Hypertension overrides the protective effect of female hormones on the development of aortic aneurysm secondary to Alk5 deficiency via ERK activation

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Schmit BM, Yang P, Fu C, DeSart K, Berceli SA, Jiang Z. Hypertension overrides the protective effect of female hormones on the development of aortic aneurysm secondary to Alk5 deficiency via ERK activation. Am J Physiol Heart Circ Physiol 308: H115–H125, 2015. First published November 11, 2014; doi:10.1152/ajpheart.00521.2014.—The prevalence of aortic aneurysm is five times higher in men than women among the general population. Similar sexual dimorphism also exists in syndromic aortic aneurysms triggered by TGF-β signaling disorders. To understand the responsible mechanisms, we developed an animal model where inducible deletion of the type I TGF-β receptor, Alk5, specifically in smooth muscle cells (Alk5αα) causes spontaneous aortic aneurysm formation. This model recapitulated an extreme scenario of the dimorphism in aortic aneurysm development between genders. In a comparative experiment, all Alk5αα males (n = 42) developed aortic aneurysms and 26% of them died prematurely from aortic rupture. In contrast, the Alk5αα females (n = 14) presented only a subclinical phenotype characteristic of scarcely scattered elastin breaks. Removal of male hormones via orchiectomy (n = 7) resulted in only minimal influence on aortic pathology. However, reduction of female hormones via ovariectomy (n = 15) increased the phenotypic penetrance from zero to 53%. Finally, an elevation of systolic blood pressure by 30 points unmasked the subclinical phenotype of Alk5αα females (n = 17) to 59%. This exaggerated phenotypic penetrance was coupled with an early intensification of ERK signaling, a molecular signature that correlated to 100% phenotypic penetrance in normotensive Alk5αα males. In conclusion, aortic aneurysm induced by Alk5αα exhibits dimorphic incidence between genders with females less susceptible to aortic disease. This sexual dimorphism is partially the result from the protective effects of female hormones. Hypertension, a known risk factor for aortic disease, is able to break the female sex protective effects through mechanisms associated with enhanced ERK activity.

Hypertension; sex hormones; aortic aneurysm; sexual dimorphism; TGF-β

AORTIC ANEURYSM IS ONE of the leading causes of cardiovascular morbidity and mortality in the United States. The incidence of death from ruptured aneurysms has continued to rise and is currently reported between 1% and 2% per year in the United States (30). There is a striking difference in prevalence of the disease, with it affecting men more frequently in a 4 to 6:1 ratio (22, 25). Furthermore, it has increasingly been recognized that when aortic aneurysm does develop in females, the course is more aggressive with a worse prognosis (31). It has been documented that females have a greater chance of presenting with ruptured aneurysm (6) and also suffer from more complications following surgical repair (44). Such a sexual dimorphism has potentiated the need for gender-specific research into the development and pathogenesis of this aortic disease. In addition to gender, hypertension, tobacco use, and hypercholesterolemia have been identified as common risk factors for the development of aortic aneurysms (9).

Recent evidence has implicated disorders in TGF-β signaling pathways as being a prime contributor to development of aortic disease. These disorders have been reported in syndromic aneurysms (4, 5, 26), familial thoracic aortic aneurysm and dissection (33), and even the sporadic forms (23). Mutations at all levels of the TGF-β signaling cascade including TGF-β itself (4), the TGF-β receptors (26), and SMAD proteins (41), which potentiate the downstream signal, all have been identified. Alterations in these pathways have been implicated in several syndromes with aortic aneurysm being life-threatening phenotypic traits. These include defects in Fibrillin-1 as seen in Marfan syndrome, defects in the TGF-β receptors themselves as seen in Loeys-Dietz syndrome type 1 and 2 (26), and defects in the downstream signaling proteins as seen in Loeys-Dietz type 3 and 4 (4, 41). A recent study monitoring patients with TGFBR1 mutations discovered gender-specific differences in the severity of vascular pathology, with earlier onset of aortic aneurysm in males but more diseased arteries in females (40).

Using a Cre-loxP system driven by a smooth muscle cell (SMC) specific promoter Myh11, we selectively deleted Alk5, the mouse homolog of human TGFBR1, in SMCs of adult mice. As is seen in human disease, a striking sexual dimorphism was observed between male and female mice, where males developed aortic pathology with 100% penetrance but no females presented gross evidence of aortic disease. Although the dimorphic incidence of aortic aneurysm has been repeatedly documented in clinical investigations and experimental studies, mechanisms underlying the phenotypic disparity between genders remain poorly understood. Both male and female hormones have been implicated to modulate aortic phenotype in experimental models. However, the biological effects of manipulating the level of sex hormones varied depending on experimental models (10, 18, 21, 45). Extending from our recent observation with a novel experimental model, this study was set both to evaluate the role of sex hormones in modulating the aortic phenotype and to explore the interaction of gender,
biomechanical forces, and TGF-β biology on the development of aortic aneurysm in female mice. We hypothesized that endogenous sex hormones play a pivotal role in phenotypic expression of aortas deficient of SMC-specific Alk5, and females harbor a subclinical phenotype that could be unmasked by the biologic alterations that accompany changes in endogenous hormones and/or risk factors such as systemic hypertension.

