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Expression levels of cathepsin L and cystatin C in a hyperglycemic environment were associated with aortic aneurysm development in a mouse model

Yang Liu¹*, Yang Jiao²*, Yuxiang He³, Xiangjiu Ding¹, Qingbo Su¹, Yan Zhao¹ and Jianjun Jiang¹

Abstract

Objectives: Diabetes mellitus (DM) attenuates the development of aortic aneurysms (AA). This study investigated the expression of cathepsin L and cystatin C in a hyperglycemic environment, and the influence of these proteins on AA development.

Methods: Mice were divided into AA and DM+AA groups (n=30 per group). DM was induced by injection of streptozotocin; AA was induced by injection of angiotensin II. Doppler examination was used to measure aortic diameter, and Weigert's elastic stain was used to detect elastin degradation. Cathepsin L and cystatin C in aortic tissue were examined by western blotting, immunohistochemistry, and polymerase chain reaction.

Results: Aortic diameter in the DM+AA group was less than that in the AA group, and elastin fragmentation grade of the aortic wall was reduced in the DM+AA group. More cathepsin L-positive cells were observed in the AA group than in the DM+AA group; conversely, more cystatin C-positive cells were observed in the DM+AA group than in the AA group. Both protein and mRNA levels of cathepsin L and cystatin C showed similar trends to those observed in immunohistochemistry.

¹Department of Vascular Surgery, Qilu Hospital of Shandong University, Jinan, Shandong, China
²Department of General Surgery, Qilu Hospital of Shandong University (Qingdao), Qingdao, Shandong, China
³Department of Vascular Surgery, Shandong Provincial Hospital affiliated to Shandong University, Jinan, Shandong, China

*These authors contributed equally to this work.

Corresponding author:
Jianjun Jiang, Department of Vascular Surgery, Qilu Hospital of Shandong University, No. 107, Road Wen Hua Xi, Jinan, Shandong 250012, China.
Email: jiangjianjun1995@163.com
Conclusions: Expression levels of cathepsin L and cystatin C in a hyperglycemic environment were associated with AA development in a mouse model.

Keywords
Hyperglycemia, cathepsin L, cystatin C, aortic aneurysm, mice, elastin, streptozocin, angiotensin II, diabetes mellitus

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Introduction
Diabetes mellitus (DM) is one of the strongest risk factors for the development of cardiovascular disease; however, epidemiologic evidence suggests a protective role for DM against the development of aortic aneurysm (AA).\(^1\) Diabetes-related suppression of matrix metalloproteinases (MMPs) has been shown to alleviate experimental development of AA.\(^2\) However, the clinical use of MMP inhibitors alone could not attenuate AA development,\(^3\) which suggests that MMPs may not be the sole tissue matrix-degrading enzymes suppressed by DM.

Cathepsins, a class of cysteine proteases, have been shown to play important roles in AAs in humans; overexpression of cathepsins L, S, K, and V in plasma, as well as deficiency in cystatin C, a cysteine protease inhibitor, have been associated with the development and progression of atherosclerosis and AAs in humans.\(^4\) Cathepsin L has been shown to participate in extracellular matrix remodeling by strong elastolytic and collagenolytic activities. High glucose reduces cathepsin L activity and gene expression in skeletal muscle and macrophages from mice with type 2 DM.\(^5\) Moreover, plasma cystatin C may be associated with the incidence of type 2 DM.\(^6\) However, the roles of cathepsin L and cystatin C in a hyperglycemic environment, in the context of an experimental AA model, are unclear. Therefore, we hypothesized that the expression of cathepsin L and cystatin C might be modulated by exposure to a hyperglycemic environment, thereby attenuating the development of AA. This study used a mouse model of AA, which could provide a new avenue for further research regarding the interaction between DM and AA.

Materials and methods

Animals and ethical approval
ApoE\(^{-/-}\) C57BL/6 mice (male, 8 to 10 weeks of age, weighing 20–30 g) were provided by Shandong University Animal Center and randomly divided into the AA group (\(n = 30\)) and DM+AA group (\(n = 30\)). Animal care and experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.\(^7\) The experimental protocol was approved by the Ethical Review Board of Shandong University.

