Knockdown of VAMP8 attenuates atherosclerosis and enhances the effect of simvastatin in APOE-deficient mice

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Sent for review: 4 February 2023 Revised accepted: 3 May 2023

Abstract

Purpose: To investigate the regulatory effects of vesicle-associated membrane protein 8 (VAMP8) in atherosclerosis (AS).

Methods: VAMP8 expression was assessed using quantitative real time-polymerase chain reaction (qRT-PCR) and western blot, while H&E staining was used to examine the morphology of arterial glandular tissues in AS mice. Lipid accumulation in mice was determined with the aid of Oil Red O staining, whereas the apoptosis of aortic cells was evaluated by TUNEL assay.

Results: VAMP8 was highly expressed in the advanced-stage AS samples, and was also elevated in AS mice (p < 0.01). VAMP8 protein level rose in AS mice (p < 0.01). Moreover, the aorta showed atherosclerotic lesions with intima thickening and atherosclerotic plaques in AS group (p < 0.01). However, these changes were alleviated in VAMP8-silenced group (p < 0.01). VAMP8 silencing decreased lipid accumulation and alleviated inflammation and oxidative stress in AS mice (p < 0.01). It was observed that depletion of VAMP8 reduced aortic cell apoptosis in AS mice (p < 0.05). Furthermore, VAMP8 knockdown enhanced the effect of simvastatin on atherosclerosis (p < 0.01).

Conclusion: Knockdown of VAMP8 alleviates AS in ApoE-deficient mice. This finding suggests that this might be a potential strategy for the prevention and treatment of AS.

Keywords: VAMP8, Atherosclerosis, ApoE-deficient mice, Lipid accumulation

INTRODUCTION

As one of the major reasons for various cardiovascular diseases, atherosclerosis (AS) exhibits high morbidity and mortality all over the world [1]. Previously, the pathogenesis of AS has been widely studied. Some researchers proposed that the progression of AS might be due to the disordered proliferation of vascular smooth muscle cells (VSMCs) and endothelial cells [2]. Some other studies also demonstrated that increased levels of blood lipids containing cholesterol and low-density lipoprotein (LDL), as well as the accumulation of oxidized low-density lipoprotein (ox-LDL) are involved in the aggravation of AS [3,4]. Due to the increased levels of plasma LDL, oxygen-free radicals are produced, which then oxidize LDL to ox-LDL [5]. Although some studies have investigated the pathogenesis of AS, the detailed pathological

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process of AS including the ox-LDL accumulation, inflammation, oxidative stress and apoptosis of aortic cells remains unclear. Identifying the more effective biomarkers for AS is of essential importance in the management and prevention of AS. The vesicle-associated membrane protein 8 (VAMP8) is one of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, and is a vital part responsible for the release of secretory granules in exocrine glands [6]. Importantly, VAMP8 has been identified as the first SNARE to join in the secretion of platelet granules [7]. Additionally, VAMP8 is upregulated in the phenotypes of hyper-reactive platelets [8]. In previous studies, VAMP8 was shown to be involved in a variety of biological processes and diseases. Although growing numbers of studies have focused on investigating the function of VAMP8 in different diseases, it is not clear whether it is implicated in the development of AS.

In this study, the roles of VAMP8 in AS were investigated, and the effect of knockdown of VAMP8 on AS in ApoE-deficient mice was assessed.

EXPERIMENTAL

ApoE mice model of AS

The ApoE (−/−) male mice (6-8 week-old, n = 12) were purchased from Beijing Weilong Lihua Laboratory Animal Technology Company (Beijing, China). All mice were fed for 7 days. 6 mice were fed with high lipid chow (1.25 % cholesterol and 15 % fat) to induce AS model. Another 6 mice were fed with regular chow. The serum lipid levels were measured from the samples of orbital blood drawn from the mice each month. Three months later, the mice were painlessly sacrificed and their aortae isolated, frozen in liquid nitrogen or fixed in 4 % formalin and embedded in paraffin prior to use. This work had obtained the approval from the Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine (approval no. 20210310), and was conducted based on the U.S. National Institutes of Health Guidelines for the Care and Use of Laboratory Animals [9].

