Synergistic effect of angiotensin II and nitric oxide synthase inhibitor in increasing aortic stiffness in mice

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Synergistic effect of angiotensin II and nitric oxide synthase inhibitor in increasing aortic stiffness in mice. Am J Physiol Heart Circ Physiol 290: H1190–H1198, 2006. First published November 4, 2005; doi:10.1152/ajpheart.00327.2005.—Although they are implicated on their own as risk factors for cardiovascular disease, the potential link between nitric oxide (NO) deficiency, ANG II, and vascular stiffening has not been tested before. We evaluated the role of chronic ANG II treatment and NO deficiency, alone and in combination, on aortic stiffness in mice and tested parameters contributing to increases in active or passive components of vascular stiffness, including blood pressure, vascular smooth muscle contractility, and extracellular matrix components. Untreated (control) mice and mice treated with a NO synthase (NOS) inhibitor [Nω-nitro-L-arginine methyl ester (L-NAME), 0.5 g/l] were implanted with osmotic minipumps delivering ANG II (500 ng kg⁻¹ min⁻¹) for 28 days. Aortic stiffness was then measured in vivo by pulse wave velocity (PWV) and ex vivo by load-strain analysis to obtain values of maximal passive stiffness (MPS). Blood pressure and aortic contractility ex vivo were measured. ANG II treatment or NOS inhibition with L-NAME did not independently increase vascular stiffness; however, the combined treatments worked synergistically to increase PWV and MPS. The combined treatments of ANG II + L-NAME also significantly increased aortic wall collagen content while decreasing elastin. These novel results suggest that NO deficiency and ANG II act synergistically to increase aortic stiffness in mice predominantly via changes in aortic wall collagen/elastin ratio.

Blood pressure; vascular smooth muscle; collagen; elastin; Nω-nitro-L-arginine methyl ester

Vascular stiffness is a risk factor for cardiovascular disease and associated mortality (2, 17, 19). A stiffened aorta impairs heart function by increasing afterload during systole and decreasing cardiac perfusion during diastole (25). Age, atherosclerosis, diabetes, hypertension, and renal failure are contributing factors to increased aortic stiffness (32, 33, 41). The passive biomechanical properties that contribute to vessel stiffness are largely determined by extracellular matrix (ECM) proteins, such as collagen and elastin, serving as the vessel’s scaffold. Collagen provides tensile strength, while elastin enables vascular elasticity (45). Active stiffness of the aorta is a function of vascular smooth muscle tone, which is modified by various mediators, many of which come from the endothelium (1, 25).

Endothelial dysfunction, characterized by endothelial nitric oxide (NO) deficiency, contributes to initiation and progression of vascular diseases. NO is a potent vasodilator that also inhibits vascular smooth muscle cell (VSMC) proliferation, platelet activation, and leukocyte adhesion. We and others (8, 30, 46) have recently shown that endothelial NO deficiency may contribute to vascular stiffness.

ANG II has been implicated in several cardiovascular diseases as evidenced by the therapeutic benefits of angiotensin converting enzyme (ACE) inhibitors and ANG II receptor antagonists (5, 44). ANG II is a vasoconstrictor that stimulates vascular remodeling, including VSMC growth and increased ECM production (13, 39). We have shown that in hypercholesterolemic mice, ANG II contributes to increased aortic stiffness (36). However, the direct impact of ANG II on vascular stiffening is still unknown.

The signaling pathways of NO and ANG II interact, such that they antagonize each other on vascular tone, VSMC growth, and signaling (47). In addition, NO inhibits ACE activity and downregulates ANG II type-1 (AT₁) receptor, while the NO synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) increases ACE activity (47). ANG II increases endothelial NOS mRNA but decreases total NO, perhaps through uncoupling of the endothelial NOS enzyme (20).

However, the link between NO, ANG II, and vascular stiffness has not been tested before. Therefore, in the present study, we evaluated the role of NO in ANG II-induced increase in aortic stiffness and tested the hypothesis that NO deficiency “enables” ANG II to increase aortic stiffness. In testing our hypothesis, we investigated aortic stiffness in vivo and ex vivo and the interaction of ANG II and NO deficiency on various components contributing to active and passive vascular stiffness.