Animal model
Hyperglycemia in mice of the DM+AA group was induced by intraperitoneal injections of streptozotocin (STZ: 50 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) dissolved in citrate buffer (1 injection per day for 5 consecutive days), as specified by the Animal Models of Diabetic Complications Consortium protocol.\(^8\) Mice in the AA group underwent the same intraperitoneal
injection protocol, in which they received citrate buffer without STZ. Serum glucose level and body weight measurements were monitored for at least 3 weeks after STZ injection. Glucose level was measured in tail vein blood by using a portable blood glucose meter (SelectSimple, Johnson & Johnson Corp., New Brunswick, NJ, USA). Hyperglycemia (>16.7 mmol/L) was confirmed before induction of the AA model and maintained until the end of the experiment. Induction of AA was performed at 3 weeks after STZ or buffer injection. Angiotensin II (Ang II: 1000 ng/kg/min; Sigma-Aldrich) was administered subcutaneously by Alzet osmotic minipumps (model 2004; ALZA Scientific Products, Inc., Vacaville, CA, USA) continuously for 28 days. Mice were sacrificed after 28 days of Ang II infusion and aortic tissue was harvested. The entire length of aortic tissue between the origin of the thoracic artery and the point of iliac artery bifurcation was harvested; only the AA lesion with maximum diameter was examined in this study.

**Aortic diameter and elastin fragmentation**

Maximum aortic diameter was measured in each mouse before AA induction and prior to sacrifice via transabdominal ultrasound imaging at 40 MHz (Vevo770; Visualsonics, Toronto, Canada). All examiners were blinded to the study group assignment. Weigert’s elastic stain was used to analyze elastin fragmentation in accordance with the manufacturer’s instructions (Solarbio, Beijing, China). Aortic wall elastin fragmentation was graded based on the degree of elastin filament breaks.

**Immunohistochemistry analysis**

Samples of aneurysms were fixed in 4% paraformaldehyde overnight, then embedded in paraffin and cut into 5-μm sections. In accordance with the streptavidin-peroxidase method (Zhongshan Biotechnology Inc., Beijing, China), endogenous peroxidase activity was blocked by using 3% hydrogen peroxide for 15 minutes. Sections were incubated with anti-cystatin C antibody (1:500; Abcam, Cambridge, UK) or anti-cathepsin L antibody (1:2000; Abcam) at 4°C overnight, then with secondary HRP-conjugated antibody (Abcam) for 30 minutes at room temperature. Stained specimens were exposed to 3, 3-diaminobenzidine (Abcam) and counterstained with hematoxylin. Primary antibodies were replaced with phosphate-buffered saline for negative control. Numbers of positive cells were counted by two independent observers.

**Western blotting**

Aortic tissues were extracted in a lysis buffer in the presence of protease inhibitor cocktail (Beyotime, Shanghai, China). Protein samples were quantified with the Bio-Rad Dc protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein extracts (30 μg/lane) were separated on a 12% SDS–PAGE gel, transferred to nitrocellulose membrane, and probed with anti-cystatin C antibody (1:10,000; Abcam) and anti-cathepsin L antibody (1:1000; Abcam). Immunoblotting of a housekeeping protein, β-actin, was performed to assure equal protein loading. Detection and quantification were performed using the LI-COR Odyssey Infrared imaging system (Lincoln, NE, USA).

**Real-time polymerase chain reaction**

Total RNA was extracted from aortic samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Equal amounts (1 μg) of RNA were reverse transcribed using the M-MLV RTase cDNA Synthesis Kit (Takara, Shiga, Japan). Real-time polymerase chain reaction (PCR) was
performed with SYBR-Green PCR Master Mix (Toyobo, Osaka, Japan) using the ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). β-actin was used as an internal control. The comparative Ct method (2^(-ΔΔCt)) was used to analyze relative gene expression. The primers for qRT-PCR were as follows: cystatin C (152 bp), forward, 5'-CCATGACCAGCCCCATCTGAT-3', and reverse, 5'-CACAAGTAAAGGAGCAGTCTGC-3'; cathepsin L (66 bp), forward, 5'-CAATAAGTAATAATATTGGCTTGTCA-3', reverse, 5'-TGATGCTCCTTCCATACCCCATT-3'; and β-actin (141 bp), forward, 5'-GACAGGATGCAGAA GGAGATTACT-3', reverse, 5'-TGAT CCACATCTGCTGGAAGGT-3'.

