Vascular Biology, Atherosclerosis, and Endothelium Biology

Prolonged Infusion of Angiotensin II in *apoE*^{-/-} Mice Promotes Macrophage Recruitment with Continued Expansion of Abdominal Aortic Aneurysm

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Angiotensin II (AngII) infusion initiates abdominal aortic aneurysm (AAA) development due to medial disruption and results in luminal dilation and thrombus formation. The objective of this study was to determine whether AAA progressed during protracted AngII infusion. Male *apoE*^{-/-} mice were infused with AngII using miniosmotic pumps. On day 27, suprarenal aortic luminal diameters were ultrasonically measured to identify mice exhibiting AAAs. Mice were designated to three groups with similar mean luminal dilation. Group 1 mice were sacrificed on day 28. Group 2 and 3 mice were subsequently infused with saline or AngII, respectively, for an additional 56 days. In Group 2, saline infusion—after the initial 28 days of AngII infusion—led to an immediate decrease in systolic blood pressure. Over the subsequent 56 days of saline infusion, there were no aneurysm-related deaths or significant changes in luminal diameter. In contrast, continuous AngII infusion in Group 3 maintained persistently increased systolic blood pressure, with aneurysmal rupture-associated deaths, increased luminal diameters, and tissue remodeling. Aortic aneurysmal segments that expanded during continuous AngII infusion exhibited macrophage accumulation in regions of medial disruption, predominantly on the adventitial aspect. Macrophages immunostained for CD206 more than for iNOS, consistent with an M2 phenotype. In conclusion, prolonged AngII infusion promotes AAA expansion, and is associated with enhanced rupture rates and increased macrophage infiltration. (*Am J Pathol* 2011, 179:1542–1548; DOI: 10.1016/j.ajpath.2011.05.049)

within 14 days.^{1–11} Initiation of AngII-induced AAA is associated with medial disruption leading to a localized thrombus.¹² There is a rapid luminal dilation between days 3 and 8 of AngII infusion with subsequent, gradual luminal expansion.¹² Although many laboratories have demonstrated that AngII consistently initiates AAA development in both hyper- and normocholesterolemic mice,^{13–16} its role in aneurysmal progression, expansion, and rupture has not been defined in prospective human studies. However, there are conflicting data from retrospective analyses on AAA expansion and rupture in humans.^{17–19}

Many factors have been associated with the development of AAAs in animal models.²⁰ Most of these studies have invoked mechanisms that prevent the initiation phase of AAAs. However, pharmacological treatment of AAAs in humans would be initiated in aortas with established disease. Currently, it is unknown whether pharmacological approaches that suppress initiation of disease would also inhibit progression of established aneurysms. Therefore, there is a need to determine mechanisms of AAA progression, expansion, and rupture since these may differ from disease initiation.

The purpose of this current study was to determine whether chronic AngII infusion led to AAA expansion and rupture. This was accomplished by prolonged infusion of AngII for 84 days with sequential luminal diameter measurements and histological characterization of aortas at termination. This group was compared to one in which AAAs were initiated by AngII infusion, but were subsequently implanted with miniosmotic pumps infusing saline. The data demonstrate that continuous AngII infusion increased aortic dilation and aneurysmal rupture. Sequential sectioning of aneurysmal tissues demonstrated morphological heterogeneity, and increased

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Infusion of angiotensin II (AngII) into mice promotes rapid formation of abdominal aortic aneurysms (AAAs)

macrophage recruitment localized to regions of medial disruption.

