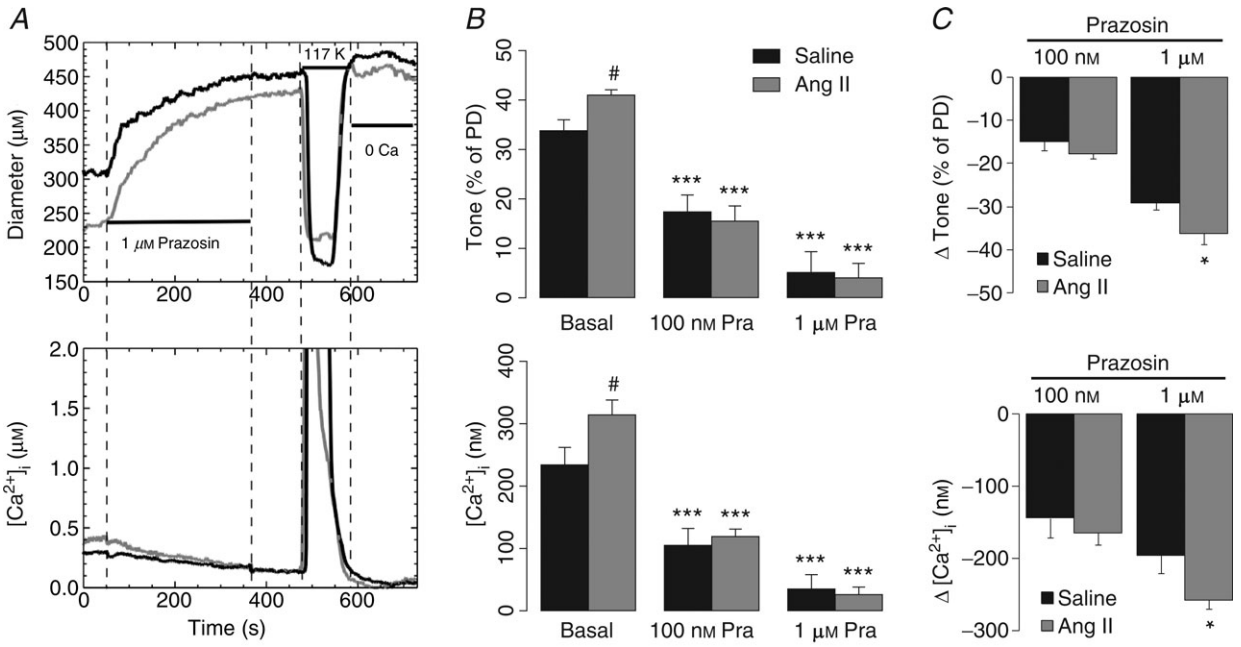

Figure 7. Local blockade of α_1-adrenergic receptor abolishes the difference in arterial tone and $[Ca^{2+}]_i$ between FAs of saline- and Ang II-infused mice
A, representative recordings of diameter (top) and $[Ca^{2+}]_i$ (bottom) changes in response to prazosin (1 μM) in FAs of saline- (black line) and Ang II (grey line)-infused mice. *B*, summary of basal vascular tone (top) and basal $[Ca^{2+}]_i$ (bottom) in the presence of 100 nM and 1 μM prazosin in FAs of saline- (filled bars) and Ang II- (shaded bars) infused mice. $^{***}P < 0.001$ *vs.* basal tone. $^{\#}P < 0.05$ *vs.* saline. *C*, comparison of prazosin-sensitive basal tone (top) and $[Ca^{2+}]_i$ changes ($\Delta[Ca^{2+}]_i$, bottom) in FAs of saline- and Ang II-infused mice. $^{*}P < 0.05$ *vs.* saline; $n = 6$–9 arteries.

Rationale of use of FAs

Increased total peripheral resistance in hypertension is almost certainly the result mainly of decreased diameter of small arteries and/or arterioles, not of decreased diameter of conduit arteries, such as the FAs studied here. Nevertheless, we postulate that FAs can be useful in studies of hypertension, as their contractile state ($[Ca^{2+}]_i$) would be expected to reflect systemic factors that may be involved in hypertension. Clearly, FAs will be exposed similarly to resistance arteries to circulating substances that might be involved in hypertension. These would include the exogenous, pump-derived Ang II used here, or natural endogenous vasoconstrictors whose secretion is stimulated by Ang II, such as AVP. Secondly, contractile activation and diameter of FAs were known to be under continuous control of SNA (Zacharia *et al.* 2013), a characteristic that could help reveal CNS-induced changes in SNA in hypertension. Finally, conduit arteries are of interest because they are importantly involved in the pathophysiology of hypertension, where increased conduit stiffness (possibly due to increased contractile activation) contributes to increased cardiac work (Sparks *et al.* 2011; Sudano *et al.* 2011).

