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Local renin-angiotensin system mediates endothelial dilator dysfunction in aging arteries

Sheila Flavahan, Fumin Chang, and Nicholas A. Flavahan

Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University, Baltimore, Maryland

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Flavahan S, Chang F, Flavahan NA. Local renin-angiotensin system mediates endothelial dilator dysfunction in aging arteries. *Am J Physiol Heart Circ Physiol* 311: H849–H854, 2016. First published July 15, 2016; doi:10.1152/ajpheart.00422.2016.—Aging impairs endothelium-dependent NO-mediated dilatation, which results from increased production of reactive oxygen species (ROS). The local generation of angiotensin II (ANG II) is increased in aging arteries and contributes to inflammatory and fibrotic activity of smooth muscle cells and arterial wall remodeling. Although prolonged in vivo ANG II inhibition improves the impaired endothelial dilatation of aging arteries, it is unclear whether this reflects inhibition of intravascular or systemic ANG II systems. Experiments were therefore performed on isolated tail arteries from young (3–4 mo) and old (22–24 mo) F344 rats to determine if a local renin-angiotensin system contributes to the endothelial dilator dysfunction of aging. Aging impaired dilatation to the endothelial agonist acetylcholine but did not influence responses to a nitric oxide (NO) donor (DEA NONOate). Dilatation to acetylcholine was greatly reduced by NO synthase inhibition [nitro-L-arginine methyl ester (L-NAME)] in young and old arteries. In isolated arteries, acute inhibition of angiotensin-converting enzyme (ACE) (perindoprilat), renin (aliskiren), or AT_1 receptors (valsartan, losartan) did not influence dilatation to acetylcholine in young arteries but increased responses in old arteries. After ANG II inhibition, the dilator response to acetylcholine was similar in young and old arteries. ROS activity, which was increased in endothelium of aging arteries, was also reduced by inhibiting ANG II (perindoprilat, losartan). Renin expression was increased by 5.6 fold and immunofluorescent levels of ANG II were confirmed to be increased in aging compared with young arteries. Exogenous ANG II inhibited acetylcholine-induced dilatation. Therefore, aging-induced impairment of endothelium-dependent dilatation in aging is caused by a local intravascular renin-angiotensin system.

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keywords. angiotensin ii; AT_1 receptors; aging; endothelium

NEW & NOTEWORTHY

The study demonstrates for the first time that the impaired activity of endothelial nitric oxide-dependent dilatation in aging arteries is mediated by a local renin-angiotensin system and pathological signaling of angiotensin II. Inhibition of angiotensin synthesis or activity in isolated arteries rapidly and completely restores normal endothelial dilatation in old arteries.

AGING IS ASSOCIATED WITH STRUCTURAL and functional deterioration of the arterial system that precipitates cardiovascular disease, organ dysfunction, and organ injury (19, 20). Endothelial dysfunction is a key contributor to this process of vascular aging, which includes diminished nitric oxide (NO) dilator activity as a result of increased reactive oxygen species (ROS) (8, 17, 31). Aging arteries have intimal fibrous lesions with deposition of poorly distensible proteins, collagen and fibronectin and degradation of highly distensible elastin fibers (19, 35). This arterial stiffening, which is a hallmark feature of vascular aging, is mediated by expression and activation of a local intravascular angiotensin II (ANG II) system (19, 33, 34). Immunofluorescence for ANG II is increased four- to sixfold in the endothelium and intima of aged compared with young arteries from rats, humans, and nonhuman primates and is paralleled by increased expression of ACE and AT_1 receptors that are colocalized with ANG II (36–38). Aging-induced arterial wall remodeling is characterized by activation of ANG II signaling cascades and is mimicked in young arteries by ANG II exposure (12, 19, 34, 35, 38). Moreover, the increased activity of inflammatory and fibrotic mediators in aged arteries or smooth muscle cells (SMCs) is reduced by blocking local ANG II activity, achieved by inhibiting AT_1 receptors in isolated cultured arteries or SMCs (12, 16, 37, 38). Chronic in vivo inhibition of ANG II activity is also highly beneficial in aging, including reducing arterial wall remodeling, increasing arterial compliance, and extending lifespan (3, 6, 35).

