Hypoxia-Inducible Factor-1α in Smooth Muscle Cells Protects Against Aortic Aneurysms—Brief Report

Masaki Imanishi, Yoichi Chiba, Noriko Tomita, Shinji Matsunaga, Toshitaka Nakagawa, Masaki Ueno, Kazuhiro Yamamoto, Toshiaki Tamaki, Shuhei Tomita

Objective—The purpose of this study was to determine the role of smooth muscle cell–derived hypoxia-inducible factor-1α (Hif-1α) in the pathogenesis of aortic aneurysms.

Approach and Results—Control mice and smooth muscle cell–specific hypoxia-inducible factor-1α–deficient mice were infused with β-aminopropionitrile for 2 weeks and angiotensin II for 6 weeks to induce aortic aneurysm formation. Mutant mice experienced increased levels of aneurysm formation of the thoracic or abdominal aorta with more severe elastin disruption, compared with control mice. Smooth muscle cell–specific hypoxia-inducible factor-1α deficiency did not affect matrix metalloproteinase-2 activity; however, the activity of lysyl oxidase and the levels of tropoelastin mRNA in the angiotensin II– and β-aminopropionitrile–treated aortae, associated with elastin fiber formation, were suppressed. Furthermore, we observed reduced volumes of mature cross-linked elastin in the thoracoabdominal aorta after treatment with angiogenin II and β-aminopropionitrile.

Conclusions—Deficiency of smooth muscle cell–derived hypoxia-inducible factor-1α augments aortic aneurysms, accompanied by disruption of elastin fiber formation, but not changes of elastin fiber degradation. (Arterioscler Thromb Vase Biol. 2016;36:2158-2162. DOI: 10.1161/ATVBAHA.116.307784.)

Key Words: aneurysm ■ angiotensin II ■ elastin ■ extracellular matrix ■ hypoxia ■ smooth muscle ■ vascular remodeling

Aortic aneurysmal lesions exhibit fragmentation and disruption of elastic laminae, inflammatory cell infiltration, and loss of vascular smooth muscle cells (VSMCs) with aortic wall expansion.1,2 Elastic lamina plays key roles in maintaining the elasticity of aortae; therefore, the disruption of elastin fiber is a major cause of aortic aneurysm formation. Degradation of elastin fiber, one of the extracellular matrix, is mainly caused by induction of matrix metalloproteinase (MMP)-2 or MMP-9 from VSMCs and inflammatory cells.3 However, dysfunction of elastin fiber maturation causes matrix failure, contributing significantly to aneurysm formation.

See accompanying editorial on page 2138

Tropoelastin, an uncross-linked soluble form of elastin, is a major component of elastin fiber. Elastin haploinsufficiency reduces the volume of the elastin cross-linking structure desmosine and enhances elastin fiber fragmentation.4 Fibrillin-1 is a major component of the microfibrils that form a sheath surrounding elastin; mutations in the fibrillin-1 gene cause Marfan syndrome, which is characterized by matrix failure, and links to aneurysm formation.5 Lysyl oxidase (LOX) is a crucial enzyme involved in elastin cross-linking and elastin coacervation; inactivation of the LOX gene leads to aortic aneurysms.6 Additionally, studies involving the administration of the LOX inhibitor β-aminopropionitrile (BAPN) to adult rat or mouse models of aortic aneurysm7,8 indicate that the mechanisms underlying elastin fiber formation, especially elastin cross-linking, protect against aortic wall expansion in mature aortae.

Our previous report showed that smooth muscle cell–specific hypoxia-inducible factor-1α (Hif-1α) deficiency suppressed angiotensin II (Ang II)–induced medial thickening by suppressing VSMC hypertrophy and vascular fibrosis via decreased aortal mRNA expression of extracellular matrix–related genes, such as collagen I.9 However, it remains unclear whether suppression of vascular remodeling and extracellular matrix metabolism is a protective compensatory reaction against vascular vulnerability, which occurs in response to chronic exogenous stimuli. Here, we investigated the role of smooth muscle cell–derived Hif-1α in pharmacologically induced aortic aneurysms, using a mouse model of vascular vulnerability. Our results indicate that deficiency of smooth muscle cell–derived Hif-1α augments aortic aneurysms, accompanied by disruption of elastin fiber formation, but not changes of elastin fiber degradation.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

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2158
Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>Ang II</td>
<td>angiotensin II</td>
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<tr>
<td>BAPN</td>
<td>β-aminopropionitrile</td>
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<tr>
<td>Hif-1α</td>
<td>hypoxia-inducible factor-1α</td>
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<tr>
<td>LOX</td>
<td>lysyl oxidase</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>SMKO</td>
<td>smooth muscle cell–specific Hif-1α–deficient</td>
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<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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Results

