Elevated pressure causes endothelial dysfunction in mouse carotid arteries by increasing local angiotensin signaling

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Zhao Y, Flavahan S, Leung SW, Xu A, Vanhoutte PM, Flavahan NA. Elevated pressure causes endothelial dysfunction in mouse carotid arteries by increasing local angiotensin signaling. Am J Physiol Heart Circ Physiol 308: H358–H363, 2015. First published December 5, 2014; doi:10.1152/ajpheart.00775.2014.—Experiments were performed to determine whether or not acute exposure to elevated pressure would disrupt endothelium-dependent dilatation by increasing local angiotensin II (ANG II) signaling. Vasomotor responses of mouse-isolated carotid arteries were analyzed in a pressure myograph at a control transmural pressure (P_{TM}) of 80 mmHg. Acetylcholine-induced dilatation was reduced by endothelial denudation or by inhibition of nitric oxide synthase (NOS) or nitric oxide donor diethylamine NONOate. Elevated P_{TM} also increased endothelial reactive oxygen species, and the pressure-induced endothelial dysfunction was prevented by the direct antioxidant and NADPH oxidase inhibitor apocynin (100 μM). The increase in endothelial reactive oxygen species in response to elevated P_{TM} was reduced by the ANG II type 1 receptor (AT_{1R}) antagonists losartan (3 μM) or valsartan (1 μM). Indeed, elevated P_{TM} caused marked expression of angiotensinogen, the precursor of ANG II. Inhibition of ANG II signaling, by blocking angiotensin-converting enzyme (1 μM perindoprilat or 10 μM captopril) or blocking AT_{1R} prevented the impaired response to acetylcholine in arteries exposed to 150 mmHg but did not affect dilatation to the muscarinic agonist in arteries maintained at 80 mmHg. After the inhibition of ANG II, elevated pressure no longer impaired endothelial dilatation. In arteries treated with perindoprilat to inhibit endogenous formation of the peptide, exogenous ANG II (0.3 μM, 180 min) inhibited dilatation to acetylcholine. Therefore, elevated pressure rapidly impairs endothelium-dependent dilatation by causing ANG expression and enabling ANG II-dependent activation of AT_{1R}s. These processes may contribute to the pathogenesis of hypertension-induced vascular dysfunction and organ injury.

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IN HEALTHY HUMAN VOLUNTEERS, short-term increases in arterial blood pressure cause long-lasting inhibition of endothelium-dependent dilatation (11, 17, 22). Likewise, in isolated arteries, acute exposure to pathological increases in transmural pressure (P_{TM}) impairs endothelium-dependent relaxation (10, 21, 26). As occurs with chronic increases in pressure (hypertension), endothelial dysfunction caused by acute exposure to elevated P_{TM} appears to be mediated by activation of NADPH oxidase and production of reactive oxygen species (ROS), causing disruption of endothelial nitric oxide (NO)-mediated dilatation (13, 24, 26). Angiotensin II (ANG II) signaling plays a key role in the development and the pathological consequences of hypertension, including the occurrence of endothelial dysfunction (9, 16, 25). Indeed, ANG II is a powerful inducer of endothelial dysfunction, including diminution in endothelial NO dilator activity (7, 14, 24). ANG II activity in hypertension may reflect a generation of the peptide by systemic and/or local ANG systems (2, 19). It is currently unknown whether or not local ANG signaling contributes to the endothelial dysfunction caused by acute increases in pressure. Prolonged organ culture of rabbit aortae at high P_{TM} (3 days, 150 mmHg) causes an ANG II-dependent increase in fibronectin expression (3), indicating that pressure can induce local ANG II signaling in the blood vessel wall. Furthermore, ANG II type 1 receptors (AT_{1R}s) can be directly activated by mechanical stretch, independently of ANG II (1, 23, 28). The goal of the present experiments was to determine whether or not an acute, transient increase in P_{TM} causes endothelial dysfunction in carotid arteries via ANG II-dependent or ANG II-independent activation of AT_{1R}s.