Materials and Methods

Mice and Study Design

Male *apoE*^{-/-} mice (8 weeks old, 10 times backcrossed into the C57BL/6 strain; colony bred from original source stock #2052; The Jackson Laboratory, Bar Harbor, ME) were infused with AngII (1000 ng/kg/min; Catalog number A-9525; Sigma Chemicals, St. Louis, MO) via miniosmotic pumps (Alzet osmotic pump 2004; Durect Corporation, Cupertino, CA) for 28 days. On day 27, the abdominal aortas of all mice were scanned by ultrasound, and mice were stratified and divided into 3 groups. Mice of each stratum were randomly assigned so the three groups had an equivalent mean luminal diameter. Group 1 (*n* = 11) was terminated at day 28. Group 2 (*n* = 11) was infused with saline for an additional 56 days; while Group 3 (*n* = 15) was infused continuously with AngII for an additional 56 days (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

Since the Alzet pump model 2004 infuses for 28 days, pumps were removed from the mice, and primed replacements were positioned in the same site at 28-day intervals. Mice were fed a normal mouse diet (Catalog # 2918 Irradiated Global Diet rodent diet; Harlan Teklad, Madison, WI). Mice that died during the study were subjected to necropsy to determine cause of death. All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Blood Pressure

Systolic blood pressure was measured on conscious mice via tail cuff using Visitech BP2000 (Visitech Systems, Inc., Apex, NC). Platforms were pre-warmed, and blood pressure was measured the same time each day for 5 consecutive days during each interval of 28 days (Day -5 to -1; 23 to 27; 51 to 55; and 79 to 83).¹⁶

In Vivo Ultrasonic Measurements

Abdominal aortas of the mice were visualized with high-frequency ultrasound (Vevo 660; VisualSonics, Toronto, ON, Canada) on day 0, 27, 56, and 84, as described previously.²¹ Luminal diameters and areas were measured on images with the maximal dilation.

Serum Characteristics

Serum total cholesterol concentrations were measured with enzymatic assay kits (Cholesterol E; Wako Chemical Co, Richmond, VA). Lipoprotein distributions were separated by fast protein liquid chromatography, and cholesterol was measured enzymatically as described previously.²²

Vascular Characterization

At termination, blood was collected via cardiac puncture. Mice were perfused with saline and then with paraformaldehyde at a pressure of approximately 100 mm Hg. After fixation, aortas were filled with low melting point agarose containing green dye.²³ Aortas were dissected free, fixed overnight, and extraneous tissue was removed. Maximal diameters of suprarenal aortas were measured using ImagePro (MediaCybernetics, Bethesda, MD). Three suprarenal aortas from each group, chosen as those nearest the mean external diameter of the group, were embedded in optimal cutting temperature compound and serially sectioned using a cryostat. Whole aneurysms were sectioned throughout in serial sets consisting of 10 slides with nine serial tissue sections per slide. This resulted in approximately 400 to 1000 sections depending on aneurysmal length (see Supplemental Figure S2 at <http://ajp.amjpathol.org>).

Immunostaining was performed to identify macrophages [rat anti-mouse CD68, Cat# MCA1957; AbD Serotec, Raleigh, NC, and rat anti-mouse CD206, Cat# MCA2235; AbD Serotec; rabbit polyclonal inducible nitric oxide synthase (iNOS), Cat# ab3523; Abcam, Cambridge, MA; and rat anti-mouse monocyte/macrophage (MOMA2), Cat# ab33451; Abcam] as described previously.²⁴ Positive reactivity was visualized using 3-amino-9-ethylcarbazole as chromogen. Nuclei were counterstained with hematoxylin. Negative controls included substitution of the primary antibody with a nonimmune rat or rabbit IgG, and absence of primary and secondary antibodies. Macrophages were manually counted on sections at 100- μ m intervals and represented as number of positive-stained cells per section. Cell counts were quantified in intimal, medial, and adventitial areas to determine localization of macrophages. Cells were counted by two observers for each section as the number of nuclei associated with chromogen development. Proximal sections of aortas (ascending aorta to descending aorta at the level of the diaphragm) were cut open, pinned, and photographed. Aortic arch areas and atherosclerosis were measured using Image-Pro (Media Cybernetics, Bethesda, MD) as described previously.²⁵

Statistics

Systolic blood pressure data and (logarithmically transformed) abdominal aortic luminal diameter data were analyzed via linear mixed models with mouse-specific random effects; whereas (logarithmically transformed) macrophage count data were analyzed via linear mixed models with slide-specific random effects. For other response variables, data from a specific time point and/or data obtained at mouse termination were subjected to a one-way analysis of variance (or a suitable nonparametric alternative); follow-up tests used adjustments for multiple comparisons as indicated in the figure legends. A *P* value < 0.05 was considered statistically significant. Data are summarized in the figures as mean $\pm$ SEM. Version 3.5 of SigmaStat (Systat Software, San Jose, CA) and Version 9.1 of SAS (SAS Institute, Cary, NC) were used to perform data analyses.