Chronic administration of Ang II and BAPN caused higher incidence of aortic aneurysm (thoracic aortic aneurysm or abdominal aortic aneurysm) formation in smooth muscle cell–specific Hif-1α–deficient mice (SMKO mice) than in control mice; 13% of SMKO mice died as a result of ruptured aortic aneurysms after Ang II and BAPN administration, whereas the control mice did not die during 6-week administration period (Figure 1A). Thoracic aortic aneurysms were formed in the ascending aorta, aortic arch, or descending aorta, whereas abdominal aortic aneurysms were consistently formed above the right renal artery in both control mice and SMKO mice (Figure 1B; Figure I in the online-only Data Supplement). Five of the 9 thoracic aortic aneurysms in SMKO mice, which occurred after Ang II and BAPN administration, formed in the descending aortic region, 3 in the aortic arch and 1 in the ascending aortic region. Two of the 4 thoracic aortic aneurysms in the control mice formed in the descending aortic region, the third in the aortic arch and the fourth in the ascending aortic region. Elastic van Gieson staining and Azan staining showed that Ang II and BAPN administration caused aortic expansion, elastin breaks, and fibrosis in aneurysmal areas in both groups (Figure 1C; Figure II and Figure III in the online-only Data Supplement). The outer diameter of the thoracic aortae was significantly larger in SMKO mice than that in control mice (Figure IV in the online-only Data Supplement). Visualization of elastic van Gieson staining in high-power fields, as well as electron micrographs of abdominal aortae, indicated that a higher level of fragmentation and disruption of elastic laminae occurred in SMKO mice, after Ang II and BAPN administration, than in control mice (Figure 1D). Electron microscopy additionally revealed the loss of VSMCs and cytoplasmic vacuolization in these cells in SMKO mice after the administration of Ang II and BAPN. In the present study, Hif-1α mRNA expression in the aorta was induced by Ang II and BAPN administration, and this expression was reduced in SMKO mice (Figure V in the online-only Data Supplement). In addition, smooth muscle cell–specific Hif-1α deficiency did not affect blood pressure elevation (Figure VI in the online-only Data Supplement), suggesting that smooth muscle cell–derived Hif-1α plays a protective role against aortic aneurysm formation via mechanisms that are independent of changes in blood pressure.

The administration of Ang II and BAPN elicited elastin breaks not only in thoracic aorta and abdominal aorta but also in thoracoabdominal aorta, although aortic aneurysms were not formed in this region (Figure 2A). This observation supported the disruption of elastin fiber as a critical contributor to aortic expansion when blood pressure is elevated. The degree of disruption of elastin fiber in the thoracoabdominal aorta in SMKO mice, after Ang II and BAPN treatment, was greater than that in control mice subjected to the same treatment (Figure 2A; Figure VII in the online-only Data Supplement). Additionally, the volume of total elastin (insoluble cross-linked elastin and soluble uncross-linked elastin) in the aortae of SMKO mice treated with Ang II and BAPN was lower than in those of control mice undergoing the same treatment (Figure 2B). These results indicate that Ang II–induced and BAPN-induced disruption of elastin fiber in the aortae of SMKO mice was more severe than that in control mice.

Gelatin zymography showed that 1 week after administration of Ang II and BAPN, there was no difference in MMP-2 activity in the aortae of control mice and SMKO mice (Figure VIII in the online-only Data Supplement). MMP-2 and MMP-9 mRNA expression was induced in the aortae of control mice but not in those of SMKO mice, 6 weeks after administration of Ang II and BAPN (Figure IX in the online-only Data Supplement). In addition, the mRNA expression of the macrophage marker, F4/80, was induced in the aortae of control mice but not in those of SMKO mice (Figure X in the online-only Data Supplement). On the contrary, tropoelastin mRNA expression in the aorta of SMKO mice treated with Ang II and BAPN was lower than that in control mice subjected to the same treatment (Figure 2C). In vitro cell culture analysis additionally showed that VSMCs from SMKO mice exhibited lower tropoelastin mRNA expression than those from control mice at day 21 (Figure XI in the online-only Data Supplement). Additionally, the activity of the enzyme required for tropoelastin cross-linking in the aorta (LOX) was lower in SMKO mice than that in control mice (Figure 2D). Inverted black-and-white micrographs of elastic van Gieson staining showed that the volume of elastin fiber, in the form of mature cross-linked elastin, measured in the aortic media of SMKO mice treated with Ang II and BAPN was lower than in those of control mice undergoing the same treatment (Figure 2E and 2F; Figure XII in the online-only Data Supplement). Although smooth muscle cell–specific Hif-1α deficiency affected the Ang II–induced and BAPN-induced mRNA expression of LOX and LOXL-1, other genes implicated in elastin fiber formation were not affected (Figure XIII in the online-only Data Supplement). Additionally, LOX mRNA expression in the aortae of SMKO mice was lower than in those of control mice, even 1 week after administration (Figure XIV in the online-only Data Supplement). These results suggest that deficiency of smooth muscle cell–derived Hif-1α augments aortic aneurysms, accompanied by disruption of elastin fiber formation, but not changes of elastin fiber degradation.

