The role of lysyl oxidase family members in the stabilization of abdominal aortic aneurysms

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ABDOMINAL AORTIC ANEURYSMS (AAAs) are a major cause of morbidity and mortality in the United States today. We employed a model for AAA development using apolipoprotein E knock out mice fed a high-fat diet and treated with ANG II and β-aminopropionitrile (β-APN) for 4 wk. ANG II induces hypertension and atherosclerotic disease, whereas β-APN inhibits the activity of the lysyl oxidase/lysyl oxidase-like protein (LOX/LOXL) family members. LOX/LOXL family members catalyze the conversion of lysine residues in collagen and elastin to allysine. The allysine residue spontaneously condenses to form highly stable cross-links that impart additional mechanical strength to collagen and elastin. LOX enzyme deficiency causes a 90% AAA incidence, whereas ANG II and β-APN caused 50% and 40% AAA incidence, respectively. These data demonstrate the importance of LOX/LOXL to the stability of the vessel wall. Therapeutic strategies to overexpress LOX/LOXL enzymes or to support the crosslinking of soluble matrix proteins in a polymeric scaffold are a promising opportunity to achieve stabilization of AAAs.

catalytic crosslinking; extracellular matrix; vascular remodeling

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Brunswick, NJ) and either treated with or without ANG II (0.75 mg·kg⁻¹·day⁻¹) using modified Paigen's atherogenic diet (Research Diets, New Brunswick, NJ) were fed either a control low-fat diet or a high-fat diet and treated with ANG II for 7 days. Mice treated with ANG II infusion and fed a high-fat diet exhibited significant increase in blood pressure. *P < 0.001.

**Table 1. LOX/LOXL and 18S primer sequences and annealing temperatures.**

<table>
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<tr>
<th>cDNA Targets</th>
<th>Primer Sequences</th>
<th>Annealing, °C</th>
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<tbody>
<tr>
<td>LOX</td>
<td>5′ TAGCGAACGCTAGCAGATGTTG 3′</td>
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</tr>
<tr>
<td>LOXL1</td>
<td>5′ TCTCGGGATGCGAATTGCAAGT 3′</td>
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<tr>
<td>LOXL2</td>
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<tr>
<td>LOXL4</td>
<td>5′ GCCATGCTTACACTTCTTCTC 3′</td>
<td>64</td>
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<td>64</td>
</tr>
<tr>
<td>18S</td>
<td>5′ Gaagctcgtcctatcaact 3′</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>5′ ccaagcttccataactgacgt 3′</td>
<td>64</td>
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LOX/LOXL, lysyl oxidase/lysyl oxidase-like proteins.

**MATERIALS AND METHODS**

**Animals.** ApoE⁻/⁻, C57BL/6, and BalbC mice age 6–8 wk (Jackson Laboratory, Bar Harbor, ME) were fed either a control low-fat diet (standard low-fat rodent chow Purina 5001) or a high-fat diet using modified Paigen’s atherogenic diet (Research Diets, New Brunswick, NJ) and either treated with or without ANG II (0.75 mg·kg⁻¹·day⁻¹) via an osmotic mini-pump. Some animals were also treated with β-APN at a dose of 100 mg·kg⁻¹·day⁻¹ via subcutaneous osmotic mini-pumps (22). All housing, surgical procedures, and experimental protocols were approved by the Institutional Animal Care and Use Committee of Emory University. Animals had free access to chow and water during all experiments. Noninvasive systolic blood pressure measurements were obtained by using the Visitech Systems BP 2000 tail-cuff system as previously described (32). Aortic atherosclerotic lesion area was measured as previously described by pressure perfusing the vasculature at −100 mm Hg with normal saline and subsequent fixation with 10% formalin (32). The aortic length was cleaned of periadventitial fat and connective tissue and then removed with the heart and kidneys attached. The thoracic-abdominal aorta was pinned on a wax dish and dissected on face longitudinally. Dissected aortas were digitally imaged and analyzed for lesion area and aortic circumference using ImageJ 1.61 software (National Institutes of Health, Bethesda, MD).

**Immunostaining.** Pressure-frozen aortas were embedded in paraffin, and sections were obtained at 5-μm intervals. Immunostaining for the LOX/LOXL family members was carried out by using rabbit polyclonal antibodies (supplied by Drs. Fong and Csiszar from University of Hawaii At Manoa) and goat anti-rabbit biotinylated secondary antibodies. Imaging was accomplished using streptavidin tagged quantum dots (emission 605 nM) as previously reported (6).

