Rapid dilation of the abdominal aorta during infusion of angiotensin II detected by noninvasive high-frequency ultrasonography

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Background: **Infusion of angiotensin II (AngII) via subcutaneous osmotic pumps into mice promotes the development of abdominal aortic aneurysms (AAAs). These AngII-induced AAAs develop via a complex process in which there is a transmedial break, lumen dilation, thrombus formation, inflammation involving cells of both the innate and acquired immune systems, and remodeling. The recent development of a high-frequency ultrasound machine has permitted the noninvasive detection of murine abdominal aortas. We assessed the ability of a Visualsonics Vevo 660 high-resolution imaging system to detect AAAs and sequentially quantify the aortic luminal diameter. This system had 100% accuracy in detecting AngII-induced AAAs in vivo, with intrauser and interuser variation coefficients of less than 10% for quantification of the aortic lumen diameter.**

Methods: **Male apolipoprotein E (apoE)$^{-/-}$ mice were infused subcutaneously with either saline or AngII and were monitored with this ultrasonic system to define the temporal changes in aortic lumen diameter. Aortic luminal diameters were measured in the aneurysm-susceptible region of the suprarenal aorta. For internal controls, abdominal aortic diameters were measured at the level of the left renal branch, because this landmark region did not dilate during AngII infusion.**

Results: **Luminal diameters of the suprarenal aorta did not change significantly in saline-infused mice over 28 days of measurement ($P = .71$). In contrast, AngII infusion led to rapid dilation of suprarenal aortas during the initial 7 days of infusion (0.071 mm/d; $P = .0037$ for the change in the initial expansion rate). Further luminal diameter expansions occurred for the remaining 21 days of observation at a more modest rate (0.023 mm/d; $P = .0001$ for continued expansion after day 7). Within the initial 14 days of AngII infusion, some apoE$^{-/-}$ mice died as a result of rupture of the aorta in the suprarenal region. We had previously assumed that aortic dilation and rupture occurred simultaneously. However, in the AngII-infused mice that succumbed to aortic rupture, luminal diameters increased several days before death.**

Conclusions: **High-frequency ultrasonography demonstrated that suprarenal aortic expansion occurs rapidly after the initiation of AngII infusion into apoE$^{-/-}$ mice. (J Vasc Surg 2006;44:372-6.)**

Clinical Relevance: **Angiotensin II has been inferred to have an important role in the development of human aortic diseases. Infusion of angiotensin II into mice leads to the development of abdominal aortic aneurysms. Definition of the natural history of abdominal aortic aneurysm development in animal models of the disease may provide insight into the factors associated with initiation and propagation in the human disease. The recent development of high-frequency ultrasonography has permitted the sequential noninvasive detection of mouse aortic luminal dimensions during angiotensin II infusion. The convergence of studies on aortic dimensions, in association with pathologic characterization of the tissue, should provide a means to define mechanisms of abdominal aortic aneurysm formation.**

Infusion of angiotensin II (AngII) via subcutaneous osmotic minipumps leads to the development of abdominal aortic aneurysms (AAAs) in low-density lipoprotein receptor$^{-/-}$ and apolipoprotein E (apoE)$^{-/-}$ mice.[1-10] AngII infusion also promotes AAAs in normolipidemic mice, albeit with a lesser incidence.[11] The development of AngII-induced AAAs is a complex process.[12] On the basis of pathologic analysis of aortic tissues acquired at different intervals of AngII infusion, the initial discernible change in the aneurysm-susceptible region is medial macrophage accumulation. This precedes a transmedial break that produces luminal dilation. The ensuing thrombus formation at the site of aortic dilation promotes an inflammatory response that predominantly involves the innate immune system. The aorta is gradually remodeled, with increased deposition of extracellular matrix and formation of atherosclerotic lesions.[12] In some mice infused with AngII, death occurs within the first 14 days as a result of aortic rupture in the suprarenal region.

