Apelin protects against abdominal aortic aneurysm and the therapeutic role of neutral endopeptidase resistant apelin analogs

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Abdominal aortic aneurysm (AAA) remains the second most frequent vascular disease with high mortality but has no approved medical therapy. We investigated the direct role of apelin (APLN) in AAA and identified a unique approach to enhance APLN action as a therapeutic intervention for this disease. Loss of APLN potentiated angiotensin II (Ang II)-induced AAA formation, aortic rupture, and reduced survival. Formation of AAA was driven by increased smooth muscle cell (SMC) apoptosis and oxidative stress in Apln\textsuperscript{−/−} aorta and in APLN-deficient cultured murine and human aortic SMCs. Ang II-induced myogenic response and hypertension were greater in Apln\textsuperscript{−/−} mice, however, an equivalent hypertensive phenotype was observed in Ang II receptor (ACE2) deficient Ang II\textsuperscript{nociceptive} mice. We further identified Ang converting enzyme 2 (ACE2), the major negative regulator of the renin-Ang system (RAS), as an important target of APLN action in the vasculature. Using a combination of genetic, pharmacological, and modeling approaches, we identified neutral endopeptidase (NEP) that is up-regulated in human AAA tissue as a major enzyme that metabolizes and inactivates APLN-17 peptide. We designed and synthesized a potent APLN-17 analog, APLN-NMeLeu9-A2, that is resistant to NEP cleavage. This stable APLN analog ameliorated Ang II-mediated adverse aortic remodeling and AAA formation in an established model of AAA, high-fat diet (HFD) in Ldlr\textsuperscript{−/−} mice. Our findings define a critical role of APLN in AAA pathogenesis based on loss-of-function and gain-of-function approaches and included human vascular SMCs and AA tissue obtained from patients. We identified NEP as a key enzyme that degrades and inactivates the active APLN-17 peptide, developed a stable APLN-17 analog resistant to NEP degradation, and established the therapeutic potential of this analog.

\section*{Significance}

Vascular diseases remain a major health burden, and AAs lack effective medical therapy. We demonstrate a seminal role for APLN in AAA pathogenesis based on loss-of-function and gain-of-function approaches and included human vascular SMCs and AA tissue obtained from patients. We identified NEP as a dominant inactivating enzyme for native APLN-17. This allowed us to design and synthesize a stable and bioactive APLN analog resistant to NEP degradation that showed profound therapeutic effects against AAA. Our study clearly defines the APLN pathway as a central node in the pathogenesis of AAA and elucidates a therapeutic strategy of enhancing the APLN pathway by using a stable APLN analog to treat AAA.

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Conflict of interest statement: Our apelin analogs have been submitted for patenting.

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therapeutic effects of this developed stable APLN analog in preventing vascular disease and formation of AAA.

Results

Loss of APLN Enhances Susceptibility to AAA. Histological analyses of human AAA revealed severely disrupted medial structure characterized by fragmented elastin fibers associated with the loss of SMCs and increased cell death in AAA specimens compared with the nondiseased aorta (NDA) (Fig. 1 A and B and SI Appendix, Fig. S1 and Table S1). These structural changes in the aneurysmal aorta were associated with increased APLN levels compared with nonaneurysmal aorta (Fig. 1 C and D and SI Appendix, Fig. S1B) and APLN was increased in Ang II-infused wild-type (WT) (Apln−/y) mice aorta (Fig. 1 E and F). A similar pattern was also seen in the thoracic aorta from patients with bicuspid aortic valve and aortopathy (SI Appendix, Fig. S2A).