**Quantitative RT-PCR.** RNA was extracted from snap-frozen aortic tissue 7 days after treatment by using the RNeasy kit (Qiagen, Valencia, CA) according to the protocol and quantified by using a spectrophotometer. cDNA was prepared and purified from aortic RNA by using standard protocol with primers unique for each of the five lysyl oxidase isoforms, 18S and plasminogen activator inhibitor-1 (PAI-1). (Table 1).

**Western blot analysis.** Mouse aortas were harvested and snap frozen in liquid nitrogen. After homogenization in radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, Santa Cruz, CA), protein was extracted, sonicated, and quantified by using the Bradford assay. Samples were then boiled for 5 min at 95°C, and aliquots of the lysate were subjected to 8% SDS-PAGE. Proteins in the gel were transferred to a nitrocellulose membrane by electro blotting. The membranes were treated with LOX/LOXL antibodies procured from Novus (LOX) and Santa Cruz (LOXL1, LOXL2, and GAPDH) or made in-house (LOXL3). Anti-rabbit and anti-goat secondary antigens conjugated to horseradish peroxidase were procured from Santa Cruz and Bio-Rad. Immunoreactive proteins were detected by an enhanced chemiluminescence system (GE).

**LOX/LOXL activity.** Aorta tissue was isolated immediately and homogenized in 4 M urea in 0.02 M borate buffer (pH 8.0) at 4°C and centrifuged at 15,000 g for 30 min at 4°C, as previously reported (2). The supernatants were analyzed immediately for LOX assay with and without β-APN (13). Activity assays for detection of LOX were performed as previously described (23). Briefly, 500 μg of protein was added to the final reaction mixture supplied by Amplite Fluorometric Lysyl Oxidase Assay kit (AAT Bioquest) according to manufacturer’s instructions in the presence or absence of 1,000 μM β-APN. The LOX/LOXL reaction produces H₂O₂. Horseradish peroxidase catalyzed oxidation of N-acetyl-3,7-dihydroxyphenoxazone by H₂O₂ produces fluorescent resorufin, which is measured at an excitation of 530/25 nm and emission of 580/50 nm. The fluorescence was recorded at 30 min at 37°C. A standard graph was constructed using both H₂O₂ (0.2–5.0 μM) and recombinant lysyl oxidase (R&D) as a measure for detectable activity. The relative fluorescence units obtained in test samples were calculated based on the difference between β-APN-treated and nontreated samples normalized to total protein.

**Additional reagents.** β-Aminopropionitrile (β-APN), ANG II, and PCR primers were obtained from Sigma Chemicals, St. Louis, MO. Osmotic mini-pumps were obtained from Alzet, Cupertino, CA.

**Statistical analysis.** Systolic blood pressure, cholesterol, and percent atherosclerotic lesion area are presented as means ± SD. ANOVA was performed using GraphPad Prism Software. Post hoc analyses were performed using the Bonferroni test. P values <0.05 were considered to be significant.

**RESULTS**

LOXL3 mRNA expression is significantly upregulated in the aortas of ANG II-treated ApoE⁻/⁻ mice fed a high-fat diet. To quantitatively assess if LOX/LOXL family member expression was regulated in our model of AAA formation, LOX/LOXL mRNA levels were measured by quantitative real-time PCR using mRNA extracted from the aortas of control ApoE⁻/⁻ mice fed a low-fat diet mice and compared with those fed a high-fat diet and treated with ANG II for 7 days. Mice treated with ANG II infusion and fed a high-fat diet exhibited significant increase in blood pressure. Mean systolic blood pressures were taken in apolipoprotein E knockout (ApoE⁻/⁻) mice before treatment. Animals were then fed a high-fat diet and treated with ANG II for 2 wk, resulting in a significant increase in blood pressure. *P = 0.0001.
Significant increases in blood pressure (Fig. 1). At baseline LOX expression in aortic tissue was highest compared with the other family members, with expression of LOXL1, LOXL2, and LOXL3 much lower and LOXL4 expression undetectable (Fig. 2, A–D). Comparison of mRNA from our AAA mouse model to the low-fat control mice after 7 days resulted in significantly increased expression of LOXL3 mRNA. (Fig. 2D) This increase observed with regard to LOXL3 was specific to a high-fat diet in the presence of ANG II, whereas no change was observed in LOXL3 expression on a low-fat diet ± ANG II. LOXL4 expression remained undetectable after treatment. Interestingly, Western blot analysis on protein harvested from aortic tissue 7 days after treatment with ANG II on a high-fat diet failed to show any significant difference in the detected isoforms (LOX and LOXL3) over control tissue (low-fat diet) (Fig. 2E). In addition, other family members that were previously detected by quantitative RT-PCR and immunohistochemistry (LOXL1 and LOXL2) were not detected at 7 days in

