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Investigation on the effect of ulinastatin on the apoptosis of vascular smooth muscle cells in rats with aortic dissection based on the Sirt1/FoxO3a pathway

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ABSTRACT

This study aimed to investigate the effects of ulinastatin on the apoptosis and (Sirt1/FoxO3a) pathway of vascular smooth muscle cells (VSMC) in aortic dissection (AD) rats. For this purpose a rat model of aortic dissection (AD) was constructed by giving drinking water containing 0.08% β-aminopropionitrile (BAPN) to rats, HE staining was used to observe the pathological changes of the aorta in AD rats; the diseased blood vessels of AD rats were taken for primary culture and passage of VSMCs, the morphology of VSMCs was observed, and VSMCs were identify with immunofluorescence staining; VSMCs were treated with culture media containing 0, 1000, 2000, 3000, 4000, 5000, 6000, 7000 U/mL ulinastatin, and MTT kit was used to determine the effect of ulinastatin on VSMC proliferation in AD rats; the VSMC of AD rats were divided into blank group (normal culture), ulinastatin group (medium containing 5000 U/mL ulinastatin), Sirt1 inhibitor group (medium containing 1 μmol/L EX527), ulinastatin + Sirt1 inhibitor group (medium containing 5000 U/mL ulinastatin, 1 μmol/L EX527), flow cytometry was used to detect the VSMC apoptosis in each group, WB was used to detect the expression of VSMC apoptosis-related proteins and Sirt1/FoxO3a pathway-related proteins in each group. Findings suggested that the aortic wall of AD rats was thickened, and the dissection false cavity appeared; VSMC mostly presented different shapes such as triangles and stars, the immunofluorescence staining results showed that α-SMA was arranged in the cytoplasm in the form of myofilaments, showing green fluorescence, and the nucleus showed blue fluorescence, and the rate of positive cells was more than 95%; various doses of ulinastatin had a certain inhibitory effect on the proliferation of VSMC, and 5000 U/mL ulinastatin had a higher proliferation inhibition rate; compared with the blank group, the VSMC apoptosis rate, Caspase-3, Bax protein, Sirt1/FoxO3a pathway related protein expression in the ulinastatin group were significantly increased, and the Bcl-2 protein expression was significantly decreased ($P<0.05$), the VSMC apoptosis rate, Caspase-3, Bax protein, Sirt1/FoxO3a pathway related protein expression in the Sirt1 inhibitor group were significantly decreased, and the Bcl-2 protein expression was significantly increased ($P<0.05$); compared with the ulinastatin group, the VSMC apoptosis rate, Caspase-3, Bax protein, Sirt1/FoxO3a pathway related protein expression in the ulinastatin + Sirt1 inhibitor group were significantly decreased, and the Bcl-2 protein expression was significantly increased ($P<0.05$). It was concluded that ulinastatin can inhibit the proliferation of VSMCs in AD rats and promote their apoptosis, which may be achieved by activating the Sirt1/FoxO3a pathway.

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Introduction

Artery dissection (AD) is an acute and severe disease in which the aortic wall is separated by true and false channels. Often with adverse manifestations such as cardiac tamponade and arrhythmia, it has a high mortality rate and seriously threatens the health and life of patients (1,2). AD involves complex pathogenesis, which is closely related to hypertension, cardiovascular disease, severe trauma, etc. Data show that the excessive proliferation, migration, and appearance changes of vascular smooth muscle cells (VSMC) are involved in the development of AD. Therefore, inhibiting the excessive proliferation of VSMC means great significance for the treatment of AD (2,3). Ulinastatin is a glycoprotein extracted from fresh human urine, which can inhibit the activity of a variety of proteolytic enzymes, and is widely used as a rescue adjuvant for acute pancreatitis and acute circulatory failure (4). Xu et al. (5) found that high-dose ulinastatin has a certain protective effect on inflammatory response and lung function damage in patients with type A AD, but there is no report about its effect on VSMC apoptosis. Silent mating type

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information regulation 2 homolog 1 (Sirt1) is a deacetylase that plays an important role in processes of cell proliferation, apoptosis, and aging, mainly by promoting the expression of forkhead protein O3a (FoxO3a) (6). Yu et al. (7) found that increasing FoxO3a expression can significantly induce VSMC apoptosis, so it is speculated that Sirt1/FoxO3a pathway has a certain relationship with AD. Accordingly, this paper explores the effect of ulinastatin on VSMC apoptosis in AD rats and its possible mechanism based on previous studies, with a view to providing a theoretical basis for the clinical treatment of AD.