MATERIALS AND METHODS

Animals. Adult mice with age ranging from 7 to 15 wk were used for all groups. This study conformed to the American Physiological Society’s Guiding Principles for the Care and Use of Vertebrate Animals and the Guide for the Care and Use of Laboratory Animals (National Research Council, Revised 2010). The University of Florida Institutional Animal Care and Use Committee approved this study. All mouse strains were back-crossed onto a C57BL/6 background for at least five generations. Surgeries were performed under asceptic conditions, and animals were anesthetized via continuous inhalation of 1% to 2% isoflurane. Buprenorphine was given (0.05 mg/kg body wt sc) 30 min before surgical procedures, and no antibiotics were administered postoperatively.

Inducible deletion of Alk5. Gene deletion was accomplished through a tamoxifen inducible Cre-loxP system driven by SMC-specific promoter Myh11. The Myh11-CreER<sup>+/−</sup> strain was kindly provided by Dr. Weiser-Evans (University of Colorado) with an agreement from Dr. Stefan Offermanns (University of Heidelberg). The Alk5<sup>loxP</sup> mouse line was kindly provided by Dr. Stefan Karlsson (Lund University). The Myh11-CreER<sup>+/−</sup> strain was bred with Alk5<sup>loxP</sup> strain, and offspring littermates were screened with primers (34) located at position as indicated in Fig. 1A. Animals with a genotype of Alk5<sup>loxP</sup>/Myh11-CreER<sup>+/−</sup> were kept for conditional SMC-specific Alk5 deletion (Alk5<sup>△</sup>), and littermates carrying a genotype of Alk5<sup>loxP</sup>/Myh11-CreER<sup>+/−</sup> (Alk5<sup>−</sup>) were saved as experimental controls. Animals received 0.1 ml (25 mg/ml) tamoxifen via intraperitoneal injection for 5 consecutive days. The day following the first dose of tamoxifen injection was counted as day 1 (d1) of gene deletion. To examine the conditional Alk5 deletion, we isolated primary SMCs from wild-type (WT; C57BL/6), Alk5<sup>−</sup>, and Alk5<sup>△</sup> aortas using method we have previously reported (13), genotyped SMCs with primers indicated in Fig. 1A, and examined the production of Alk5 protein in these cells.

Tissue collection. Mice were injected with 0.2 ml Evans Blue dye and euthanized for tissue harvest 20 min after injection. Aortas were flushed with 1.0 ml saline via left ventricle injection and divided into five segments: ascending thoracic aorta (ATA); proximal descending thoracic aorta (dT1A), from the takeoff of the left subclavian artery to the mid-thoracic aorta; distal descending thoracic aorta (dT2A), from the midthoracic aorta to proximal to the diaphragm; suprarenal aorta (SRA), distal to the diaphragm and proximal to the right renal artery; and the infrarenal aorta (AA). Tissues were either snap-frozen in liquid nitrogen for protein assays or perfusion fixed with 10% neutral buffered formalin for morphometry. Fixed tissue specimens were dehydrated and embedded in paraffin, and cross sections (5 μm) were collected for histology.

Morphology. The phenotypic penetrance was evaluated both grossly and microscopically. Evaluation of the gross morphology was performed under an operating microscope by noting the presence and location of lesions in each aorta. In addition to areas with obvious aneurysmal morphology, aortic segment showing Evans blue extravasation was also considered as being diseased. Phenotypic penetrance for a given group of animals was calculated based on the presence or absence of any aortic lesion per aorta. Similarly, lesion incidence for a given segment was calculated based on the presence or absence of any aortic lesion per segment. Masson’s trichrome and Movat’s staining protocols were used for histologic evaluation. Zeiss Axiovision (V4.8.2) was used for image acquisition and morphometric measurements. Elastic fiber breaks per high power field were calculated from four different regions in one section per sample. The first field with elastin fragmentations was randomly noted, and then the other three fields were assigned to areas around the points evenly spaced on the cross section. The four fields were chosen to cover the majority of elastin breaks appearing in the untreated female Alk5<sup>△</sup> aortas.

Scanning electron microscopy. Scanning electron microscopy was used to examine and characterize early pathologic changes in the Alk5<sup>△</sup> aortas. Aortic tissue was collected for histology.