ANG II is a powerful inducer of endothelial dysfunction, including inhibiting NO dilator activity as a result of increased ROS (10, 21). Prolonged in vivo inhibition of ANG II activity reverses the impaired endothelium-dependent dilatation of aging arteries, which is associated with reduced oxidant stress (24, 27). No previous studies have assessed whether such beneficial effects of ANG II inhibition on endothelial dilatation reflect inhibition of the intravascular or the systemic circulating ANG II system. The present experiments were therefore performed to evaluate the role of a local renin-angiotensin system in the endothelial dilator dysfunction of aging.

MATERIALS AND METHODS

Animals. Young (3–4 mo) and old (22–24 mo old) male F344 rats were obtained from Charles River and their NIA colony and killed by CO_2 asphyxiation. Tail arteries were rapidly removed and placed in cold Krebs-Ringer bicarbonate solution (control solution) (28). Animal use was approved by the Johns Hopkins University Institutional Animal Care and Use Committee and complied with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Vasodilatation. Tail arteries were cannulated with micropipettes, secured within a microvascular chamber, and maintained at a transmural pressure of 60 mmHg in the absence of flow, i.e., without perfusion (Living Systems, VT) (28). The arteries were superfused with control solution (37°C, pH 7.4, 16% O_2-5% CO_2-balance N_2), and the chamber was placed on the stage of an inverted microscope (28). Arterial diameter was continuously monitored and recorded (BIOPAC, Santa Barbara, CA) (28). Concentration-effect curves to vasodilators were obtained during constriction to phenylephrine, and acetylcholine responses were determined in paired arteries with one segment serving as control and the other treated with [nitro-L-arginine

Address for reprint requests and other correspondence: N. A. Flavahan, Dept. of Anesthesiology, Johns Hopkins Univ., 720 Rutland Ave., Ross-1159, Baltimore, MD 21205 (e-mail: nflavah1@jhmi.edu).

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methyl ester (L-NAME)] (100 μM) to inhibit NO synthase, perindoprilat (1 μM) to inhibit angiotensin-converting enzyme (ACE), aliskiren (0.1 μM) to inhibit renin, or valsartan (1 μM) or losartan (3 μM) to inhibit AT_1 receptors, as previously described (40). Arteries were incubated with the inhibitors for 60–90 min before and during exposure to acetylcholine.

Real-time PCR. Arteries were disrupted in RLT lysis buffer using the Bullet Blender (Next Advance) and total RNA isolated using the RNeasy Plus mini kit (Qiagen) following the manufacturer's directions. The quality and quantity of RNA samples were analyzed using a Nanodrop spectrophotometer (ThermoFisher Scientific). Reverse transcription was performed using the iScript Reverse Transcription super mix kit (Bio-Rad), following the manufacturer's directions. Real-Time PCR was performed in duplicate on an Applied Biosystems 7500 Real-Time PCR System. Relative abundance of renin mRNA in young and aging arteries, compared with GAPDH mRNA, was determined by the comparative cycle threshold (Ct) method using TaqMan probes and primers designed and supplied by Applied Biosystems: rat GAPDH (Rn 01775763_g1) and rat renin (Rn02586313_m1).

Immunofluorescence. Arteries were fixed with paraformaldehyde, permeabilized in Triton-X and then incubated in donkey serum to reduce nonspecific binding, as described previously (13). They were incubated overnight with a rabbit primary antibody to ANG II (1:500; Peninsula Labs), as performed previously (2, 36–38), and then incubated (120 min) with an AlexaFluor 488 donkey anti rabbit antibody (1:200; Jackson ImmunoResearch) and Draq5 (5 μM, nuclear stain; Biostatus). The endothelium was imaged (1024 × 1024 pixels) with a Leica SP5 LSM using a ×63 objective (NA 1.4), pinhole of 1 Airy unit, scan speed of 400 Hz, 6-line averaging, optical zoom of 3.0, and excitation/emission settings for Alexa 488 (488 nm/492–541 nm) and Draq5 (633 nm/659–758 nm). For quantitative comparison, young and old arteries were processed at the same time using the same instrument settings. For each artery, the fluorescence intensity from three Z-stacks of the endothelial layer (five 0.25-μm slices) was averaged to obtain the arterial fluorescence ($n = 1$) and is expressed as detector units (40).