Currently, the quantification of AngII-induced AAAs requires the death of the mouse and acquisition of the aortic tissue for AAA analysis using intact ex vivo or sec-

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tioned tissue. Definition of the sequence of events leading to AAA formation in mice would be enhanced by the availability of noninvasive techniques. Previous approaches have used nondestructive techniques such as transrectal ultrasonography.[13] Ultrasonic imaging of AngII-induced AAAs has been attempted previously, but available instruments were not optimal for the small dimensions of mouse aortas.[3] Recently, a high-frequency ultrasound machine has become commercially available that has characteristics amenable to imaging mice. The Visualsonics Vevo 660 (Visualsonics Inc, Toronto, Ontario, Canada) machine uses probes with a sufficiently high frequency of 40 MHz to permit a resolution of 30 μm at a focal length of 6 mm. These characteristics have the capability of imaging the abdominal aorta in mice. The practical application of this instrument to quantify changes in the dimensions of the abdominal aorta of mice in vivo has been demonstrated recently by Martin-McNulty et al.[14]

Using this instrument, we performed a validation process to measure aortic dimensions and determine intrauser and interuser variability in this measurement. High-frequency ultrasonography was then used to quantify the temporal evolution of luminal aortic expansion in the suprarenal region of AngII-infused male apoE$^{-/-}$ mice, which have a high propensity to develop AAAs.[15] These studies demonstrated that the aortic expansion occurred rapidly after the initiation of AngII infusion. In addition, an unanticipated finding was that death due to aortic rupture occurred several days after luminal dilation.

MATERIALS AND METHODS

Mice. Male ApoE$^{-/-}$ mice (bred in house from stock originally obtained from the Jackson Laboratory, Bar Harbor, ME; 10 times backcrossed into the C57BL/6 background), 8 to 12 weeks old, were housed in a pathogen-free environment. Water and regular mouse diet were available ad libitum. Three separate groups of apoE$^{-/-}$ mice were used to acquire data. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Infusions. Alzet (Durect Corp; Cupertino, CA) osmotic pumps (model 2004) were filled with either AngII (infusion rate of 1000 ng $\cdot$ kg^{-1} $\cdot$ min^{-1}) or saline, as described previously.[1,2] Pumps were implanted subcutaneously on the right flank via an incision in the scapular region. Mice were observed twice daily after the implantation of pumps.

Ultrasound imaging. The high-resolution ultrasound imaging system performed two-dimensional imaging (B mode) by using a real-time microvisualization scanhead (RMV 704) with a central frequency at the mechanical transducer of 40 MHz (frame rate of 30 Hz), a focal length of 6 mm, and an 8 $\times$ 8-mm field of view (Visualsonics; Toronto, Canada).

To perform ultrasound scans, mice were anesthetized with a mixture of ketamine and xylazine, and hair was removed from the abdomen by using a depilatory cream (Nair; Church & Dwight Co, Inc; Princeton, NJ). Mice were laid supine on a heated table, and warmed ultrasound transmission gel was placed on the abdomen. Short-axis scans of aortas were performed on the abdominal aorta from the level of the left renal arterial branch through to the suprarenal region. Aortic images were acquired at selected intervals (0, 2, 5, 7, 12, 16, 21, and 28 days) after the initiation of infusions. Cine loops of 300 frames were acquired throughout the renal region of the abdominal aorta and used to determine the maximal diameters of the abdominal aorta in the suprarenal region. To define user variability, three operators independently acquired and analyzed images from the same group of mice to calculate the coefficient of variation.

Aortic tissue acquisition. Twenty-eight days after implantation of the pumps, sedated mice were killed and perfused briefly with saline at a pressure of approximately 100 mm Hg, followed by prolonged infusion with paraformaldehyde (4% wt/wt solution in phosphate-buffered saline). Photographs were acquired of aortas in situ. Some suprarenal aortas were sectioned at 10 μm throughout the region (8-10 mm). Sections were visualized after staining with hematoxylin, and aortic lumen dimensions were measured with Image-Pro (Media Cybernetics Inc; Silver Springs, MD). Necropsies were performed on all mice that died during the 28-day infusion interval, to determine the cause of death.

Statistics. Summary data are expressed as mean $\pm$ SEM. To quantify the relationship of aortic measurements in vivo and ex vivo, linear regression was used (SigmaStat; Point Richmond, CA). A linear mixed model was fit to the aortic diameter data by using version 8.2 of SAS (SAS Institute, Cary, NC). In particular, the mean aortic diameter was modeled as a quadratic function of time. Estimates of model parameters were used to make inferences about the time courses of dilation. For all analyses, $P < .05$ was considered to be statistically significant.