Discussion

The major finding of this study was that smooth muscle cell–specific Hif-1α deficiency elicited an increase in the formation of pharmacologically induced aortic aneurysms by suppressing elastin expression and cross-linking. We first demonstrated that Ang II–induced and BAPN-induced disruption of elastin fiber formation in the aortae of SMKO...
mice was more severe than in the aortae of control mice; however, no difference in MMP activity was observed between the groups 1 week after treatment. In addition, Ang II- and BAPN-induced mRNA expression of MMP-2 and MMP-9 in the aortae was suppressed in SMKO mice. This result is consistent with those of previous studies showing that MMP-2 expression is regulated by Hif-1α. The expression of tissue inhibitors of metalloproteinase-3, which suppresses activity of MMPs, was unchanged in both groups (Figure IX in the online-only Data Supplement). In addition, there was no difference in the expression of inflammation-related genes between the groups after 6 weeks. These results suggest that deficiency of smooth muscle cell–derived Hif-1α augments aortic aneurysms, accompanied by disruption of elastin fiber formation, but not changes of elastin fiber degradation.

Figure 1. The effects of smooth muscle cell–specific hypoxia-inducible factor-1α (Hif-1α) deficiency in pharmacologically induced aortic aneurysm formation. A, Angiotensin II (Ang II)–induced (1000 ng/kg/min, 6 wk) and β-aminopropionitrile (BAPN)–induced (150 mg/kg/d, 2 wk) incidence of aneurysms in the thoracic or abdominal aorta was higher in smooth muscle cell–specific Hif-1α–deficient (SMKO) mice (n=31) than in control mice (n=29). Aneurysms were defined as a localized dilation of the aortic wall with maximal outside diameter >50% of its adjacent intact portion. B, Arrow heads indicate thoracic or abdominal aortic aneurysms, and white arrows indicate aortic rupture regions. Scale bar=1 mm. (23–28 images were scanned in each group.) C, Elastic van Gieson (EVG) staining of the section of the thoracic and abdominal aorta showed aortic expansion (arrow heads) and elastin breaks (black arrows) induced by Ang II and BAPN. Ruptured aortic sections show thinning adventitia in SMKO mice. Scale bar=0.5 mm. (3–6 images were scanned in sham group, and 13–17 images were scanned in Ang II+BAPN group.) D, (a and b) High-power field of the EVG stain of the abdominal aortic section showing more extensive fragmentation and disruption of the elastic lamina in SMKO mice treated with Ang II and BAPN relative to that observed in control mice under the same treatment. Scale bar=0.1 mm. (c and d) Electron micrographs of the medial area showing elastic fragmentation and rupture (arrow heads), the loss of VSMCs, and cytoplasmic vacuolization in VSMCs (black arrow) in SMKO mice treated with Ang II and BAPN. Scale bar=10 µm. (3–4 electron micrographs were scanned in each group.) *P<0.05 compared with the control. BT indicates brachiocephalic trunk; CCA, common carotid artery; H, heart; Lt, left; RA, renal artery; Rt, right; and SA, subclavian artery.
Tropoelastin, which is the major source of elastin fiber, binds to elastin-binding protein on the cell surface of VSMCs. Elastin-binding protein stabilizes tropoelastin and transports tropoelastin aggregates to adjacent microfibrils involved in elastin fiber assembly. LOX catalyzes elastin cross-linking and coacervation to produce mature elastin fiber. Our results show that smooth muscle cell–specific Hif-1α deficiency suppressed tropoelastin and LOX mRNA expression in the aortae of mice treated with Ang II and BAPN and reduced LOX activity in the aorta but did not affect the expression of other genes associated with elastin fiber formation. These data indicate that smooth muscle cell–derived Hif-1α is essential for tropoelastin expression and elastin fiber formation, which is promoted by elastin cross-linking, in the medial area (Figure XVIII in the online-only Data Supplement). These results were supported by the observation of reduction in the level of mature cross-linked elastin, even in the thoracoabdominal aortic region, which did not experience aortic aneurysm formation, in SMKO mice treated with Ang II and BAPN relative to control mice (Figure 2E and 2F; Figure XII in the online-only Data Supplement). Aortic aneurysm formation had not been observed in SMKO mice that were not subjected to Ang II and BAPN treatment, despite decreased levels of LOX activity in the aorta. Untreated SMKO mice exhibited high level of tropoelastin mRNA expression as a LOX substrate, indicating that its high tropoelastin expression may be sufficient to maintain normal elastin cross-linking activity, despite the low levels of LOX enzyme activity. The high levels of tropoelastin mRNA expression in the aortae of SMKO mice compared with those observed in the control mice may be attributable to a compensatory reaction to the lower levels of LOX enzyme activity. Furthermore, this could explain the discrepancy between the levels of tropoelastin mRNA expression in untreated aortae and untreated VSMCs of SMKO mice. Several previous studies suggested that the disruption of other crucial matrix proteins, such as collagen or fibronectin, is also linked to aortic aneurysm formation. The induction of collagen I mRNA expression in the aortae of control mice, but not in those of SMKO mice, suggests that lower levels of collagen I expression might also contribute to aortic aneurysm formation in the latter. Fibronectin expression did not affect aortic aneurysm formation in SMKO mice (Figure XV in the online-only Data Supplement) in the present study. We also found that the lower α-smooth muscle actin (SMA)–positive vascular area in abdominal aortic aneurysmal sections of SMKO mice treated with Ang II and BAPN accompanied with greater incidence of aortic aneurysm, relative to the control (Figure XVI in the online-only Data Supplement). Because VSMC apoptosis has previously been shown to be a factor in aneurysm formation by multiple groups, this result may reflect the reduced number of VSMCs in aneurysmal regions in SMKO mice. However, we could not confirm so many apoptotic VSMCs in abdominal aortic aneurysmal regions in control mice and SMKO mice in this model (data not shown). We supposed that VSMC apoptosis also contributes to aneurysm formation, but it does not play a huge role in the exacerbation of aneurysm formation in SMKO mice. The result of α-SMA immunostaining is accorded with our previous report of suppressed Ang II–induced medial thickening and suppressed Ang II–induced VSMC hypertrophy in SMKO mice. We found cytoplasmic vacuolization in the VSMCs of aneurysmal regions in SMKO mice, but we could not elucidate whether that morphological change linked to a cause of aortic aneurysm formation or...
was an aortic structural abnormality accompanied with aortic aneurysm formation. The lack of difference in the expression of neovascularization-related genes mRNA expression between control mice and SMKO mice suggests that neovascularization did not contribute to aortic aneurysm formation in the latter (Figure XVII in the online-only Data Supplement).