Infusion of norepinephrine. After the day when tamoxifen injections were finished, adult female mice were infused with norepinephrine (NE) (Sigma-Aldrich, St. Louis, MO) at a rate of 5.6 mg/kg/24 h.
by osmotic mini-pump (model 2004; ALZET, Cupertino, CA) for a period of 28 days as described by others (8). This dosage leads to an increase of mean blood pressure by 30 mmHg, thus creating a hypertensive (HTN) environment for Alk5\(^{ik0}\) aortas (Alk5\(^{ik0}\)-HTN). Controls included tamoxifen-injected and NE-treated Alk5\(^{f/f}\) (Alk5\(^{f/f}\)-HTN) and tamoxifen-injected but not NE-treated or normotensive (NTN) Alk5\(^{ik0}\) (Alk5\(^{ik0}\)-NTN) female mice. Blood pressure of these animals was measured with a non-invasive tail-cuff method (CODA; Kent Scientific, Torrington, CT) at time points before Alk5 deletion and the day of tissue collection.

**Western blot.** To elucidate mechanisms by which hypertension breaks the female-sex protective effects, we evaluated early activation of ERK signaling pathway in both male and female aortas under various conditions. For males, the Alk5\(^{ik0}\) and Alk5\(^{f/f}\) ATAs (n = 5 per group) were examined on d5. The Alk5\(^{ik0}\) and Alk5\(^{f/f}\) females were treated with or without NE infusion (n = 3 per group), and ATAs of the Alk5\(^{ik0}\)-NTN, Alk5\(^{ik0}\)-HTN, Alk5\(^{f/f}\)-NTN, and Alk5\(^{f/f}\)-HTN aortas were evaluated 14 days after NE treatment. Total proteins were extracted with Tris·HCl buffer containing a cocktail of phosphatase inhibitor, separated with SDS-PAGE gels, and transferred to nitrocellulose membranes. Primary antibodies applied to the assays were rabbit antiserum to pSmad2 (ser465/ser467, No. 3101), pERK (Thr22/Tyr24/Thr204, No. 4370), ERK (No. 4696), and phospho-NF-

**RESULTS**

The Cre-LoxP system driven by Myh11 promoter abrogates production of Alk5 receptors in SMCs. PCR assays on genomic DNA extracted from SMCs were performed with primers shown in Fig. 1A. Primers with primers β and γ revealed a null (iko), floxed (ff), and WT Alk5 allele for SMCs isolated from Alk5\(^{ik0}\), Alk5\(^{f/f}\), and WT SMCs, respectively (Fig. 1B, top). Successful removal of exon 3 was confirmed in assays with primers α and γ (Fig. 1B, bottom). To evaluate the deletion of Alk5 receptor in SMCs, we examined the production of Alk5 protein with Western blotting assays. As shown in Fig. 1C, although abundant Alk5 receptor is made by Alk5\(^{f/f}\) SMCs, a band specific to Alk5 was not detected in Alk5\(^{ik0}\) SMCs, indicating effective Alk5 deletion.

**SMC-specific Alk5 deletion induces aortic aneurysm formation with phenotypic penetrance differing between genders.** After tamoxifen induction, all Alk5\(^{ik0}\) males displayed evidence of aortic pathology. In an observation of 42 males, gross lesions such as dissection, intramural hematoma, and/or fusiform dilation were evident in all aortas at d28 (Fig. 2A), with an incidence of 74%, 26%, 11%, 32%, and zero for ATA, DTA1, DTA2, SRA, and AA segments, respectively. Histological evaluation with Masson’s staining revealed randomly distributed deep intimal/medial tears, dissections, medial thinning, and occasionally false lumen formation (Fig. 2B). In a sharp contrast, the Alk5\(^{ik0}\) females (n = 14), just like their Alk5\(^{f/f}\) controls, presented no evidence of gross pathology at d28 (Fig. 2A; see Supplemental Material, Fig. S1). The structure of each tunica layer appeared largely normal when evaluated with Masson’s staining (Fig. 2C, left), although breaks of elastic fibers were occasionally observed (Fig. 2C, right). Of the 42 Alk5\(^{ik0}\) males, 26% died prematurely from autopsy confirmed aortic rupture and dissection, whereas none of the Alk5\(^{f/f}\) females experienced early death from rupture (P = 0.02; Fig. 2D).

Ovariectomy blunts the sex-protective effect, whereas orchiectomy brings minimal change to Alk5\(^{ik0}\) aortas. To explore the role of endogenous sex hormones on the development of aortic aneurysm, we performed ovariectomy and orchiectomy in adult Alk5\(^{f/f}\)/Myh11-CreER\(^{+/0}\) mice to lower the level of sex hormones and evaluated their aortic pathology 28 days after Alk5 deletion. Ovariectomy was performed in seven males with 19 noncastrated littersmates included as controls. As expected, all noncastrated Alk5\(^{ik0}\) controls displayed aortic lesions with an incidence at each anatomic location similar to that obtained in our initial observation (Fig. 3A). After ovariectomy, although ATA and SRA remained the vulnerable regions, a trend of increase in lesion incidence was noted for DTA1 and DTA2 (Fig. 3A). However, both castrated and noncastrated animals experienced a similar rate of aortic rupture during the 28-day observation period (Table 1). Severe aneurysmal degeneration with features of deep intimal/medial tears and intramural dissections remained evident with no notable differences between castrated and noncastrated groups under microscopic evaluation (Fig. 3B and C).