RESULTS

To reproduce the recent results of Martin-McNulty et al,[14] we performed initial studies in which the lumen diameters of aortas were determined in saline- and AngII-infused mice by using both an ultrasound approach in vivo and a pathologic analysis of the vessel at necropsy. Examples of the ultrasonic images obtained by this approach are shown in Fig 1 with photographs of the corresponding aortas. The coefficient of variance in luminal diameter measurements of the suprarenal aorta among operators was 7.64% $\pm$ 0.94% during saline infusion and 8.45% $\pm$ 1.09% during AngII infusion. These measurements were recorded in a nongated mode that did not take into account the pulsatile nature of the aorta. In highly dilated aortas, a contribution to variance is the level of difficulty in sharply delineating the lumen/aortic wall boundary in grossly diseased vessels. However, these variances were relatively minor compared with the extent of lumen dilation that occurs during AngII infusion.

To quantify the fidelity of the aortic measurements by ultrasonography, we performed studies similar to those

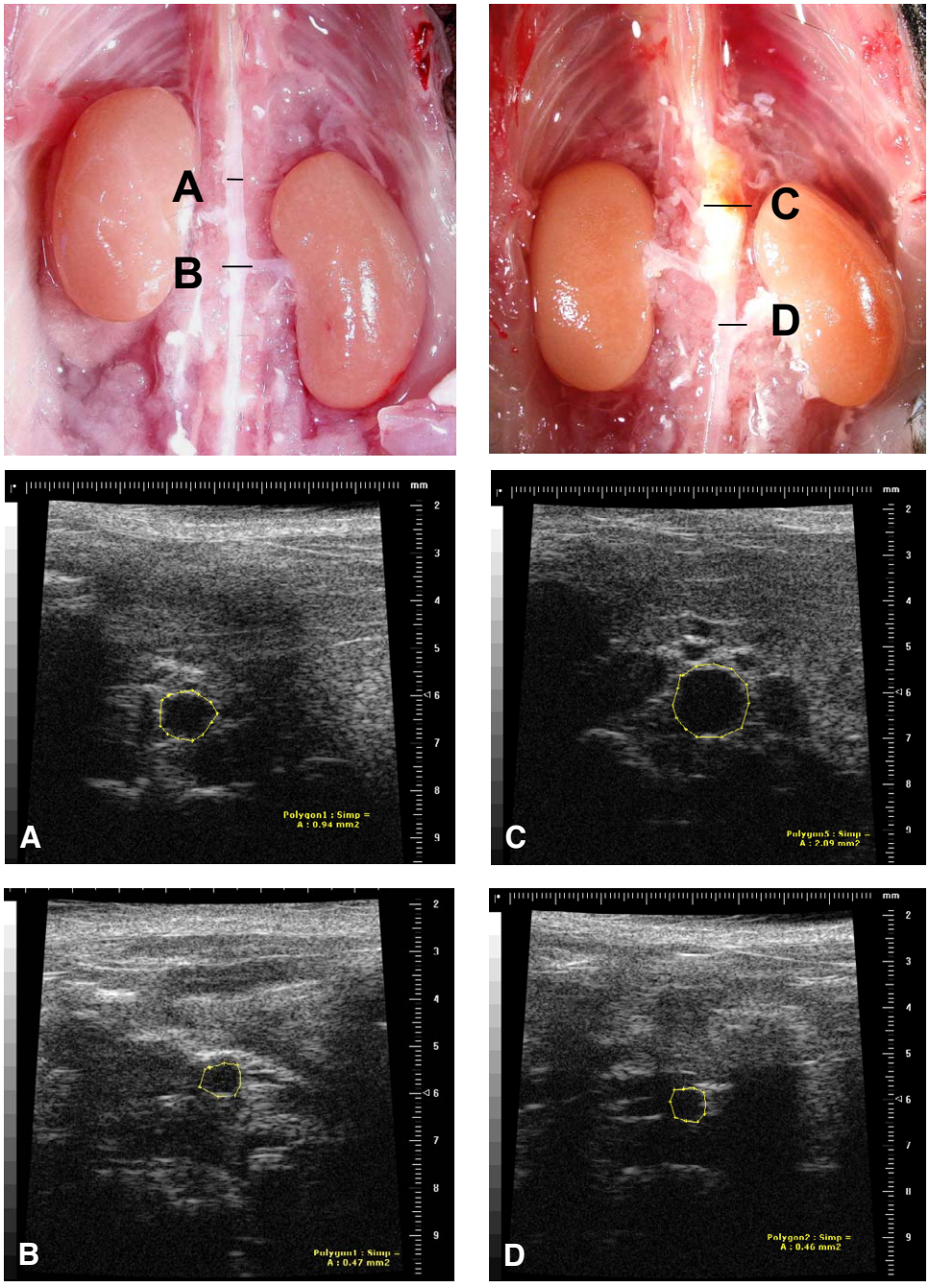

Fig 1. Measurement of murine aortic luminal dimensions in vivo by ultrasonography. Photographs are shown of apolipoprotein $E^{-/-}$ mice infused with either saline *(left)* or angiotensin II *(right)* for 28 days. The regions used to acquire images are indicated on these photographs. Samples of short-axis ultrasonic images at these abdominal aortic regions are shown (**A** and **C**, suprarenal region; **B** and **D**, at the level the left renal branch). The perimeter of the aortic lumen is outlined by *yellow dotted lines.*

previously reported by Martin-McNulty et al.[14] We also analyzed the lumen dimensions by both ultrasound and histologic analysis of the same regions. This comparison of the maximal aortic lumen diameters measured in vivo by ultrasonography and on tissue sections is shown in Fig 2. In accord with Martin-McNulty et al, there was a close agreement between ultrasound and histological measurements ($r = 0.9897$).

To determine the temporal characteristics of lumen diameters during 28-day infusions of saline or AngII, mice

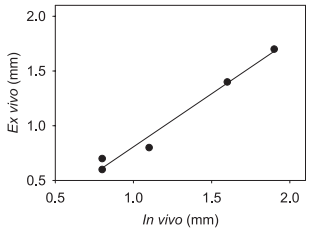

