Research Article

One amino acid change of Angiotensin II diminishes its effects on abdominal aortic aneurysm

Ya Wang1, Yinchuan Xu1, Congqing Wu2, Hongguang Xia2, Yingchao Wang1, Jinliang Nan1, Jinghai Chen1, Hong Yu1, Wei Zhu1, Peng Shi1, Alan Daugherty2,4, Hong S. Lu2,4 and Jian’an Wang1

1Department of Cardiology, The Second Affiliated Hospital, Cardiovascular Key Laboratory of Zhejiang Province, College of Medicine, Zhejiang University Hangzhou, Zhejiang, China; 2Saha Cardiovascular Research Center, University of Kentucky, Lexington, KY, U.S.A.; 3Department of Biochemistry and Molecular Biology, Zhejiang University College of Medicine, Hangzhou, Zhejiang, China; 4Department of Physiology, University of Kentucky, Lexington, KY, U.S.A.

Correspondence: Jian’an Wang (wangjianan111@zju.edu.cn)

Angiotensin (Ang) A is formed by the decarboxylation of the N terminal residue of AngII. The present study determined whether this one amino acid change impacted effects of AngII on abdominal aortic aneurysm (AAA) formation in mice. Computational analyses implicated that AngA had comparable binding affinity to both AngII type 1 and 2 receptors as AngII. To compare effects of these two octapeptides in vivo, male low-density lipoprotein receptor (Ldlr) or apolipoprotein E (Apoe) deficient mice were infused with either AngII or AngA (1 μg/kg/min) for 4 weeks. While AngII infusion induced AAA consistently in both mouse strains, the equivalent infusion rate of AngA did not lead to AAA formation. We also determined whether co-infusion of AngA would influence AngII-induced aortic aneurysm formation in male Apoe−/− mice. Co-infusion of the same infusion rate of AngII and AngA did not change AngII-induced AAA formation. Since it was reported that a 10-fold higher concentration of AngA elicited comparable vasoconstrictive responses as AngII, we compared a 10-fold higher rate (10 μg/kg/min) of AngA infusion into male Apoe−/− mice with AngII (1 μg/kg/min). This rate of AngA led to abdominal aortic dilation in three of ten mice, but no aortic rupture, whereas the 10-fold lower rate of AngII infusion led to abdominal aortic dilation or rupture in eight of ten mice. In conclusion, AngA, despite only being one amino acid different from AngII, has diminished effects on aortic aneurysmal formation, implicating that the first amino acid of AngII has important pathophysiological functions.

Introduction

The renin-angiotensin system (RAS) plays critical roles in many physiological and pathophysiological functions [1,2]. The major bioactive peptide, angiotensin (Ang)II, exerts its effects through activation of AngII type 1 (AT1) and type 2 (AT2) receptors, with AT1 receptor being the major receptor mediating aortic aneurysmal formation [3–5]. Subcutaneous infusion of AngII at rates of 0.5–2.5 μg/kg/min in mice leads to abdominal aortic aneurysm (AAA) formation, as demonstrated by many investigators [6–10].

We chose to investigate AngA because AngA is an endogenous octapeptide found in human plasma by Jankowski and colleagues, although its concentration is much lower than AngII [11]. However, knowledge about the effect of AngA in physiological and pathological processes is very limited. The sequence of AngII is Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, whereas the aspartate of AngII was replaced by alanine in AngA [11]. Previous studies suggest that higher dose of AngA has a similar vasoconstrictive activity as AngII through AT1 receptor as demonstrated by in vitro study or a bolus injection of AngA [11–14]. Since AT1 receptor activation plays a critical role in AngII-mediated aortic aneurysm formation [4,15]. The present study determined whether AngA would have an equivalent effect as AngII on aortic aneurysm formation.
Materials and methods