Ovariectomy was performed in 15 Alk5\(^{f/f}\)/Myh11-CreER\(^{+/0}\) females, with 10 age-matched but not operated (non-Ovx) Alk5\(^{f/f}\)/Myh11-CreER\(^{+/0}\) females included as controls. After tamoxifen induction, aortic pathology was not detected in the controls under gross evaluation (Fig. 3D). In contrast with the relatively normal aortic structure presented by the non-operated controls, gross lesions were evident in ATA of eight

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**Fig. 1.** The inducible Cre-loxP system driven by Myh11 promoter promotes homogenous Alk5 deletion in smooth muscle cells (SMCs). A: position of the primers applied for screening animals carrying floxed Alk5 (β and γ) and examining the recombiant excision of Alk5 exon 3 (α and γ). B: genotyping results of primary SMCs isolated from Alk5\(^{ik0}\), Alk5\(^{f/f}\), and wild-type (WT) aortas. Note the presence of null (iko), floxed (ff), and WT Alk5 alleles. C: Western blotting assay for Alk5 protein in SMCs with indicated genotypes. Arrow points to the position of WT Alk5 receptor. Note the absence of Alk5 protein in Alk5\(^{ik0}\) SMCs.
Unlike the surface of the lesions (Fig. 3I) and aggregation of platelets and white blood cells on luminal exposure of the subendothelial structure to the bloodstream (Fig. 3I). Scanning electronic microscopy revealed blood flow (Fig. 3I). -test). Areas stained dark blue by Evans Blue (Fig. 3I), noted in Ovx females compared with the non-Ovx controls. Overall, Ovx females displayed significantly more elastic fiber breaks than non-Ovx controls (Fig. 3G, P < 0.001, unpaired t-test). Areas stained dark blue by Evans Blue (Fig. 3H) corresponded to intimal/medial tears with depth varying from superficial intima to full thickness of media (Fig. 3I). All tears were found consistently oriented parallel to the direction of blood flow (Fig. 3I). Scanning electronic microscopy revealed exposure of the subendothelial structure to the bloodstream and aggregation of platelets and white blood cells on luminal surface of the lesions (Fig. 3I). Unlike the Alk5f/f males where aortic lesions were noted in various anatomic sites proximal to the renal arteries, the Ovx females presented gross lesions only in ATA segment. In locations other than ATA, lesions were not detected in all Ovx females except one that displayed severe dissection spreading from the ATA all the way down to the SRA region.

(53%) ovariectomized females (Table 1), evidenced by dilation, intramural dissections, and/or focalized areas of Evans Blue extravasation (Fig. 3D). Histological features of these gross lesions were characterized by intramural hematoma, intimal/medial tears, and adventitial thickening and inflammation (Fig. 3E). In ATAs that appeared grossly normal under surgical evaluation, elastic fiber breaks were more frequently noted in Ovx females than in the non-Ovx controls. Overall, Ovx females displayed significantly more elastic fiber breaks than non-Ovx controls (Fig. 3G, P < 0.001, unpaired t-test). Areas stained dark blue by Evans Blue (Fig. 3H) corresponded to intimal/medial tears with depth varying from superficial intima to full thickness of media (Fig. 3I). All tears were found consistently oriented parallel to the direction of blood flow (Fig. 3I). Scanning electronic microscopy revealed exposure of the subendothelial structure to the bloodstream and aggregation of platelets and white blood cells on luminal surface of the lesions (Fig. 3I). Unlike the Alk5f/f males where aortic lesions were noted in various anatomic sites proximal to the renal arteries, the Ovx females presented gross lesions only in ATA segment. In locations other than ATA, lesions were not detected in all Ovx females except one that displayed severe dissection spreading from the ATA all the way down to the SRA region.

NE-induced hypertension breaks the protective effects of female sex to structural degeneration of Alk5f/f aortas. In Alk5f/f-HTN females, infusion of NE increased systolic blood pressure by a mean of 30 points (P < 0.001, paired t-test) from a baseline of 94 ± 2.26 (mmHg) to 124 ± 3.57 (mmHg). In the Alk5f/f-HTN controls, there was an increase of systolic blood pressure by a mean of 36 points (P < 0.001, paired t-test) from a baseline of 85 ± 3.15 to 121 ± 2.26. The difference in the elevation of the systolic blood pressure was insignificant between Alk5f/f-HTN and Alk5f/f-HTN groups (P = 0.35, unpaired t-test). Untreated Alk5f/f-HTN mice had no significant change in blood pressure over the 4-wk observation period. Systolic blood pressure of these mice averaged 103 ± 4.68 and 100 ± 4.69 mmHg at time points before Alk5 deletion and the day of tissue collection (P = 0.77, paired t-test), respectively.