METHODS

Animals. Experiments were carried out in 6-mo-old male C57Bl/6J mice obtained from Jackson Laboratories (Bar Harbor, ME). Animals were kept in a room at controlled temperature (24°C) and lighting (14:10 h light-dark cycle) with free access to food and tap water. All experimental procedures were approved by the Animal Care Committee at Berlex Biosciences, in agreement with the recommendations of the American Association for the Accreditation of Laboratory Animal Care. Animals were randomly assigned to groups and given identification numbers. Only these numbers were used to identify animals during all measurements, so that the investigator remained blinded during the study.

Osmotic minipumps (model 2004, Alzet, Palo Alto, CA) containing 500 ng kg⁻¹ min⁻¹ ANG II (CalBiochem, La Jolla, CA) were implanted subcutaneously in isoflurane-anesthetized mice. A group of
C57Bl/6J mice were also given water containing 0.5 g/l of the NOS inhibitor L-NAME (Sigma, St. Louis, MO) ad libitum. The concentration of L-NAME used in the drinking water was based on published reports that have shown impaired NO-mediated endothelium-dependent vasorelaxation in mouse aorta (6, 15). After 28 days administration of ANG II, L-NAME, or ANG II + L-NAME, in vivo vascular stiffness was determined noninvasively by measuring pulse wave velocity (PWV) by using a Doppler probe. Systolic blood pressure was measured noninvasively by the tail-cuff method. The animals were then euthanized with CO2 asphyxiation. The right atrium of the heart was snipped, and the left ventricle was perfused slowly with 10 ml cold PBS to flush blood from the vascular system. Microdissection of the thoracic aorta was carefully performed to cut away adjacent fat and intercostal branches without significantly impairing the structural integrity of the vessel. The vessel was then carefully extracted for histology, ex vivo functional studies, and biochemical analysis.

**Aortic stiffness measured by PWV in vivo.** Noninvasive Doppler measurement of PWV was developed for the determination of aortic stiffness in mice and has been used repeatedly in our laboratory (12, 36, 43). Anesthesia was induced by placing mice in a closed chamber ventilated with 1.5% isoflurane for 3–5 min (IMPAc 6, VetEquip, Pleasanton, CA). After induction, the mouse was taped supine to an electrocardiogram (ECG) electrodes incorporated into a temperature-controlled printed circuit board. The temperature of the mouse was monitored with a rectal probe (Physitemp, Clifton, NJ), and body temperature was maintained at 35°C throughout the study. The ECG electrodes were connected to a high-fidelity ECG amplifier with a 0.1- to 2-KHz bandwidth set to record lead II. Anesthesia was maintained during measurements by placing coaxial tubing from the anesthesia machine loosely over the face of the mouse. A 20-MHz Doppler probe with a 4-mm focal distance was placed just left of the sternum and angled to record velocity in the aortic arch moving toward the probe at a depth of 2–4 mm. A mark was made on the chest at the aortic arch measurement site, and a second mark was made 40 mm distal on the abdomen. A measurement was then taken at the second mark for the abdominal aortic waveform. Pulse waves and ECG were recorded and analyzed on a Doppler Signal Processing Workstation (version 1.41e; Indus Instruments, Houston, TX). Aortic PWV was calculated by dividing the separation distance (40 mm) by the difference in arrival times of the Doppler Signal Processing Workstation (version 1.41e; Indus Instruments). In preparation for each stretch, the aortic segments were conditioned three times to a standard strain (10% of maximal strain) that was determined in previous experiments. The vessel was stretched until breakage. Load-strain curves were generated for each vessel. MPS is defined as the maximal slope of the load-strain relationship. MPS was measured in three adjacent 1-mm segments, and the result from each was averaged for each aorta.