Mice and diets

Twenty male low-density lipoprotein receptor deficient (Ldlr−/−) mice were purchased from The Jackson Laboratory (Stock #2207; Bar Harbor, ME, U.S.A.) and maintained in individually vented cages (maximally five mice/cage) on a light : dark cycle of 14:10 h. The cage bedding was Teklad Sani-Chip bedding (Cat # 7090A; Harlan Teklad; Madison, WI, U.S.A.). Mice were fed a normal rodent laboratory diet (Diet # 2918, Harlan Teklad; Madison, WI, U.S.A.) and given drinking water from a reverse osmosis system ad libitum. During experiments, Ldlr−/− mice were fed a Western diet (Diet # TD.88137; Harlan Teklad) containing 21% wt/wt (equals 42% calories/calories) saturated fat extracted from milk and 0.2% (wt/wt) cholesterol (0.15% supplemented, and 0.05% from the fat source) 1 week prior to and during 4 weeks of AngII or AngA infusion. Seventy male Apoe−/− mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Mice were housed at a density of fewer than five per cage with a light:dark cycle of 12:12 h at the Department of Cardiology, Second Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, P.R. China. These mice were fed a normal rodent laboratory diet and given free access to water during the study. All procedures were performed with the approval of the University of Kentucky (IACUC protocol #2006-0009) or Zhejiang Institutional Animal Care and Use Committee.

Osmotic mini-pump implantation

AngII (Cat# H-1705) and AngA (Cat# H-6498) were purchased from Bachem. They were infused subcutaneously via Alzet osmotic pumps (Model 2004 or 1004; Durect Corporation, Cupertino, CA, U.S.A.) as described previously [16]. AngII or AngA was dissolved in sterile saline. Mice were sedated with isoflurane and pumps were implanted subcutaneously on the right flank of each mouse. Surgical staples were used to close the incision site immediately.

Necropsy

Necropsies were performed for mice died prior to the termination of each study. Aortic rupture was defined as observation of blood clots in either the thoracic cavity (thoracic aortic rupture) or retroperitoneal cavity (abdominal aortic rupture).

Quantification of abdominal aortic aneurysms

At termination, after blood collection, right atrium was cut open, and saline was perfused through the left ventricle to remove blood from the systemic circulation. Subsequently, aortas were dissected and placed in 4% paraformaldehyde (Sinoparmac Chemical Reagent Co., Ltd.) overnight at room temperature. After fixation, periaortic adventitia was carefully removed thoroughly. Maximal outer diameter of the suprarenal aorta was measured ex vivo as a parameter for AAA quantification using Image-Pro software (Media Cybernetics Inc.). Definition of an AAA includes (1) aortic rupture of the abdominal aortic region, and (2) maximal outer diameter is at least 1.5 times of the mean maximal outer diameter of mice infused with saline or greater than 1.35 mm if saline group was not available.

Statistical analyses

Statistical analyses were conducted using SigmaPlot software version 12.5 (Systat Software, Inc.). Comparisons between two groups were performed using unpaired two-tailed Student’s t-test for normally distributed variables with equal variance and Mann–Whitney rank sum test for data that did not pass normality test. AAA incidence between groups was analyzed using Fisher Exact test. The P<0.05 was considered statistically significant.

Results

Equivalent rate of AngA infusion to AngII infusion did not induce aortic aneurysm formation in two hypercholesterolemic mouse models

There are reports that AngA and AngII had similar affinity to AT1 receptor [11,12]. Our computational analysis also implicated that AngA bound to AT1 and AT2 receptors (Supplementary Figure SI in the online-only Data Supplement). To compare effects of the two octapeptides in vivo, we infused AngA versus AngII in either Ldlr or Apoe deficient mice at a rate of 1 μg/kg/min for 4 weeks. Six of ten mice (60%) had AAA formation in AngII-infused Ldlr−/− mice. Four mice died of aortic rupture and five of the remaining six mice had AAA formation (AAA incidence: 90%) in AngII-infused Apoe−/− mice. An equivalent rate of AngA infusion did not lead to aortic rupture or AAA formation in either Ldlr−/− or Apoe−/− mice (Figure 1A–D and Supplementary Figures SII and III in the online-only Data Supplement).
Figure 1. Equivalent rates of AngA infusion to AngII infusion did not influence an AAA in both Ldlr−/− and Apoe−/− mouse models

Male Ldlr−/− mice fed a Western diet (A and B) or Apoe−/− mice fed a normal diet (C–F) were infused with AngII (1 μg/kg/min), AngA (1 μg/kg/min), or both AngA and AngII (1 μg/kg/min, respectively) for 4 weeks. Incidence of AAA was defined by calculating ratio (%) of abdominal aortic rupture and AAA formation in each group (A, C, and E) and analyzed by Fisher exact test. Maximum widths of suprarenal aortas (B, D, and F) were measured on ex vivo images. Triangles represent values of individual mice (N = 6–10/group). Lines in boxes represent medians, and the boxes span the 25th–75th percentiles, with the bars representing the 5th and 95th percentiles, respectively. Maximal width data were analyzed using Mann–Whitney rank sum test.