The Alk5f/f-HTN females exhibited both gross and microscopic evidence of aortic aneurysm and dissection, whereas both Alk5f/f-HTN and Alk5f/f-HTN controls presented grossly normal aortic structure (Fig. 4A). In Alk5f/f-HTN females, aortic lesions were found along the aortas (Fig. 4, A and B) with an incidence similar to that of Alk5f/f males at each anatomic location. Aortic pathology was not detected in AA of all animals with and without hypertension. By the Alk5f/f female being made hypertensive, the penetrance of aortic aneurysm and dissection increased from zero to 59% with two
premature deaths (Table 2). When compared with the Alk5\(^{+/+}\) HTN and Alk5\(^{-/-}\)NTN controls where the aortic structure remained relatively normal, the Alk5\(^{+/+}\)HTN females displayed typical aortic aneurysm degeneration, characterized by dilation, intramural dissection, intramural hematoma, and adventitial fibrosis/remodeling (Fig. 4B).

The Alk5\(^{+/+}\)HTN females demonstrated a significant aortic dilation. Ultrasound imaging uncovered that the dilation in thorax often extended from aortic root all the way down to aortic arch. In abdomen, it was exclusively limited to the region above the renal arteries (Fig. 5A). The cross-sectional area was measured 1.63 ± 0.05 (mm) and 0.55 ± 0.02 (mm) before NE infusion and 2.16 ± 0.04 (mm) and 1.13 ± 0.17 (mm) 28 days after NE infusion for ATA and SRA, respectively (Fig. 5B). On the other hand, the aortic size remained unchanged in Alk5\(^{+/+}\)HTN and Alk5\(^{-/-}\)NTN females throughout the study period, as reflected by similar ultrasound measurements at locations of ATA and SRA (Fig. 5B). Overall, Alk5\(^{+/+}\)HTN females experienced significant aortic dilation at both ATA (P < 0.001) and SRA (P < 0.001) segments over the 28-day period. Such aortic dilation increased the cross-sectional area of Alk5\(^{+/+}\)HTN aortas and made it significantly larger than Alk5\(^{-/-}\)NTN and Alk5\(^{+/+}\)HTN controls when measured at both ATA (P < 0.001) and SRA (P < 0.001) locations.

Abolishment of the female-sex protective effects by hypertension is associated with an early activation of ERK signaling. Activity of key components of the canonical TGF-\(\beta\) signaling pathway, noncanonical TGF-\(\beta\) signaling pathway, and inflammatory pathways was measured by Western blot analysis on protein harvested from fresh aortic tissue. In normotensive males, Alk5\(^{+/+}\), which leads to 100% phenotypic penetrance of aortic aneurysm, elevated early ERK signaling to over three-folds of the baseline levels compared with the Alk5\(^{+/+}\) male controls (Fig. 6A). In females it was noted that both hyperten-

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Table 1. Incidence of aortic lesions in castrated and noncastrated Alk5\(^{-/-}\)mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Aortic Rupture (%)</th>
<th>Gross Pathology</th>
<th>Phenotypic Penetrence, %</th>
</tr>
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<tbody>
<tr>
<td>Alk5(^{-/-}) female</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Non-ovariectomy</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>15</td>
<td>0</td>
<td>8</td>
<td>53*</td>
</tr>
<tr>
<td>Alk5(^{-/-}) male</td>
<td>19</td>
<td>4 (21)</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>Alk5(^{-/-}) male orchiectomy</td>
<td>7</td>
<td>1 (14)</td>
<td>7</td>
<td>100</td>
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Ovariectomy vs. non-ovariectomy: *P = 0.008, by Fisher’s Exact Test.