**Organ chamber studies.** Aortic rings from the proximal region of the descending thoracic aorta were isolated from each mouse, cut under microscope into 4-mm-long rings, and mounted in organ chambers (Radnoti). The chambers were filled with physiological saline solution with the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.17 H3PO4, 25 NaHCO3, 0.026 EDTA, and 11 glucose. The solution was continuously gassed (95% O2-5% CO2) and maintained at 37°C. Changes in isometric tension were measured with force transducers (FT03; Grass Instruments, Quincy, MA) and recorded by a Powerlab 16/S data-acquisition system. Optimal resting tension was determined from length-tension curves done in previous experiments. The aortic rings were allowed to equilibrate for 90 min in the organ chamber with increasing resting tension every 20 min (0.25 g, 0.5 g, 1.0 g, and 1.5 g) and then were maintained at 1.5 g throughout the rest of the experiment. The vessels were challenged with 70 mM KCl for 20 min just before the start of the experiment. Vessels were tested for contractile response to KCl (70 mM) and in a dose-dependent manner (0.1 mM–3 μM) to phenylephrine (PE), 5-hydroxytryptamine (5-HT), and the thromboxane A2 analog U-46619. Force (mN) was normalized to cross-sectional area (mm2), where cross-sectional area was determined as previously described: cross-sectional area = [2 × wet weight (in mg)]/[1.06 mg/mm2 × circumference (in mm)] (27, 38). Circumference of the aorta was determined by cutting open the aorta segment; laying it flat, lumen side down; and measuring its circumference under microscope with a micrometer.

**Determination of dry weight and protein, collagen, and elastin content.** To determine protein, collagen, and elastin content of dissected thoracic aortas, first the vessel length was measured with a micrometer under microscope, then dried (40°C for 24 h), weighed, and hydrolyzed in 6 N HCl at 110°C for 24 h. Hydrolysates were filtered with Acrodisc HPLC filters (0.2 μm) and evaporated with a Savant Automatic Environmental SpeedVac system for 30 min. The samples were resuspended in 200 μl deionized water and stored at −20°C. Dry weight, protein, collagen, and elastin content were expressed per vessel length (mm).

A modified Lowry protein assay (Bio-Rad DC Protein Assay Kit) was used to quantify total protein in hydrolysates. Collagen content was quantified by measuring hydroxypyroline in hydrolysates using a method described by Stegemann and Stalder (34). Elastin content was assessed by measuring the unique amino acids desmosine (DES) and isodesmosine (IDE), which form the crosslinks of elastin (37). They have identical molecular weights (526.5 atomic mass units) as well as fragmentation patterns in tandem mass spectrometry (MS/MS), so DES and IDE were quantified as one entity by using a rapid liquid chromatography (LC)/MS/MS method on a triple quadrupole API3000 mass spectrometer (Applied Biosystems, Foster City, CA) with a dual-pump HPLC system (Shimadzu, Kyoto, Japan). An internal standard consisting of 100 μl of 1 μM pyridylethyl-cysteine (Sigma) in water with 1% heptfluorobutyric acid was added to 100 μl
significantly increased PWV in mice treated with the nitric oxide synthase
was used to retain DES and IDE to a Varian Polaris C18 column (5
of each aorta hydrosylate sample. Ion-pairing reversed-phase HPLC
Fig. 1. Aortic stiffness measured by pulse wave velocity (PWV) in 6-mo-old
are shown as means ± SE. Significant differences (one-way ANOVA followed
by Bonferroni post hoc test): *P < 0.05, ANG II + l-NAME vs. l-NAME; †P < 0.05, ANG II + l-NAME vs. ANG II.
of each aorta hydrosylate sample. Ion-pairing reversed-phase HPLC
was used to retain DES and IDE to a Varian Polaris C18 column (5
μm) (Varian, Palo Alto, CA). The organic solvent used was methanol
with 1% heptafluorobutyric acid (the ion-pairing agent), and the
aqueous solvent was water with 1% heptafluorobutyric acid. The
gradient used was as follows: 5% organic solvent to 23% over 3 min,
with 1% heptafluorobutyric acid (the ion-pairing agent), and the
aqueous solvent was water with 1% heptafluorobutyric acid. The
gradient used was as follows: 5% organic solvent to 23% over 3 min,
then ramped up to 85% for 3 min to wash, then returned to 5% to
equilibrate for 2 min. Values of total DES and IDE were calculated
from a dose-response curve created with purified DES and IDE
obtained from Sigma.