Figure 1. Systolic blood pressure and mortality during prolonged infusion of either AngII or saline. **A:** Systolic blood pressure was measured continuously for 5 days per week at the indicated intervals. Means of groups are represented by symbols and SEM by bars. * $P < 0.0001$ for comparison of Groups 2 and 3 during the 28- to 84-day interval using a linear mixed model. **B:** Symbols represent the number of surviving mice during the 56-day infusion of either AngII or saline.

Results

Continuous AngII Infusion Promotes Aneurysmal Rupture and Aortic Luminal Expansion

AngII infusion at a rate of 1000 ng/kg/min increased systolic blood pressure by approximately 40 mm Hg during the initial 28 days. In Group 2, in which miniosmotic pumps were replaced with saline after the 28 days of AngII infusion, systolic blood pressure returned near baseline within 24 hours (Figure 1A). In contrast, sequential replacement of primed miniosmotic pumps continuously delivering AngII at 1000 ng/kg/min continued to sustain the increase in systolic blood pressure to approximately 160 mm Hg throughout the infusion interval. Although body weight increased over time, no significant difference in serum cholesterol concentration was observed among these three groups of *apoE*^{-/-} mice at experimental termination (see Supplemental Table S1 at <http://ajp.amjpathol.org>). Lipoprotein distribution of cholesterol was similar among all groups (see Supplemental Figure S3 at <http://ajp.amjpathol.org>).

Previous studies have demonstrated that some mice die of abdominal aortic rupture during the initial 10 days of a 28-day AngII infusion, and subsequent deaths are infrequent for the remaining 18 days.²⁶ In the present study, mice were stratified into groups after the initial 28-day infusion of AngII. In Group 2 mice, there were no deaths during the interval in which saline was infused. In

contrast, four mice died of aneurysmal rupture in Group 3 with continuous infusion of AngII for 84 days (Figure 1B).

The maximal luminal diameter of suprarenal aortas was determined sequentially *in vivo* using high-frequency ultrasound. As noted in *Materials and Methods*, mice were stratified into three groups on day 27 based on this measurement (see Supplemental Figure S4 at <http://ajp.amjpathol.org>). In Group 2 mice, the implantation of pumps that infused saline led to no significant change in the luminal diameter during the additional 56 days of observation. In contrast, the continuous infusion of AngII led to a gradual increase in luminal diameter (Figure 2A). Despite considerable variance in the luminal diameters within the groups, Group 3 had a significantly larger mean of luminal diameter at the termination of the experiment compared to time of stratification (Figure 2B).

Figure 2. *In vivo* and *ex vivo* dimensions of suprarenal aortas measured in mice infused with either AngII or saline. **A:** Luminal diameters of the suprarenal aortas were measured *in vivo* ultrasonically before AngII infusion and at 28-day intervals. Symbols represent means, and bars are SEM. * $P = 0.0014$, ** $P < 0.0001$ for comparisons of Groups 2 and 3 using a linear mixed model. **B:** Individual suprarenal aortic diameters, measured *in vivo* ultrasonically, are represented by triangles and circles, mean data are represented by diamonds, and SEMs are represented by bars. * $P < 0.001$ for comparisons of Group 3 to Groups 1 and 2 by analysis of variance followed by post hoc pairwise multiple comparison procedures using the Holm-Sidak method. **C:** Individual suprarenal aortic widths, measured *ex vivo*, are represented by triangles and circles, mean data are represented by diamonds, and SEMs are represented by bars. * $P = 0.004$ for comparisons of Group 3 to Groups 1 and 2 using Kruskal-Wallis analysis of variance on ranks followed by post hoc multiple comparison procedures using the nonparametric Dunn's method.