ROS activity. Tail arteries were incubated (control solution, 37°C) for 180 min in the absence or presence of the AT_1 receptor antagonist losartan or the ACE inhibitor perindoprilat before being incubated with the ROS-sensitive fluorescent probe 5-(and 6)-chloromethyl-29, 79-dichlorodihydro-fluorescein diacetate (DCDHF; 5 μg/ml; Life Technologies) and Draq5 (5 μM) for 30 min (37°C, control solution) (40). They were then placed in cold control solution (4°C) and the endothelium imaged as in *Immunofluorescence* using laser-scanning microscopy (×20 air objective, 0.7 NA). The endothelium was visualized using an intensity filter, and optical slices were captured at the highest level of DCDHF fluorescence. For each arterial segment, the fluorescence intensity from multiple images was averaged to obtain the arterial fluorescence ($n = 1$), which is expressed as detector units (40).

Drugs. Acetylcholine, L-NAME, and ANG II were from Sigma-Aldrich, losartan and valsartan from Tocris Biosciences, DEA-NONOate from Enzo Life Sciences, perindoprilat from Santa Cruz Biotechnology, and aliskiren from Selleck Chemicals.

Data analysis. Vasomotor responses were expressed as a percent change in baseline diameter. Agonist concentrations causing 50% dilatation of the phenylephrine constriction (EC_{50}) were calculated by regression analysis and compared as $-\log EC_{50}$. Maximum responses were determined as the maximal observed dilatation of the constriction to phenylephrine. Data are expressed as means ± SE, where "*n*" equals the number of animals from which arteries were studied. Statistical evaluation of the data was performed by Student's *t*-test for paired or unpaired observations. When more than two means were compared, ANOVA was used. If a significant *F* value was found, then the Tukey-Kramer test for multiple comparisons was employed to identify differences among groups. Values were considered to be statistically different when $P < 0.05$.

RESULTS

Endothelial dilator dysfunction in aging arteries. Dilatation to acetylcholine was reduced in old compared with young arteries, reflecting a decrease in the maximal response and a rightward shift in the concentration-effect curve (maximums of 108.1 ± 1.1 and 89.6 ± 3.0%, $-\log EC_{50}$ of 7.32 ± 0.06 and 6.87 ± 0.07 in young, $n = 17$, and old, $n = 25$, respectively, $P < 0.001$ for each comparison; Fig. 1). Inhibition of NO synthase with L-NAME (100 μM) suppressed responses to