Fig 2. Comparison of aortic lumen measurements acquired in vivo by ultrasonography and ex vivo by histology. Apolipoprotein $E^{-/-}$ mice infused with either saline or angiotensin II for 28 days were subjected to ultrasonography to determine the maximum lumen diameter in the suprarenal region. At the termination of the study, mice were perfusion-fixed at approximately 100 mm Hg, and their entire suprarenal aortas were sectioned to determine the region of maximal dilation. Points represent the maximal lumen diameter for individual mice for ex vivo and in vivo measurements. The two measurements were highly correlated with each other ($r = 0.9897$).

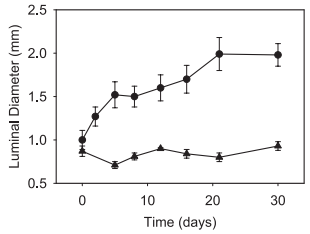

Fig 3. Sequential measurements of abdominal aortic luminal diameters of apolipoprotein $E^{-/-}$ mice during infusion with either saline or angiotensin II (AngII). Mice were infused with saline or AngII for 28 days and monitored at frequent intervals. Data are shown for AngII-infused mice that developed abdominal aortic aneurysms and survived the infusion period. Abdominal aortic luminal diameters were measured in the suprarenal region of mice infused with saline (*triangles*; n = 8 at all time points) or AngII (*circles*; n = 12). Data are based on the measurement of diameters at each time point by at least two independent operators. *Symbols* represent means, and *bars* represent SEMs.

were implanted with Alzet pumps. Ultrasound measurements of aortic diameters were recorded at frequent intervals over the 28-day infusion period. In saline-infused mice (n = 8), there was no significant change in the dimensions of suprarenal aortas ($P = .71$; Fig 3). In addition, there was no change in abdominal aortic luminal diameters at the level of the left renal arterial branch, where dilations do not occur during AngII infusion (data not shown). In contrast, AngII promoted a prompt expansion of lumen diameters of suprarenal aortas of apo$E^{-/-}$ mice. In the mice that developed AAAs and survived throughout the infusion interval (n = 12), the expansion rate of the luminal diameter was greatest during the first 7 days (0.071 mm/d; $P = .0037$ for the change in the initial expansion rate). The rate of increase in aortic luminal diameters was more modest during the remaining 21 days, but expansion persisted throughout the infusion period (0.023 mm/d; $P = .0001$ for the continued expansion rate after day 7; Fig 3). This mode of aortic expansion occurs irrespective of the magnitude of the diameter increase of the aorta at 28 days (data not shown).

