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Therapeutic effects of the soluble epoxide hydrolase (sEH) inhibitor AUDA on atherosclerotic diseases

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In this study, we aimed to detect the effects of the soluble epoxide hydrolase (sEH) inhibitor 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA) on atherosclerotic diseases and to explore its mechanism. The atherosclerosis animal model was constructed by ApoE$^{-/-}$ mice. To determine the optimal therapeutic concentration of AUDA, different concentrations of AUDA were infused into ApoE-/- mice, with controls receiving infusions of normal saline alone. Mouse body weight and serum total cholesterol, triglyceride, LDL and HDL levels were measured. The western blotting (WB) method was used to detect the expression of TLR4 and NFκB in the aortic wall of the AUDA-treated and control mice. After the animals were sacrificed, we performed Oil Red O staining of the aortic sinus atherosclerotic plaque area followed by quantitative analysis of the aortic atherosclerotic plaque size and the percentage of lumen area in the two groups of mice. The expression levels of inflammatory cytokines, adhesion molecules and chemokines in the AUDA group were significantly decreased compared to the saline-treatment group ($P < 0.05$). The optimal AUDA concentration was found to be 0.35 ml/mg. AUDA significantly inhibited the expression of TLR4 and NFκB in ApoE$^{-/-}$ mouse aortas and reduced the aortic sinus plaque area of the ApoE$^{-/-}$ mouse group ($P < 0.05$). In conclusion, AUDA can regulate blood lipid balance, which may be one of the mechanisms for its protective effects on the cardiovascular system.

1. Introduction

Atherosclerosis is a chronic inflammatory disease associated with immune response throughout the whole course of its pathogenesis. Atherosclerosis pathogenesis doctrines include the lipid infiltration theory, thrombosis doctrine, endothelial injury inflammation doctrine and several others (Libby et al. 2010). Recent studies suggest that inflammation and innate immunity play important roles in the development of atherosclerosis (Rudd et al. 2002). Atherosclerosis is based on chronic inflammatory processes and leads to the accumulation of natural and acquired immune cells in the intima of the arterial wall (Tobias and Curtiss 2005). However, the specific mechanism is not fully understood. The role of soluble epoxide hydrolase (sEH) is to convert ethylene-eicosatrienoic acid (EET) to dihydroxyeicosatrienoic acid (Morisseau and Hammock 2005). EET is considered to be an endogenous protective factor against atherosclerosis, hypertension and other vascular diseases (Moghaddam et al. 1997; Morisseau and Hammock 2005; Morisseau et al. 1999). Because sEH can metabolize endogenous fatty acid oxides and the regulation of sEH is altered in a variety of cardiovascular diseases, sEH is considered to be closely related to cardiovascular function (Pinot et al. 1995). Recent studies indicate that sEH plays an important role in the regulation of blood pressure and the inhibition of sEH may be an effective method for the treatment of hypertension, atherosclerosis, stroke and kidney failure (Ameshima et al. 2003; Zhao et al. 2004).

The sEH inhibitor 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA) has been confirmed to have significant anti-inflammatory effects (Imig and Hammock 2009; Schmelzer et al. 2005). Other studies showed that sEH inhibitors prevented the expression of cell adhesion molecules induced by TNFa and IL6 by inhibiting NFκB. In effect, this reduces leukocyte adhesion to the vessel wall, which may play an anti-atherosclerotic role (Zhang et al. 2007). However, the changes in sEH and the mechanism of its effects in human atherosclerotic development are unclear.

In our study, physical and chemical serum indicators were detected by enzyme-linked immunosorbent assay (ELISA). The expression of TLR4 and NFκB in the aortic wall of AUDA-treated ApoE-/- mice was detected by western blotting. The results show that AUDA treatment may have an effect on atherosclerotic lesions. Our results may provide new specific targets for the treatment of atherosclerosis and a theoretical basis for clinical application.