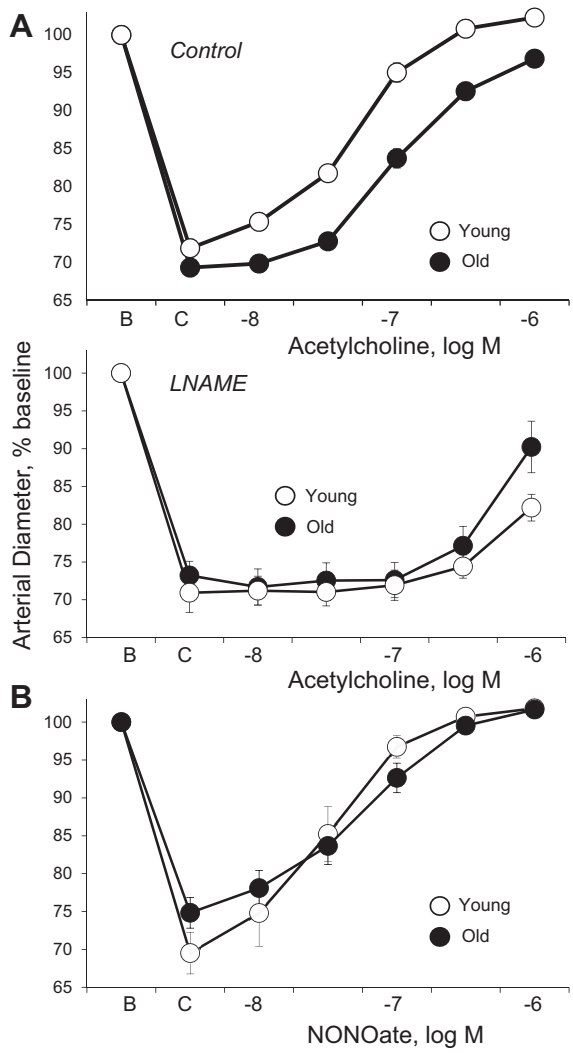

Fig. 1. Dilatation of young and old rat isolated tail arteries to the endothelial agonist acetylcholine (*A*) or the nitric oxide (NO) donor DEA NONOate (*B*). Responses to acetylcholine (*A*) were assessed under control conditions (*top*) and after inhibition of NO synthase with L-NAME (*bottom*). Dilator responses were analyzed in pressurized arteries during constriction to phenylephrine (*C*). Data are expressed relative to baseline diameter (*B*) and presented as means ± SE for *A*, $n = 25$ (control old), $n = 17$ (control young), or $n = 6$ {[nitro-L-arginine methyl ester (L-NAME)]-treated arteries}; *B*, $n = 6$. Aging decreased the maximal dilator response to acetylcholine ($P < 0.001$) and caused a rightward shift in the concentration-effect curve ($P < 0.001$) (*A*). After L-NAME, the residual dilatation to acetylcholine (1 μM) was greater in old compared with young arteries ($P < 0.05$; *B*). Aging did not significantly affect the concentration-effect curve to NONOate (*C*).

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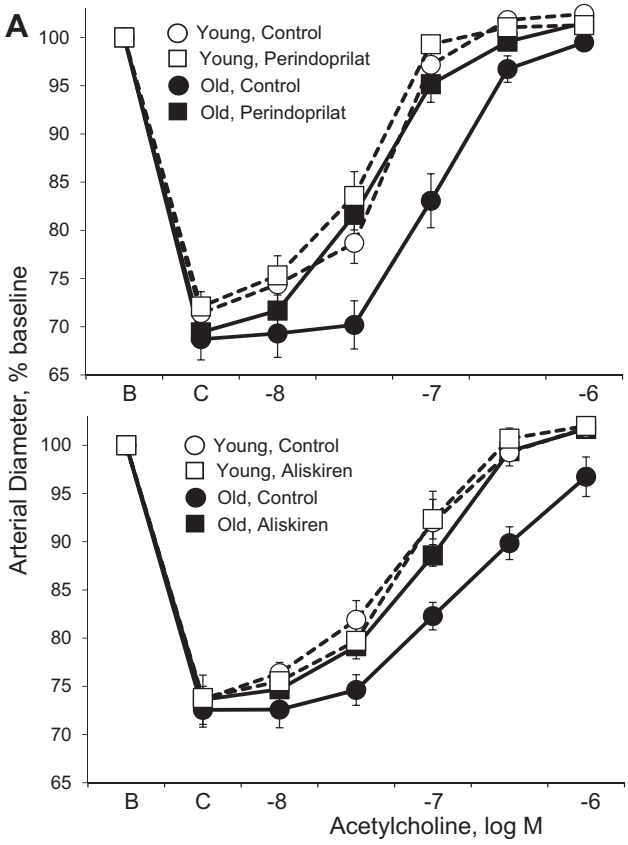