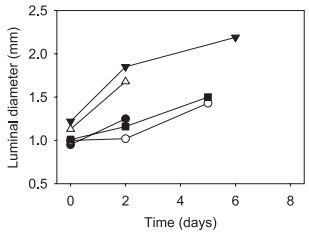

Fig 4. Lumen diameters of the suprarenal aortas of angiotensin II–infused mice that subsequently died of abdominal aortic rupture. Each curve represents an example of temporal changes in luminal diameters of individual mice. In all of these examples, aortic rupture of the suprarenal region occurred 1 or 2 days after the final luminal measurement.

As noted previously,[12,16] infusion of AngII into hypercholesterolemic male mice results in some mortality within the initial 14 days; this is usually attributable to rupture of the suprarenal aorta. In this study, death due to aortic rupture within this interval occurred in eight AngII-infused mice (40% incidence). On the basis of previous pathologic characterization of evolving AAAs,[12] we had assumed that the transmedial breaks, luminal dilation, and aortic rupture in this region were simultaneous events. However, in the course of this study, we observed that the luminal diameters of suprarenal aortas of several mice expanded before mice died by aortic rupture. Figure 4 represents examples of the aortic diameters of mice that subsequently died from aortic rupture in the suprarenal region. All these mice had discernible aortic dilations before aortic rupture. In some cases, the aortic dilation occurred many days before the rupture.

DISCUSSION

This study used a high-frequency ultrasound technique[14] to determine the changes in aortic dimensions during the infusion of saline or AngII into hypercholesterolemic mice. In addition, we demonstrated that this technique can be performed with relatively small intraindividual and interindividual variance; this further supports the validity of the measurements. Using this approach, we determined that the dilation of the suprarenal abdominal aorta during AngII infusion occurred rapidly. This study also determined that the occasional AngII-induced fatal aortic rupture in this region occurs subsequent to luminal dilation.

Our previous study characterized temporal pathologic features of AngII-induced AAAs as determined by the acquisition and staining of tissues at selected intervals after initiation of AngII.[12] These results implied that AngII-infused AAAs were initiated by macrophage accumulation in the medial layer of the suprarenal aorta that preceded transmedial breaks, luminal dilation, and subsequent

thrombus formation. These data were obtained by using excised tissues from a relatively small number of mice that were killed at selected intervals. This previous study implicated that there were rapid pathologic changes of the suprarenal aorta during AngII infusion. The current study used a noninvasive technique that was amenable to sequential measurement of the aortic luminal dimensions of a large number of mice. This technique demonstrated that the rate of aortic lumen expansion is rapid, with the greatest rate of dilation occurring during the first 7 days. Thereafter, the aortic lumen diameter expanded at a slower rate. These data also demonstrated that the AAA size at the termination of the infusion interval was largely determined by the extent of expansion in this early phase. These results demonstrate that the lumen diameter determined noninvasively by ultrasonography can be used as a measure of both AAA incidence (percentage increase over baseline) and severity (absolute magnitude of lumen diameter).

Several studies have demonstrated that infusion of AngII into hyperlipidemic mice can lead to death, usually in the first 14 days, as a result of rupture of the suprarenal aorta. The rupture is usually in the retroperitoneal region on the left side of the suprarenal aorta and leads to death as a result of exsanguination. We had previously assumed that aortic rupture occurred simultaneously with a transmedial break.[12] This was based on the premise that aortic rupture occurred because of the inability of the adventitia to constrain blood after formation of a transmedial break. However, we were surprised to find that dilation of the suprarenal aorta preceded aortic rupture by several days. Thus, on the basis of the ultrasonic characterization of these mice, aortic rupture occurred in the region of luminal dilation, but it was precipitated by a mechanism that occurred subsequent to the transmedial breaks.

In conclusion, high-frequency ultrasonography has enabled the quantification of the rapid expansion of suprarenal aortic luminal dimensions in response to AngII infusion. The elucidation of the mechanism of this rapid AngII-induced dilation will provide insight into the processes of AAA initiation.