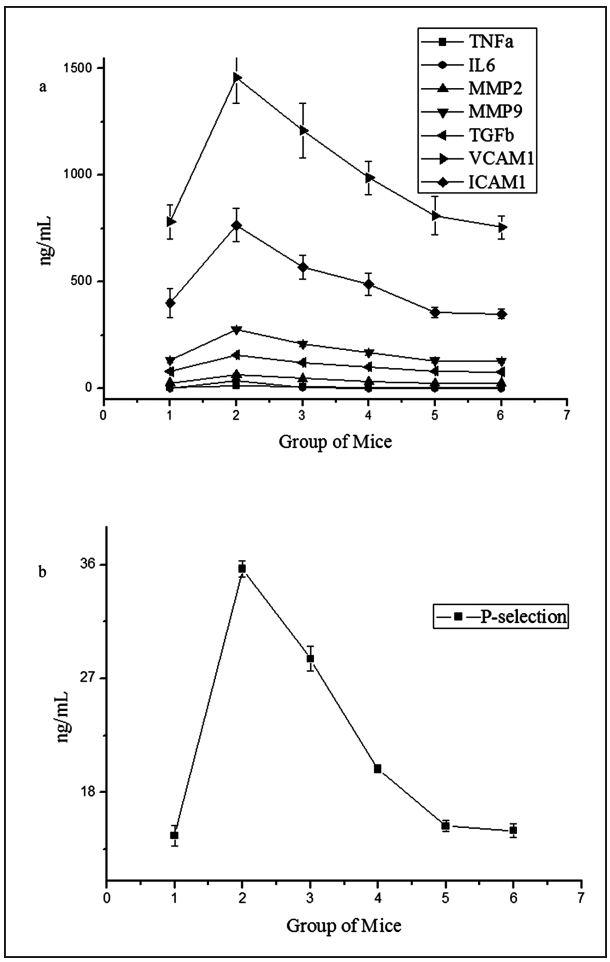

Fig. 1: The trend in the expression of serum cytokines in ApoE-/- mice treated with different concentrations of AUDA. A, changes in the expression of various cytokines in the serum; B, change in the expression of P-selectin in the plasma.

2. Investigations and results

2.1. Effect of AUDA on cytokine expression levels in the serum and plasma of ApoE-/- mice analyzed by the ELISA assay

Detailed results of ELISA are shown in the Table. The cytokine level in ApoE-/- mice perfused with normal saline was highest, and there were significant differences in some cytokines compared to the other five groups. The expression of some cytokines in ApoE-/- mice perfused with 0.15 ml/kg AUDA was different from the other five groups, and were significantly reduced compared to the ApoE-/- group that was perfused with normal saline. The expression of some cytokines in ApoE-/- mice perfused with 0.25 ml/kg AUDA was different from the other five groups. It was significantly reduced compared to that of the ApoE-/- mice perfused with 0.15 ml/kg AUDA. When compared to normal mice, the difference in the cytokine levels of ApoE-/- mice perfused with 0.35 ml/kg AUDA and 0.45 ml/kg AUDA was not significant. However, the expression of cytokines was significantly lower than in the other three groups. The trends in the expression of serum cytokines in ApoE-/- mice treated with different concentrations of AUDA are shown in Fig. 1 The optimal concentration of AUDA was found to be 0.35 ml/kg.

2.2. Weight and blood lipid levels in ApoE-/- mice

As we can see from Fig. 2, the weight of ApoE-/- mice perfused with 0.35 ml/kg AUDA was significantly reduced compared to

Table 1: The expression of various cytokines in serum and plasma (n = 3)