Materials and Methods

Animals

10 SD male rats (Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd.), SPF grade, production license number SCXK (Zhejiang) 2016-0006, were about 8 weeks old, weighing about 300 g. All rats were fed in the animal room of our hospital alternatingly day and night for 12 h. During this period, rats could eat and drink freely. The animal room temperature was about 25℃ and the relative humidity was about 50%. The animal room environment and rat cage were kept clean during the experiment. Humanitarian care was given in accordance with the "3R" principle for animals during the experiment. This study was approved by the animal ethics committee.

Main reagents and instruments

β-Aminopropionitrile (BAPN), EX527 (Sirt1 inhibitor group), ulinastatin (article numbers: T13475, T6111, T8802) were purchased from Topscience; Annexin V-fluorescein isothiocyanate/ propidium iodide (Annexin V-FITC/PI) apoptosis double staining kit, Polyethylene Glycol Octylphenyl Ether (Triton X-100) reagent (Lot Numbers: P-CA-201, SA -209) were purchased from Wuhan Punuosai Life Technology Co., Ltd.; HE staining kit, MTT kit, RIPA lysate, BCA protein determination kit (article numbers: G1120, M1020, R0010, PC0020) were purchased from Beijing Solarbio Life Sciences Co., Ltd.; rabbit source Caspase-3, Bax, Bcl-2, Sirt1, FoxO3a, p27, GAPDH primary antibody, goat anti-rabbit IgG secondary antibody (article numbers: ab181286, ab76003, ab184787, ab189494, ab23683, ab32034, ab9485, ab6721) were purchased from Abcam.

The dual scanning laser confocal microscope (model: FV1200) was purchased from Olympus; the manual rotary microtome (model: RM2125RTS) was purchased from Leica, Germany; the optical microscope (model: SMZ745) was purchased from Nikon, Japan; microplate reader (model: XElx800) was purchased from Perkin Elmer, USA; protein electrophoresis instrument (model: 1659001), semi-dry transfer instrument (model: Trans-Blot SD) were purchased from Bio-Rad, USA; gel imager (model: GIS-500)) was purchased from Miulab.

AD rat model construction and model identification

AD rat model was built with reference to literature (8). 10 SD rats were randomly divided into a control group and a model group, 5 in each group. The control group was fed normally, and the model group rats were given drinking water containing 0.08% BAPN every day for 40 d. When the rats died during the experiment, spare rats were selected for modeling treatment in time.

The rat was fixed on a platform in a supine position, intraperitoneally injected with 10% chloral hydrate and then sacrificed. The skin was disinfected and then dissected layer by layer. The thoracic aorta was separated along the spine. The heart and aorta were carefully taken out, and the redundant blood vessels were washed away with saline. The blood vessels were observed with suspicious dissection; they were fixed in 10% paraformaldehyde for 48 h. The rat vascular tissue was placed in a dehydrator for dehydration, and the dehydrated tissue was treated with xylene and embedded in melted paraffin. After the paraffin solidification, the wax block was trimmed and cut into about 4 μm thick sections. The paraffin sections were deparaffinized and stained according to the instructions of HE staining kit. For identification of the construction status of the AD rat model, a HE stained image of the rat aorta was observed.

VSMC culture and identification of AD rats

The blood vessels of AD rats were taken, washed with normal saline, and placed in petri dishes containing DMEM. The tunica media was separated under a microscope, moved to a petri dish containing DMEM, then cut with scissors and transferred to a culture flask. DMEM culture medium containing 20% fetal bovine serum was added, and it was cultured in a 37°C, 5% CO2 incubator. The culture medium was replaced once every five days. After observing cell adhesion to the flask wall, digestion and passage were performed.

The shape and size of fifth-generation cells were observed under an inverted microscope after 48h, 72h, and 96h of culture. The cells were selected in good growth conditions and placed in a laser-confocal petri dish. A 4% paraformaldehyde fixative was used. It was blocked with goat serum and rabbit-derived α-SMA primary antibody (1:100) was added. FITC-labeled goat anti-rabbit IgG secondary antibody (1:100) was also added. The DAPI working solution, a fluorescence quencher, was added, observed under a confocal laser scanning microscope, and pictured.