There are reports implicating that AngA had AngII-antagonizing effects [11]. We then determined whether AngA would attenuate AngII-induced AAA by infusing AngII alone or co-infusing AngII and AngA at the same rate (1 μg/kg/min) for 4 weeks in male Apoe−/− mice. Two mice from AngII infusion group died of aortic rupture, one mouse died of aortic rupture in co-infusion group, and the incidence of abdominal aortic aneurysm formation were not significantly different between groups (Figure 1E,F and Supplementary Figure SIV in the online-only Data Supplement).

A high-rate of AngA infusion had modest effects on aortic aneurysm formation in hypercholesterolemic mouse model

It was reported that a 10-fold higher concentration of AngA had similar vasoconstrictive effects as AngII [11,12]. Therefore, we compared effects of AngA (10 μg/kg/min) infusion with AngII (1 μg/kg/min) infusion on AAA formation in male Apoe−/− mice. AngII infusion led to aortic rupture in two of ten mice, and six of the remaining eight
Figure 2. A high rate of AngA infusion had modest effects on aortic aneurysm formation in Apoe<sup>−/−</sup> mouse model

Male Apoe<sup>−/−</sup> mice fed a normal diet were infused with AngII (1 μg/kg/min) or AngA (10 μg/kg/min) for 4 weeks. (A) Ex vivo aortic images of whole aortas. (B) Incidence of AAA was defined by calculating ratio (%) of abdominal aortic rupture and AAA formation and analyzed by Fisher exact test. (C) Maximum widths of suprarenal aortas were measured on ex vivo images. Triangles represent values of individual mice (N = 8–10/group). Lines in boxes represent medians, and the boxes span the 25th–75th percentiles, with the bars representing the 5th and 95th percentiles, respectively. Data were analyzed by Mann–Whitney rank sum test.

Discussion

In the present study, we found that infusion of AngA with the same rate of AngII did not induce AAA in the two commonly used hypercholesterolemic mouse models. A 10-fold infusion rate of AngA (10 μg/kg/min) led to AAA formation, but with a much lower incidence than AngII at 1 μg/kg/min. On the basis of low plasma ratio of AngA/AngII (~0.2 in normal subjects and up to approximately 0.8 in patients with end stage of chronic kidney disease), we hypothesize that the pathophysiological processes of AngII decreased after decarboxylation of the Asp [1] to Ala [1] in vivo.

Jankowski and colleagues reported that AngA and AngII had similar binding affinity to AT1 receptor in cultured cells [11]. Our computational analysis (Supplementary Figure S1) also predicts that AngA binds to AT1 receptor, which is comparable to AngII binding to AT1 receptor. However, AngA had much less effects on AAA induction, even when its infusion rate was 10-folds of AngII (Figure 2B). This finding implicates that AngA might have much
lower affinity to AT1 receptor, although the mechanism is unclear. Indeed, there are also reports that the dose of AngA required to achieve comparable vasoconstrictive effect as AngII was ten times higher than AngII [11,12]. Our future study will aim to define whether the less effect of AngA on AAA is mediated through its lower affinity with AT1 receptor.

Jankowski and colleagues also found that AngA had higher affinity to AT2 receptors than to AngII [11]. Our previous studies have demonstrated that genetic deficiency of AT2 receptor has no effects on AngII-induced AAA development [5]. Hence, the present study does not rule out that AngA may have physiological and pathophysiological functions by binding to AT2 receptor. However, AT2 receptor is abundant during fetal development, but diminishes largely in adults. Therefore, potential physiological or pathophysiological effects of AngA through its binding to AT2 receptor might be modest. AngA is the direct precursor of alamandine, a newly identified member of the RAS, which has several physiological actions that resemble Ang-(1-7), but antagonizes effects of AngII, such as antihypertensive and vasodilation [17]. There is no direct evidence that alamandine or Ang-(1-7) protects against AngII-induced AAA. Taken together, diminished effects of AngII on AAA formation by replacing its first amino acid from aspartate to alanine cannot be explained by its interaction with AT2 receptor or its downstream product alamandine. It remains unclear why a single amino acid change on a not conserved position of AngII would diminish its pathophysiological effects on AAA. It is possible that decarboxylation of the first amino acid aspartate changes the structure of AngII, decreasing the intrinsic activity of binding to AT1 receptor, thereby reducing the harmful effects to cardiovascular system.