Group	TNF (ng/ml)	IL6 (ng/ml)	MMP-2(ng/ml)	MMP-9 (ng/ml)	TGF-b (ng/ml)	VCAM-1 (ng/ml)	ICAM-1 (ng/ml)	P-selection (ug/mL)
1	5.2 ± 0.56^{a}	0 ± 0.00^{a}	25.67 ± 1.09^{a}	132.78 ± 12.98^{a}	78.56 ± 1.98^{a}	780.45 ± 78.98^{a}	398.78 ± 67.56^{a}	14.57 ± 0.80^{a}
2	$10.67 \pm 0.89^{**}$	$35.78 \pm 1.24^{**}$	$65.34 \pm 2.67^{*}$	$276.34 \pm 10.09^{*}$	$156.89 \pm 2.12^{**}$	$1458.76 \pm 123.90^{**}$	$765.9 \pm 78.98^{*}$	$35.67 \pm 0.64^{*}$
3	$8.7 \pm 0.45^{*}$	$3.78 \pm 0.11^{*a}$	45.68 ± 2.09	$208.65 \pm 11.21^{*}$	$121.34 \pm 2.67^{*}$	$1208.23 \pm 129.09^{*}$	$567.94 \pm 56.90^{*}$	$28.56 \pm 0.99^{*}$
4	$5.8 \pm 0.23^{*}$	0.65 ± 0.01^{a}	31.09 ± 1.08	167.45 ± 9.87	100.09 ± 1.90	987.54 ± 78.65	487.65 ± 52.34	19.87 ± 0.32
5	4.5 ± 0.32^{a}	0.034 ± 0.008^{a}	24.67 ± 0.99^{a}	129.8 ± 8.04^{a}	79.86 ± 1.02^{a}	809.9 ± 89.98^{a}	356.23 ± 24.98^{a}	15.34 ± 0.45^{a}
6	4.6 ± 0.21^{a}	0.009 ± 0.001^{a}	25.89 ± 0.87^{a}	127.87 ± 8.99^{a}	76.54 ± 1.03^{a}	754.9 ± 54.90^{a}	348.79 ± 22.67^{a}	14.98 ± 0.56^{a}

Compared with group 1 in the same column, * indicates a significant difference ($P < 0.05$), ** indicates a extremely significant difference ($P < 0.01$); Compared with group 2, indicates a significant difference ($P < 0.05$). Group 1, three normal mice perfused with normal saline; Group 2, three ApoE-/- mice perfused with normal saline; Group 3, three ApoE-/- mice perfused with 0.15 ml/kg AUDA (American caymm company, 1mg/mL); Group 4, three ApoE-/- mice perfused with 0.25 ml/kg AUDA; Group 5, three ApoE-/- mice perfused with 0.35 ml/kg AUDA; Group 6, three ApoE-/- mice perfused with 0.45 ml/kg AUDA.

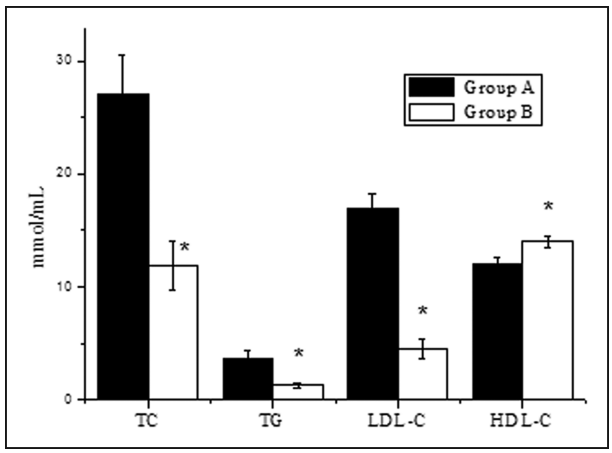

Fig. 2: Measurements of weight and blood lipid levels in ApoE-/- mice. * $P < 0.05$ indicated that there was a significant difference between group A and group B; Group A, three ApoE-/- mice perfused with normal saline; Group B, three ApoE-/- mice perfused with 0.35 ml/kg AUDA.

that of ApoE-/- mice perfused with normal saline. The total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), triglyceride (TG) levels in ApoE-/- mice perfused with 0.35 ml/kg AUDA were also significantly reduced compared to those in ApoE-/- mice perfused with normal saline ($p < 0.05$). The high density lipoprotein cholesterol (HDL-C) was significantly increased ($p < 0.05$).

2.3. Western blotting to detect changes in the expression of TLR4 and NFκB in the aortic wall of ApoE-/- mice perfused with AUDA

A western blot showing changes in TLR4 and NFκB protein expression in the aortic wall of ApoE-/- mice perfused with AUDA is shown in Fig. 3. The expression of TLR4 and NFκB in ApoE-/- mice perfused with AUDA was lower compared to control mice ($p < 0.05$).