Histology. The arch of the thoracic aorta was dehydrated through a
graded ethanol series, cleared with xylene, infiltrated with warm
paraffin, and embedded in paraffin blocks. The embedded aorta
segment was cut just distal to the left common carotid artery into
5-μm-thick sections which were mounted on gelatin-coated glass
slides. Sections were stained with Movas’s pentachrome stain (21). The
cross-sectional area of the aortic lumen, media, and adventitia were
measured with histomorphometric analysis of stained sections using the
Olympus C.A.S.T.-Grid system (Olympus Denmark A/S, Alberslund,
Denmark). The media is defined as the space between internal and
external elastic lamina, and the adventitia is defined as the space between
the external elastic lamina and the outer limit of the vessel, which is
the outer edge between tightly packed, well-organized tissue and sur-
rounding loose tissue with a clear loss of organization and structure.

Calculations and statistical analysis. In the majority of animals, PWV,
MPS, systolic blood pressure, collagen, elastin, protein, and
histomorphometry were measured in the same animal. For ex vivo
contractility and aortic dry weight determination, separate animals were
used because for the MPS measurements most of the aorta was used and
treated in a way that precluded the above measurements. Invasive blood
pressure measurements were also conducted in separate animals.

Data are expressed as means ± SE. Statistical analysis was performed
between groups using one-way ANOVA (with a Bonferroni’s post hoc test) for normally distributed populations. Nonnormal data,
as determined by Shapiro-Wilk normality test of residuals, were
transformed to normally distributed data. Unequal variances between
groups, as determined by Levene’s test, were remedied with weighted
least squares as suggested by Neter et al. (24). P < 0.05 was
considered statistically significant.

RESULTS

Aortic stiffness. Aortic stiffness measured in vivo by PWV
was not different between untreated (control) mice (3.4 ± 0.1
m/s), mice given l-NAME alone (3.5 ± 0.1 m/s), or ANG II
alone (3.4 ± 0.2 m/s) (Fig. 1). In contrast, ANG II infusion
significantly increased PWV in l-NAME-treated mice (4.8 ±
0.3 m/s; P < 0.05).

Aortic stiffness measured ex vivo by MPS was similar in
control (0.7 ± 0.1 N), l-NAME (0.7 ± 0.1 N), and ANG
II-infused mice (1.2 ± 0.3 N) (Fig. 2). In good agreement with
the PWV measurements in vivo, ANG II infusion significantly
increased MPS in l-NAME-treated mice (3.4 ± 1.0 N; P < 0.05).

Blood pressure. Systolic blood pressure measured (noninva-
sively by the tail-cuff method) in mice, where PWV meas-
urements were also performed in most of the same mice, was not
significantly different between control (143 ± 8 mmHg, n = 8)
animals and mice treated with ANG II (153 ± 5 mmHg, n = 8),
l-NAME (150 ± 6 mmHg, n = 10), or ANG II + l-NAME
(163 ± 5 mmHg, n = 10) (one-way ANOVA, P = 0.15).

In a separate group of animals, blood pressure was measured
invasively in isoflurane-anesthetized mice to obtain central
arterial pressure. Mean arterial pressure (MAP), systolic arte-
rial pressure (SAP), and PP measured directly in the aorta were
not significantly different between control, ANG II-, or L-
NAME-treated mice (Fig. 3, A, B, and D). However, MAP,
SAP, and PP were significantly greater in the ANG II + l-NAME-treated mice compared with control, and SAP was
also significantly greater in the ANG II + l-NAME-treated
mice compared with l-NAME treatment alone. Diastolic arte-
rial pressure (DAP) was not significantly different between any
of the groups studied (Fig. 3C).