Contractile activation and $[Ca^{2+}]_i$

The discussion above supports the conclusion that a real, small, change in contractile activation was present in the FAs of the Ang II-infused mice, rather than structural remodelling. As mentioned previously, this change in contractile activation could be due to increased $[Ca^{2+}]_i$ and increased activation of MLCK, and/or due to decreased activity of myosin light chain phosphatase. The changes in smooth muscle $[Ca^{2+}]_i$ that we observed were small, ∼78 nM, but available information suggests that such changes are adequate to drive significant changes in arterial diameter, due to increased Ca^{2+}-dependent contractile activation. These results are in accordance with our previous study (Zhang *et al.* 2010) in which we showed that the method was capable of detecting changes in $[Ca^{2+}]_i$ as small as 29 nM between two groups of 12 mice. Here, we used groups of 20 and 21 mice (Fig. 2). In contrast to our previous study, however, we provided a true intracellular 'calibration' curve of exMLCK here (Fig. 1). The calibration curve was obtained using mesenteric small arteries *ex vivo*, as it could not be obtained on FA *in vivo* (as the procedure requires artery permeabilization). Importantly, the Hill coefficient and

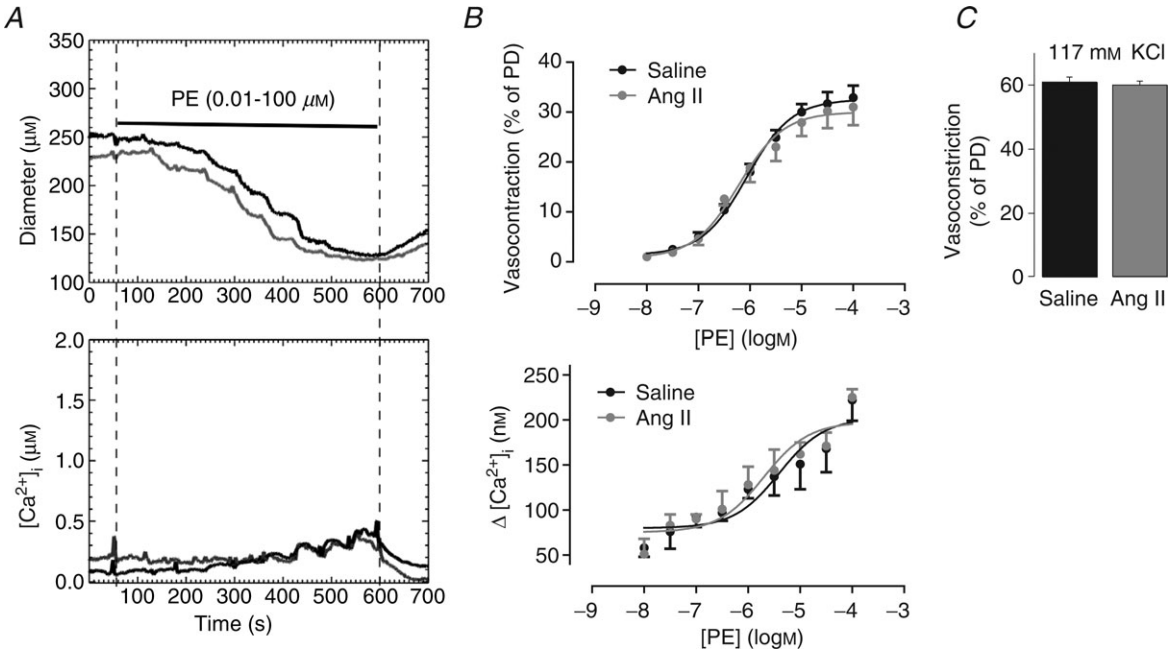

Figure 8. Vasoconstriction and $[Ca^{2+}]_i$ elevation induced by locally applied agonists are not different between arteries of saline- and Ang II-infused mice
A, representative recordings of diameter (top) and $[Ca^{2+}]_i$ (bottom) changes in response to α-adrenoceptor activation by PE (0.01–100 μM) in FAs of saline- (black line) and Ang II (grey line)-infused mice. *B*, summary of dose-dependent vasoconstriction (top) and $[Ca^{2+}]_i$ elevation (bottom) to PE in FAs of saline- ($n = 7$) and Ang II ($n = 5$)-infused mice. $P > 0.05$, two-way ANOVA. *C*, comparison of vasoconstriction induced by 117 K solution in FAs of saline- ($n = 20$) and Ang II- ($n = 21$) infused mice.