2.4. Effects of AUDA on aortic sinus atherosclerotic lesions in ApoE-/- mice

Figs. 4 and 5 show that the atherosclerotic plaque area of ApoE-/- mice perfused with AUDA was significantly lower than the control. The atherosclerotic plaque area of ApoE-/- mice perfused with normal saline was $2.78 \pm 0.09\ mm^2$, which was significantly lower than that of ApoE-/- mice perfused with AUDA, in which the plaque area was $0.89 \pm 0.07\ mm^2$ ($p < 0.05$). The percentage of atherosclerotic plaque area per lumen area of the AUDA-treated ApoE-/- mice was $3.5 \pm 1.0\,\%$, which was significantly lower ($p < .05$) than the control ($20.5 \pm 0.9\,\%$).

3. Discussion

Atherosclerosis is an inflammatory disease of the immune system in which an artery wall thickens as a result of accumulation of macrophages, white blood cells and fat, such as cholesterol, triglycerides, low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Vink et al. 2002; Schoneveld et al. 2008). In this study, we examined the serum cytokine levels of AUDA-treated ApoE-/- mice. We found the optimal concentration of AUDA to be 0.35 ml/kg. The weight, TC, TG and LDL-C levels of ApoE-/- mice treated with 0.35 ml/kg AUDA were significantly lower than those of the saline-perfused control ApoE-/- mice. In addition, HDL-C was significantly increased compared to the controls. Western blotting showed that the expression of

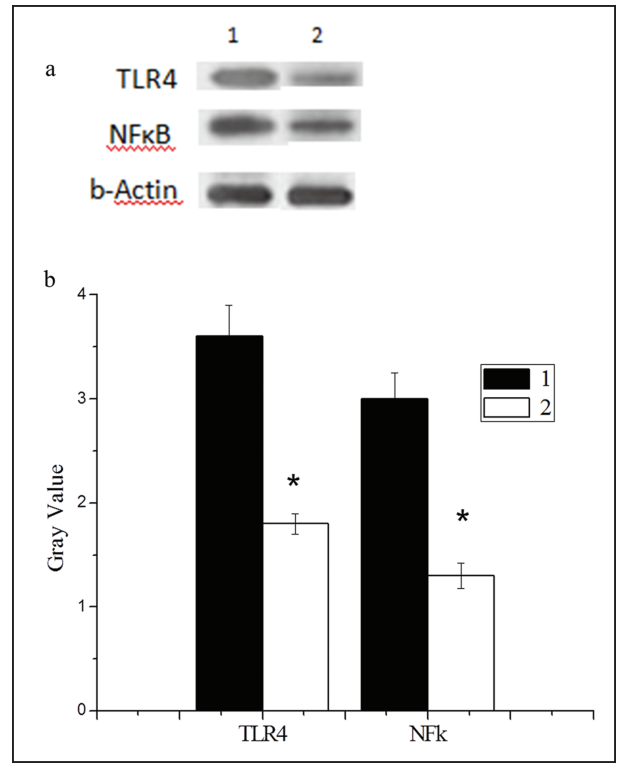

Fig. 3: Western blotting to detect changes in the expression of TLR4 and NFκB in the aortic wall of ApoE-/- mice perfused with AUDA. A, The protein expression of TLR4 (73 ku) and NFκB (65 ku) in ApoE-/- mice shown by western blotting; B, the histogram showed the gray value of TLR4 and NFκB, * $P < 0.05$ indicated a significant difference (n = 6) between the two groups; Group 1, three ApoE-/- mice perfused with normal saline; Group 2, three ApoE-/- mice perfused with 0.35 ml/kg AUDA.