Figure 3. Examples of suprarenal aortas of *apoE*^{-/-} mice infused with different intervals of AngII (1000 ng/kg/min). After 28 days of AngII infusion, the suprarenal aorta is dilated and frequently contains overt thrombotic material. After 84 days of AngII infusion, the suprarenal aorta can be greatly dilated (over 4 mm from a baseline of 0.8 mm) with thinned wall and resolution of thrombotic material. To facilitate cryosectioning of these thinned and dilated tissues, they are routinely perfusion fixed and filled with low melting point agarose containing green dye to assist visualization of the tissue in the optimal cutting temperature compound.

The size of AAAs was also determined by measurement of the maximal external diameter of the suprarenal region following excision of perfusion-fixed tissue. Similar to data derived from ultrasonic measurement of luminal diameters, external diameters of the suprarenal aorta increased in Group 3, but not in Group 2 (Figure 2C). In age-matched *apoE*^{-/-} mice ($n = 10$) in which no AngII was infused, there were no significant changes in suprarenal aortic dimensions over the same intervals (data not shown).

AAA Expansion Associates with Adventitial Macrophage Infiltration in Areas of Medial Disruption

AngII infusion generates AAAs that are morphologically heterogeneous in both external appearance and tissue characteristics. Given this heterogeneity, entire aneurysms were sectioned and examined every 100 μ m. Compared to AAAs formed after 28 days of AngII infusion, the histological characteristics of AAAs harvested at 84 days usually had less thrombotic material and exhibited aortic wall thinning (Figure 3).

Serial sections were stained with Movat's pentachrome at 100- μ m intervals throughout entire aneurysmal tissue to account for the considerable heterogeneity along the length of the aorta (Figure 4). The lumen of aorta was normal proximal to dilations (Figure 4, A, B, and C). Adjacent to dilated areas, adventitial regions were thickened and contained fibrous material and thrombus (Figure 4, B and C). In areas with lumen dilation, transmural breaks of elastin fibers were present in the media (Figure 4, D and E). Distally, aortic lumen diameters were equivalent to mice infused with saline (Figure 4F).

CD68-positive cells were present in many sections of aneurysmal tissue retrieved at 28 days of AngII infusion (Group 1). In tissues harvested after an additional 56 days of AngII infusion, there were many more

Figure 4. Movat's pentachrome staining of an entire abdominal aortic aneurysm. The full length of an AAA was serial sectioned, and every tenth slide was stained with Movat's pentachrome. Section (A, B, and C) starts proximally to the AAA and sections continue through it (D and E), finishing distally to the dilation (F). Arrows point to elastin breaks (D and E). T, thrombus. Original magnification, 400 $\times$.

CD68-positive cells, which were particularly apparent in regions of medial disruption. To quantify immuno-stained macrophages, the numbers of CD68-positive cells present in each section and within aortic regions were counted (Figure 5A). Macrophages present in aneurysmal tissues were predominantly located in the adventitia of all three groups (Figure 5B). This accumulation was focused at the medial break (Figure 6) on the adventitial aspect of the break (see Supplemental Figure S5 at <http://ajp.amjpathol.org>).

Figure 5. Macrophage counting throughout the suprarenal aneurysmal aorta. **A:** The mean number of macrophages per section of all samples in each group. Histograms represent means, and bars represent SEM. * $P = 0.0005$ for comparison of Group 3 to Group 1; † $P = 0.0002$ for comparison of Group 3 to 2 using a linear mixed model. **B:** The mean number of macrophages located in the adventitia of all samples in each group. Histograms represent means, and bars represent SEM. * $P = 0.02$, † $P = 0.002$ for comparisons of Group 3 to Groups 1 and 2, respectively, using a linear mixed model.