EC_{50} for free $[Ca^{2+}]$ (no added calmodulin) are virtually identical to that of endogenous MLCK (Geguchadze *et al.* 2004). These data can be used to conclude that a change in fractional activation of MLCK was ∼4.4% to alter $[Ca^{2+}]_i$ by ∼78 nM. Clearly, however, we cannot exclude that increased contractile activation was not also due to decreased levels of myosin light chain phosphatase activity. Indeed, such an effect is probably a result of α_1-adrenergic receptor activation, the mechanism we think most likely to account for the increased contractile activation of FAs in Ang II-infused mice.

Vascular AT_1Rs

One possible mechanism of increased $[Ca^{2+}]_i$ and decreased diameter would be a direct effect of the infused Ang II on vascular AT_1Rs. Although indirect evidence (Huang *et al.* 2010) already suggests that this is not likely to be the case, the possibility had never been tested before. Here, local application of the AT_1R blocker losartan did reveal a small direct effect of the exogenous Ang II. The concentration used was adequate to block the effects of exogenously applied Ang II. Acute application of AT_1R agonists (e.g. Ang II) to segments of exposed FA showed that the artery was highly sensitive to exogenous Ang II: at a concentration of Ang II as low as 10 nM, vasoconstriction could be easily detected in saline-infused mice (data not shown). Losartan, a specific AT_1R antagonist, at the concentration that effectively blocks 100 nM Ang II-induced vasoconstriction and $[Ca^{2+}]_i$ elevation, had no effect on basal tone or $[Ca^{2+}]_i$ when applied in the absence of Ang II. This suggests that local arterial AT_1R activation seems to play a negligible role under basal conditions in control mice. Systemic application of losartan does decrease arterial BP dramatically (Xu & Brooks, 1996; Collister & Osborn, 2005; Bek *et al.* 2006). Thus, we speculate that the effect of systemic losartan on BP is not due, directly, to the blockade of vascular AT_1Rs in saline or normal control mice. In the chronic Ang II infusion model of hypertension, however, elevated plasma Ang II does act, to a small extent (∼9%), through activation of AT_1Rs on the systemic arteries, as acute local application of losartan did reduce tone and $[Ca^{2+}]_i$. This activation of AT_1R is probably due directly to the infused, circulating plasma Ang II.

Sympathetic nerve activity

We have shown previously (Mauban *et al.* 2013; Zacharia *et al.* 2013) that ∼90% of the vascular tone in FAs in anaesthetized biosensor mice is attributable to SNA and specifically to activation of α_{1A} and α_{1D} receptor