The effect of ulinastatin on the proliferation of VSMC in AD rats

The VSMCs of AD rats were divided into 8 groups and inoculated into 96-well plates at a cell concentration of 4×105 cells/mL. The control group received no treatment, and the remaining 6 groups were treated with culture medium containing 0, 1000, 2000, 3000, 4000, 5000, 6000, and 7000 U/mL ulinastatin in an incubator for 48 h, added with MTT reagent and further incubated for 4 h. Dimethyl sulfoxide was added and shaken well. The absorption of cell A in each well was measured using an automatic microplate reader. Cell proliferation inhibition rate (%)=(A1-A2)/(A1-A3)×100%, A1: contains cells, medium, MTT solution, without ulinastatin; A2: contains cells, MTT solution, ulinastatin, medium; A3: contains medium, MTT solution, without ulinastatin and cells.

Cell grouping and VSMC apoptosis of AD rats in each group

VSMCs of AD rats were divided into blank group (normal culture), ulinastatin group (medium containing 5000 U/mL ulinastatin), Sirt1 inhibitor group (medium containing 1 μmol/L EX527) (9), ulinastatin + Sirt1 inhi-

bitor group (medium containing 5000 U/mL ulinastatin, 1 μmol/L EX527), and then cultured in a 37 ℃, 5% CO2 incubator for 48 h. VSMCs from each group were collected, cell density was adjusted to 1×106 cells/mL, and AnnexinV-FITC/PI cell apoptosis double staining kit was used to determine cell apoptosis in each group. The main operation refers to the requirements stated in the kit (10). 1 mL of cell liquid was added to an EP tube, then fixed with ethanol, followed by RNase treatment and the addition of Triton X-100 to increase cell membrane permeability. After staining with PI solution, cell apoptosis was analyzed using a cell flow cytometer. There were 5 parallel wells in each group, and the experiment was repeated 3 times (n=3).

VSMC apoptosis, Sirt1/FoxO3a pathway-related protein expression in AD rats of each group

VSMC was Taken from each group of AD rats, and protein was extracted using RIPA lysate. The protein concentration was measured using a BCA protein determination kit. The protein concentration of each group was adjusted with the same protein loading solution, and for protein separation, SDS polyacrylamide gel electrophoresis was performed. The protein was transferred to the NC membrane by wet transfer method, sealed with 5% skimmed milk powder, and added with rabbit-derived Caspase-3, Bax, Bcl-2, Sirt1, FoxO3a, p27, GAPDH primary antibodies (1:1000). After washing with TBST buffer, horseradish peroxide-labeled goat anti-rabbit IgG secondary antibody (1:5000) was added. After incubation at room temperature, a gel imager was used for analysis of the expression of related proteins.

Statistical analysis

SPSS 22.0 software was used for statistical analysis of the experimental data. Measurement data are expressed as mean ± standard deviation. One-way analysis of variance is made for pairwise comparison, and LSD-t test is used for further pairwise comparison. P<0.05 indicates a statistically significant difference.

Results

Identification of AD rat model

The aortic wall structure of rats in the control group was normal; compared with the control group, the aortic wall of the model group was thickened, the membrane structure in the vascular wall was destroyed, and the dissection false channel was visible (Figure 1).

VSMC identification for AD rat

After 48 h culture, rat VSMCs began to adhere to the wall, and after 72 h, they migrated around with fragmented tissue masses as the center, showing an "island" shape. After 96 h culture, the primary cells gradually fused, showing a "peak-valley" appearance, with passaged cells presenting different shapes such as triangles and stars. α-SMA immunofluorescence staining results indicate that α-SMA was arranged in the cytoplasm like myofilaments, showing green fluorescence, while the nucleus showed blue fluorescence, with a positive cell rate>95% (Figure 2).

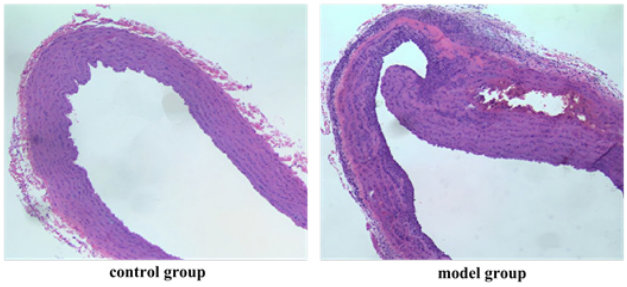

Figure 1. HE staining of aortic dissection rats (×400).