Ang II is a well-known mediator of adverse vascular remodeling and is widely used in AAA models (18–21). The up-regulation of APLN levels in the diseased aorta suggest that the APLN pathway is responsive to disease. To determine the role of APLN in AAA, we tested the effects of Ang II in WT (Apln+/y) and APLN knockout (Apln−/y) mice. Four weeks of Ang II infusion resulted in high incidence of severe AAA in the Apln−/y but not in parallel WT mice (Fig. 2A). The AAA in Apln−/y mice was associated with aortic dissection, intramural hematoma, and increased mortality due to aortic rupture (Fig. 2 B and C). Among the 23 Apln−/y mice that received Ang II, 5 died from AAA rupture, 18 survived, and 12 of the survivors developed AAA (Fig. 2 A–C). Vascular ultrasound imaging showed progressive greater dilation, localized aneurysm formation, and decreased compliance (aortic expansion index) in the abdominal aorta of Ang II-infused Apln−/y compared with Apln+/y mice, whereas no difference was observed between the genotypes at baseline (Fig. 2D). Consistent with the phenotypic changes in the abdominal aorta, thoracic aorta also displayed adverse remodeling in Apln−/y compared with Apln+/y mice (SI Appendix, Fig. S2B). Histological analyses confirmed disruption of the elastin lamellae in the aortic media and excess fibrotic deposition in the adventitia in Apln−/y mice compared with the uniform thickening of the aortic wall in Apln+/y mice in response to Ang II (Fig. 2E). Overall, our findings demonstrate that APLN is a major determinant in the pathogenesis of AAA.

APLN Deficiency Promotes Ang II-Induced Hypertension and VSMC Stress. We next explored the mechanism for the enhanced susceptibility of APLN-deficient mice to Ang II-induced AAA. We

Fig. 1. Up-regulation of APLN levels in vascular disease. (A and B) Adverse structural remodeling in surgical resected AAA specimens from patients as revealed by Movat’s pentachrome (A) and anti-calponin staining to visualize SMCs (red, B) of NDA and AAA. The arrow heads in AAA images point to elastin fiber fragments. L = aortic lumen. (B) Elastin fiber autofluorescence appears green. DAPI staining (blue) was used to visualize the nuclei. Averaged SMC content (calponin-positive staining), and apoptotic SMCs (positive for TUNEL in green and DAPI staining) in the NDA and AAA are shown as boxes with scatter plots on the right. n = 6/group. The arrows in AAA images point to apoptotic cells. (C) Immunostaining for APLN (red) with DAPI nuclear staining (blue), and Western blots for APLN (D) in NDA and AAA specimens with averaged quantification of APLN levels shown in boxes with scatter plots; n = 7/group in C, n = 4/group in D. (E) Immunostaining for APLN (red) with DAPI nuclear staining (blue), and Western blots (F) in abdominal aorta from WT mice receiving saline as vehicle (Veh) or Ang II for 4 wk (1.5 mg/kg/d) with averaged quantification of APLN levels shown in boxes and scatter plots; n = 4/group. *P < 0.05 compared with the NDA group; #P < 0.05 compared with the Veh group; A.U., arbitrary units.
determined the impact of Apln deficiency on vascular function and showed stronger Ang II-induced vasoconstriction in Apln−/− mesenteric resistance arteries compared with Apln+/− arteries associated with marked suppression of basal phospho-eNOS (Ser1177) levels (Fig. 3A, B) and shows the presence of aneurysms (A), aortic dissection, and rupture (B) leading to hemorrhage in the peritoneal cavity in the Apln−/− Ang II group. The white arrow points to a hemorrhage. RK = right kidney, LK = left kidney, and the red arrows point to ruptured ends of elastin lamellae. (C) Kaplan Meir survival curve showing mortality due to aortic rupture assessed by logarithm-rank testing. n = 12–18/group. (D) Ultrasound B-mode images of the AA in Ang II-infused WT and Apln−/− mice. *K* indicates the top of the left kidney as a reference, and the red lines show where measurements of aortic diameter were obtained (suprarenal). (E) Histological analysis (Movat’s pentachrome staining) showing disruption of the elastin lamellae in the medial aortic wall with fibrotic deposition in Apln−/− mice compared with the uniform thickening of the aortic wall in WT mice exposed to Ang II. *P < 0.05 compared with the Veh group; *P < 0.05 compared with the WT-Ang II group.