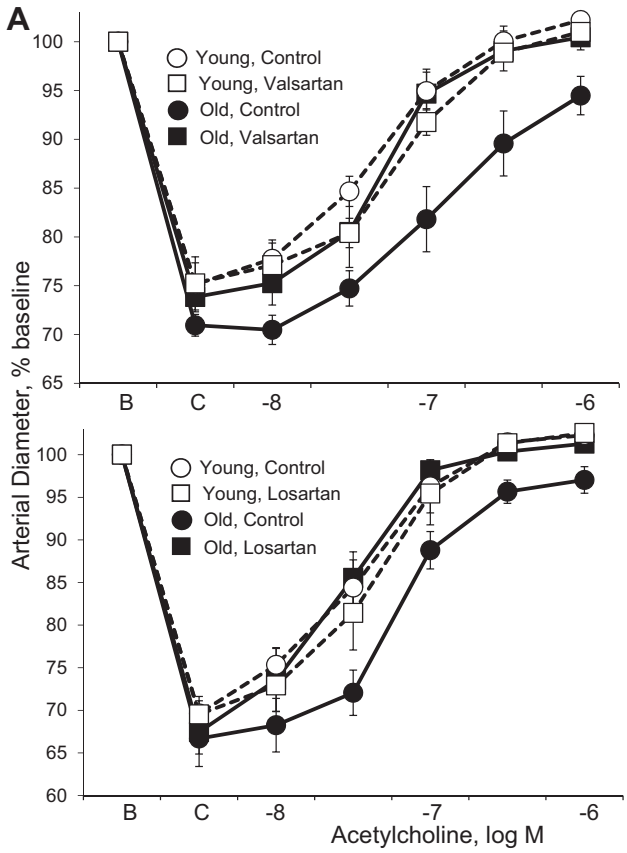
B

		Maximum	-Log EC_{50}
OLD	Control (6)	98.8 ± 1.7@@	6.96 ± 0.07@@
	Perindoprilat (6)	105.1 ± 2.2*	7.38 ± 0.05**
YOUNG	Control (6)	108.6 ± 1.5	7.32 ± 0.03
	Perindoprilat (6)	104.8 ± 1.0	7.44 ± 0.07
OLD	Control (7)	88.8 ± 7.2@	6.68 ± 0.13@
	Aliskiren (7)	107.2 ± 3.2*	7.07 ± 0.05**
YOUNG	Control (6)	106.8 ± 1.5	7.22 ± 0.13
	Aliskiren (6)	107.6 ± 3.8	7.18 ± 0.07

Fig. 2. Effects of the ACE inhibitor perindoprilat (*top*) or the renin inhibitor aliskiren (*bottom*) on dilatation to acetylcholine in young and old rat isolated tail arteries. Dilatation was analyzed in pressurized arteries during constriction to phenylephrine (*C*). Data are expressed relative to baseline diameter (*B*) and presented as means ± SE for $n = 6$ or 7 (see parenthesis in *B*). Concentration-effect curves are presented in *A* and statistical analysis of the curves are presented in *B*. In *B*: "Maximum" is maximal dilatation; $^{@}P < 0.05$, $^{@@}P < 0.01$, statistically significant difference from the corresponding group in young arteries; $^{*}P < 0.05$, $^{**}P < 0.01$, statistically significant difference between the corresponding paired control group. Inhibition of ANG II production augmented dilatation to acetylcholine in old arteries, increasing the maximal response and causing a leftward shift in the concentration-effect curve but had no significant effect in young arteries. After ANG II inhibition, there was no longer any significant difference in the concentration-effect curves to acetylcholine between young and old arteries.

acetylcholine in young and old arteries (Fig. 1). After L-NAME, the residual dilatation to acetylcholine was greater in old arteries (1 μM caused 35.9 ± 8.1 and 66.0 ± 10.7% dilatation in young and old arteries, respectively, $n = 6$, $P < 0.05$) (Fig. 1). Dilatation to the NO donor NONOate was not significantly different between young and old arteries (maximums of 106.0 ± 2.4 and 106.9 ± 1.4%, −log EC_{50} of 7.58 ± 0.14 and 7.31 ± 0.10, respectively, $n = 6$, P = NS) (Fig. 1).

Role of ANG II in aging-induced endothelial dilator dysfunction. Inhibition of endogenous ANG II activity by blocking ACE (perindoprilat), renin (aliskiren), or AT_1 receptors (valsartan, losartan) did not affect dilatation to acetylcholine in young arteries but significantly increased responses in old arteries, increasing the maximal dilatation and causing leftward shifts in the concentration-effect curve (Figs. 2 and 3). After ANG II inhibition, there was no longer any significant difference in the concentration-effect curves to acetylcholine



B

		Maximum	-Log EC_{50}
OLD	Control (6)	81.5 ± 6.9@@	6.73 ± 0.18@
	Valsartan (6)	103.0 ± 5.6**	7.31 ± 0.12***
YOUNG	Control (6)	108.7 ± 5.0	7.30 ± 0.11
	Valsartan (6)	105.1 ± 3.1	7.14 ± 0.07
OLD	Control (6)	89.9 ± 5.5@@	7.16 ± 0.06@
	Losartan (6)	104.0 ± 0.9*	7.61 ± 0.10***
YOUNG	Control (6)	107.3 ± 1.8	7.45 ± 0.11
	Losartan (6)	108.9 ± 2.7	7.42 ± 0.12

Fig. 3. Effects of the AT_1 receptor antagonists valsartan (*top*) or losartan (*bottom*) on dilatation to acetylcholine in young and old rat isolated tail arteries. Dilatation was analyzed in pressurized arteries during constriction to phenylephrine (*C*). Data are expressed relative to baseline diameter (*B*) and presented as means ± SE for $n = 6$. Concentration-effect curves are presented in *A* and statistical analysis of the curves are presented in *B*. In *B*: "Maximum" is maximal dilatation; $^{@}P < 0.05$, $^{@@}P < 0.01$, statistically significant difference from the corresponding group in young arteries; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, statistically significant difference between the corresponding paired control group. Inhibition of AT_1 receptors augmented dilatation to acetylcholine in old arteries, increasing the maximal response and causing a leftward shift in the concentration-effect curve, but had no significant effect in young arteries. After ANG II inhibition, there was no longer any significant difference in the concentration-effect curves to acetylcholine between young and old arteries