The development and progression of AAA involve activation of the RAS [18–20]. One significant impact of our study is the potential to prevent or treat AAA. If AngII can be converted to AngA in vivo by enzymatic manipulation, it is possible to prevent or even treat AAA.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

Funding
The research work reported in this manuscript was supported by the National Basic Research Program of China [973 Program, grant number 2014CB965103 (to J.W.)]. Grants from National Natural Science Foundation of China [grant number 81320108003, 31371498 (to J.W.)]; Grants from National Key Research and Development Program of China [grant number 81500876 (to Y.C.X.)]; and Grants from Natural Science Foundation of Zhejiang Province [grant number LQ16H020003 (to Y.C.X.)]. Ldlr−/− mouse study was supported by NIH grants of the United States [HL133723 and HL139748 (to A.D. and H.S.L.)].

Author Contribution
Y.W. performed most experiments. Y.X. and C.W. performed the experiment using LDL receptor −/− mice and edited the manuscript. Y.W. and Y.X. contributed to data analysis and manuscript drafting. H.L. and J.W. designed the experiments, interpreted the data, and edited the manuscript. All other authors collected some of the data and contributed to the critical revision of the manuscript. All the authors read and approved the final manuscript.

Abbreviations
AAA, abdominal aortic aneurysm; Ang, Angiotensin; Apoe, apolipoprotein E; AT1, AngII type 1; Ldlr, low-density lipoprotein receptor; RAS, renin-angiotensin system.

References
1 Unger, T., Paulis, L. and Sica, D.A. (2011) Therapeutic perspectives in hypertension: novel means for renin-angiotensin-aldosterone system modulation and emerging device-based approaches. Eur. Heart J. 32, 2739–2747, https://doi.org/10.1093/eurheartj/ehr253
2 Simko, F., Simko, J. and Fabryova, M. (2003) ACE-inhibition and angiotensin II receptor blockers in chronic heart failure: pathophysiological consideration of the unresolved battle. Cardiovasc. Drugs Ther. 17, 287–290, https://doi.org/10.1023/A:1026215712983
3 Cassis, L.A., Rateri, D.L., Lu, H. and Daugherty, A. (2007) Bone marrow transplantation reveals that recipient AT1a receptors are required to initiate angiotensin II-induced atherosclerosis and aneurysms. Arterioscler. Thromb. Vasc. Biol. 27, 380–386, https://doi.org/10.1161/01.ATV.0000254680.71485.92
4 Poduri, A., Owens, 3rd, A.P., Howatt, D.A., Moorleghen, J.J., Balakrishnan, A., Cassis, L.A. et al. (2012) Regional variation in aortic AT1b receptor mRNA abundance is associated with contractility but unrelated to atherosclerosis and aortic aneurysms. PLoS ONE 7, e48462, https://doi.org/10.1371/journal.pone.0048462
5 Daugherty, A., Rateri, D.L., Howatt, D.A., Chernigo, R. and Cassis, L.A. (2013) PD123319 augments angiotensin II-induced abdominal aortic aneurysms through an AT2 receptor-independent mechanism. PLoS ONE 8, e61849, https://doi.org/10.1371/journal.pone.0061849

© 2019 The Author(s). This is an open access article published by Portland Press Limited on behalf of the Biochemical Society and distributed under the Creative Commons Attribution License 4.0 (CC BY).
6 Daugherty, A., Manning, M.W. and Cassis, L.A. (2000) Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. J. Clin. Invest. 105, 1605–1612, https://doi.org/10.1172/JCI7818

7 Daugherty, A., Rateri, D.L., Charo, I.F., Owens, A.P., Howatt, D.A. and Cassis, L.A. (2010) Angiotensin II infusion promotes ascending aortic aneurysms: attenuation by CCR2 deficiency in apoE-/- mice. Clin. Sci. 118, 681–689, https://doi.org/10.1042/CS20090372

8 Liu, J., Lu, H., Howatt, D.A., Balakrishnan, A., Moorleghen, J.J., Sorci-Thomas, M. et al. (2015) Associations of ApoAI and ApoB-containing lipoproteins with AngII-induced abdominal aortic aneurysms in mice. Arterioscler. Thromb. Vasc. Biol. 35, 1826–1834, https://doi.org/10.1161/ATVBAHA.115.305482