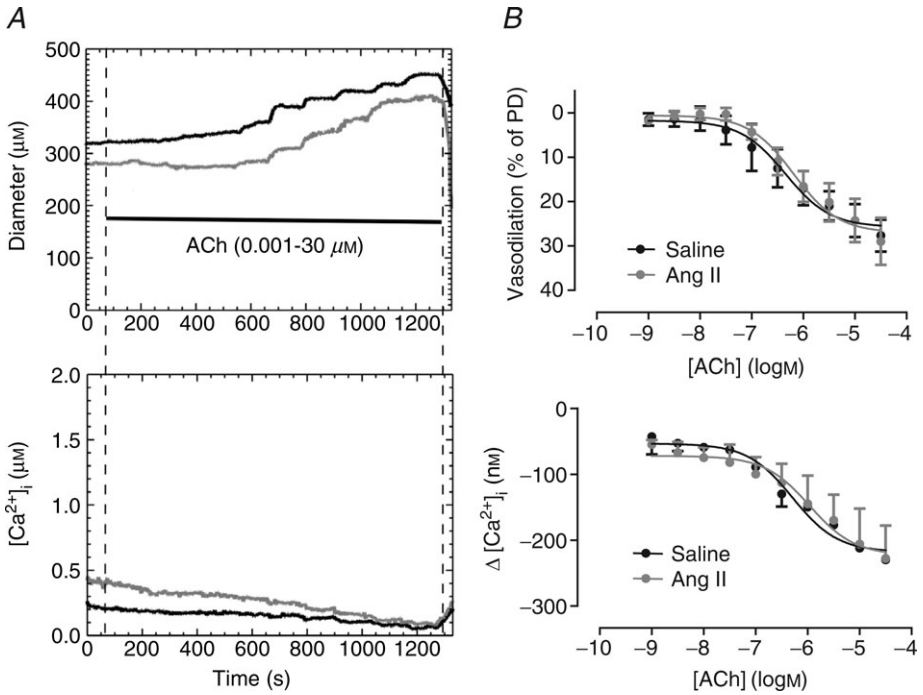

Figure 9. Vasodilation and $[Ca^{2+}]_i$ reduction induced by locally applied ACh are not different between arteries of saline- and Ang II-infused mice
A, representative recordings of diameter (top) and $[Ca^{2+}]_i$ (bottom) changes in response to ACh (0.001–30 μM) in FAs of saline- (black line) and Ang II- (grey line) infused mice. *B*, dose-dependent vasodilation (top) and $[Ca^{2+}]_i$ reduction (bottom) in response to ACh in FAs of saline- ($n = 4$) and Ang II- ($n = 3$) infused mice. $P > 0.05$, two-way ANOVA.

subtypes (Zacharia *et al.* 2013). Total autonomic ganglionic blockade (Fig. 5) has the disadvantage that arterial BP falls rapidly, producing changes in arterial diameter that are probably the result of both decreased SNA to the artery and decreased distending forces. In Ang II–salt hypertension also, an increased 'depressor' (decrease in mean arterial BP) response to systemic block of autonomic ganglionic transmission can be demonstrated (Kuroki *et al.* 2012), suggesting increased SNA-induced vascular tone. In contrast, local application of sympathetic neurotransmitter receptor blockers (Figs. 6 and 7) does not change arterial BP (Zhang *et al.* 2010). While our results indicated clearly that the additional FA tone in mice infused with Ang II for 2–3 weeks was associated with increased sympathetic α_1-adrenergic receptor activation, other mechanisms that mediate BP increase during early phase (i.e. hours to days) Ang II infusion, which were not tested here, might also exist. Indeed, Feng *et al.* (2010) reported that brain-targeted angiotensin-converting enzyme type 2 overexpression attenuated the development of hypertension during the 2nd but not the 1st week of Ang II infusion, suggesting a non-neurogenic mechanism in the early stage of BP elevation in response to Ang II infusion.

Arterial reactivity

An increased 'depressor' response or increased dilation after abolition of sympathetic neuromuscular transmission (local receptor block) could arise either from increased SNA or from an increased vascular response to an unchanged SNA (i.e. increased 'vascular reactivity') (Anning *et al.* 2005; Touyz *et al.* 2005; Hercule *et al.* 2007; Ding *et al.* 2007; Kirabo *et al.* 2011; Kharade *et al.* 2013). As SNA was not measured directly in our experiments, it was important to determine whether FA might have increased reactivity to sympathetic neurotransmitters. Local application of PE activated the α-adrenergic receptor, without potentially confounding changes in arterial BP or baroreflexes (Zhang *et al.* 2010). No differences in the ability of PE to increase $[Ca^{2+}]_i$ and/or decrease diameter were found (Fig. 8). Altered vascular reactivity, e.g. enhanced vasoconstriction, either myogenic or induced by agonists (e.g. PE and endothelin), or impaired endothelial-dependent vasodilation (Huang *et al.* 1995; Anning *et al.* 2005), also contributes to Ang II-induced hypertension (Ding *et al.* 2007; Hercule *et al.* 2007). In the present study, altered vascular reactivity, however, was not evident in FAs of Ang II-infused mice, as neither vasoconstrictions in response to acute application of PE, AVP or 117 K solution, nor vasorelaxation to ACh, appeared to be different in arteries of Ang II-infused mice from those effects in arteries of saline-infused mice. Although vascular reactivity to vasoactive stimuli, e.g. PE, was not altered, alterations in the expression level of α_1-adrenoceptor cannot be excluded, but such changes would have to have been compensated for by other mechanisms.