The effect of ulinastatin on VSMC proliferation in AD rats

Compared with 0 U/mL ulinastatin, 1000, 2000, 3000, 4000, 5000, 6000, and 7000 U/mL ulinastatin had significantly increased inhibition rate against VSMC proliferation in AD rats (P<0.05). Where, 5000 U/mL ulinastatin had a higher proliferation inhibition rate, so 5000 U/mL ulinastatin was selected for follow-up research (Table 1).

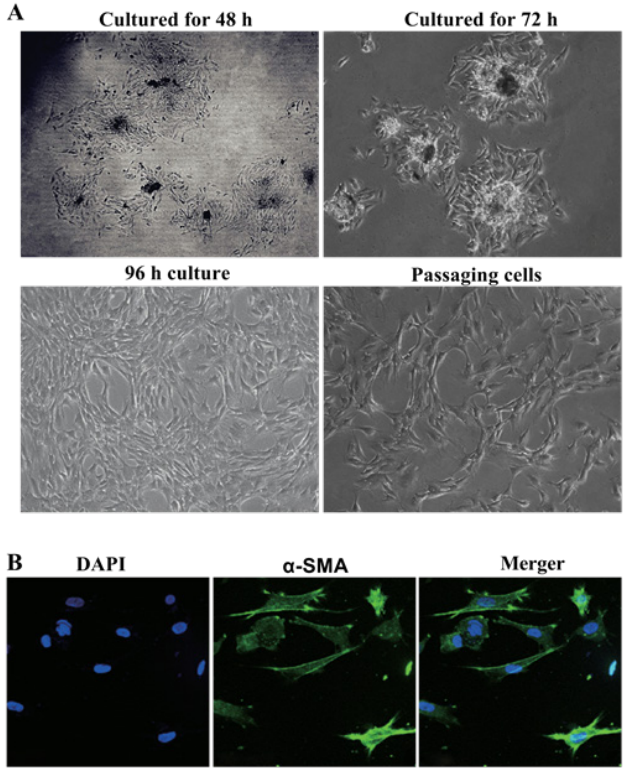

Figure 2. A: Morphological observation of VSMC in AD rats; B: α-SMA immunofluorescence staining of VSMC in AD rats.

Table 1. Effect of ulinastatin on VSMC proliferation in AD rats ($\bar{x}\pm s$, n = 3).

Ulinastatin concentration	Inhibitory rate of cell proliferation (%)
0 U/mL	0
1000 U/mL	15.41±2.12[a]
2000 U/mL	19.13±2.39[a]
3000 U/mL	21.22±3.33[a]
4000 U/mL	23.16±4.34[a]
5000 U/mL	25.35±4.12[a]
6000 U/mL	25.06±4.32[a]
7000 U/mL	24.89±4.06[a]

Note: Compared with 0 U/mL, [a]P<0.05.

Comparison of VSMC apoptosis in AD rats of each group

Compared with the blank group, AD rats in the ulinastatin group had significantly increased VSMC apoptosis rate, Caspase-3 and Bax protein expression, and significantly decreased Bcl-2 protein expression (P<0.05); AD rats in the Sirt1 inhibitor group had significantly reduced VSMC apoptosis rate, Caspase-3 and Bax protein expression, and significantly increased Bcl-2 protein expression (P<0.05). Compared with ulinastatin group, AD rats in the ulinastatin + Sirt1 inhibitor group had significantly reduced VSMC apoptosis rate, Caspase-3 and Bax protein expression, and significantly increased Bcl-2 protein expression (P<0.05) (Figure 3 and Table 2).

Comparison of Sirt1/FoxO3a pathway-related protein expression in VSMC of AD rats in each group

Compared with the blank group, AD rats in the ulinastatin group have significantly increased expression of Sirt1, FoxO3a, and P27 protein in VSMC (P<0.05), AD rats in the Sirt1 inhibitor group had significantly decreased expression of Sirt1, FoxO3a, and P27 protein in VSMC (P<0.05); Compared with the ulinastatin group, AD rats in the ulinastatin+Sirt1 inhibitor group had significantly reduced expression of Sirt1, FoxO3a and P27 protein in VSMC (P<0.05) (Figure 4 and Table 3).