Recently, several studies have suggested that hypoxic conditions and the induction of Hif-1α during aneurysms contribute to MMP activation,17 and Hif-1α contributes to arterial aneurysm formation via abnormal matrix metabolism involving MMP activation and inflammation.18 Our results of MMP mRNA expression in aortae of SMKO mice were consistent with those observed in these previous studies. However, our results directly suggest that smooth muscle cell-derived HIF-1α protects against aneurysm formation. Furthermore, we think that this protection is provided by tropoelastin expression and LOX expression and a potential therapeutic strategy for the prevention of aortic aneurysm formation. Furthermore, we think that this protection is provided by tropoelastin expression and LOX expression and a potential therapeutic strategy for the prevention of aortic aneurysm formation.

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Disclosures
None.

References
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SUPPLEMENTAL MATERIAL

Detailed Methods

Mouse model of pharmacologically induced aortic aneurysm
All experimental procedures conformed to the guidelines for animal experimentation administered by Tottori University, Tottori, Japan, and this investigation conformed to US National Institutes of Health guidelines (NIH Publication, 8th Edition, 2011). Smooth-muscle cell-specific Hif-1α-deficient mice (SMKO mice) and control mice were used, with the same genotype, as described previously.1 We used SM22α-Cre+/-;Hif-1αflox/flox mice as SMKO mice, and SM22α-Cre+/-;Hif-1α+/- mice as control mice. β-aminopropionitrile fumarate salt (BAPN; A3134; Sigma-Aldrich, St. Louis, MO, USA) at 150 mg/kg/day for 2 weeks and angiotensin II (Ang II; A9525; Sigma-Aldrich) at 1000 ng/kg/min for 6 weeks were continuously infused via subcutaneously implanted osmotic minipumps (Alzet model 2002, Alzet model 2006, respectively; Alza Corp., Mountain View, CA, USA) into 13–15-week-old male control mice (n = 29) and SMKO mice (n = 31) to induce aortic aneurysms pharmacologically.2 The mice were anesthetized with an intraperitoneal injection of both pentobarbital sodium (Somnopentyl; Kyoritsu Seiyaku Corporation, Tokyo, Japan; dosage: 50 mg/kg) and xylazine (Selactar; Bayer, Osaka, Japan; dosage: 10 mg/kg), and 1% lidocaine hydrochloride (Xylocaine; AstraZeneca, London, UK) was injected subcutaneously for local analgesia. The absence of pedal withdrawal reflex was checked frequently to ensure adequacy of anesthesia, with plans to use more if the mice exhibited any signs of pain during the operation. Systolic blood pressure was measured noninvasively by a computerized tail-cuff system (BP-98A; Softron, Tokyo, Japan; n = 12–16). After a 1-week or a 6-week administration, the mice were euthanized with an intraperitoneal injection of pentobarbital sodium (150 mg/kg) and the aortae were isolated for further analysis at the end of the experiments (quantitative real-time PCR, histomorphology, and immunofluorescence).