Relation to hypertension

Abundant data indicate that Ang II plays an essential role in the pathogenesis of hypertension. Chronic systemic Ang II infusion induces experimental hypertension, which involves numerous changes in the CNS (Zimmerman *et al.* 2004; Kuroki *et al.* 2012; Young *et al.* 2012), immune system (Guzik *et al.* 2007; Marvar *et al.* 2010), cardiovascular system (Kim & Iwao, 2000; Touyz *et al.* 2005; Ding *et al.* 2007; Hercule *et al.* 2007; Kirabo *et al.* 2011; Kharade *et al.* 2013), and the peripheral salt and water regulation organs, including the kidneys (Kim & Iwao, 2000; Bek *et al.* 2006; Gonzalez-Villalobos *et al.* 2009; Navar, 2010). Increased central SNA has been implicated in various hypertension models, particularly in models involving Ang II (Zimmerman *et al.* 2004; Marvar *et al.* 2010; Yoshimoto *et al.* 2010; Kuroki *et al.* 2012; Young *et al.* 2012). A vigorous debate has focused recently on the relative importance of the sympathetic nervous system *vs.* the kidneys (Esler *et al.* 2010; Navar, 2010) in essential hypertension. A current concept about the pressor effect of chronic systemic (e.g. subcutaneous) Ang II infusion (Zimmerman *et al.* 2004; Marvar *et al.* 2010; Young *et al.* 2012; Lob *et al.* 2013) is that elevated levels of plasma Ang II acts in the CNS, perhaps in a region of the brain that lacks the blood brain barrier (namely the subfornical organ) to modify hormonal output (e.g. AVP) and sympathetic nerve drive to the periphery, thus influencing the function of kidneys, adrenals, heart and blood vessels. In the present study, however, we have shown that the increased tone in FAs of Ang II-infused mice was not attributable to an increased V_{1A} receptor activation. This is consistent with the reported primary anti-diuretic function of AVP in the kidney by affecting renal handling of water via V_2 receptors (den Ouden & Meinders, 2005). Interestingly, however, V_{1A} receptor activation does appear to contribute a small component to the basal tone, as V_{1A} receptor antagonist attenuated slightly the basal tone even in FAs of saline-infused mice (Fig. 4).

The increased SNA is thought to be region-specific, and to vary during experimental hypertension (Yoshimoto *et al.* 2010; Kuroki *et al.* 2012). Also, increased SNA has been postulated to be regulated by both fast and slow signalling pathways in Ang II/salt hypertensions (Blaustein *et al.* 2012; Gabor & Leenen, 2012). Despite the consensus that SNA is strongly involved in Ang II-induced hypertension (Zimmerman *et al.* 2004; Esler *et al.* 2010; Yoshimoto *et al.* 2010; Young *et al.* 2012), however, actual demonstration of increased SNA has been difficult. Microneurography to record SNA directly in a region-specific

manner is useful (Campese *et al.* 2004), but is difficult practically. Measurement of noradrenaline spillover (King *et al.* 2008) in plasma usually lacks spatial information and may not reflect well the postulated region-specific changes in SNA.

In summary, to address some of the uncertainty regarding the involvement of sympathetic nervous system in Ang II experimental hypertension, we used a new method (Zhang *et al.* 2010) and accomplished quantification of changes in $[Ca^{2+}]_i$ and outer diameter in FAs in living mice. Local application of receptor blockers and ligands allowed determination of local receptor activation and determination of the contributions of the sympathetic neurotransmitters, as well as that of circulating hormones on the arterial tone *in vivo*. The results show that murine FA does exhibit high vascular tone, which appears to be due almost entirely to SNA. These results suggest that FA tone *in vivo* might be a direct assessment of SNA. Increased contractile tone of these arteries would be expected to increase arterial stiffness, affecting cardiovascular parameters such as arterial pulse pressure, pulse wave velocity and cardiac work. The elevated $[Ca^{2+}]_i$ and vascular tone were mediated primarily by α_1-adrenergic neurotransmitter release as a result of increased central SNA.

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Additional information

Competing interests

None.

Author contributions

Y.W. performed the experiments, collected and analysed the data and made the figures. L.C. installed Ang II pumps, collected and analysed the BP data, and edited the manuscript. W.G.W. analysed $[Ca^{2+}]_i$ data and interpreted the results, and revised the article critically for important intellectual content. J.Z. designed the experiments, analysed the data, interpreted the results, and drafted and revised the manuscript. All authors approved the final submission.

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