Statistical analysis
Results are presented as mean ± standard deviation and were analyzed by Student’s t-test using SPSS software (version 20.0, SPSS, Inc., Chicago, IL, USA). The aneurysm induction rate in the two groups was compared by using the χ² test. P < 0.05 was considered to be statistically significant.

Results
Plasma glucose levels in the DM+AA group increased significantly, compared with levels in the AA group, both before Ang II infusion (21.5 ± 0.8 mmol/L vs. 8.1 ± 0.6 mmol/L, p < 0.05) and before sacrifice (27.8 ± 1.0 mmol/L vs. 8.4 ± 0.5 mmol/L, p < 0.05). AAs were successfully established in both groups; most occurred in the thoracoabdominal aorta or infrarenal abdominal aorta (Figure 1A, B). There were no significant differences in aortic diameters between groups before Ang II perfusion (P < 0.05, Figure 1C). Hyperglycemia did not influence the aneurysm induction rate (DM+AA: 21/30 mice vs. AA: 19/30 mice). Aortic diameter and elastin degradation decreased in the DM+AA group, compared with that in the AA group. (A) AA was established in 21/30 mice (black rectangle). (B) AA+DM was established in 19/30 mice (black rectangle). (C) There was no difference between groups in baseline aortic diameter (before ANG infusion), whereas the final aortic diameter of the DM+AA group was significantly less than that of AA group (after ANG infusion). (D–F) The elastin fragmentation grade of the aortic wall in the DM+AA group was significantly reduced, compared with that in the AA group. (Original magnification ×400). * = P < 0.05.

AA, aortic aneurysm; ANG, angiotensin II; DM, diabetes mellitus.
vs. AA: 19/30 mice). In the AA group, four mice died of aneurysm rupture and one mouse died of infection; AA was not induced in the remaining four mice. In the DM+AA group, three mice died of aneurysm rupture; AA was not induced in the remaining eight mice. Dead mice were not included in subsequent examinations. Notably, aortic diameter in the DM+AA group was significantly less than that in the AA group (P < 0.05, Figure 1C). Weigert’s elastic stain revealed that the elastin fragmentation grade of the aortic wall in the DM+AA group was significantly reduced, compared with that in the AA group (P < 0.05, Figure 1D–F). Immunohistochemical analysis showed more cathepsin L-positive cells in the AA group than in the DM+AA group (P < 0.05, Figure 1A–C). Conversely, there were more cystatin C-positive cells in the DM+AA group than in the AA group (P < 0.05, Figure 2D–F). Western blotting and real-time PCR also revealed results that were similar to those of immunohistochemistry analyses (P < 0.05, Figure 2G–I): the cathepsin L expression level in the AA group was higher than that in the DM+AA group, while the expression of cystatin C was higher in the DM+AA group than in the AA group.

**Discussion**

Cathepsin L is present in late endosomes and lysosomes, and is activated upon hydrolysis of an inactive zymogen precursor in an acidic environment; AA risk factors, such as smoking and atherosclerosis, can contribute to elevated lysosomal membrane