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between young and old arteries (Figs. 2 and 3). In old arteries treated with perindoprilat to inhibit endogenous production of the peptide, exogenous ANG II (300 nM, 120 min) inhibited dilatation to acetylcholine, decreasing the maximal response and causing a rightward shift in the concentration-effect curve (maximums of 101.4 ± 2.6 and 94.0 ± 4.2%, $P < 0.05$, and $-\log EC_{50}$ values of −7.13 ± 0.09 and 6.78 ± 0.07, $P < 0.01$, in perindoprilat and perindoprilat plus ANG II-treated arteries, respectively, $n = 6$).

ROS activity was significantly increased in old compared with young endothelium (Fig. 4). Antagonism of AT_1 receptors (losartan) or inhibition of ACE (perindoprilat) significantly reduced ROS activity in old arterial endothelium (Fig. 4). After ANG II inhibition, ROS activity in old endothelium was no longer significantly different from young endothelium.

Previous studies demonstrated increased immunofluorescent staining for ANG II in the endothelium and intima of old arteries, which was paralleled by increased expression of ACE and AT_1 receptors (36–38). Immunofluorescent levels of ANG II were confirmed to be increased in old compared with young endothelium (Fig. 4). Moreover, expression of renin was increased in old compared with young arteries (Fig. 4).

DISCUSSION

The present study demonstrates that aging-induced impairment of endothelial NO-dependent dilatation is mediated by ANG II generated from a local arterial renin-angiotensin system. Indeed, acute local inhibition of the synthesis (ACE, renin) or activity (AT_1 receptors) of ANG II completely restored endothelial dilator activity in aging arteries. Lakatta and colleagues previously demonstrated the presence of an intravascular ANG II signaling system in aging arteries and highlighted its role in the fibrotic and inflammatory activity of intimal SMCs and in arterial remodeling (19, 33, 34). They observed a four to sixfold increase in ANG II immunofluores-