Fig. 2. Lysyl oxidase/lysyl oxidase-like protein (LOX/LOXL) family member expression 7 days after ANG II infusion in the presence of a high-fat diet. Quantitative RT-PCR was performed on cDNA from aortic tissue of ApoE−/− mice. A–C: LOX, LOXL1, and LOXL2 expression were not affected by ANG II in the presence of a high-fat diet. *P = 0.005. D: LOXL3 expression increased with ANG II on an atherogenic diet. E: Western blot analysis on ApoE−/− mice 7 days after treatment with low-fat diet or high-fat diet + ANG II. By Western analysis, LOX and LOXL3 were the only detectable family members in the low-fat and high-fat diet + ANG II setting 7 days post-treatment. Neither LOX nor LOXL3 showed a statistically significant change in expression with treatment (n = 3).
Aneurysmal dilation, defined as suprarenal circumference doubling, was most pronounced in animals treated with ANG II + high-fat and β-APN (Fig. 4, A and B). Within these groups the frequency of large aneurysm formation was greater than 90%, and the high-fat/ANG II/β-APN group was associated with 60% mortality (Fig. 4B). Necropsies revealed aortic rupture as the cause of death. Mice fed a low-fat diet alone for 4 wk ± vehicle or β-APN did not demonstrate an increased frequency of aneurysm formation. Longer time points were not studied because of the high rate of mortality in the animals treated with β-APN. In addition to evaluating the contribution of LOX/LOXL family members to ANG II-induced vessel stability using β-APN inhibition, we also monitored total LOX/LOXL activity in ApoE−/− mice 7 days following treatment with either low-fat or high-fat diet + ANG II. We found a trend (P = 0.0576) toward decreased LOX/LOXL activity after treatment with ANG II and a high-fat diet, as compared with low fat controls (Fig. 4D). These data suggest that LOX/LOXL activity is critical for the crosslinking mechanisms that preserve vessel wall integrity during ANG II infusion.

**LOX/LOXL activity protects against ANG II-induced atherosclerotic lesion formation.** To determine the functional importance of lysyl oxidase family member activity in maintaining the structural organization of the vasculature and in protection against disease, we measured the atherosclerotic lesion area in the aorta after 4 wk of control diet and after treatment with ANG II + high-fat with and without β-APN in ApoE−/− mice. ANG II + high-fat treatment induced a significant increase in atherosclerotic lesion formation above control (Fig. 4C). Conversely, mice infused with β-APN alone did not demonstrate an increase in lesion formation. However, when β-APN infusion was combined with ANG II + a high-fat diet, lesion formation increased significantly over control mice, covering nearly 35% of the aortic surface area. This dramatic increase in lesion formation demonstrates the deleterious effects of a high-fat diet and ANG II treatment.

**LOX/LOXL proteins are expressed in the aortic wall.** Immunostaining was performed to determine the cellular localization of individual LOX/LOXL family members within the aortic wall of ApoE−/− mice fed a high-fat diet and treated with ANG II for 8 wk. LOX, LOXL1, LOXL2, and LOXL3 expression was diffuse throughout the aortic wall (Fig. 3B). LOX, LOXL1, and LOXL2 displayed robust expression in the medial and intimal layers of the aortic wall (Fig. 3B). LOXL2 expression was most prominent in the endothelial cell layer (Fig. 3B). Interestingly, LOXL1 and LOX3 had significant expression in the neointimal layer, suggesting certain LOX/LOXL family members are expressed in migratory smooth muscle cells. LOX/LOXL family member localization was similar between low-fat and high-fat + ANG II treatment groups at 8 wk, suggesting that diet and treatment did not alter individual family member expression patterns (Fig. 3A). However, differential LOX/LOXL family member localization and expression profiles further illustrate the importance of this family of matrix crosslinking enzymes to the structural integrity of the vessel wall. Each member was clearly expressed in the vessel wall, underscoring the potential role of the LOX/LOXL family in maintaining vascular stability.