Aortic smooth muscle contractility. Chronic l-NAME treat-
ment in vivo significantly reduced maximal contractions of
isolated aortic rings (mounted on organ chambers) to U-46619
(26 ± 1 mN/mm², P < 0.05) and attenuated (but not signifi-
cantly) maximal contraction to 70 mM KCl (12 ± 2 mN/mm²),
PE (17 ± 2 mN/mm²), and 5-HT (17 ± 2 mN/mm²) compared
with aortic rings isolated from untreated (control) mice (U-
46619, 37 ± 4 mN/mm²; 70 mM KCl, 17 ± 2 mN/mm²; PE,
20 ± 3 mN/mm²; 5-HT, 27 ± 4 mN/mm²) (Fig. 4). Chronic
ANG II treatment in vivo significantly reduced maximal aortic
contractions to 70 mM KCl (7.7 ± 0.9 mN/mm²), PE (10 ± 1
Fig. 3. Blood pressure measured in the aortic arch of isoflurane-anesthetized control, ANG II-treated, L-NAME-treated, and ANG II + L-NAME-treated mice. Mean arterial pressure (MAP; A), systolic arterial pressure (SAP; B), diastolic arterial pressure (DAP; C), and pulse pressure (PP; D) were determined from blood pressure waveforms. *P < 0.05 vs. control, †P < 0.05 vs. L-NAME.

Fig. 4. Maximal contractile responses to 70 mM KCl, phenylephrine (PE), 5-hydroxytryptamine (5-HT), and U-46619 in aortic segments isolated from 6-mo-old male mice (n = 6–8 animals/group) ex vivo. Chronic ANG II (500 ng·kg⁻¹·min⁻¹) treatment in mice significantly impaired vascular smooth muscle contractile responses to KCl, PE, 5-HT, and U-46619. Chronic treatment with NOS inhibitor (L-NAME, 0.5 g/l) also impaired vascular smooth muscle contractile responses to KCl, PE, 5-HT, and U-46619. Combined chronic treatment with ANG II + L-NAME significantly reduced maximal vascular smooth muscle contractile responses to KCl, 5-HT, and U-46619, to a greater extent than ANG II or L-NAME treatment alone. Results are shown as means ± SE. Significant differences (one-way ANOVA followed by Bonferroni post hoc test): *P < 0.05 for ANG II vs. control and for ANG II + L-NAME vs. L-NAME; †P < 0.05 for L-NAME vs. control and for ANG II + L-NAME vs. ANG II.
mN/mm²), 5-HT (14 ± 1 mN/mm²), and U-46619 (17 ± 2 mN/mm²) (P < 0.05). The ANG II-induced decrease in maximum aortic contractility was more pronounced in L-NAME-treated mice with 70 KCl (4 ± 1 mN/mm²), 5-HT (7 ± 1 mN/mm²), and U-46619 (9 ± 1 mN/mm²) compared with ANG II alone (P < 0.05).

Aortic dry weight, total protein, and collagen and elastin content. Thoracic aorta hypertrophy, measured by thoracic aorta dry weight and protein content (Fig. 5), was similar between control (untreated) (63 ± 1 and 16 ± 1 μg/mm, respectively), ANG II-treated (108 ± 8 and 21 ± 2 μg/mm, respectively), and L-NAME-treated (60 ± 1 and 16 ± 1 μg/mm, respectively) mice. However, the combined treatment with ANG II + L-NAME significantly increased both thoracic aorta dry weight and protein content (274 ± 26 and 37 ± 3, μg/mm, respectively; P < 0.05) compared with untreated mice or mice treated with ANG II or L-NAME alone.

Chronic ANG II infusion alone (3.8 ± 0.7 μg/mm) or L-NAME alone (2.2 ± 0.2 μg/mm) did not significantly increase hydroxyproline levels compared with untreated control Fig. 5. Thoracic aorta dry weight and protein levels from 6-mo-old male mice (n = 4–8 animals/group). ANG II (500 ng·kg⁻¹·min⁻¹) significantly increased thoracic aorta dry weight and protein level in mice chronically treated with NOS inhibitor (L-NAME, 0.5 g/l). Results are shown as means ± SE. Significant differences (one-way ANOVA followed by Bonferroni post hoc test): *P < 0.05, ANG II + L-NAME vs. L-NAME; †P < 0.05, ANG II + L-NAME vs. ANG II.

Fig. 5. Thoracic aorta dry weight and protein levels from 6-mo-old male mice (n = 4–8 animals/group). ANG II (500 ng·kg⁻¹·min⁻¹) significantly increased thoracic aorta dry weight and protein level in mice chronically treated with NOS inhibitor (L-NAME, 0.5 g/l). Results are shown as means ± SE. Significant differences (one-way ANOVA followed by Bonferroni post hoc test): *P < 0.05, ANG II + L-NAME vs. L-NAME; †P < 0.05, ANG II + L-NAME vs. ANG II.