MATERIALS AND METHODS

Animals

Male C57BL6 mice (10–12 wk old) were obtained from Jackson Laboratories (Bar Harbor, ME) and euthanized by CO2 asphyxiation. Animal use was approved by the Institutional Animal Care and Use Committee and complied with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

Blood Vessel Analysis

Carotid arteries were isolated and placed in cold Krebs-Ringer bicarbonate solution (control solution) (20). They were cannulated with glass micropipettes, secured within a microvascular chamber, and maintained at a control P_{TM} of 80 mmHg in the absence of flow (Living Systems, Burlington, VT). The chamber was superfused with control solution (37°C, pH 7.4, 16% O2−5% CO2-balanced N2) and placed on the stage of an inverted microscope (20). The artery image was captured by a video camera, and the internal diameter was continuously determined by a video dimension analyzer and recorded using a data acquisition system (BiOPAC, Santa Barbara, CA) (20). To optimize the role of NO, dilatation was assessed in arteries constricted with 34 mM KCl (in equimolar replacement of NaCl) after inhibition of cyclooxygenase with indomethacin (10 μM). Concentration-effect curves to dilator agonists were generated by increasing their concentration in half-log increments once the response to the preceding concentration had stabilized. Acetylcholine (1 μM) caused 69.2 ± 2.9% dilatation of KCl-constricted arteries under control conditions and 7.6 ± 1.5% dilatation after inhibition of NO synthase (NOS-nitro-l-arginine methyl ester, 100 μM) (P ≤ 0.001, n = 3).

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Endothelial denudation by wire abrasion (70 μm) (20) abolished acetylcholine-induced dilatation. Following washout (with control solution) and return of the arterial diameter to basal levels, \( P_{TM} \) was maintained at 80 mmHg or increased to 150 mmHg for up to 3 h (12, 13). \( P_{TM} \) was then returned to 80 mmHg, the arteries were constricted again with KCl (34 mM), and responses to dilator agonists were reassessed. There was no significant time-dependent change in dilator responses in arteries maintained under control conditions at 80 mmHg. When inhibitors were assessed, arteries were incubated for 30 min with the drugs before and during exposure to acetylcholine and during exposure of the arteries to elevated \( P_{TM} \) (inhibitors were reintroduced into the superfusate if required, following washout and recovery of the arteries). The inhibitors did not significantly affect constriction to KCl. The effect of exogenous ANG II (0.3 μM, 180 min) was studied in arteries treated with perindoprilat (1 μM, to inhibit endogenous production of the peptide) and exposed to increased pressure (150 mmHg).

**Western Blot Analysis**

Isolated carotid arteries (maintained at 80 mmHg or exposed to 150 mmHg for 180 min) were frozen in liquid nitrogen. Because of their small size, three carotid arteries were pooled to obtain one sample (\( n = 1 \)). Arteries were disrupted in cold radioimmunoprecipitation assay lysis and extraction buffer (Thermo Fisher, Rockford, IL) containing protease inhibitor cocktail (Thermo Fisher) and homogenized (5 min) using Tissuelyser LT (QIAGEN, Valencia, CA). The lysate was then cleared by centrifugation (14,000 g, 5 min, 4°C).

**ROS Activity**

Isolated carotid arteries that had been maintained at 80 mmHg or exposed to 150 mmHg for 15 or 180 min were rapidly removed from the chamber and incubated under nonpressurized conditions with the ROS-sensitive fluorescent probe 5-(and 6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate (5 μg/ml) (Life Technologies, Grand Island, NY) and Draq5 (5 μM, nuclear stain) (Biostatus, Leicestershire, UK) for 20 min (37°C, control solution) (20). They were then placed in control solution (4°C), and the endothelial surface was viewed using a Leica SP5 laser-scanning microscope (×20 air objective; 0.7 numerical aperture). Images (1024 × 1024 pixels) were obtained using sequential acquisition, a pinhole of 1 Airy unit, scan speed of 400 Hz, six line averaging and an optical zoom of 3.0. For 5-(and 6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, excitation was 488 nm and emission captured from 520-541 nm, and for Draq5, excitation was 633 nm and emission captured from 650–750 nm. Arteries were processed at the same time, using the same approach and instrument settings. Fluorescence intensity is therefore expressed as detector units. The entire endothelial surface of each carotid artery was scanned (~7 images/artery), with the average fluorescent intensity representing the arterial fluorescence (\( n = 1 \)).