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Fig. 3. Endogenous female, but not male, sex hormones modulate the phenotypic expression of Alk5\(^{-/-}\) aortas. A: incidence of aortic lesions at the indicated anatomic locations in castrated and noncastrated Alk5\(^{-/-}\) males. *P = 0.012 by Fisher’s exact test for castrated vs. noncastrated animals. B and C: histological evaluation of aortic pathology at d28 with Masson’s staining. B and C represent typical pathology observed in ATAs of castrated and noncastrated Alk5\(^{-/-}\) aortas, respectively. Arrows point to full thickness of medial tears under both conditions. Scale bars, 200 \(\mu\)m. D: gross specimen representative of ovariectomy (Ovx) and non-Ovx Alk5\(^{-/-}\) females at d28. Arrow points to a grossly evident lesion. E and F: Masson’s (top row) and Movat’s (bottom row) staining of ATAs from Ovx (E) and non-Ovx (F) Alk5\(^{-/-}\) females. Scale bars, 200 \(\mu\)m. G: elastic fiber breaks (EFBs) per high power field (hpf). *P < 0.001 by unpaired t-test. H: gross specimen of an Ovx Alk5\(^{-/-}\) female at d28. Note the dark blue dots in ATA, indicating focalized endothelial destruction that leads to Evans blue extravasation. I: en-face evaluation of luminal surface of the aortic specimen shown in H. Arrow denotes the direction of blood flow. Note the tear highlighted by Evans blue staining. Scale bars, 250 \(\mu\)m. J: scanning electronic microscopy of lesions detected by Evans blue staining. A few white blood cells (arrow head) adhered to luminal surface of the tear. Scale bars, 15 \(\mu\)m. DTA, descending thoracic aorta.
sion and Alk5 deficiency was required to cause an early ERK activation and subsequent development of pathology. Both Alk5<sup>5ko</sup>-HTN and Alk5<sup>iko</sup>-NTN aortas displayed levels of phosphorylated ERK similar to Alk5<sup>5f/f</sup> controls (Fig. 6, A and B), indicating hypertension or Alk5<sup>5ko</sup> alone is not sufficient to promote ERK signaling in the aortic wall. In contrast with the single factor scenario, aortas exposed to both hypertension and Alk5<sup>5ko</sup>, namely the Alk5<sup>5ko</sup>-HTN female group, had increased production of p-ERK. The differences are statistically significant compared with that of both Alk5<sup>5f/f</sup>-HTN (P = 0.01) and Alk5<sup>iko</sup>-NTN (P < 0.05) females (Fig. 6, C and D). Therefore, hypertension, when acting in conjunction with Alk5 deficiency (Alk5<sup>5ko</sup>), intensifies ERK signaling in the aortic wall. Importantly, in the female group, it was only the mice with combined effects of hypertension and Alk5 deficiency that developed clinically significant aortic pathology.

Unlike its stimulatory effect on ERK signaling activation, the joined treatment with both hypertension and Alk5 deletion had insignificant impact on the production of p-SMAD2 or p-p65. When compared with those subject to NE-induced hypertension (e.g., Alk5<sup>5f/f</sup>-HTN) or Alk5 deletion (e.g., Alk5<sup>5ko</sup>-NTN), aortas insulted by both (e.g., Alk5<sup>5ko</sup>-HTN) displayed a similar level of p-SMAD2 (Fig. 7, A and B) and p-p65 (Fig. 7, C and D).

**DISCUSSION**

Sexual dimorphism in the development of cardiovascular disease and aortic aneurysm in particular is a well-known phenomenon with males being more susceptible than females. In the present study we found that removing Alk5 in SMCs of adult mice leads to aortic aneurysm and dissection in a dichotomous manner between genders, with all male mice displaying gross evidence of disease by 28 days and all females being unaffected. This model produces an extreme example of the sexual dimorphism seen in human aortic aneurysm development. In addition, we provided evidence suggesting that this sexual dimorphism is, at least in part, the result of protective effect of ovary-derived sex hormones to the structural degeneration triggered by SMC specific Alk5 deletion. Hypertension, one of the modifiable risk factors for aortic aneurysm formation, can break this female sex protective effect and is coupled with enhanced activation of ERK signaling pathway in the aortic wall.

Several theories have been postulated to explain sexual dimorphism in the development of aortic aneurysm. Most of these theories center on either the protective effects of estrogens or the deleterious effects of testosterone. The most prominent hypotheses include the effect of estrogens on inflammatory responses (39), lipid profile (7), and structural homeostasis of the blood vessels themselves (29). Interestingly, in our model, ovariectomy provoked the phenotypic penetrance of aortic aneurysm in Alk5<sup>5ko</sup> females, characteristic of elastin fragmentation, intimal/medial tears and defects, and intramural dissection. However, orchietomy could not ameliorate the aortic pathology; nor did it decrease the incidence of aortic rupture. These results suggest that endogenous female sex hormones modulate the phenotype of Alk5<sup>5ko</sup> aortas to a favorable form, whereas endogenous male sex hormones have modest impact on the phenotypic expression of Alk5<sup>5ko</sup> aortas.