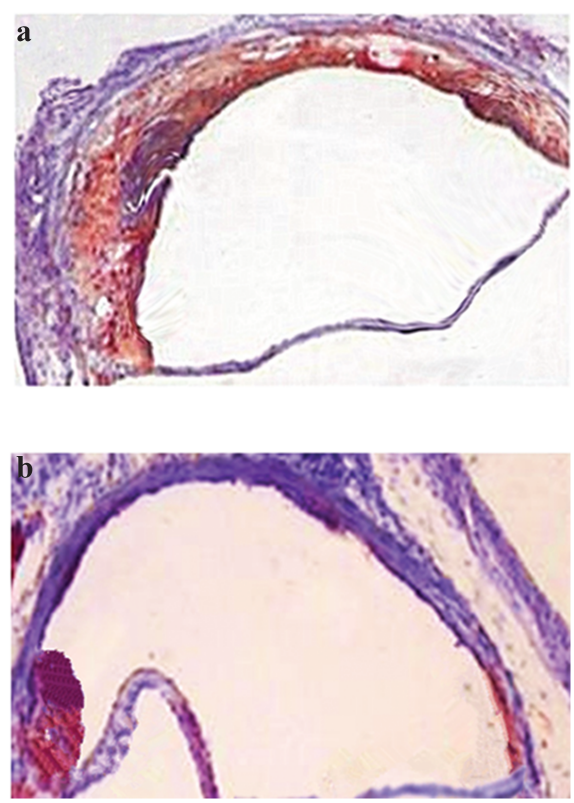

Fig. 4: The effects of AUDA on aortic sinus atherosclerotic lesions in ApoE-/- mice. A, Aortic sinus atherosclerotic lesions of ApoE-/- mice infused with normal saline; B, aortic sinus atherosclerotic lesions of ApoE-/- mice infused with AUDA.

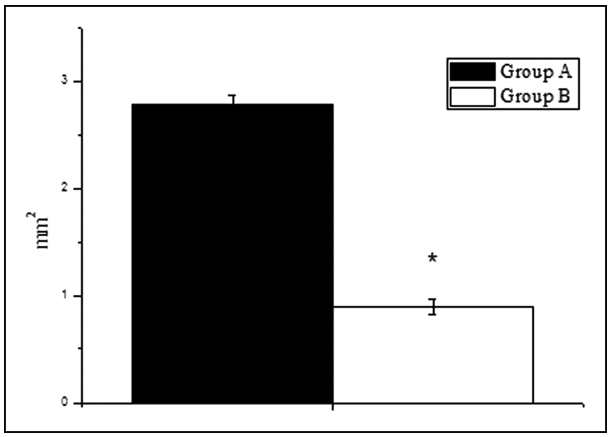

Fig. 5: The percentage of atherosclerotic plaque area per lumen area in ApoE-/- mice. * $P < 0.05$ indicated a significant difference between the two groups (n = 6); Group 1, three ApoE-/- mice perfused with normal saline; Group 2, three ApoE-/- mice perfused with 0.35 ml/kg AUDA.

TLR4 and NFκB in ApoE-/- mice perfused with AUDA was significantly lower than that of ApoE-/- mice perfused with normal saline alone.

Finally, a large amount of data has shown that lipid levels, especially cholesterol and triglyceride levels, are closely related to atherosclerosis. Our studies have shown that AUDA can significantly reduce TC, TG and LDL-C levels in ApoE-/- mice, while elevating the HDL-C level, thereby reducing atherosclerotic lesions. This implies that AUDA can regulate the blood lipid balance. This could be one of the mechanisms by which AUDA protects the cardiovascular system. sEH plays an important role in animal models of atherosclerosis development; thus, inhibition of sEH may be a promising clinical treatment.

The results of this study showed that sEH inhibitors reduced the expression of TNFa, IL6, MMP-2, MMP-9, VEGF, TGF-b, VCAM-1 and ICAM-1 in the serum and P-selectin in the plasma. This study determined the optimal concentration of AUDA (Inokubo et al. 2001). Other studies have confirmed that the expression of MMP-2 and MMP-9 in atherosclerosis was significantly higher than in controls (Chen et al. 2011). The matrix metalloproteases secreted by macrophages within an atherosclerotic plaque can degrade various types of collagen and gelatin within the plaque. This is an important contributing factor in the formation of atherosclerosis and plaque rupture. P-selectin is mainly present in the resting platelet granule membrane and can mediate cell-cell adhesion and cell-matrix adhesion and participate in inflammation and thrombosis (Barbaux et al. 2010). P-selectin is a molecular marker for the degree of platelet activation (Robinson et al. 2006) and plays a key role in inflammation and atherosclerosis formation. Antiplatelet therapy can reduce P-selectin levels and improve atherosclerosis treatment.