Figure 6. Macrophage counting and medial breakage throughout the suprarenal aneurysmal aorta. Examples (three per group, **A**, **B**, and **C**) of macrophage numbers per section throughout the aneurysmal suprarenal aorta of all of the three groups described in this study. Symbols represent macrophage counts per section. **Shaded areas** represent regions of elastin breaks associated with luminal dilation.

A panel of antibodies was used to examine macrophage heterogeneity. CD68-positive macrophages were also immunostained for MOMA2. Immunostaining for CD206 was more common than iNOS in aneurysmal tissue sections from Group 3 mice (see Supplemental Figure S6 at <http://ajp.amjpathol.org>).

Continuous AngII Infusion Promotes Other Vascular Pathologies

Previous studies have demonstrated that AngII infusion into *apoE*^{-/-} mice for 28 days increases atherosclerosis.^{2,27} We determined whether continuous AngII infusion promotes a persistent increase in atherosclerotic area. In Group 3 (AngII infusion for 84 days), there was a significant increase in atherosclerotic lesion area compared to Groups 1 and 2 (see Supplemental Figure S7A at <http://ajp.amjpathol.org>).

Previous studies have demonstrated that AngII infusion also promotes pronounced dilation of ascending aortas.^{27,28} In mice not infused with AngII, the intimal area of the aortic arch was $18.8 \pm 0.5 \text{ mm}^2$. Infusion of AngII for 28 days (Group 1) increased intimal area to $25.3 \pm 1.1 \text{ mm}^2$ ($P < 0.001$). There was no further increase in this area in the saline-infused mice of Group 2 ($24.0 \pm 0.6 \text{ mm}^2$; $P = 0.646$). In contrast, there was a marked expansion to $31.1 \pm 1.3 \text{ mm}^2$ ($P < 0.001$) in the ascending aortas of Group 3 during continuous AngII infusion (see Supplemental Figure S7B at <http://ajp.amjpathol.org>).

Discussion

Many studies have demonstrated that AngII infusion into mice leads to the rapid formation of AAAs with large

luminal dilations present within 10 days of initiating the disease. The basis for initiation of human AAAs has not been defined, and thus, the concordance of initiation processes between animal models and human disease will be difficult to establish. However, after 28 days of AngII infusion, aneurysmal tissue has many features of the human disease, including luminal dilation, extracellular matrix fragmentation, leukocyte accumulation, and thrombus.¹² This study demonstrated that prolonged AngII infusion led to several changes, including progressive luminal expansion, increased propensity to rupture, and greater numbers of CD68⁺ cells.

This study demonstrates that continuous infusion of AngII into *apoE*^{-/-} mice led to gradual luminal expansion of the suprarenal aorta. We have also demonstrated similar responses during prolonged infusion of AngII into low-density lipoprotein (LDL) receptor-deficient mice (unpublished observations). In contrast, infusion of saline into previously AngII-infused mice led to no further increase in suprarenal lumen diameter. This is in agreement with Yoshimura et al,⁶ who demonstrated that a shorter interval of saline infusion (28 days) after 28 days of AngII infusion into *apoE*^{-/-} mice led to no changes in lumen diameter of the formed AAAs.

The present study demonstrates that the suprarenal aorta does not revert to a normal appearance after the cessation of AngII infusion. This was an expected result since the generation of AngII-induced AAAs is characterized by dramatic changes in the tissue appearance that includes regional destruction of the medial tissue with pronounced inflammation and fibrosis. This lack of reversal to normal appearance contrasts with the findings of Ayabe et al.⁸ However, their study was performed in female mice that had a low aneurysm incidence of 10% in the abdominal segment (4 from a group of 40), as noted previously.^{26,29,30} Although no AAAs were observed in their additional 24 female mice that were infused with AngII for 14 days and tissues excised 98 days later, no ultrasonic screening was performed to determine the presence of AAAs in their mice. Importantly, the continued progression of AAAs with prolonged AngII infusion suggests that studies can be designed to test effects of interventions on aneurysm progression using this experimental model.