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected from the mice, and then supplemented with regional citrate anticoagulation (RCA), followed by centrifugation at 2500 × g at 4 °C for 20 min. Next, the ELISA kit (Cat. No. 210-A-050, R&D Systems, Minneapolis, USA) for interleukin-6 (IL-6) (eBioscience), interleukin-1β (IL-1β) (eBioscience), tumor necrosis factor-α (TNF-α) (eBioscience) total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) was applied. The concentrations of IL-6, IL-1β, TNF-α, TG, TC, HDL and LDL were evaluated.

Assessment of oxidative stress status

The concentrations of oxidative stress indices including superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH-px) were examined using SOD Activity Assay Kit (BioVision, Milpitas, USA), MDA Assay Kit (Beyotime) and GSH-px Assay Kit (Beyotime).

Terminal deoxynucleotidyl transferase dUTP Nick end labeling (TUNEL) assay

The aortic tissue sections were incubated with TUNEL reaction mixture at 37 °C for 60 min in a humidified dark room. The frozen sections were subjected to DAPI staining. The number of DAPI- and TUNEL-positive nuclei were photographed using an Olympus DP50-CU digital camera (Tokyo, Japan) to examine the images. The number of TUNEL-positive nuclei were defined using a percentage of the total cell number in the lesion for each mouse.

Hematoxylin and eosin (H&E) staining

H&E staining was performed to explore the pathological characteristics of AS lesions,. The specimens were cut into 3-μm serial sections, and then subjected to hematoxylin and eosin staining. A light microscope was applied to examine the H&E-stained specimens to find lesions and evaluate the lesion location and pathological features.

Quantitative real-time polymerase chain reaction analysis (qRT-PCR)

TRizol reagent (Invitrogen, USA) was utilized for isolating the RNAs from the arterial tissues of mice, and the PrimeScript RT Reagent Kit (Takara, Dalian, China) was utilized for reverse-transcribing RNA into cDNA. The cDNA served as a template, and RT-qPCR analysis was conducted using SYBR Premix Ex Taq™ II commercial kit (Takara). The relative expression of VAMP8 was calculated using 2^ΔΔCt method, with GAPDH serving as an internal reference.

Western blot analysis

Total protein was extracted from the arterial tissues of mice using radioimmunoprecipitation
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(RIPA) lysis buffer (Invitrogen). The samples of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and transferred onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, USA). After blocking with non-fat milk, the primary antibodies including VAMP8 (ab76021, 1:10000, Abcam, Shanghai, China), Bax (ab32503, 1:1000, Abcam), cleaved-caspase3 (ab32351, 1:5000, Abcam) and cleaved-PARP (ab32064, 1:1000, Abcam) were added to the PVDF membrane. The horseradish peroxidase (HRP)-labeled secondary antibody was then added. Enhanced chemiluminescent visualization (ECL) system (Pierce Biotechnology, Rockford, IL, USA) was employed for assessing protein bands, and the quantification was made with Image J software.

Oil Red O staining

Intact aortas and adventitial fat were extracted from the AS mice, and treated with paraformaldehyde solution for over 24 h. Washing was done using PBS, and the tissues unfolded along the longitudinal axis with ophthalmic dissection scissors, followed by staining with saturated Oil Red O for 15 min, and differentiation in 75 % alcohol three times. An optical microscope (Nikon Eclipse, E100) was used to examine the images.

Statistical analysis

SPSS 21.0 software (SPSS Inc, Chicago, IL) was employed for statistical analysis. All data were presented as mean ± standard deviation (SD, n = 3). Student’s t test was utilized for comparisons between two groups, while one-way ANOVA was used for comparison among multi-groups. P < 0.05 was set as statistically significant.