Chronic ANG II infusion alone (3.8 ± 0.7 μg/mm) or L-NAME alone (2.2 ± 0.2 μg/mm) did not significantly increase hydroxyproline levels compared with untreated control...
Combination of L-NAME treatment with ANG II significantly increased hydroxyproline levels (7.8 \pm 1.2 \mu g/mm; \( P < 0.05 \)) compared with untreated mice or mice treated with ANG II or L-NAME alone.

Chronic ANG II treatment (110 \pm 12 ng/mm) significantly elevated the elastin content compared with control (61 \pm 9 ng/mm; \( P < 0.05 \)) (Fig. 6), whereas L-NAME treatment alone (62 \pm 12 ng/mm) did not. ANG II and L-NAME treatment combined (43 \pm 5 ng/mm; \( P < 0.05 \)) significantly lowered elastin content compared with ANG II treatment alone.

The ratio of aortic collagen and elastin content was similar between untreated control (0.05 \pm 0.01), ANG II-treated (0.04 \pm 0.01), and L-NAME-treated mice (0.05 \pm 0.01) (Fig. 6). Chronic ANG II + L-NAME combined treatment significantly increased the aortic collagen/elastin ratio fourfold (0.2 \pm 0.03; \( P < 0.05 \)).

Histology. The media-to-lumen ratio was not different between control animals and mice treated with ANG II, L-NAME, or ANG II + L-NAME (Figs. 7 and 8). The cross-sectional area of adventitia was not different between control animals and mice treated with ANG II or L-NAME alone (Figs. 7 and 8). However, combined treatment with ANG II + L-NAME significantly increased adventitial cross-sectional area (0.25 \pm 0.01 mm\(^2\); \( P < 0.05 \)) compared with ANG II (0.10 \pm 0.01 mm\(^2\)) or L-NAME treatment (0.06 \pm 0.01 mm\(^2\)) alone.

DISCUSSION

The aim of the present study was to evaluate the role and potential interactions of NO deficiency and ANG II on aortic stiffening in mice and to investigate the biomechanical and...
synergistic interaction between ANG II and NO deficiency in aortic stiffening. MPS is a biomechanical measure of arterial stiffness determined by the passive components of the vascular wall (4, 26, 35) where previously frozen, rehydrated thoracic aorta rings were subjected to a load-strain apparatus. The fourfold increase in MPS observed in the ANG II-infused and L-NAME-treated mice suggests that changes in passive stiffness largely contribute to the increases in PWV observed in vivo.

Factors contributing to passive stiffness. Passive stiffness is determined to a large extent by two biomaterials in the vascular wall, collagen, and elastin (25). Collagen is a very stiff biomaterial having a high elastic modulus ($9 \times 10^8$ dyn/cm$^2$) that adds strength to the structure of the vascular wall (25). Chronic ANG II infusion was shown to induce hypertrophy and increase the protein content and collagen synthesis in VSMC via the AT$_1$ receptor (9, 11, 14, 23). Previous studies have also shown that NO donors and endothelium-derived NO inhibit protein and collagen synthesis in VSMC and endothelial cells (16, 22) in vitro. Endothelin-induced increase in collagen and protein synthesis was inhibited by exogenous NO in VSMC in culture (29). In our studies, chronic inhibition of NOS by itself did not increase mouse aorta collagen or protein levels in vivo. In contrast, NOS inhibition facilitated ANG II-induced increase in aortic wall collagen and total protein levels, suggesting that NO has an inhibitory effect on ANG II-induced VSMC hypertrophy and increased collagen synthesis.

Elastin is a vessel wall component with a low elastic modulus ($5 \times 10^6$ dyn/cm$^2$) that contributes to aortic distensibility (25). ANG II combined with L-NAME treatment decreased total elastin; however, ANG II, but not L-NAME alone, significantly increased elastin levels. Significant elevation of aortic wall collagen/elastin ratio in response to combined ANG II + L-NAME treatment provided a biochemical mechanism that contributes to the observed increase in aortic stiffness among all known factors contributing to passive stiffness because it has been established that changes in collagen/elastin ratio strongly predict corresponding changes in vessel stiffness in vivo (31).