**Drugs**

Acetylcholine, bradykinin, indomethacin, N^2^-nitro-L-arginine methyl ester, and ANG II were from Sigma-Aldrich (St. Louis, MO); apocynin, losartan, valsartan, and captopril were from Tocris Biosciences (Bristol, UK); diethylamine NONOate was from Enzo Life Sciences (Farmingdale, NY); and perindoprilat was from Santa Cruz Biotechnology.

**Data Analysis**

Vasomotor responses were expressed as a percent change in baseline diameter. Agonist concentrations causing 30% dilatation of the KCl constriiction (EC_{30}) were calculated by regression analysis and compared as –log EC_{30}. Data are expressed as means ± SE, where \( n \) equals the number of animals from which blood vessels were studied (except as stated for Western blot analysis). Statistical evaluation of the data was performed by Student’s t-test for paired or unpaired observations. When more than two means were compared, analysis of variance was used. If a significant F-value was found, then Student-Newman-Keuls or Dunnett tests for multiple comparisons were employed to identify differences among groups. Values were considered to be statistically different when \( P \) was <0.05.

**RESULTS**

**Increased Pressure Inhibits Endothelium-Dependent Dilatation to Acetylcholine**

Acetylcholine-induced dilatation was significantly inhibited following transient exposure to elevated \( P_{TM} \) (150 mmHg) (Fig. 1). After 60 min, elevated \( P_{TM} \) caused a significant rightward shift in the concentration-effect curve to acetylcholine (log shift of 0.20 ± 0.08, 1.6-fold, \( n = 7 \), \( P < 0.05 \)) with no significant change in maximal dilatation. After 180 min, elevated \( P_{TM} \) exerted more profound depression, causing a larger rightward shift in the concentration-effect curve and a significant depression in the maximal dilatation (Fig. 1). Elevated \( P_{TM} \) (150 mmHg, 180 min) did not significantly affect dilatation to the NO donor diethylamine NONOate (data not shown).

**Mechanisms Contributing to Pressure-Induced Disruption of Endothelial Dilatation**

**ROS**

**ANGER Signal**

The AT_{1}R antagonist losartan (3 μM) did not influence the immediate increase in endothelial ROS activity in response to elevated \( P_{TM} \) (150 mmHg, 15 min) but significantly reduced the ROS increase occurring after more prolonged exposure to elevated \( P_{TM} \) (150 mmHg, 180 min) (Fig. 2). Similar results were obtained with the AT_{1}R antagonist valsartan (1 μM) (data not shown).

**ANG expression.** Immunoblot analysis of arterial lysates selectively identified molecular species consistent with glycosylated forms of ANGogen in blood vessels exposed to elevated \( P_{TM} \) (150 mmHg, 180 min) (Fig. 2). The exposure to elevated \( P_{TM} \) caused significant induction of this ANGogen-like activity in mouse carotid arteries (Fig. 2).

**ANG Converting enzyme inhibition.** Perindoprilat (1 μM) or captopril (10 μM) did not significantly affect...
dilatation to acetylcholine in arteries maintained at 80 mmHg but amplified responses to the agonist in arteries exposed to 150 mmHg (180 min), significantly increasing the maximal dilatation and causing significant leftward shifts in the concentration-effect curves (Fig. 4). After AT$_1$R antagonism, increased pressure no longer significantly inhibited dilatation to acetylcholine (Fig. 4).

Exogenous ANG II. ANG II (0.3 μM, 180 min) depressed acetylcholine-induced dilatation, causing significant inhibition of the maximal response (from 97.3 ± 1.0 to 93.7 ± 0.3% dilatation of KCl constriction, n = 7, P < 0.05) and a significant rightward shift in the concentration-effect curve (log shift of 0.25 ± 0.03, 1.8-fold, n = 7, P < 0.001). ANG II did not cause constriction of the arteries and did not significantly affect constriction to KCl (data not shown).