Definition of aortic aneurysm
Thoracic and abdominal aortic aneurysms were defined as previously described.2 Thoracic and abdominal aortic aneurysms were defined as a localized dilation of the aortic wall with maximal outside diameter greater than 50% of its adjacent intact portion of aorta. The outer diameter of the aortic wall was quantified using ImageJ v1.48 software (National Institute of Health, Bethesda, MD, USA; n=21-24).

Quantitative real-time PCR
After a 1-week or a 6-week treatment, the mice were euthanized and aortae were resected and placed in 0.8 mL of ISOGEN (NIPPON GENE, Tokyo, Japan). Total RNA was extracted and cDNA was prepared as previously described.1 The PCR mixture contained cDNA equal to 6.25 ng or 12.5 ng total RNA, forward and reverse primer mix, and THUNDERBIRD SYBR qPCR Mix (QPS-201; TOYOBO, Osaka, Japan). PCR reactions were performed with ViiA7 (Applied Biosystems, Foster City, CA, USA). Amplification included one stage of 1 min at 95°C, followed by 45 cycles of a two-step loop: 15 s at 95°C and 35 s at 60°C. Mouse β-actin was used to normalize sample amplification and 10 to 11 independent samples in each group were used for real-time PCR. The mouse-specific primers used in this study are listed on Table 1.

Total RNA was extracted from vascular smooth muscle cells (VSMCs) isolated from mouse aortae by the modified acid-guanidinium-phenol-chloroform method with ISOGEN. The mRNA expression levels in VSMCs were measured in the same way as was done for samples from the mouse aortae. Three independent samples in each group were used for real-time PCR.
**Elastic-van Gieson (EVG) staining**
Thoracic aorta, abdominal aorta, and thoracoabdominal aorta (Figure XIX in the online-only Data Supplement) were excised and placed into 4% neutral-buffered formalin. After fixation, tissues were paraffin embedded. For morphometric analysis, transverse sections (2–3 μm thickness) were stained by EVG staining (Elastica van Gieson staining kit; MERCK, Kenilworth, NJ, USA). Aortae were sectioned at the same height as the diaphragm for thoracoabdominal aorta section, between left common carotid artery and left subclavian artery for thoracic aorta section, and above the right renal artery for abdominal aorta section. To assess the elastin breaks, disruption of elastic lamina or branching elastic lamina within the medial area of entire thoracoabdominal aortic sections were counted.

**Mature cross-linked elastin volume**
EVG staining of thoracoabdominal aorta sections was also used to assess the volume of mature cross-linked elastin, elastic lamina. Tissue imaging was performed using a microscope (Leica DMD108; Leica MICROSYSTEMS, Wetzlar, Germany). To assess the volume of mature cross-linked elastin fiber, intensity of EVG staining of elastic lamina, using inverted black-and-white micrographs, was quantified using ImageJ v1.48 software (National Institutes of Health, Bethesda, MD, USA; n = 4-8). We used entire thoracoabdominal aortic sections, because the disruption and vanishment of elastic lamina of thoracic aortic aneurysmal sections or abdominal aortic aneurysmal sections were too severe to assess the elastin breaks and mature cross-linked elastin volume.

**Electron microscopy**
The samples of abdominal aorta were removed, immersed in a fixative containing 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4°C for 2 days, and washed in 0.1 M phosphate buffer at 4°C. The samples were immersed in 1% osmium tetroxide in 0.1 M phosphate buffer at 4°C for 2 h, dehydrated, and embedded in EPON 812. Sections, 1-μm thick, were taken from each block and stained with 0.5% toluidine blue. For ultrastructural observation, ultrathin sections were prepared, counterstained with uranyl acetate and lead citrate, and observed with a JEM-1400 electron microscope (JEOL, Tokyo, Japan).

**Elastin volume of aortae**
The mouse aortic elastin content was quantified by using the Fastin Elastin assay kit (F2000; Biocolor, Carrickfergus, County Antrim, UK) following the manufacturer’s instructions. We used half of thoracic aorta and half of abdominal aorta per mouse for this measurement. Briefly, the mouse aorta samples were homogenized in 0.25 mol/L oxalic acid and were digested twice at 100°C for 60 min. After centrifugation, the supernatants were treated with an elastin-precipitating reagent and centrifuged again. After discarding the supernatants, the elastin pellet was treated with a dye reagent that binds to elastin. Lastly, the bound dye-elastin complex was resuspended with a dye dissociation reagent and the dye was dissociated from the elastin. The absorbance of each sample was measured at 510 nm and compared with a set of standard elastin samples. The elastin content of each mouse aorta sample was expressed as elastin volume (μg) to wet tissue weight (mg) (n = 7–8).