**LOX/LOXL activity protects against AAA formation.** To determine the contribution of total LOX/LOXL activity to the stabilization of the aortic wall during AAA formation, we measured AAA incidence 4 wk after treatment in the ApoE−/− mice. In addition to evaluating the contribution of LOX/LOXL family members to ANG II-induced vessel stability using β-APN inhibition, we also monitored total LOX/LOXL activity in ApoE−/− mice 7 days following treatment with either low-fat or high-fat diet + ANG II. We found a trend (P = 0.0576) toward decreased LOX/LOXL activity after treatment with ANG II and a high-fat diet, as compared with low fat controls (Fig. 4D). These data suggest that LOX/LOXL activity is critical for the crosslinking mechanisms that preserve vessel wall integrity during ANG II infusion.
Fig. 4. Effect of β-aminopropionitrile (β-APN) on abdominal aortic aneurysms (AAA) incidence and atherosclerotic lesion area in ApoE−/− mice on a high-fat diet with ANG II infusion. A: representative examples of ApoE−/− mice treated with the lysyl oxidase inhibitor β-APN + ANG II. B: incidence of AAA in all groups. C: percent lesion area per total en face aortic area. *P < 0.001. D: total LOX/LOXL activity in aortic tissues of ApoE−/− mice after 7 days of treatment. Total LOX/LOXL activity showed a trend toward decreased LOX/LOXL activity (P = 0.0576) after treatment with a high-fat diet and ANG II. RLU, relative light units, detected as a readout for H₂O₂ produced. All data are normalized to 500 μg of total protein and normalized to the β-APN inhibitable signal. LF, low fat; HF, high fat.
damaged matrix on vascular disease and illustrates the protective action of LOX/LOXL against atherosclerotic lesion formation driven by an ANG II-induced mechanism.

We also investigated the less atheroprone but not atheroprotected C57BL/6 mice in the same manner as the atherogenic ApoE<sup>−/−</sup> mice. The results of these experiments follow a similar trend as the ApoE<sup>−/−</sup> mice in that ANG II was sufficient to cause AAAs in some of the mice on a high-fat diet, but dual treatment with ANG II and β-APN caused further induction of AAAs, although not to the same degree as in the ApoE<sup>−/−</sup> mice (Fig. 5B). Lesion area was collectively much lower in these mice in all treatment groups, as compared with the ApoE<sup>−/−</sup> mice, and was not affected by β-APN treatment (Fig. 5, A and C).

To further investigate the mechanism by which LOX/LOXL family members protect against AAA, the previously established atheroprotected BalbC mouse model (30) was treated using the same experimental strategy as described above for the atherogenic ApoE<sup>−/−</sup> mice. As expected, neither ANG II alone nor β-APN alone induced lesion formation. ANG II alone was not sufficient to produce any AAAs in the BalbC mice. However, the combination of both ANG II and β-APN induced significant aortic dilation above control (Fig. 6B), suggesting further that LOX/LOXL activity strongly counteracts ANG II-induced vascular remodeling at the matrix level and is protective against AAA formation. These data also suggest that although the presence of atherosclerotic lesion formation greatly increases the incidence of AAA formation, it is not necessary, since AAAs were induced in C57Bl/6 and BalbC mouse models, which have minimal or no atherosclerotic disease.

**DISCUSSION**

We have shown that LOX/LOXL family members are expressed in the aortic wall of normal and ANG II-treated mice. We have further demonstrated that although there may be spatial differences in protein expression, global LOXL3 mRNA is upregulated at the mRNA level but not at the protein level in ApoE<sup>−/−</sup> mice fed an atherogenic diet 7 days after treatment with ANG II. To elucidate the functional significance of the LOX/LOXL family members in the ApoE<sup>−/−</sup> mice fed a high-fat diet + ANG II, we inhibited LOX/LOXL activity...
with β-APN and observed accelerated AAA development in the setting of atherosclerosis. This suggests that the crosslinking activity of LOX/LOXL family members is a necessary compensatory mechanism for vessel stability during vascular disease. To further demonstrate that LOX/LOXL family members are crucial for maintaining matrix stability, we recapitulated this model in less atherogenic prone C57BL/6 and BalbC mice. These mice developed AAAs, although their atherosclerotic lesion area was limited. These results point to the critical significance of LOX/LOXL family members in vessel stability independent of atherosclerotic disease.

The connection between ANG II, LOX/LOXL, and AAA formation has yet to be completely elucidated. However, we can speculate on how these factors work synergistically to promote AAA formation. It is noteworthy that lesion area in the C57BL/6 and BalbC mice treated with ANG II was low (<10%) compared with the ApoE−/− mice treated with ANG II (20–30%). It is plausible that the contribution of ANG II to AAA is through the induction of atherosclerotic lesion formation, which ultimately destabilizes the vessel wall leading to dilation. This instability was more pronounced in our most atheroprone mice, which could explain why more ApoE−/− mice develop AAAs with ANG II treatment alone. The rank order of atherosclerotic potential in our mouse models is ApoE−/− > C57BL/6 > BalbC. As such, we saw AAA formation with ANG II alone only in more atheroprone models and when lesion area was more severe. Inhibition of LOX/LOXL was also capable of causing instability in the vessel wall, but not in healthy adult mice. The combination of these two factors (ANG II + high-fat diet and β-APN) promoted AAA formation regardless of the atherosclerotic potential of the mouse model. Lesion area and AAA formation reported within this manuscript are lower than previously reported numbers, which is likely attributable to differences in the ages of mice (8 wk) at the onset of treatment, as previously demonstrated by Weiss et al. (32). The use of more juvenile aged mice required the combination of the high fat + ANG II diet to detect lesion