Sexual dimorphism has been previously studied in various animal models of aortic aneurysm with results differing among
studies using different experimental models. In an angiotensin II infusion model, Zhang and colleagues (17, 46) demonstrated that neonatal exposure of female mice to testosterone increased angiotensin receptor expression in the aorta and thus severity of aortic pathology (17, 46), suggesting that it is the harmful effect of testosterone that accounts for sexual dimorphism. For adult animals, reports by the same group demonstrated that ovariectomy had no effect on aortic pathology; however, orchietomy of male mice was protective (18, 45), further implicating testosterone. In an elastase perfusion model of aortic aneurysm, Ailawadi et al. (1) demonstrated that estrogen-treated male rats had decreased inflammatory infiltration and decreased MMP-9 production, translating to less severe aortic pathology. In a separate study from the same group, they showed no changes in development of aortic disease in females treated with testosterone or undergoing ovariectomy but attenuated aortic dilation in males treated with estrogens (10), thus implicating testosterone. In an elastase perfusion model of aortic aneurysm, Ailawadi et al. (1) demonstrated that estrogen-treated male rats had decreased inflammatory infiltration and decreased MMP-9 production, translating to less severe aortic pathology. In a separate study from the same group, they showed no changes in development of aortic disease in females treated with testosterone or undergoing ovariectomy but attenuated aortic dilation in males treated with estrogens (10), thus implicating the protective effects of estrogens. More recently they have shown that decreasing endogenous estrogens through aromatase knockout in mice diminishes the protective effects of the female sex on development of aortic disease (21).

Another group had similar findings in an angiotensin II infusion model, in which mice treated with estrogens demonstrated decreased activation of NF-κB inflammatory pathways and decreased severity of aortic disease (27).

Our results obtained with ovariectomized females support the concept that female sex hormones have protective effects on aortic aneurysm formation. However, data generated with castrated males hold potential conflict with studies that have demonstrated deleterious effects of male hormones on aortic aneurysm formation. In our study, aortic aneurysm was induced via smooth muscle cell-specific \( \text{Alk5} \) deletion, which is different from the models that others have used to study sex dimorphism of aortic aneurysm formation. It appears that model difference is a dominant factor that dictates a differential modulatory effect for sex hormones on the phenotypic expression of the insulted aortas. For example, in the angiotensin infusion model, the deleterious effect of testosterone is linked to increased expression of the AT1a receptor in the aortic wall (18, 35), whereas in models created by elastase perfusion, estrogen protect aortic wall from degeneration through inhibiting inflammatory cell infiltration and MMP production (10, 21). In the model used by our current study, how sex hormones modulate the biological outcome of smooth muscle cell-specific \( \text{Alk5} \) deletion remains to be defined.

The widely accepted TGF-\( \beta \) signaling dogma asserts that the type II receptor \( \text{Tgfbr2} \) signals in tandem with \( \text{Alk5} \) to relay TGF-\( \beta \) signal from cell surface to nucleus (28). According to this theory, mechanisms responsible for the phenotype of \( \text{Alk5} \) deficiency would mirror that of \( \text{Tgfbr2} \) deficiency. A recent study proposed that aortic pathology seen in \( \text{Tgfbr2} \) deletion is caused by the consequent defect of cell contractile apparatus and the subsequent activation of ERK signaling (24). Although these cellular and molecular events may be modulated by sex hormones in \( \text{Alk5}^{\text{iko}} \) animals, further studies remain wanted to provide direct evidence to prove this hypothesis.

Fig. 5. Hypertension breaks the resistance of \( \text{Alk5}^{\text{iko}} \) females to aortic dilation. A: ultrasound imaging of the ATA and SRA at time points before and 28 days after NE-induced hypertension. SRAs are traced with white circles. B: summarization of ultrasound measurements. Comparisons were performed with 2-way repeated measures ANOVA analysis. \( \text{Alk5}^{\text{iko}}\)-HTN vs. \( \text{Alk5}^{\text{f/f}}\)-HTN or \( \text{Alk5}^{\text{iko}}\)-NTN: \( P < 0.001 \) for both ATA and SRA; aortic dilation: \( *P < 0.001 \) for both ATA and SRA of the \( \text{Alk5}^{\text{iko}}\)-HTN mice.
In our model, animals received tamoxifen to induce SMC specific Alk5 deletion. Tamoxifen is a selective estrogen receptor modulator that can act as either antagonist or agonist to estrogen, depending on types of organs. In an enzymatic destruction model, it has been shown that tamoxifen ameliorates the aneurysmal pathology via a mechanism similar to exogenous estrogen (15). Such an off-target effect of the tamoxifen seems not a dominant factor in our model since aortic pathology still occurred in ovariectomized females while absent in females with intact ovaries following tamoxifen administration. One limitation of our work is that we only manipulated sex hormone levels by removal of the gonads. Approaches such as aromatase deletion (21) and administration of nontransformable testosterone (18) may provide more specific insights into the role of male versus female hormones in modulating the aortic phenotype following SMC specific Alk5 deletion. An interesting hypothesis is that sex-specific expression of human disease may be more attributable to X and Y chromosome dosage effect between males and females as opposed to simply differing hormone levels (32). Mechanisms responsible for the dimorphic incidence of aortic aneurysm between genders may go beyond the biological effects of sex hormones.