**Gelatinzymography**
Mouse aortae after a 1-week administration were homogenized in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM AEBSF (Sigma-Aldrich), and 10 μg/mL leupeptin (Peptide Institute, Osaka, Japan). Soluble extracts were separated by centrifugation and subsequently stored at ~80°C. Gelatin-substrate zymography was used to detect and characterize proteinase activity in the soluble extract.
Aortic-tissue soluble extract (20 μg total protein) was mixed with the sample buffer containing 10% SDS, 0.25 M Tris-HCl, 35% glycerol, and 0.25% bromophenol blue at pH 6.8, and electrophoresed on 8% SDS-polyacrylamide gels co-polymerized with gelatin (1 mg/mL). After electrophoresis, gels were washed twice for 30 min in 2.5% Triton X-100, incubated for 24 h at 37°C in 50 mM Tris-HCl, 10 mM CaCl₂ (pH 7.6) and 1% Triton X-100, and then stained with Coomassie blue R-250 and destained in a solution containing 5% acetic acid and 30% methanol. The enzymatic activity of the bands was quantified using ImageJ v1.48 software (n = 5–6).

**Cell culture**
Smooth muscle cells isolated from control mouse aortae (control VSMC) and SMKO mouse aortae (SMKO VSMC) were cultured as described previously. The cells (passage 3–5) used in this study were added to collagen-coated 6-well plates (40,000 cells/well), and the next day, the culture medium was changed to a new medium containing 10% fetal-bovine serum with or without Ang II (1 µmol/L) and BAPN (25 µg/mL). The culture medium and the stimulation of Ang II and BAPN were changed every week until day 21.

**LOX activity of aortae**
LOX activity of aortae was measured by using Lysyl Oxidase Activity Assay Kit – Red Fluorescence (ab112139; Abcam, Cambridge, UK). We used half of thoracic aorta and half of abdominal aorta per mouse for this measurement. After measuring aortic tissue weight, the sample was homogenized in 0.1% bovine serum albumin/phosphate-buffered saline. After centrifugation, 50 µL of the supernatant was used for measuring LOX activity. Recombinant human lysyl oxidase homolog 2/LOXL2 (2639-AO; R&D Systems, Minneapolis, MN, USA) was used to generate a standard curve, and aortic LOX activity was expressed as the level of equivalent activity of recombinant human lysyl oxidase homolog 2/LOXL2 protein per tissue weight (n = 7–11).

**α-SMA immunostaining of abdominal aortic aneurysmal sections**
Paraffin embedded abdominal aortae were sectioned and deparaffinized. The tissue samples were blocked with 2% goat serum for 30 min, and incubated with actin, smooth muscle antibody (RB-9010-R7; Thermo SCIENTIFIC, MA, USA) at room temperature for 3 hours, followed by incubation with anti-rabbit antibody conjugated to a peroxidase-labeled dextran polymer (Histofine Simplestain MAX-PO(R), Nichirei, Japan). Color was developed by reacting the sections with dianaminobenzidine peroxide substrate (Simplestain DAB, Nichirei), and weakly counterstaining with hematoxylin. The α-SMA positive area was quantified using ImageJ v1.48 software (n = 6–7).

**Azan staining**
Paraffin embedded abdominal aortae were sectioned and deparaffinized. The tissue samples were placed into the solution equal parts of 10% K₂Cr₂O₇ and 10% trichloroacetic acid for 10 minutes, and 0.1% azocarmine G solution containing 1% acetic acid for 30 minutes after 5 minutes of wash. The samples were next placed into 95% ethanol containing 0.1% aniline after the wash. The samples were placed into 5% phosphotungstic acid for 30 min following 1 minute of 95% ethanol containing 1% acetic acid and 5 minutes of wash. Finally, the samples were placed into 4% acetic acid containing 0.25% aniline and 1% orange G for 30-60 min following brief wash. The sections were dehydrated, cleared and mounted.

**Statistical Analysis**
The χ² test was used to analyze categorical data. We tested the normality and equal variance before parametric data analysis. To analyze the data corresponding to the small number of
aortic ruptures, the $\chi^2$ test was used with Yates’ continuity correction. Data are presented as means ± standard error of the mean. Statistical significance was assessed by Student's $t$ test for two comparisons, by a two-way analysis of variance followed by Bonferroni post-hoc testing for multiple comparisons, where values of $p < 0.05$ or $p < 0.01$ were considered significant. The number of individual experiments is represented by $n$. 
Table 1. Mouse oligonucleotide primers used for quantitative Real-Time PCR.