We investigated the cellular basis for the enhanced susceptibility to AAA formation in Apln−/− mice and found reduced Apln−/− mice after 4 wk of PE infusion (Fig. 3D–F). Apln−/− mice were concordant with a marked suppression of survival signaling pathways, Akt and Erk1/2 pathways, whereas preventing Ang II-mediated phosphorylation of p38 and JNK1/2 MAPK (SI Appendix, Fig. S6). These changes were associated with elevated oxidative stress as evident by the increased number of dihydroethidium (DHE)-positive cells in the aortic wall coupled with elevated NADPH oxidase (Fig. 3E and F and SI Appendix, Fig. S5B) and in situ gelatinase activities reflecting the action of matrix metalloproteases 2 and 9 (SI Appendix, Fig. S7).

Next, we characterized the impact of APLN deficiency on VSMCs in response to Ang II in vitro. In cultured primary aortic SMCs from human and mouse aorta (SI Appendix, Fig. S8), APLN expression was knocked down using specific APLN-siRNA (siAPLN), whereas scrambled siRNA (siNS) was used as the control (Fig. 4A). Ang II treatment increased Apln mRNA levels in control human and mouse SMCs (siNS) but induced a markedly higher rate of apoptotic cell death in the siAPLN-expressing SMCs (siAPLN). These results demonstrate that APLN-deficient vasculature is intrinsically susceptible to the adverse effects of Ang II-induced vascular remodeling.

To test whether the Ang II-induced higher blood pressure in Apln−/− mice accounted for AAA formation, we used another hypertensive agent, phenylephrine (PE), to induce the same degree of hypertension. Interestingly, no AAA was observed in either Apln−/− mice or their parallel control Apln+/− mice after 4 wk of PE infusion (SI Appendix, Fig. S4). These results demonstrate that the APLN-deficient vasculature is intrinsically susceptible to the adverse effects of Ang II-induced vascular remodeling.

We investigated the cellular basis for the enhanced susceptibility to AAA formation in Apln−/− mice and found reduced VSMC density, increased apoptotic cell death, and cleaved caspase 3 levels following 2 wk (SI Appendix, Fig. S5A) and 4 wk of Ang II infusion (Fig. 3C and D). These cellular phenotypes were concordant with a marked suppression of survival signaling pathways, Akt and Erk1/2 pathways, whereas preventing Ang II-mediated phosphorylation of p38 and JNK1/2 MAPK (SI Appendix, Fig. S6). These changes were associated with elevated oxidative stress as evident by the increased number of dihydroethidium (DHE)-positive cells in the aortic wall coupled with elevated NADPH oxidase (Fig. 3E and F and SI Appendix, Fig. S5B) and in situ gelatinase activities reflecting the action of matrix metalloproteases 2 and 9 (SI Appendix, Fig. S7).
Native APLN peptides are easily degraded by NEP, and re-sensitization of the vasculature to Ang II infusion follows. To test if NEP re-sensitizes the vasculature to Ang II, we used a biochemical assay and found that ex vivo infusion of NEP into APLN-17 treated with a NEP inhibitor, sacubitrilat, elevated steady-state APLN levels compared with the WT-Ang II group.

**NEP is a Key Enzyme that Inactivates APLN Peptides.** We next examined the ability of NEP in inactivating APLN peptides that could provide a fundamental mechanism for the pathogenesis of AAA. Computer modeling and simulation demonstrated a feasible model of APLN-17 binding with the active catalytic site in NEP (His583, His587, and Glu646) resulting in the cleavage of APLN-17 at 2 distinct sites, Arginine8-Tyr697, Val710, His711, and Arg717 (Fig. 5C and SI Appendix, Fig. S10). Other active site residues in NEP that facilitate the binding of APLN-17 in the catalytic pocket are Arg102, Arg110, Glu533, Val541, Ser546, Ser547, Ile585, Glu646, Ile648, Gly655, Ala657, Tyr697, Val710, His711, and Arg717 (Fig. 5C). To confirm this prediction, we used a biochemical assay and found that ex vivo incubation of APLN-17 in human plasma with recombinant NEP resulted in efficient degradation of APLN whereas the application of a NEP inhibitor, sacubitrilat, elevated steady-state APLN levels (Fig. 6A) with corresponding inverse changes detected in plasma APLN 17 products, APLN 9–17 and APLN 10–17 peptides (SI Appendix, Fig. S11). The APLN degradation products were completely inactive demonstrating a key functional role of NEP in degrading APLN (Fig. 6D). We next tested the in vivo role of NEP in metabolizing APLN-17. Genetic loss or pharmacological inhibition (by sacubitrilat) of NEP potentiated the hypotensive action of APLN-17 (Fig. 6C) and markedly elevated plasma levels of APLN-17 (Fig. 6D). These results highlight a dominant role for NEP in metabolizing and inactivating the endogenous APLN-17 peptide, which implied the NEP-resistant APLN analog is much needed for therapeutic use in vivo.