Systolic blood pressure immediately decreased on cessation of AngII infusion, despite extensive arterial modeling during this interval. Previous studies have demonstrated that AngII infusions initiate AAAs in the absence of measurable increases in systolic blood pressure.^{2,31} Furthermore, pharmacological agents that had no effect on systolic blood pressure changed AAA formation.^{18,29–34} Thus, the changes in systolic blood pressure, per se, may not be responsible for the initiation of AAAs. Similarly, increased blood pressure is not a major factor in AngII-induced augmentation of atherosclerosis.³¹ Additional studies are necessary to determine whether the maintenance of systolic blood pressure during continuous infusion of AngII has a significant impact on progression of AAAs.

The heterogeneity of AAA tissue provides a challenge in the characterization of the tissue. In a previous publi-

cation, we observed that AngII-induced AAAs are initiated by medial disruption soon after initiation of the infusion.¹² This led to abrupt luminal expansion and thrombus formation. Thrombi were present at the region of the medial break and extruded both distally and proximally into the adventitia. These thrombi provoked pronounced macrophage recruitment and gradually resolved during a remodeling process. This leads to regions of pronounced luminal expansion adjacent to other regions of intact media that are surrounded by adventitial thickening. After protracted AngII infusion, thrombus was not commonly seen, except in those mice that succumbed to rupture during the continuous AngII infusion. The present study has demonstrated that the regions of medial disruption, as indicated by elastin fragmentation, provided stimuli for macrophage accumulation during continuous AngII-infusion.

Previous studies have demonstrated that T helper 2 (Th2) cytokines are more common in human AAAs and have been shown to function in development of experimental aneurysms.³⁴ Conversely, inhibition of Th1 cytokine effects, such as deficiency of gamma interferon or its receptors, leads to augmentation of experimental AAAs.^{33,34} These cytokines are major mediators of macrophage phenotypes. Although macrophage phenotypes have both heterogeneity and plasticity, iNOS and CD206 have been used as markers at opposite ends of the M1 and M2 phenotype, respectively.³⁵ In agreement with previous studies on cytokines that modulate these phenotypes, we demonstrated that macrophages accumulating in aneurysmal tissue during continuous AngII infusion predominantly expressed CD206. The functional significance of this macrophage phenotype in the progression of AAAs remains to be determined.

In addition to promoting the formation of AAAs, AngII infusion into hypercholesterolemic mice also augments development of atherosclerosis. This has been uniformly demonstrated in both *apoE*^{-/-} and *LDL receptor*^{-/-} mice. Termination of AngII infusion after 28 days led to a modest increase in lesion area during the subsequent 56 days in which saline filled miniosmotic pumps were implanted. However, there was a clear increase in the lesion area of mice that were infused continuously with AngII (Group 3). Therefore, AngII promoted a continuous process for lesion growth. AngII infusion also led to expansion of ascending aortic arches. The pathological appearance of ascending aortic tissues differs substantially from that of AngII-induced AAAs. After the initial 28 days of AngII infusion, the ascending aorta had expanded compared to age-matched controls not implanted with miniosmotic pumps (data not shown), as defined by the luminal area of this region. Termination of AngII infusion after 28 days led to no further expansion whereas continuous infusion led to further increases in aortic arch diameter. Thus, as with other vascular diseases quantified in this study, continuous AngII infusion led to progressive dilation of the ascending aorta. Currently, the mechanisms responsible for AngII-induced ascending aortic expansion have not been defined, but warrant further study with the recent recognition of a pivotal role of

AT1 receptor antagonism in ascending aortic dilation of Marfan's patients.²⁴

In conclusion, the present study has demonstrated that continuous AngII infusion leads to progressive aneurysmal expansion, an increased propensity to rupture, and cellular changes. These AngII-mediated changes were associated with a marked increase in macrophages present at the sites of medial disruption. The development of mice that permit conditional macrophage ablation that can be controlled temporally will facilitate the determination of whether macrophage accumulation in aneurysmal tissue is responsible for the changes noted during protracted AngII infusion. Moreover, the continued progression of AngII-induced AAAs should facilitate testing of interventions to slow progression of formed AAAs.

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