RESULTS

VAMP8 was highly expressed in advanced-stage AS

To assess genes that might be associated with the development of AS, R language was used to analyze the differential genes in early-stage AS and advanced-stage AS in the data. It was found that 23 genes were down-regulated, while 154 genes up-regulated (Figure 1 a). Heat map was drawn for the first 30 of all the differential genes (GSM714070-73 was the advanced-stage AS samples while GSM714086-89 was the early-stage AS samples), and it was also found that VAMP8 was highly expressed in the advanced-stage AS samples (Figure 1 b).

Figure 1: VAMP8 is highly expressed in AS. (a): Volcano map showing the expression of genes in AS tissues; (b): Heat map was drawn for the first 30 of the differential genes

VAMP8 was highly expressed in AS mice

The Gene Set Enrichment Analysis data revealed that all differential genes were mainly enriched in inflammation, TYROBP causal network in microglia, regulation of cell activation, and innate immunity (Figures 2 a - c). Quantitative RT-PCR was applied to measure the expression of VAMP8 in AS mice, and the results revealed that VAMP8 expression was elevated in AS mice (Figure 2 d). The protein level of VAMP8 was also enhanced in AS mice (Figure 2 e). On the whole, VAMP8 was highly expressed in AS.

VAMP8 knockdown improved pathological conditions in AS mice

The data from western blot analysis revealed that the transfection of sh-VAMP8 in AS mice decreased VAMP8 expression (Figure 3 a). Pathological analysis of arterial glandular tissues in AS mice also revealed that the intima of the aorta was not thick and there was no stenosis in the lumen in the control group, while the aorta had obvious atherosclerotic lesions with intima thickening and atherosclerotic plaques in the AS group, but these changes were alleviated in the AS+sh-VAMP8 silencing group (Figure 3 b). VAMP8 knockdown mitigated the pathological conditions of AS mice.

VAMP8 silencing decreased lipid accumulation in AS mice

The results of Oil Red O staining revealed that the atherosclerotic plaques were localized in the aortic arch and around the branched regions of the aorta in the AS mice, while VAMP8 downregulation reduced the atherosclerotic plaque area (Figure 4 a). In addition, it was found that the elevated TC, TG, LDL levels, and the reduced HDL level in AS mice were reversed.
after VAMP8 knockdown (Figure 4 b). Thus, inhibition of VAMP8 reduced lipid accumulation in AS mice.

**Figure 2:** (a-c): Go-analysis of the enrichment of genes; (d): RT-qPCR unveiled the level of VAMP8; (e): Western blot revealed the protein level of VAMP8. "P < 0.01

**Figure 3:** VAMP8 knockdown was used to ameliorated the pathological conditions of AS mice. (a): The protein level of VAMP8 was detected through western blot analysis; (b): The pathological analysis of arterial glandular tissues was measured through H&E staining. "P < 0.01

**Figure 4:** VAMP8 knockdown mitigated lipid accumulation in AS mice. (a): The lipid accumulation in mice was measured through Oil Red O staining; (b): The concentrations of TG, TC, HDL and LDL were examined through ELISA. "P < 0.01

VAMP8 deficiency alleviated inflammation and oxidative stress in AS mice

The results of ELISA revealed that inflammation index (based on IL-6, TNF-α and IL-1β concentrations) were enhanced in AS mice, but VAMP8 silencing ameliorated the effects (Figure 5 a). Besides, the enhanced concentrations of MDA and the reduced concentrations of SOD and GSH-Px were apparently offset as a result of VAMP8 downregulation in AS (Figure 5 b). All in all, VAMP8 deficiency alleviated inflammation and oxidative stress in AS mice.

**Figure 5:** VAMP8 knockdown decreased inflammation and oxidative stress in AS mice. (a): The concentrations of IL-6, IL-1β, and TNF-α were detected through ELISA; (b): The concentrations of SOD, MDA and GSH-Px were examined through the respective kit. "p < 0.01
Figure 6: VAMP8 knockdown reduced aortic cell apoptosis in AS mice. (a): Apoptosis of aortic cells was examined through TUNEL assay; (b): The protein levels of Bax, cleaved-caspase3 and cleaved PARP were measured through western blot analysis. *P < 0.05, **P < 0.01

Depletion of VAMP8 reduced aortic cell apoptosis in AS mice

In the AS mice, it was observed the aortic cell apoptosis was elevated, but this effect was counteracted by VAMP8 attenuation (Figure 6 a). In addition, VAMP8 suppression decreased the enhanced level of Bax, cleaved-caspase3 and cleaved-PARP protein (Figure 6 b). These results indicated that depletion of VAMP8 reduced aortic cell apoptosis in AS mice.