**APLN Analogs Have Improved Pharmacokinetics and Equivalent Pharmacodynamics.** Native APLN peptides are easily degraded and have short half-lives (14, 24, 25). Therefore, we designed and tested 35 different analogs and were able to identify and develop a long-lasting stable APLN-17 analog NMeLeu9Nle15Aib16BrPhe17-APLN-17 (abbreviated as APLN-NMeLeu9A2) (Fig. 7A) and confirmed a marked improvement in plasma levels and hypotensive effects (Fig. 7B and C). The APLN receptor (formerly known as APJ) is the only known native receptor for APLN peptides in mammals (26). Binding studies with the murine APLN receptor showed that murine Gi activation and receptor internalization were maintained by APLN-NMeLeu9A2 at similar plasma levels compared with native APLNs, minimizing the possibility of off-target effects of APLN analogs (Fig. 7D–G). Our NEP resistant APLN-17 analog (APLN-NMeLeu9A2) represents a therapeutic approach for AAs.
To test the therapeutic potential of our synthetic APLN analog designed to be resistant to NEP-mediated degradation, we utilized a well-established model of an AAA. We used a murine model lacking low-density lipoprotein receptors (Ldlr−−) given a HFD and Ang II infusion (21, 27). Although the placebo-treated...
group (Ldlr−/−-Ang II + placebo) showed a 50% mortality mainly due to aortic rupture in the abdominal region, treatment with the APLN analog (Ldlr−/−-Ang II + APLN-NMeLeu9-A2) had no incidence of aortic rupture after 4 wk of Ang II infusion (Fig. 8A). Vascular ultrasound showed that the administration of APLN-NMe17A2 prevented aortic lumen dilation and preserved aortic

![NEP/Elastin/DAPI and NEP/Elastin/DAPI](image)

Fig. 5. NEP is up-regulated in the diseased aorta, and computer modeling predicts NEP mediates proteolytic cleavage of APLN-17. (A and B) Immunofluorescence staining and Western blot analysis of NEP in diseased human (A) and murine (B) aorta illustrating increased levels of NEP. Nep−/− aorta used as a negative control. ND, not detected; n = 4/group in (A), n = 6/group in B. (C) Computer-based in silico modeling showing the interaction between the catalytic site of NEP and cleavage sites in native APLN-17 peptide. *P < 0.05 compared with the NDA or Veh group.

![Fig. 6](image)

Fig. 6. NEP plays a key role in the inactivation of APLN-17: synthesis of NEP resistant APLN analogs. (A and B) In vitro assay using human plasma and human recombinant NEP demonstrating the ability of NEP to efficiently cleave APLN-17 (A) and in vivo blood pressure assay in WT mice demonstrating that the cleaved products of NEP action on APLN-17 peptides are inactive (B); NEPi = sacubitrilat; n = 5 to 6/group. (C) In vivo blood pressure assay in WT mice examining the vasodepressor activity of the NEP-mediated cleavage of the APLN-17 peptide product; n = 6/group; Averaged values represent mean ± SEM. MABP; SBP = systolic blood pressure; DBP = diastolic blood pressure. (D) Plasma APLN-17 levels in WT and Nep−/− mice and in response to pharmacological inhibition of NEP using sacubitrilat in WT mice; n = 12/group. *P < 0.05 compared with the Veh group; #P < 0.05 compared with the NEP group without sacubitrilat; $P < 0.05 compared with the WT group without sacubitrilat.
compliance (expansion index) (Fig. 8B). Structural analysis of the abdominal aorta provided definitive evidence that Ang II-mediated aortic pathology in \textit{Ldrr}−/− mice was prevented by treatment with APLN-NMe17A2. Importantly, mice receiving APLN-NMeLeu9-A2 preserved SMC density and elastin structure, and reduced apoptosis (TUNEL and cleaved caspase 3 levels) in the aortic wall in response to 2 and 4 wk of Ang II infusion (Fig. 8C and D). Intriguingly, APLN analog supplementation increased ACE2 levels in the aortic wall (Fig. 9A and B), which has been reported to have vasculoprotective effects (15). In isolated VSMCs, Ang II-mediated production of reactive oxygen species determined by DHE fluorescence and NADPH oxidase activity were markedly attenuated by APLN-NMe17A2 (Fig. 9C and D). Our results highlighted a dominant role of the APLN pathway in AAA and support the use of a stable APLN analog as a therapy for AAA (Fig. 9E).