The potential cause of adventitia expansion is unknown. It is possible that it is due to the effect of ANG II on perivascular fibroblasts. ANG II may stimulate NAD(P)H-mediated production of superoxide anions in aortic adventitial fibroblasts, which is mitigated by the presence of NO, scavenging superoxide, and forming peroxynitrite as a result (47). Indeed, it was demonstrated that ANG II increases the expression of p67phox [component of the NAD(P)H oxidase] in adventitial fibroblasts (10, 40) and immunohistochemical staining for 3-nitrotyrosine (indicative of peroxynitrite generation) in adventitia of aortas from ANG II-infused animals (10, 40). Therefore, in NO deficiency, ANG II treatment may expand aortic adventitia through superoxide-mediated signaling that is uninhibited by NO.

Factors contributing to active stiffness. Active stiffness as assessed by changes in systolic blood pressure and aortic contractility does not appear to contribute to aortic stiffening in NO-deficient, ANG II-infused mice. Active stiffness refers to the functional aspect of the cardiovascular system that contributes to stiffness, such as blood pressure and vascular smooth muscle tone.

Blood pressure is an important contributor to vascular stiffness (25), which increases as blood pressure rises (8), DAP
being the critical parameter influencing vascular stiffness measured by PWV (25, 31). We measured blood pressure noninvasively by using the tail-cuff method in conscious mice to avoid the effects of anesthesia and surgery on blood pressure. However, it is important to measure blood pressure at the site where PWV is determined (i.e., aorta). We performed a separate study (invasively) where we measured blood pressure directly under isoflurane anesthesia to obtain central arterial pressure in the same experimental groups. Neither ANG II nor \( L \)-NAME significantly changed SAP measured noninvasively nor MAP, SAP, DAP, or PP measured centrally in anesthetized mice. Combined ANG II + \( L \)-NAME treatment did not significantly increase SAP measured by tail-cuff method (vs. any treatment group) but did increase MAP, SAP, and PP when measured centrally. On the other hand, DAP was not significantly different between any of the groups measured centrally in mice. The increase in SAP and PP, but not DAP, observed in the ANG II + \( L \)-NAME group may suggest these changes are the consequence, rather than the cause, of increased aortic stiffness, DAP being the critical parameter influencing vascular stiffness and affecting PWV (25, 31). However, on the basis of the results obtained with direct blood pressure measurements, we cannot rule out some contribution of elevated blood pressure to the observed increase in aortic stiffness in the ANG II + \( L \)-NAME group.

We were unable to assess aortic vascular tone directly in vivo, so we measured aortic contractility in isolated aortic rings mounted in organ baths to test vascular smooth muscle responsiveness to various vasoconstrictors. ANG II infusion, with or without NO deficiency, did not increase contractile responses in isolated rings of mouse thoracic aorta. In contrast, ANG II reduced maximum contractile responses to both nonreceptor agonists (PE, 5-HT, and U-46619). Similarly, treating mice with \( L \)-NAME for 30 days significantly reduced maximal contractile responses to U-46619. Therefore, the contribution of active component(s) to the observed increases in aortic stiffness in vivo can be ruled out.

In summary, our study for the first time describes a synergistic interaction between chronic ANG II and \( L \)-NAME treatment to increase aortic stiffness in mice. By evaluating several factors contributing to passive or active components of vascular stiffening, we conclude that an increase in aortic wall collagen/elastin ratio is the main cause of the observed phenomenon. The novel finding of adventitial enlargement and adventitial collagen deposition after combined treatment with ANG II + \( L \)-NAME may also contribute to increased aortic stiffness.

Many cardiovascular diseases are associated with imbalances in both the ANG II and the NO systems (18, 28). While ANG II and NO are both capable of altering vascular function, our in vivo data demonstrate that an imbalance between ANG II and NO causes structural changes in the aorta, leading to an increase in aortic stiffness. This finding supports the development of therapeutic strategies that target restoration of the balance between ANG II and NO, and in doing so may prevent aortic stiffening and related cardiovascular complications.

GRANTS

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