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**Figure 2.** Expression levels of cathepsin L and cystatin C differed between the AA and DM+AA groups. (A, B) Cathepsin L-positive cells in AA and DM+AA groups (original magnification ×400). (C) More cathepsin L-positive cells were observed in the AA group than in the DM+AA group. (D, E) Cystatin C-positive cells in AA and DM+AA groups (original magnification ×400). (F) More cystatin C-positive cells were observed in the DM+AA group than in the AA group. (G–I) Both protein and mRNA expression levels of cathepsin L were higher in the AA group than in the DM+AA group, while the expression levels of cystatin C were higher in the DM+AA group than in the AA group. * = P < 0.05. AA, aortic aneurysm; DM, diabetes mellitus.
permeability and induction of cathepsin secretion, eventually resulting in vascular endothelial cell damage.\textsuperscript{9} Moreover, Cathepsin L activity can be inhibited by endogenous cystatin C, which is present in nearly all organs; cystatin C can prevent potentially destructive proteolytic enzyme activity.\textsuperscript{9} In human AA, the expression of cathepsin L is elevated in plasma, whereas the expression of cystatin C is reduced in plasma.\textsuperscript{4} In a mouse model, cathepsin L participates in AA pathogenesis through regulation of the following activities: monocyte and T-cell recruitment, vascular wall matrix protein degradation, lesion cell proliferation, protease expression, and angiogenesis.\textsuperscript{10} Importantly, the absence of cystatin C has been shown to result in enlarged AA lesion areas and increased luminal diameters, possibly through enhanced cathepsin activities that promote microvascularization, apoptosis, leukocyte adhesion, and cellular proliferation.\textsuperscript{11} Our study showed similar results. In AA mice, elevated cathepsin L and reduced expression of cystatin C were observed in aortic tissue, which suggested that cathepsin L and cystatin C may contribute to AA pathogenesis.

The expression and activity of cathepsin L are reportedly closely related to the plasma glucose level: the expression of cathepsin L was attenuated in mesangial cells and endothelial progenitor cells cultured with glucose, as well as in experimental diabetic rats.\textsuperscript{12,13} The reduced expression of cathepsin L may be a result of impaired glucose metabolism, rather than the effect of an inherited trait.\textsuperscript{5} However, it has been unclear whether the negative effect of hyperglycemia on cathepsin L activity was sufficient to impact AA pathogenesis. Our study revealed that both protein and mRNA levels of cathepsin L were significantly attenuated in the aortic wall in DM+AA mice. Moreover, aortic diameter in DM+AA mice was less than that in AA mice, which had normal plasma glucose level. Immunohistochemical analysis revealed that the wavy layered structure of elastin was well preserved in the hyperglycemic environment. These results suggested that hyperglycemia attenuated aortic aneurysm pathogenesis, possibly through modulation of cathepsin L expression. However, there was no difference in aneurysm rate between the groups, which revealed that hyperglycemia could attenuate AA, but could not inhibit the factors that contributed to its initial onset.

Previous studies suggested negative correlations between plasma cystatin C levels and both abdominal aortic diameter and aortic expansion rate in humans.\textsuperscript{14} Cathepsin activity was inhibited by cystatin C, which suggests that there is an interaction between cathepsin activity and cystatin C activity that may be important in the development of AA. The inverse expression levels of cathepsin L and cystatin C in both groups in the present study support this hypothesis. Both protein and mRNA levels of cystatin C were significantly elevated in the aortic wall of hyperglycemic mice, which indicates a positive relationship between cystatin C and hyperglycemia. We suspect that hyperglycemia may lead to upregulation of cystatin C expression, and that cystatin C inhibited cathepsin L activity; hyperglycemia may also directly attenuate cathepsin L expression. The mechanism by which hyperglycemia provides a protective effect against AA pathogenesis by modulation of cathepsin L and cystatin C will be the focus of our future studies.

To the best of our knowledge, this is the first report regarding the expression of cathepsin L and cystatin C in experimental AA within a hyperglycemic environment in vivo. It may be useful to explore drugs targeting cathepsin L in clinical treatment of human patients with AA, based on these
results and further research. Moreover, the plasma levels of cathepsin L and cystatin C may be used as meaningful predictors of the development of AA, especially in patients with DM. However, this study had some limitations. Most importantly, the animal model used in this study was more similar to human type 1 (insulin-deficient) diabetes, rather than human type 2 (insulin-resistant) diabetes. Alternative models and methods are needed in the future to clarify the influence of insulin resistance on AA. In addition, inflammatory signaling pathways should be examined to clarify the mechanism by which cathepsin L, cystatin C, and hyperglycemia participate in the pathogenesis of AA.

Conclusion
This study demonstrated that the expression levels of cathepsin L and cystatin C in a hyperglycemic environment were associated with AA development, which might provide a basis for further research regarding the interaction between DM and AA.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.

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ORCID iD
Jianjun Jiang https://orcid.org/0000-0002-6722-1268

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