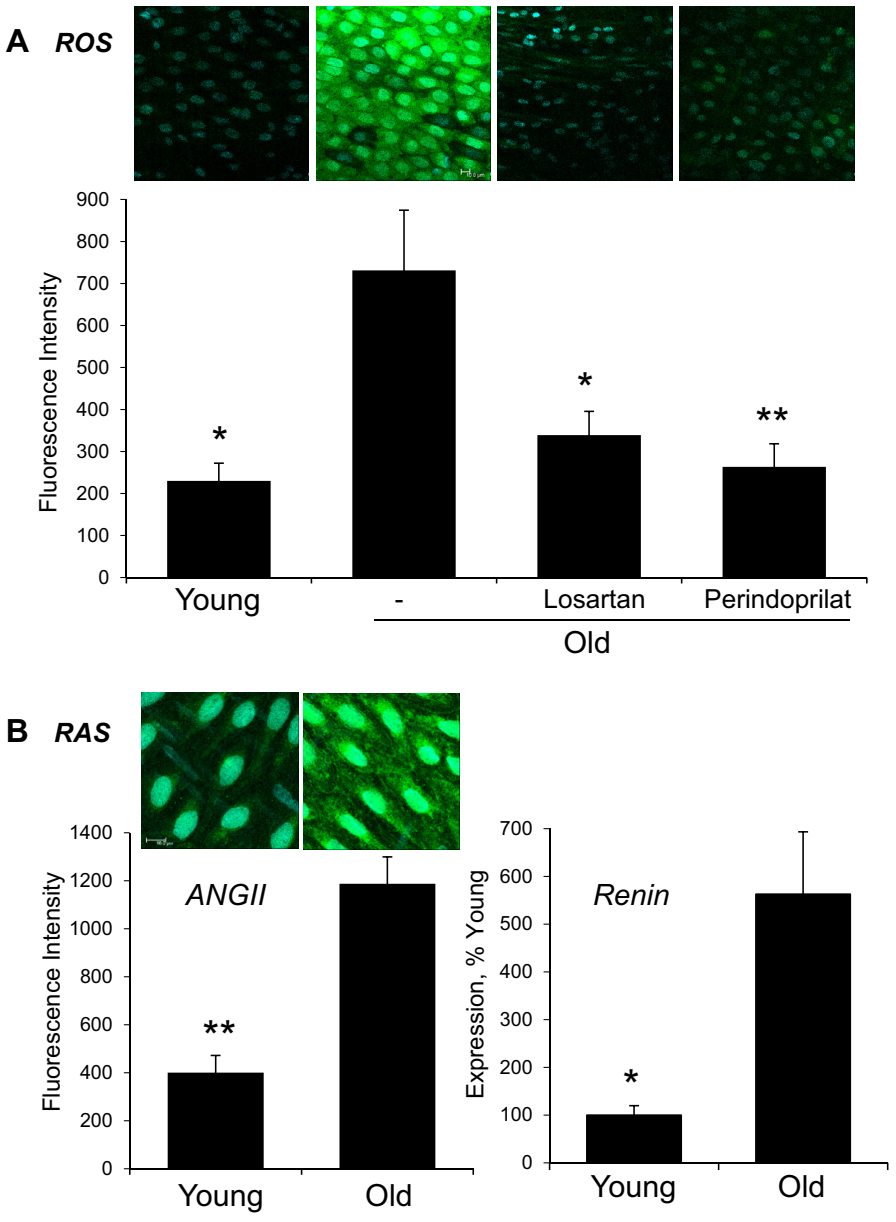

Fig. 4. *A*: reactive oxygen species (ROS) activity, assessed using DCDHF fluorescence (DCDHF, green; Draq5, light blue), in endothelium lining young and old arteries. Data is expressed as fluorescence units and is presented as means ± SE; $n = 3$. *$P < 0.05$; **$P < 0.01$, significant difference from old control arteries. DCDHF fluorescence was increased in old compared with young untreated arterial endothelium, and was reduced by inhibition of AT_1 receptors (losartan) or ACE (peridoprilat). *B*: renin angiotensin system (RAS). Expression levels of ANG II (*left*) (ANG II, green; Draq5, light blue) and renin (*right*) in young and old arteries assessed by immunofluorescence and Real-Time PCR, respectively. Fluorescent images are maximal projections of Z-stacks comprising the entire endothelial layer. Data are expressed as fluorescence units (*left*) or relative to the average value in young arteries (*right*) and is presented as means ± SE; $n = 3$. *$P < 0.05$; **$P < 0.01$, significant difference from old control arteries. Expression levels of ANG II and renin were increased in old compared with young arteries. Fluorescent images in *A* and *B* are presented in their original unprocessed state.

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cence in the endothelium and intimal SMCs of old compared with young arteries from rats, humans and nonhuman primates, which was paralleled by similar increases in ACE and AT_1 receptor expression (36–38). The present experiments confirmed an increase in ANG II immunofluorescence levels and further demonstrated a 5.6-fold increase in renin expression in old compared with young arteries. Previous studies demonstrated that prolonged in vivo inhibition of ANG II activity improves endothelium-dependent dilatation of old arteries, which was associated with a decrease in ROS activity (24, 27). The results of the present study indicate that those protective effects of ANG II inhibition may be explained by inhibition of a local rather than systemic renin-angiotensin system.

The results of the present study are consistent with the sequential action of renin and ACE to generate ANG II, which then acts on endothelial AT_1 receptors to cause endothelial dilator dysfunction. The expression of chymase, which like ACE can convert ANGI to ANG II, is increased in aortas of old monkeys (36). However, chymase expression was restricted to the adventitia, whereas both ANG II and ACE were localized to the endothelium and intima (36). Therefore, in both of these aging vascular systems, the predominant if not exclusive source of ANG II appears to be ACE (36). Aging is also reported to increase the vascular expression of (pro)renin receptors (39). Aliskiren inhibits ANGI generation by renin and by prorenin bound to the (pro)renin receptor (9). Therefore, both mechanisms could contribute to the local generation of ANG II in aging arteries. Indeed, in addition to the observed increase in vascular expression of renin, capture of (pro)renin from the circulation by (pro)renin receptors could also contribute to increased ANG II production (40). Increased expression of AT_1 receptors in the endothelium and intima of aging arteries (37) could potentially increase constitutive ligand-independent activity of the receptors. The similar effects of blocking the synthesis (renin, ACE) and activity (AT_1 receptors) of ANG II on endothelial dilatation argue against ligand-independent AT_1 receptor activity. Furthermore, although valsartan is a strong inverse agonist and can effectively inhibit ANG II-independent AT_1 receptor activity, losartan is considered a poor inverse agonist and is ineffective (40). However, both agents are powerful AT_1 receptor antagonists and inhibit responses to ANG II (40).