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AUTHOR CONTRIBUTIONS

Conception and design: AD, CB, DLR
Analysis and interpretation: AD, CB, RC, DAH, JJM, DLR
Data collection: CB, DAH, JJM, DLR
Writing the article: AD, CB, DLR
Critical revision of the article: AD, CB, RC, DAH, JJM, DLR
Final approval of the article: AD, CB, RC, DAH, JJM, DLR
Statistical analysis: RC
Obtained funding: AD
Overall responsibility: AD

REFERENCES

1. Daugherty A, Cassis L. Chronic angiotensin II infusion promotes atherogenesis in low density lipoprotein receptor −/− mice. Ann N Y Acad Sci 1999;892:108-18.
2. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. J Clin Invest 2000;105:1605-12.
3. Wang YX, Martin McNulty B, Freay AD, Sukovich DA, Halks Miller M, Li WW, et al. Angiotensin II increases urokinase-type plasminogen activator expression and induces aneurysm in the abdominal aorta of apolipoprotein E-deficient mice. Am J Pathol 2001;159:1455-64.
4. Tham DM, Martin McNulty B, Wang YX, Wilson DW, Vergona R, Sullivan ME, et al. Angiotensin II is associated with activation of NF-kappa B-mediated genes and downregulation of PPARs. Physiol Genomics 2002;11:21-30.
5. Ishibashi M, Egashira K, Zhao Q, Hiasa KI, Ohtani K, Ihara Y, et al. Bone marrow-derived monocyte chemoattractant protein-1 receptor CCR2 is critical in angiotensin II-induced acceleration of atherosclerosis and aneurysm formation in hypercholesterolemic mice. Arterioscler Thromb Vasc Biol 2004;24:e174-8.
6. Bruemmer D, Collins AQ, Noh G, Wang W, Territo M, Arias Magallona S, et al. Angiotensin II-accelerated atherosclerosis and aneurysm formation is attenuated in osteopontin-deficient mice. J Clin Invest 2003;112:1318-31.
7. Zhou Y, Chen R, Catanzaro SE, Hu L, Dansky HM, Catanzaro DF. Differential effects of angiotensin II on atherogenesis at the aortic sinus and descending aorta of apolipoprotein-E-deficient mice. Am J Hypertens 2005;18:486-92.
8. Gavrila D, Li WG, McCormick ML, Thomas M, Daugherty A, Cassis LA, et al. Vitamin E inhibits abdominal aortic aneurysm formation in angiotensin II-infused, apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 2005;25:1671-7.
9. Ayabe N, Babaev VR, Tang Y, Tanizawa T, Fogo AB, Linton MF, et al. Transiently heightened angiotensin II has distinct effects on atherosclerosis and aneurysm formation in hyperlipidemic mice. Atherosclerosis 2005;184:312-21.
10. Daugherty A, Cassis LA. Mouse models of abdominal aortic aneurysms. Arterioscler Thromb Vasc Biol 2004;24:429-34.
11. Deng GG, Martin-McNulty B, Sukovich DA, Freay A, Halks-Miller M, Thinnes T, et al. Urokinase-type plasminogen activator plays a critical role in angiotensin II-induced abdominal aortic aneurysm. Circ Res 2003;92:510-7.
12. Saraff K, Babamusta F, Cassis LA, Daugherty A. Aortic dissection precedes formation of aneurysms and atherosclerosis in angiotensin II-infused, apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 2003;23:1621-6.
13. Chiou AC, Chiu B, Oppat WF, Matsumura JS, Chisholm RL, Pearce WH. Transrectal ultrasound assessment of murine aorta and iliac arteries. J Surg Res 2000;88:193-9.
14. Martin-McNulty B, Vincelette J, Vergona R, Sullivan ME, Wang YX. Noninvasive measurement of abdominal aortic aneurysms in intact mice by a high frequency ultrasound imaging system. Ultrasound Med Biol 2005;31:746-9.
15. Henriques TS, Huang J, D'Souza SS, Daugherty A, Cassis LA. Orchiectomy, but not ovariectomy, regulates angiotensin II-induced vascular diseases in apolipoprotein E deficient mice. Endocrinology 2004; 145:3866-72.
16. Manning MW, Cassis LA, Huang J, Szilvassy SJ, Daugherty A. Abdominal aortic aneurysms: fresh insights from a novel animal model of the disease. Vasc Med 2002;7:45-54.

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