**DISCUSSION**

In the present study, an acute transient increase in P$_{TM}$ impaired endothelium-dependent, NO-mediated dilatation to acetylcholine but did not influence dilatation to an NO donor. This pressure-induced endothelial dysfunction was associated with an increase in endothelial ROS and was prevented by the direct antioxidant and NADPH oxidase inhibitor apocynin, consistent with previous reports (13, 24, 26). AT$_1$Rs are considered mechanosensitive and can be directly activated by mechanical stretch in an ANG II-independent manner (1, 18, 28). Although valsartan is a strong inverse agonist and can effectively inhibit such ANG II-independent AT$_1$R activity, losartan is considered a poor inverse agonist and is ineffective (1, 18). However, both agents are powerful AT$_1$R antagonists and inhibit activity to ANG II. The immediate pressure-induced increase in endothelial ROS (15 min) was not affected by these AT$_1$R inhibitors, indicating that the initial increase in ROS is not dependent on AT$_1$R activation. However, the increase in ROS activity following a more prolonged elevation in pressure (180 min) was reduced by either agent, suggesting that the role of AT$_1$Rs involves ANG II-dependent activation rather than direct mechanosensitive stimulation. Indeed, prolonged exposure to elevated pressure caused a dramatic induction of ANGogen expression. Moreover, the pressure-induced disruption of endothelial dilatation was prevented by antagonizing AT$_1$Rs with losartan or valsartan and by preventing ANG II formation with the ACE inhibitors captopril or perindopril. The protective effects of ACE inhibition on endothelial dilatation are unlikely to be mediated by production of endogenous kinins because bradykinin did not initiate endo-

Fig. 1. Effects of a transient increase in transmural pressure (P$_{TM}$) on acetylcholine-induced dilatation in mouse isolated carotid arteries. Dilatation was analyzed at a P$_{TM}$ of 80 mmHg during KCl (34 mM) constriction in arteries that had been maintained at 80 mmHg (P80) or exposed to a transient increase in P$_{TM}$ (150 mmHg, 180 min) (P150). **Top:** effects of elevated P$_{TM}$ in untreated arteries. Middle and bottom: influence of the direct antioxidant and NADPH oxidase inhibitor apocynin (100 μM) in arteries exposed to 150 mmHg or maintained at 80 mmHg, respectively. Data are expressed relative to baseline diameter and presented as means ± SE (n = 6). B, baseline; C, KCl constriction. Vertical arrows indicate statistical comparisons between maximal responses, whereas horizontal arrows indicate comparisons between −log 30% dilatation of the KCl constriction (EC$_{30}$) values (***P < 0.001; **P < 0.01; NS, not significant).

Exogenous ANG II. ANG II (0.3 μM, 180 min) depressed acetylcholine-induced dilatation, causing significant inhibition of the maximal response (from 97.3 ± 1.0 to 93.7 ± 0.3% dilatation of KCl constriction, n = 7, P < 0.05) and a significant rightward shift in the concentration-effect curve (log shift of 0.25 ± 0.03, 1.8-fold, n = 7, P < 0.001). ANG II did not cause constriction of the arteries and did not significantly affect constriction to KCl (data not shown).
helium-dependent dilatation and did not prevent the pressure-induced depression of responses to acetylcholine. Although inhibition of AT$_1$Rs or ACE markedly increased the depressed responses to acetylcholine in arteries exposed to elevated pressure, these interventions had no effect in preparations maintained at a control pressure. Therefore, pathological local ANG II signaling is present only at elevated pressure, consistent with the lack of ANGogen expression under control conditions. A previous report suggested that elevated pressure in isolated porcine coronary arteries caused endothelial dysfunction by direct mechanical activation of AT$_1$Rs (15). However, this was based solely on the effects of losartan (15), which would be expected to inhibit AT$_1$R responses initiated by ANG II but not direct mechanical activation (1, 18). Thus the present study indicates that acute pressure-induced endothelial dysfunction is mediated by local induction of ANG II signaling and ANG II-dependent activation of AT$_1$Rs.