VAMP8 knockdown enhanced the effect of simvastatin on atherosclerosis

VAMP8 knockdown decreased TC, TG, LDL levels, and increased HDL level in AS + Simva group, and this was more pronounced in AS+sh-VAMP8+Simva group (Figure 7 a). Moreover, the reduced concentrations of IL-6, TNF-α and IL-1β in the AS +Simva group were further deceased after VAMP8 knockdown (Figure 7 b). Thus, VAMP8 knockdown enhanced the effect of simvastatin on atherosclerosis.

DISCUSSION

Atherosclerosis (AS) is a serious and prevalent arterial inflammatory disease with high morbidity and mortality [10], and has become the second major cause of death, as well as the pathophysiological basis for cardiovascular diseases [11]. The main lesion of AS is lipid deposition in parts of the artery, the proliferation of VSMCs and fibrous matrix, gradually developing into atherosclerotic plaque formation. At present, the pathogenesis of AS is not fully
understood. A growing number of studies have established an AS animal model through using apolipoprotein E (ApoE) (−/−) mice [12]. In addition, multiple studies have been performed using the mice model. For example, in high-fat diet-fed ApoE (−/−) mice, butter-originated ruminant trans fatty acids have no impact on the alleviation of AS lesions [13]. In ApoE (−/−) deficient mice, proteins extracted from housefly (Musca domestica) maggots suppressed the development of AS via the production of bile acids and the consumption of cholesterol. In the spleen of ApoE-deficient mice, high-fat diet increases the level of splenic NOD1, the recruitment of neutrophils and the release of neutrophil extracellular traps [14]. Therefore, ApoE-deficient mice model is a reliable animal model for AS. Earlier, some studies found that numerous proteins were involved in the progression of AS. For instance, MFN2 suppression promotes the calcification of vascular via the modulation of the RAS-RAF-ERK1/2 pathway, and participate in the development of AS [15]. Downregulation of methyltransferase-like 14 inhibits AS development by regulating the m6A modification of p65 [16]. KLF7 modulates HDAC4/miR-148b-3p/NCOR1 pathway to reduce the lesions of AS and relieve glucose metabolic reprogramming in macrophages [17]. Although VAMP8 was reported to be implicated with type I interferon antiviral response, venous thrombosis and tumor progression, whether VAMP8 was involved in AS requires further investigation. In the current study, bioinformatic studies reveal that VAMP8 expression was enhanced in AS samples. In addition, VAMP8 was found to be highly expressed in the tissues of the AS mice model. Besides, the silencing of VAMP8 alleviated the pathological conditions of the AS mice. Furthermore, the results showed that suppression of VAMP8 reduced lipid accumulation in AS mice. VAMP8 knockdown was also found to decrease inflammation and oxidative stress in AS mice. VAMP8 deficiency reduced aortic cell apoptosis in AS mice. Thus, VAMP8 knockdown enhanced the effect of simvastatin on the progression of AS.

CONCLUSION

The findings of this study show that VAMP8 silencing alleviates AS in ApoE-deficient mice, and this highlights the role and function of VAMP8 in AS. However, investigations on VAMP8 in AS progression are required for deeper understanding of the processes.

DECLARATIONS

Acknowledgements

Funding

None provided.

This work was supported by the Startup Foundation of Doctor of Scientific Research of the Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine (Grant no. GZEYK-B[2021]3).

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Lei Zhan designed the study, completed the experiment and supervised the data collection; Hua Zhao analyzed and interpreted the data; Lei Zhan and Hua Zhao prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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