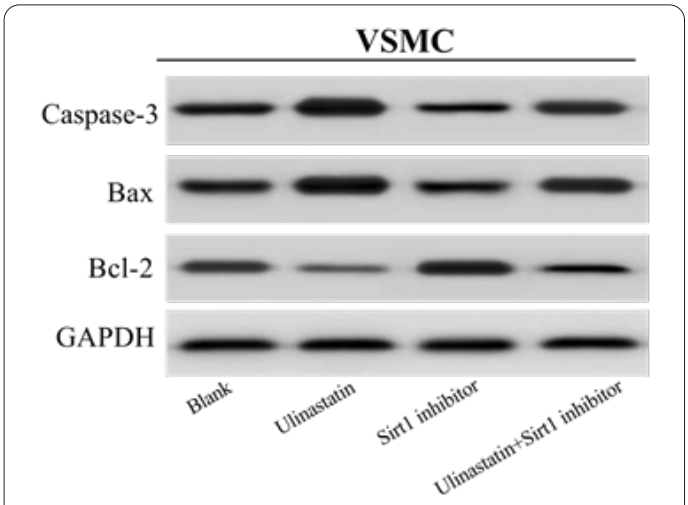

Figure 3. Comparison of apoptosis-related protein expression of VSMC in AD rats.

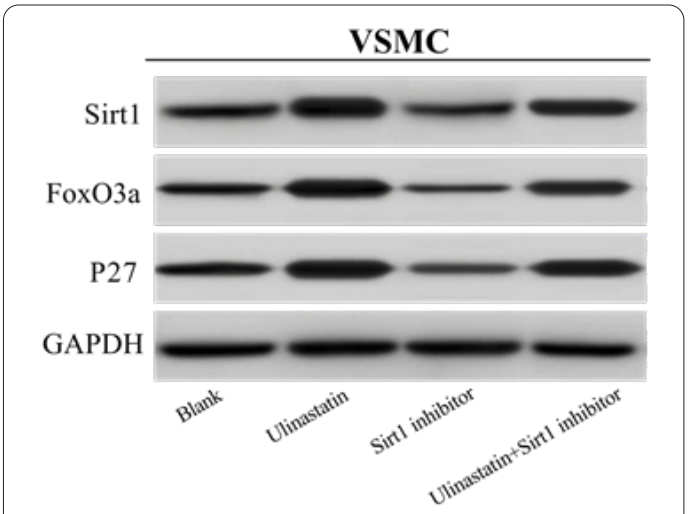

Figure 4. Comparison of Sirt1/FoxO3a pathway-related protein expression in VSMC of AD rats in each group.

Discussion

AD is usually caused by aortic intimal tear. Blood enters the tunica media from the breach, which separates the intima and adventitia of the aorta, resulting in impaired aortic valve function. In severe cases, it can cause the aortic wall to expand or even rupture, which seriously threatens patients' life safety (11,12). In research, the AD rat model is often constructed by adding BAPN. BAPN can inhibit bridging of the two in the blood vessel without affecting the concentration of protein fiber and collagen fiber, thereby destroying vascular homeostasis and causing the formation of artery dissection. AD rats constructed via this approach have the advantages of a high dissection formation rate, simple operation, low cost, and low mortality (10). In this study, AD rat model was constructed by giving

Table 2. Comparison of VSMC apoptosis in AD rats ($\bar{x}\pm s$, n = 3).

Group	Apoptosis ratio (%)	Apoptosis-related proteins		
		Caspase-3/GAPDH	Bax/GAPDH	Bcl-2/GAPDH
Blank group	14.73±2.27	0.92±0.09	0.90±0.11	0.79±0.10
Ulinastatin group	32.25±3.12[a]	1.73±0.20[a]	1.75±0.21[a]	0.25±0.06[a]
Sirt1 inhibitor group	10.48±1.16[ab]	0.68±0.11[ab]	0.64±0.13[ab]	1.18±0.19[ab]
Ulinastatin+Sirt1 inhibitor group	21.97±3.08[abc]	1.25±0.18[abc]	1.28±0.19[abc]	0.49±0.13[abc]

Note: Compared with the blank group, [a]P<0.05; Compared with ulinastatin group, [b]P<0.05; Compared with Sirt1 inhibitor group, [c]P<0.05.