**Discussion**

Vascular diseases remain a major health burden, and AAs lack effective medical therapy representing a progressive disease state with a life-threatening but unpredictable risk for rupture (1, 2). Currently, no pharmacological intervention effectively inhibits the progressive expansion of human AAAs or prevents aortic rupture (28, 29). In this study, we demonstrate a seminal role for APLN in AAA pathogenesis using loss-of-function and gain-of-function approaches. Using an Ang II-induced model of an AAA, loss of APLN resulted in greater adverse remodeling and propensity to develop an AAA, aortic rupture, and increased mortality. Given the short half-life of native APLN peptides, we identified NEP as a dominant inactivating enzyme for APLN-17. This allowed us to design and synthesize a stable and bioactive APLN analog that is resistant to NEP degradation, active in both blood pressure in vivo as well as in vitro APLN receptor binding studies; and it showed profound therapeutic effects for AAAs.

In aortic SMCs, APLN showed a dose-dependent protective effect against Ang II-induced apoptosis and reactive oxygen species stress, whereas loss of APLN exacerbated these responses, consistent with a dominant role of apoptotic loss of VSMCs in the progression of AAAs. A well-recognized characteristic in human AAAs is the increased abundance and activation of matrix metalloproteinases in the diseased aortic tissues that was modulated by the APLN pathway likely in response to changes in oxidative stress. APLN action on endothelial cells including promoting angiogenesis (6, 11, 12), APLN-mediated nitric oxide vasodilation.

**Fig. 7.** APLN receptor coupled Gi activation and β-arrestin recruitment by APLN peptides and APLN analogs. (A–C) Schematic of the APLN-NMeLeu9A2 with plasma levels at 5 min posti.v. administration and blood pressure response compared with native APLN-17; n = 10/group. *P < 0.05 compared with the APLN 17 group. (D–G) Concentration-response effect of endogenous APLN peptides (K17F and pE13F) and metabolically stable APLN analogs (K17FA2 and pE13FA2) on murine angiogenesis (6, 11, 12), APLN-mediated nitric oxide vasodilation.
The mesenteric artery could not be identified a unique susceptibility of the arteries without affecting passive elasticity. Indeed, we identified a unique susceptibility of the arteries without affecting passive elasticity. Apln mRNA levels were increased in the aorta compared with WT and failed to increase in response to Ang II. In Ang II-induced greater hypertension in Apiln−/− mice compared with WT mice; however, this finding also poses a complexity in understanding the role of APLN in Ang II-induced adverse aortic remodeling because of the potential involvement of hypertension. As such, we used a PE-induced hypertension model and cultured murine and human aortic SMCs to demonstrate the specific susceptibility of APLN-deficient VSMCs to the pathological effects of Ang II.