Fig. 6. Effect of β-APN on AAA incidence and atherosclerotic lesion area in BalbC mice on a high-fat (HF) diet with ANG II. A: representative examples demonstrating lack of significant atherosclerotic lesion formation. However, treatment with ANG II and β-APN did cause AAA formation. B: incidence of AAA in all groups.
formation, similar to the method used to induce lesion formation in Low density lipoprotein receptor (LDLR−/−) mice.

It is possible that the ANG II effect on LOX/LOXL mRNA expression is a compensatory mechanism invoked to stabilize aortic structure. As determined by monitoring LOX/LOXL activity at 7 days, we detected a trend toward a decrease in LOX/LOXL activity, suggesting that a compensatory mechanism is invoked within LOXL3 to upregulate expression and counteract the decrease in LOX/LOXL activity observed at 7 days post-treatment. The catalytic function of LOX/LOXL in modifying proteins responsible for tensile and elastic strength of aortic tissue suggests a potentially important role in aneurysm formation. It is likely that inhibition of LOX/LOXL family members by β-APN induced AAA in our animal model because there was a balance that exists between extracellular matrix proteolytic activity and LOX/LOXL activity, and we altered that. We suggest that tipping the balance in favor of either can lead to expansion or stabilization of the aortic wall. It has been reported that ANG II causes the activation and/or release of MMPs that are capable of degrading collagen and elastin fibers (1, 31). β-APN treatment in combination with ANG II prevented LOX/LOXL crosslinking activity, shifting the balance in favor of aortic dilation.

To date, LOX/LOXL family members are the only known enzymes responsible for the covalent crosslinking of soluble collagen and elastin into protease-resistant fibers. LOX activity has been demonstrated to be crucial for matrix development during gestation and postnatal development (20, 28). Although synthesis of elastin into concentric laminae during development is defined, synthesis of mature elastin during adulthood is quite limited (26, 27). Furthermore, our studies show that inhibition of LOX/LOXL activity in healthy adult animals is not associated with cardiovascular disease, implying that LOX/LOXL is crucially important during times of growth and remodeling. These results were previously suggested by Kanematsu using C57BL/6 mice that were treated with ANG II and β-APN, which demonstrated that the hypertensive/hemodynamic effects of ANG II along with LOX/LOXL inhibition via β-APN contribute to the formation of AAAs (12).

Using immunohistochemistry, we have shown that LOX, LOXL1, LOXL2, and LOXL3 are expressed at the protein level and are regulated at the mRNA level by ANG II in the aortic wall. Western blot analysis did not suggest regulation in the global amount of LOX/LOXL family members after 7 days of ANG II treatment. We have further demonstrated that LOX/LOXL activity is crucial in the setting of ANG II-induced vascular disease independently of atherosclerotic lesion formation. As such, therapeutic strategies to overexpress LOX/LOXL enzymes or to support the crosslinking of soluble matrix proteins in a polymeric scaffold are a promising opportunity to achieve stabilization of AAA. It is noteworthy that attempts to overexpress LOX/LOXL activity and or expression must be done carefully since heightened levels of LOX activity have been associated with tumor progression. Losses of heterozygosity in the LOX and LOXL2 genes have been associated with colonic and esophageal neoplasms, identifying these genes as tumor suppressors (10). The by-product of LOX activity, H2O2, has been associated with cell motility and migration in breast cancer (25). Furthermore, increased LOX activity has been linked to conditions of fibrosis, such as atherosclerosis (10, 11). With this knowledge, site-specific targeting and regulated activation of the LOX/LOXL family members should be a priority of future studies.

In conclusion, these data in ApoE−/− and C57BL/6 and BalbC mice establish that the activity of the LOX/LOXL family imparts critical structural integrity to the vascular wall. Inhibition of LOX/LOXL in these models generates a novel insight to study AAA development from the standpoint of LOX/LOXL activity.

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

AUTHOR CONTRIBUTIONS

REFERENCES