<table>
<thead>
<tr>
<th>(Mouse)</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hif-1α</td>
<td>5’- CTTGCAGAATGCTCAGAGG -3’</td>
<td>5’- CTGTTGTAATCCACTCTCATC -3’</td>
</tr>
<tr>
<td>Tropoelastin</td>
<td>5’-CTGGTGTTGGTCTTCCAGGT-3’</td>
<td>5’-TGGGATTCCAGAAAAGCAC-3’</td>
</tr>
<tr>
<td>LOX</td>
<td>5’-GGTTACTCCAGTACGGTCTCC-3’</td>
<td>5’-GCAGCGCATCTCAGGTTGT-3’</td>
</tr>
<tr>
<td>LOXL-1</td>
<td>5’-TTACCACAGCATGGACGAGTTCCAG-3’</td>
<td>5’-GTTATGCGATCCACTGGCAGTCA-3’</td>
</tr>
<tr>
<td>Fibulin-4</td>
<td>5’-CACGGAATGCACAGATGGCTA-3’</td>
<td>5’-CATCCACACAGCTCTCCTGTT-3’</td>
</tr>
<tr>
<td>Fibulin-5</td>
<td>5’-TGTGACCCAGGATATGAACTTGAG-3’</td>
<td>5’-AGCCCCCTTGTAGATTGTAGCA-3’</td>
</tr>
<tr>
<td>Integrin-αV</td>
<td>5’-GGCACAAGACCCTTGAGTA-3’</td>
<td>5’-GCCACTTGGTCCGAAATGAG-3’</td>
</tr>
<tr>
<td>Integrin-β3</td>
<td>5’-TGCTCCAGAGTCTATGGATGGTCC-3’</td>
<td>5’-GAGAAAGACAGGTCCATCAAGTAG-3’</td>
</tr>
<tr>
<td>Fibrillin-1</td>
<td>5’-GATCAACGGCTACCCAAAAC-3’</td>
<td>5’-GTTGGCTTCCATCTCAGACC-3’</td>
</tr>
<tr>
<td>MMP-2</td>
<td>5’-TGGGGGAGATTCTCACTTG-3’</td>
<td>5’-CCATCAGCGTTCCCATACTT-3’</td>
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<tr>
<td>MMP-9</td>
<td>5’-CAATCCTTGGCAATGGGATG-3’</td>
<td>5’-AGTAAGGAAGGGGCCCTGTA-3’</td>
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<tr>
<td>TIMP-3</td>
<td>5’-GATGCCTTCTGCAACTCCGACA-3’</td>
<td>5’-CCCAGGGTGATACGGTATTTGAG-3’</td>
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<tr>
<td>Gene</td>
<td>5'- Sequence</td>
<td>3'</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------------------------------</td>
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<td>F4/80</td>
<td>5'- CTGGCTATGGGCTTCCAGTC -3'</td>
<td>5'- GCAAGGAGGACAGAGTTTATCGTG -3'</td>
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<tr>
<td>IL-1β</td>
<td>5'- TGGGCCCTCAAAGGAAAGAAT -3'</td>
<td>5'- CAGGCTTTGTGCTCTGTTTGT -3'</td>
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<td>IL-6</td>
<td>5'- GTTCTCTGGGAAATCGTGGA -3'</td>
<td>5'- TGTACTCCAGGTAGCTATGG -3'</td>
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<td>Collagen I</td>
<td>5'- TGGATTCCCGTTCGAGTACG -3'</td>
<td>5'- AGGTGATGTGTTCTGGGAGGCC -3'</td>
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<td>Fibronectin</td>
<td>5'- TGGCTGCTTTCAACTTCTCCT -3'</td>
<td>5'- TGTGGATCTGGACTGGCAGTTT -3'</td>
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<td>VEGF</td>
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<td>5'- TTTGTTTCTTGTTCCTGCTGGATGT -3'</td>
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<tr>
<td>β-actin</td>
<td>5'- AAGTGTGACGGTCATCCG -3'</td>
<td>5'- GATCCACATCTGCTGGAAAG -3'</td>
</tr>
</tbody>
</table>
Supplemental References


Supplemental Figures and Figure Legends

Supplemental Figure I. Ang II- and BAPN-induced morphological changes in thoracic aortae and abdominal aortae from 3 or 4 separate individuals in each group. Arrow heads indicate thoracic or abdominal aortic aneurysm formations. Scale bar: 1 mm.

Imanishi et al., Supplemental Fig I
**Supplemental Figure II.** EVG staining of the sections of the thoracic and abdominal aortae, following treatment with Ang II and BAPN, from 3 separate individuals in each group. Scale bar: 0.5 mm.