Therapeutic supplementation with our stable APLN analog exhibited protective effects against AAA formation and upregulated ACE2 which promotes vascular protective remodeling. Indeed, decreased ACE2 in the Apln−/− arteries without affecting passive elasticity and constrictive response to the α-adrenergic agonist PE. The Ang II-induced atherosclerosis was potentiated in Apln−/− arteries without affecting passive elasticity and constrictive response to the α-adrenergic agonist PE. Indeed, Ang II-induced greater hypertension in Apln−/− mice compared with WT mice; however, this finding also poses a complexity in understanding the role of APLN in Ang II-induced adverse aortic remodeling because of the potential involvement of hypertension. As such, we used a PE-induced hypertension model and cultured murine and human aortic SMCs to demonstrate the specific susceptibility of APLN-deficient VSMCs to the pathological effects of Ang II.

Enhancing APLN action offers promising therapeutic effects on the aorta. We show that cleavage of APLN-17 by NEP completely inactivates this peptide, and the marked increase in mitotic calcium uniporter complex is protected from Ang II-induced apoptosis. The Ang II-induced vasoconstriction was potentiated in Apln−/−/y mice on a HFD that received Veh (saline) or Ang II for 4 wk (1.5 mg/kg/d), or Ang II−/− mice. (A) Representative ultrasound images of the AA and averaged measurement for AA diameter during systole and diastole, and aortic expansion index, a measure of aortic wall compliance; n = 6/group. (B) Representative images of immunostaining for AA sections for calponin (SMC, red), TUNEL (green), DAPI (blue), and elastin fibers autofluorescence (green) in the indicated groups. Averaged quantification for calponin levels (measure of viable SMCs), and apoptotic cells (TUNEL positive) for each group is shown on the right; n = 4/group; arrows point to apoptotic cells. (D) Western blots for cleaved and total caspase 3 and averaged cleaved-to-total ratio for caspase 3; n = 4/group. *P < 0.05 compared with the Veh group; **P < 0.05 compared with the Ang II group.

Fig. 8. NEP resistant APLN analog prevents Ang II-induced formation of AAA. (A) Survival curves showing the rate of mortality due to aortic rupture in Ldlr−/− mice on a HFD that received Veh (saline) or Ang II for 4 wk (1.5 mg/kg/d), or Ang II−/− mice. Mortality only presented in the HFD-Ang II group and is significantly higher than in other groups; n = 15/group. (B) Representative ultrasound images of the AA and averaged measurement for AA diameter during systole and diastole, and aortic expansion index, a measure of aortic wall compliance; n = 6/group. (C) Representative images of immunostaining for AA sections for calponin (SMC, red), TUNEL (green), DAPI (blue), and elastin fibers autofluorescence (green) in the indicated groups. Averaged quantification for calponin levels (measure of viable SMCs), and apoptotic cells (TUNEL positive) for each group is shown on the right; n = 4/group; arrows point to apoptotic cells. (D) Western blots for cleaved and total caspase 3 and averaged cleaved-to-total ratio for caspase 3; n = 4/group. *P < 0.05 compared with the Veh group; **P < 0.05 compared with the Ang II group.

(11), and direct antagonism of the Ang II/Ang II type 1 receptor (10) highlights a key role of endothelial homeostasis as a critical pathway protecting the aorta from AAA formation (29). Ang II increases vascular tone, and excessive activation causes systemic hypertension, which is a major risk factor for AAA, atherosclerosis, and cardiac hypertrophy. The Ang II-induced atherosclerosis was potentiated in Apln−/− arteries without affecting passive elasticity and constrictive response to the α-adrenergic agonist PE. Indeed, Ang II-induced greater hypertension in Apln−/− mice compared with WT mice; however, this finding also poses a complexity in understanding the role of APLN in Ang II-induced adverse aortic remodeling because of the potential involvement of hypertension. As such, we used a PE-induced hypertension model and cultured murine and human aortic SMCs to demonstrate the specific susceptibility of APLN-deficient VSMCs to the pathological effects of Ang II.