9 Lysgaard Poulsen, J., Stubbe, J. and Lindholt, J.S. (2016) Animal models used to explore abdominal aortic aneurysms: a systematic review. Eur. J. Vasc. Endovasc. Surg. 52, 487–499, https://doi.org/10.1016/j.ejvs.2016.07.004

10 Lu, H., Howatt, D.A., Balakrishnan, A., Moorleghen, J.J., Rateri, D.L., Cassis, L.A. et al. (2015) Subcutaneous angiotensin II infusion using osmotic pumps induces aortic aneurysms in mice. J. Vis. Exp. 103, https://doi.org/10.3791/53191

11 Jankowski, V., Vanholder, R., van der Giet, M., Tolle, M., Karadogan, S., Gobom, J. et al. (2007) Mass-spectrometric identification of a novel angiotensin peptide in human plasma. Arterioscler. Thromb. Vasc. Biol. 27, 297–302, https://doi.org/10.1161/01.ATV.0000253889.09765.5f

12 Yang, R., Smolders, I., Vanderheyden, P., Demaegdt, H., Van Eeckhaut, A., Vauquelin, G. et al. (2011) Pressor and renal hemodynamic effects of the novel angiotensin A peptide are angiotensin II type 1A receptor dependent. Hypertension 57, 956–964, https://doi.org/10.1161/HYPERTENSIONAHA.110.161836

13 Habiyakare, B., Alsaadon, H., Mathai, M.L., Hayes, A. and Zulli, A. (2014) Reduction of angiotensin A and alamandine vasoactivity in the rabbit model of atherogenesis: differential effects of alamandine and Ang(1-7). Int. J. Exp. Pathol. 95, 290–295, https://doi.org/10.1111/iexp.12087

14 Coutinho, D.C., Foureaux, G., Rodrigues, K.D., Salles, R.L., Moraes, P.L., Murca, T.M. et al. (2014) Cardiovascular effects of angiotensin A: a novel peptide of the renin-angiotensin system. J. Renin Angiotensin Aldosterone Syst. 15, 480–486, https://doi.org/10.1177/1470320312474856

15 Rateri, D.L., Moorleghen, J.J., Balakrishnan, A., Owens, 3rd, A.P., Howatt, D.A., Subramanian, V. et al. (2011) Endothelial cell-specific deficiency of AngII type 1a receptors attenuates AngII-induced ascending aortic aneurysms in LDL receptor-/- mice. Circ. Res. 108, 574–581, https://doi.org/10.1161/CIRCRESAHA.110.222844

16 Lu, H., Howatt, D.A., Balakrishnan, A., Moorleghen, J.J., Rateri, D.L., Cassis, L.A. et al. (2015) Subcutaneous angiotensin II infusion using osmotic pumps induces aortic aneurysms in mice. J. Vis. Exp., https://doi.org/10.3791/53191

17 Lautner, R.Q., Villela, D.C., Fraga-Silva, R., Silva, N., Verano-Braga, T., Costa-Fraga, F. et al. (2013) Discovery and characterization of alamandine: a novel component of the renin-angiotensin system. J. Renin Angiotensin Aldosterone Syst. 15, 480–486, https://doi.org/10.1177/1470320312474856

18 Moran, C.S., Biros, E., Krishna, S.M., Wang, Y., Tikellis, C., Morton, S.K. et al. (2017) Resveratrol inhibits growth of experimental abdominal aortic aneurysm associated with upregulation of angiotensin-converting enzyme 2. Arterioscler. Thromb. Vasc. Biol. 37, 2195–2203, https://doi.org/10.1161/ATVBAHA.117.310129

19 Phie, J., Moxon, J.V., Krishna, S.M., Kinobe, R., Morton, S.K. and Golledge, J. (2018) A diet enriched with tree nuts reduces severity of atherosclerosis but not abdominal aneurysm in angiotensin II-infused apolipoprotein E deficient mice. Atherosclerosis 277, 28–33, https://doi.org/10.1016/j.atherosclerosis.2018.08.004

20 Weiss, D., Kools, J.J. and Taylor, W.R. (2001) Angiotensin II-induced hypertension accelerates the development of atherosclerosis in apoE-deficient mice. Circulation 103, 448–454, https://doi.org/10.1161/01.CIR.103.3.448