[Images of EVG staining for control and SMKO groups]

**Supplemental Figure III.** Azan staining of the sections of the abdominal aortae, following treatment with Ang II and BAPN, from 3 separate individuals in each group. Blue-stained area shows vascular fibrotic region. Scale bar: 0.5 mm.

[Images of Azan staining for control and SMKO groups]
Supplemental Figure IV. Measurement of the outer diameter of thoracic aortae (n = 21 and n = 21, respectively) and abdominal aortae (n = 22 and n = 24, respectively) of control and SMKO mice treated with Ang II and BAPN.

* $p < 0.05$. 
Supplemental Figure V. Hif-1α mRNA expression in the aortae after a 6-week administration. 
(n = 7-8) 
** p < 0.01.

Supplemental Figure VI. Ang II- and BAPN-induced systolic blood pressure elevation in control and SMKO mice. (n = 12-16)

Supplemental Figure VII. EVG staining of the sections of EVG staining of the thoracoabdominal section, following treatment with Ang II and BAPN, from 3 separate
individuals in each group. Black arrows indicate elastin fragmentation and elastin branching in abnormal elastic lamina. Scale bar: 0.1 mm.

**Supplemental Figure VIII.** Gelatin zymography results of active MMP2, pro MMP2, and pro MMP9 showing no difference between aortae of control and SMKO mice after a 1-week administration. ‘M’ indicates the MMP marker. The graphs show the band density of gelatin zymography from aortic samples of control and SMKO mice treated with Ang II and BAPN. (n = 5-6)

**Supplemental Figure IX.** The mRNA expression of MMP-2, MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-3 in the aortae after a 6-week administration. (n = 7-11)

* $p < 0.05$.

** $p < 0.01$. 
Supplemental Figure X. The mRNA expression of F4/80, IL-1β and IL-6 in the aortae after a 6-week administration. (n = 9-11)
* p < 0.05.

Supplemental Figure XI. Lower tropoelastin mRNA expression in VSMCs isolated from SMKO aortae relative to control aortae after 21 days of cell culture with and without Ang II (1 µmol/L) and BAPN (25 µg/mL) stimulation. (n = 3)
** p < 0.01.
Supplemental Figure XII. The plot profiles of intensity of EVG staining of elastic lamina (inverted black-and-white micrographs), as shown in Figure 2E. We measured 6 independent lines (a-b) from one entire thoracoabdominal aortic cross-section per mouse aorta, and calculated the average from these 6 values per mouse aortae (n = 4-8). Three areas under the curve of the plot profile of elastic laminas were averaged per line (a-b).
Supplemental Figure XIII. The mRNA expression of elastin fiber formation-related genes in the aortae after 6 weeks following administration. Lysyl oxidase homolog 1 (LOXL-1) is encoded by a member of the LOX gene family, and also catalyzes elastin cross-linking such as LOX. Fibulin-4 and fibulin-5 are two kinds of elastin binding proteins. Integrin-αv and integrin-β3 mediate VSMC adhesion to elastin fiber. Fibrillin-1 is a major component of microfibrils. (n = 9-11)

* $p < 0.05$
** $p < 0.01$. 

Imanishi et al., Supplemental Fig XIII
Supplemental Figure XIV. The mRNA expression of tropoelastin and LOX in the aorta after a 1-week administration. \((n = 6-10)\)
* \(p < 0.05\).

Supplemental Figure XV. The mRNA expression of collagen I and fibronectin in the aorta after a 6-week administration. \((n = 7-11)\)
* \(p < 0.05\).
** \(p < 0.01\).

Supplemental Figure XVI. \(\alpha\)-Smooth muscle actin (\(\alpha\)-SMA) immunostaining of abdominal aortic aneurysmal sections after a 6-week administration. The graph shows \(\alpha\)-SMA positive area \((\text{mm}^2)\) in each aortic aneurysmal section. \((n = 6-7)\) Scale bar: 0.5 mm.
Supplemental Figure XVII. The mRNA expression of neovascularization-related genes in the aortae after a 6-week administration. \( n = 9-11 \)

* \( p < 0.05 \).

Supplemental Figure XVIII. The mechanisms involved in smooth-muscle cell-derived Hif-1\( \alpha \) induction of elastin fiber formation.
Supplemental Figure XIX. The segmentation of the artery between thoracic aorta, thoracoabdominal aorta and abdominal aorta in untreated mice.
Aortic aneurysm

HIF-1α SMKO

Aneurysm induction

Elastin-fiber degradation

Elastin-fiber formation

Aortic aneurysm

Control

Elastin-fiber degradation

Elastin-fiber formation

Aortic aneurysm

HIF-1α SMKO

Aneurysm induction

Elastin-fiber degradation

Elastin-fiber formation

Aortic aneurysm