Table 3. Comparison of the expression of Sirt1 / FoxO3a pathway-related proteins in VSMC of AD rats in each group ($\bar{x}\pm s$, n = 3).

Group	Sirt1/GAPDH	FoxO3a/GAPDH	P27/GAPDH
Blank group	0.96±0.14	0.97±0.14	0.92±0.13
Ulinastatin group	1.76±0.21[a]	1.80±0.19[a]	1.85±0.20[a]
Sirt1 inhibitor group	0.70±0.08[ab]	0.69±0.09[ab]	0.71±0.07[ab]
Ulinastatin+Sirt1 inhibitor group	1.32±0.17[abc]	1.33±0.16[abc]	1.32±0.17[abc]

Note: Compared with the blank group, [a]P<0.05; Compared with ulinastatin group, [b]P<0.05; Compared with Sirt1 inhibitor group, [c]P<0.05.

rats drinking water containing BAPN every day. The HE staining results of aortic tissue showed that AD rats' aorta wall thickened, the vascular wall media structure was destroyed, and dissection false channel appeared, which was consistent with the results of Vijayarathna et al. (10), indicating that the AD rat model was successfully constructed.

VSMC is an important part of the aorta, accounting for more than 90% of the total number of intrinsic cells. Data show that VSMC in AD tissues proliferate faster than in normal aortic tissues, and abnormal proliferation of VSMCs may be the primary cause of pathological vascular remodeling and vascular diseases, whose mechanism may be closely related to the stability of vascular medium structure and function (13,14). Therefore, searching for drugs that inhibit the abnormal proliferation of VSMC means great significance for the treatment of AD. Ulinastatin is a class of protease inhibitors, which has the functions of inhibiting inflammation, scavenging oxygen free radicals, and inhibiting the formation of aortic aneurysms (15,16). Lun et al. (17). have found that ulinastatin apoptosis pathway induced by endoplasmic reticulum stress has a certain protective effect on myocardial ischemia-reperfusion in rats, but there is no report about its effect on VSMC apoptosis. The results of this study show that different doses of ulinastatin have a certain inhibitory effect on the proliferation of VSMCs. Where, 5000 U/mL ulinastatin had a higher proliferation inhibition rate, so 5000 U/mL ulinastatin was selected for follow-up research.

Caspase-3 and Bax are important pro-apoptotic factors. Bax mainly plays a role in enhancing the expression of Caspase-3. Bcl-2 is an important anti-apoptotic factor, which plays a role in preventing cell apoptosis. Detection of Caspase-3, Bax, and Bcl-2 proteins in cells can indirectly reflect the apoptotic ability of cells (18). The results of this study showed that compared with the control group, AD rats treated with ulinastatin had significantly increased VSMC apoptosis rate, Caspase-3, Bax protein expression, and significantly decreased Bcl-2 protein expression, suggesting ulinastatin can induce VSMC apoptosis, but its possible mechanism is still unclear. Sirt1 is a class III deacetylase, which can deacetylate a variety of proteins and transcription factors and is closely related to cell proliferation, aging, apoptosis, and differentiation processes. Activated Sirt1 can promote the expression of FoxO3a, and then up-regulate the expression of cell cycle arrest-related protein P27, thereby inhibiting cell proliferation (19,20). A number of studies have shown that up-regulating the expression of FoxO3a can promote VSMC apoptosis (21,22). The results of this study showed that AD rats treated with ulinastatin had significantly increased expression of Sirt1, FoxO3a, and P27 protein in VSMC. Compared with the Sirt1 inhibitor group, AD rats of ulinastatin+Sirt1 inhibitor group had significantly increased expression of Sirt1, FoxO3a, and P27 protein in VSMC, suggesting that ulinastatin may induce VSMC apoptosis in AD rats by activating the Sirt1/FoxO3a pathway.

In summary, ulinastatin can induce VSMC apoptosis in AD rats, which may be related to the activation of the Sirt1/FoxO3a pathway. However, the action mechanism of ulinastatin is complicated. Whether it can exert its effects through other pathways is still unclear, so in-depth study is necessary.

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Declaration of conflicting interests
The authors declared no conflict of interest.

Consent for publication
All authors give consent for publication

Data availability statement
The [DATA TYPE] data used to support the findings of this study are included within the article.

Research involving human participants and/or animal
This retrospective study involving human participants was in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by our institution's ethics review board.

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