Therapeutic supplementation with our stable APLN analog exhibited protective effects against AAA formation and upregulated ACE2 which promotes vascular protective remodeling. Indeed, decreased ACE2 in the Apln−/− mesenteric artery could contribute to the increased sensitivity of these mice to Ang II-induced AAA which highlights the vasculoprotective effect of Ang II. Basal ACE2 levels were lowered in the Apln−/− aorta compared with WT and failed to increase in response to Ang II. As such, the Ang II-mediated up-regulation of APLN in WT mice, which, in turn, up-regulates ACE2 leading to the conversion of Ang II into the protective Ang 1–7 peptide (5, 30) represents a critical negative feedback mechanism to confer vascular protection. The beneficial effects of APLN extend beyond the ACE2 pathway since Ang II infusion in Acc2−/− mice does not recapitulate the severe phenotype observed in the Apln−/− mice. Indeed, we identified a unique susceptibility of the APLN-deficient VSMCs to Ang II-mediated apoptotic cell death. Apln-deficiency reduced Ang II-mediated phosphorylation of Akt and Erk1/2 in the aorta consistent with the ability of the APLN peptide to activate a classic G protein coupled receptor leading to PI3 kinase activation and phosphorylation of Akt and Erk1/2 pathways (6, 14, 31).

Enhancing APLN action offers promising therapeutic effects on the aorta. We show that cleavage of APLN-17 by NEP completely inactivates this peptide, and the marked increase in NEP in a human aorta with AAA is likely a key mechanism of the progression of AAA. Computational modeling of the interaction between NEP and APLN-17 showed that the catalytic interaction between NEP and APLN-17 showed that the catalytic residues that promote the cleavage of the peptide, and other active site residues that assist APLN-17 binding are situated in the C-terminal region of the enzyme which implicates a domain specific enzyme catalysis. MICU2, a regulatory subunit of the mitochondrial calcium uniporter complex is protected from Ang II-mediated injury to the abdominal aorta associated with a marked up-regulation of Apln expression (20), whereas APLN also mediates protective effects in atherosclerosis (10) consistent
with a vascular protective effect of APLN peptides. Our study clearly defines the APLN pathway as a central node in the pathogenesis of AAAs and the therapeutic strategy of enhancing the APLN pathway in treating AA. Enhancing APLN improves metabolic function and prevents sarcopenia and aging-related loss in muscle function (8), protects the failing heart (9, 32, 33) and pulmonary vasculature in patients with pulmonary arterial hypertension (7), and, as such, APLN analogs may confer unique therapeutic effects beyond AAAs.

Materials and Methods

All animal experiments were carried out in accordance with the Canadian Council on Animal Care Guidelines, and animal protocols were reviewed and approved by the Animal Care and Use Committee at the University of Alberta. Diseased and nondiseased human abdominal aortic specimens were collected at the University of Rochester, NY. Written consent was obtained from all participants, and our study was approved by the University of Rochester, Research Subjects Review Board. Ascending thoracic aorta from patients with bicuspid aortic valve, aortic dilation, and nondiseased aorta were collected as described before (34, 35). Materials and experimental procedures for animal models and protocols, peptide analysis and metabolism, RNA isolation, Taqman PCR, cell culture, tissue and cellular staining and immunofluorescence, flow cytometry, ultrasonic vasculography, vascular myography, blood pressure measurement, computer modeling, receptor binding, protein isolation, Western blotting, and quantification and statistical analysis are described in SI Appendix, SI Materials and Methods.

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Fig. 9. Up-regulation of ACE2 by APLN analog: role of APLN in AAA pathogenesis. (A) Immunostaining for ACE2 and quantification in the AA of Ang II−/− on HFD receiving Veh, Ang II, or Ang II + APLN-NMeLeu9-A2; n = 4/group/genotype. (B) Western blots and quantification for ACE2 levels in abdominal aorta of Ang II−/− on HFD mice receiving Veh, Ang II + placebo, or Ang II + APLN-NMeLeu9-A2. Aortic proteins from Ang II−/− mice were used as a negative control; n = 4/group/genotype. (C) DHE-based fluorescence with (D) NADPH oxidase activity in cultured human primary aortic SMCs in response to Ang II and effects of APLN-NMeLeu9-A2; n = 6/group. (E) Schematic showing the interaction among APLN, ACE2, and NEP in a pathological setting driving the formation of AAA. *P < 0.05 compared with the placebo group. A.U., arbitrary units. Averaged values represent mean ± SEM.
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