When compared with the endogenous hormone levels, NE-induced hypertension acted as a more potent modulator to the development of aortic pathology in the Alk5-deficient females. With the introduction of systemic hypertension to Alk5<sup>k/o</sup> females, penetrance of clinically relevant disease increased from zero to 59% with a 10% rate of premature death from aortic dissection and/or rupture in 28 days. In the general population, the link between hypertension and aortic aneurysm has been controversial, with some reports asserting a strong association to the development of aneurysm (20, 42) and others suggesting the contrary or at the very most a weak link (2, 3, 43). However, it has been suggested by several studies that hypertension has a greater association with development of aortic aneurysm in females than in males (12, 38). In line with these clinical observations, we found that NE-induced hypertension blunted the protection that the female sex provides toward development of aortic disease. It is important to note that in our study hypertension or Alk5<sup>k/o</sup> alone was unable to cause aortic aneurysm. Only the combination of both promoted aneurysmal degeneration in aortic wall of female mice. These results have led us to an important “double hit” phenomenon in which both Alk5 deficiency and hypertension are required in the female mice to develop aortic pathology.

To further understand the biologic alterations that accompany hypertension-induced aortic disease in Alk5<sup>k/o</sup> female mice, we performed Western blot analysis on key components of the TGF-β signaling and inflammatory pathways on these animals before and after 2 wk of NE infusion. The 2-wk time-point was chosen to avoid confounding activity that was
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A

B

C

D

Fig. 7. Female aortas produce similar amount of p-SMAD2 and p-p65 under conditions with or without aortic aneurysm development. A and B: impact of Alk5^{f/f} and hypertension on SMAD2 phosphorylation. C and D: impact of Alk5^{f/f} and hypertension on phosphorylation of NF-κB subunit p65. For comparison purpose, samples of groups to be compared were run on the same gel. Blots shown in B at top are separated because of re-arrangement of groups during figure creation. P = not significant (NS), by unpaired t-tests.

A result secondary to the development of pathology as opposed to leading to pathology, since this is typically before development of any gross pathology in NE-infused Alk5^{f/f} females. An earlier time point (e.g., d5) was chosen in experiments with Alk5^{f/f} males due to their earlier onset of aortic pathology (Fig. 2D). Our results demonstrate a consistent relationship between levels of early ERK signal and the aortic phenotype in both male and hypertensive/Alk5-deficient female mice, with enhanced early ERK signal correlating to the occurrence of aortic aneurysm. In females, which demonstrated female hormone protective effects, hypertension was able to break such sex-protective effects; and meanwhile, it coupled with an elevated early ERK signaling intensity. The ERK pathway can be activated by several growth factors and regulates a wide variety of cellular events including cell growth, differentiation, and survival (36). In the context of TGF-β signaling disorders, activation of ERK signaling pathway has been demonstrated to cause aortic aneurysm formation (14, 19). Our results, together with these reports, suggest a role for ERK activation in exaggerating the susceptibility of females to Alk5^{f/f}. NE stimulation has previously been shown to promote ERK phosphorylation in cell cultures (11). However, our data showed similar level of p-ERK in Alk5^{f/f}-NTN and Alk5^{f/f}-HTN female mice, indicating that the NE dose applied in our study is not sufficient enough to activate ERK signaling in vivo while increasing the blood pressure. Interestingly, our data show that aortas produced similar amount of p-SMAD2 under conditions with or without SMC Alk5. A well-known phenomenon of TGF-β signaling disorders is the “TGF-β paradox,” where loss-of-function mutation in TGF-β signaling components is coupled with enhanced SMAD2 phosphorylation (4, 14, 26). This paradox, however, seemingly did not occur in Alk5^{f/f} females. Such a disparity in the canonical TGF-β signaling activity indicates that aortic aneurysms forms via mechanisms differing between these conditions. Hypertension imposes hemodynamic stress that activates NF-κB pathway, elaborating expression of proinflammatory mediators such as VCAM-1 and ICAM-1 in the aortic wall (16). Shiraya et al. (37) have previously shown that hypertension-induced upregulation of NF-κB is associated with worsening pathology of aortic aneurysm induced by elastase perfusion (37). However, in our model, development of aortic phenotype by NE-induced hypertension is not associated with changes of NF-κB activity, indicating that the deleterious effect of hypertension in Alk5^{f/f} females is not maneuvered via regulating NF-κB signaling pathway.

In conclusion, we have shown that aortic aneurysm induced by SMC-specific Alk5 deletion exhibits dimorphic incidence between genders, with much lower phenotypic penetrance in females. This sexual dimorphism is due more to the protective effect of female sex hormones than the deleterious effect of male sex hormones on structural homeostasis of the aortic wall. The Alk5^{f/f} females, although not presenting clinical manifestations as their male counterparts, are vulnerable to common risk factors, particularly hypertension. An early ERK signaling is intensified only in the groups that develop evident aortic pathology, suggesting that ERK may serve as the biologic mediator to hypertension-induced aortic aneurysm in female mice.
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