Other pathological mediators are known to contribute to the endothelial dysfunction of aging. As with prolonged ANG II inhibition, chronic in vivo inhibition of TNF-α increased endothelial dilatation and decreased ROS activity in aging arteries (1, 7, 26). This may reflect the close relationship between TNF-α and ANG II in the aging arterial wall. Indeed, inhibition of TNF-α reduced the heightened expression of ACE, AT_1 receptors, and immunofluorescent ANG II in aging arteries (2), suggesting that expression of the local renin-angiotensin system is amplified by TNF-α and the inflammatory activity of aging arteries (19, 32, 33). The results of the present study also explain the parallel mechanisms contributing to endothelial dilator dysfunction in old arteries or in response to ANG II, which include increased expression and activation of arginase, increased production of ROS from NOXs and mitochondria, and uncoupling of NO synthase (5, 8, 11, 17, 30, 31). Cardiovascular effects resulting from chronic administration of ANG II, including vascular remodeling and endothelial dysfunction, can be reduced by mineralocorticoid receptor (MR) antagonism, which may reflect ANG II-induced release of aldosterone from the adrenal gland (4). MR expression is increased in aging arteries and in cultured SMCs derived from them, and an MR antagonist reduced the heightened inflammatory and fibrotic activity of cultured aging SMCs (18). Although ANG II might increase the local vascular production of aldosterone (4, 23, 29), this MR activity in aging SMCs likely results from ANG II and AT_1 receptor-dependent transactivation of MRs (15, 18). It is currently unknown if MR activation contributes to the direct effects of ANG II to cause endothelial dilator dysfunction, including in aging arteries.

Aging arterial endothelial cells are severely compromised: their production of protective NO is reduced, their barrier function is impaired, they are highly susceptible to apoptosis, and they have a prominent inflammatory phenotype (19, 32). Aging endothelial cells are therefore caught in a chronic cycle of inflammatory stress, both producing and responding to pathological mediators, and their resulting frailty is considered a key instigator of vascular aging (32, 34). Indeed, specific suppression of endothelial inflammatory activity, by transgenic inhibition of NF-κB, inhibited arterial senescence, increased locomotor activity, and prolonged lifespan in aging mice (14). Similar beneficial effects, including extended lifespan, are observed following systemic inhibition of ANG II (3, 6, 35). The present experiments were restricted to analyzing endothelial NO-mediated dilator function and did not address additional dysfunctional aspects of aging endothelium. However, because of the pleiotropic pathological effects of ANG II and the pleiotropic protective effects of NO (22, 25), ANG II likely contributes to other aspects of the aging endothelial phenotype. Indeed, some of the beneficial effects of inhibiting NF-κB in aging were mediated by increased NO activity (14). Vascular aging represents deterioration of the structure and function of the entire arterial system, from central arteries to the microcirculation (32). Although expression of the intravascular ANG II system occurs in other aging arteries and vascular beds (2, 36–38), the present experiments were restricted to proximal tail arteries and did not analyze the potential role of local ANG II signaling in other systems or in the microcirculation.

In conclusion, we have demonstrated that the aging-induced dysfunction in endothelial NO-dependent dilatation in rat tail arteries is mediated by a local arterial renin-angiotensin system and pathological ANG II activity. Increased understanding of the mechanisms regulating this intravascular system may provide novel therapeutic approaches to alleviate the devastating effects of vascular aging.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

S.F., F.C., and N.A.F. conception and design of research; S.F., F.C., and N.A.F. performed experiments; S.F., F.C., and N.A.F. analyzed data; S.F., F.C., and N.A.F. interpreted results of experiments; S.F., F.C., and N.A.F. edited and revised manuscript; S.F., F.C., and N.A.F. approved final version of manuscript; F.C. and N.A.F. prepared figures; N.A.F. drafted manuscript.

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