The concept that elevated pressure increases local vascular ANG II signaling is consistent with previous studies. Hypertension induced by aortic banding was associated with increased ANGogen expression in coronary arteries (24). Likewise, prolonged culture of rabbit aortae at elevated PTM (150 mmHg, 3 days) caused an ANG II-dependent increase in fibronectin production (3). Furthermore, cyclic stretch of cultured endothelium increased ANG expression and caused an ANG II-dependent decrease in NO activity (6). The present study demonstrates that an acute elevation in pressure causes endothelial dysfunction by rapidly increasing local ANG II signaling. A key positive regulator of ANGogen expression is NF-$\kappa$B (4). In mouse carotid arteries, elevated P$_{TM}$ (150 mmHg, 180 min) caused a peak increase in NF-$\kappa$B activity within 15 min, which appears to be mediated by the pressure-induced generation of ROS (12, 13). Pressure-induced modulation of endothelial dilatation appears to be developmentally regulated.
in the arterial wall can express ANGogen and contribute to vascular ANG II signaling, including endothelial cells, smooth muscle cells, and perivascular adipocytes (5, 6, 19). Conversion of ANGogen to ANG I, the substrate for ACE can be accomplished by renin expressed within the blood vessel wall, by (pro)renin captured from the circulation by (pro)renin receptors, or by renin-independent mechanisms (2, 19). Experiments confirmed that in arteries treated with an ACE inhibitor, endothelial dysfunction could be restored by exogenous ANG II (7). The pathological effects of ANG II on the endothelium have been extensively studied, including demonstrating a role of increased NADPH oxidase and ROS production in causing endothelial dilator dysfunction (7, 14, 24). Interestingly, exogenous ANG II did not cause constriction or change the re-

In contrast to adult carotid arteries, elevated \( P_{\text{TM}} \) increased endothelium-dependent, NO-mediated dilatation to acetylcholine in carotid arteries of newborn mice (8). Despite differences in baseline pressure at these distinct developmental stages, the elevation in \( P_{\text{TM}} \) (20–50 mmHg in newborns and 80–150 mmHg in adults) caused similar increases in internal diameter (31.0 ± 2.4% in newborns and 29.3 ± 1.9% in adults, \( n = 15; \) NS). The pressure-induced mechanical stimulus to the endothelium would therefore be of similar magnitude. Thus increased pressure can exert protective effects on endothelium-dependent dilatation, but in adult arteries this mechanism is suppressed by pressure-induced pathological expression of ANG II signaling.

The present study did not identify the cellular source of ANGogen or the mechanism(s) responsible for the initial processing of this ANG precursor. Numerous cell types located processing of this ANG precursor. Numerous cell types located...
response to KCl in mouse carotid arteries, confirming a minimal role for AT1Rs in the smooth muscle of this blood vessel (27).

In conclusion, the results of the present study demonstrate that transient exposure to elevated pressure impairs endothelium-dependent NO-mediated dilatation by increasing local ANG II signaling and causing ANG II-dependent activation of AT1Rs. Pressure-induced local ANG II signaling is likely a key mediator in the pathological effects of increased arterial blood pressure on the arterial wall.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Y.Z., S.F.L., A.X., P.M.V., and N.A.F. conception and design of research; Y.Z., S.F., S.W.L., A.X., P.M.V., and N.A.F. performed experiments; Y.Z., S.F., and N.A.F. analyzed data; Y.Z., S.F., S.W.L., A.X., P.M.V., and N.A.F. interpreted results of experiments; Y.Z., S.F., S.W.L., A.X., P.M.V., and N.A.F. edited and revised manuscript; Y.Z., S.F., and N.A.F. drafted manuscript; Y.Z., S.F., S.W.L., A.X., P.M.V., and N.A.F. approved final version of manuscript; Y.Z., S.F., S.W.L., A.X., P.M.V., and N.A.F. interpreted results of experiments; Y.Z., S.F., and N.A.F. performed experiments; Y.Z., S.F., and N.A.F. analyzed data; Y.Z., S.F., and N.A.F. interpreted results of experiments; Y.Z., S.F., S.W.L., A.X., P.M.V., and N.A.F. edited and revised manuscript; Y.Z., S.F., S.W.L., A.X., P.M.V., and N.A.F. approved final version of manuscript; N.A.F. drafted manuscript; Y.Z., S.F., S.W.L., A.X., P.M.V., and N.A.F. interpreted results of experiments; Y.Z., S.F., S.W.L., A.X., P.M.V., and N.A.F. edited and revised manuscript; Y.Z., S.F., S.W.L., A.X., P.M.V., and N.A.F. approved final version of manuscript; Y.Z., S.F., S.W.L., A.X., P.M.V., and N.A.F. interpreted results of experiments; Y.Z., S.F., S.W.L., A.X., P.M.V., and N.A.F. edited and revised manuscript; Y.Z., S.F., S.W.L., A.X., P.M.V., and N.A.F. approved final version of manuscript; N.A.F. prepared figures; N.A.F. drafted manuscript.

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