The present study demonstrated that AUDA can significantly reduce the expression levels of TLR4 and NFκB in the aortas of ApoE-/- mice. These results indicated that sEH inhibitors may be associated with the activation of Toll-like receptor signaling pathways (Vabulas et al. 2001). AUDA may suppress TLR4 in some way and lead to the significant decline of NFκB expression in its downstream signal transduction pathway. The activation of this signal transduction pathway significantly reduced the expression of MCP-1 and TNF-a, which reveals that sEH inhibitors may inhibit the TLR4/NFκB pathway. Several studies have reported the relationship between TLR4 and atherosclerosis (Aksoy et al. 2012). The proposed mechanism involves TLR4 binding to ligands (such as lipopolysaccharide) on the pathogen, thereby initiating intracellular signaling pathways. This leads to a series of enzyme activation events, including activation of the NFκB pathway, which causes the release of TNFa, IL-1, IL-6 and other inflammatory mediators that have proinflammatory effects (Papatriantafyllou 2012). The expression of adhesion molecules has been shown to be regulated by the transcription factor NFκB, and the up-regulation of VCAM-1, ICAM-1 and MCP-1, which cause monocyte aggregation, results in further inflammation (Stoll et al. 2004) and the formation of atherosclerotic plaques. The suppression of a variety of inflammatory cytokines, chemokines and adhesion factors in the serum may be associated with the inhibition of TLR4, NFκB and other TLR pathways. Whether reducing the expression of these inflammatory cytokines and chemokinesis is the only way to inhibit the activation of the TLR4 signal transduction pathways needs to be investigated further.

In conclusion, we demonstrated that sEH inhibitors may inhibit the activation of the TLR4/NFκB pathway and reduce the expression of inflammatory cytokines, chemokines, and adhesion factors. These factors play key roles in the treatment of atherosclerosis. The fact that AUDA can regulate blood lipid levels might be the mechanistic explanation for its cardiovascular protective effects.

4. Experimental

4.1. Construction and grouping of animal models

All animal studies were approved by the China Ethics Committee and were performed in accordance with the corresponding ethical standards. Twenty 8-week-old ApoE-/- mice and twenty normal mice (Department of Experimental Animals, Beijing University) were maintained on the western diet (21% fat and 0.15% cholesterol) for 10 weeks, and divided into six groups: Group 1, three normal mice perfused with normal saline; Group 2, three ApoE-/- mice perfused with normal saline; Group 3, three ApoE-/- mice perfused with 0.15 ml/kg AUDA (l mg/mL; Cayman, MI, USA); Group 4, three ApoE-/- mice perfused with 0.25 ml/kg AUDA; Group 5, three ApoE-/- mice perfused with 0.35 ml/kg AUDA; Group 6, three ApoE-/- mice perfused with 0.45 ml/kg AUDA. The liquid was injected into the esophagus of each mouse *via* the mouth with a 1 ml syringe equipped with a gavage needle. Infusions were performed every other day.

4.2. Detection of serum physical and chemical indicators by ELISA

The mice perfused with AUDA for 10 weeks were sacrificed, and the serum samples were taken from their veins. The primary antibodies against TNFa, IL6, MMP-2, MMP-9, VEGF, TGF-b, ICAM-1 and VCAM-1 (1:1000; DuoSet R&D Systems, Minneapolis, MN, USA), as well as antibodies against TC, HDL-C, low density lipoprotein cholesterol LDL-C, triglyceride TG and plasma platelet P-selection (1:500; Antibody Online, Atlanta, GA, , USA) were added to 96-well plates and incubated overnight at 4 °C. The plates were then washed and blocked with 2% bovine serum albumin (BSA). Then, secondary antibodies (1:500; Antibody-online Company, USA) were added to the wells and were incubated at 4 °C for 30 min. Plates were washed five times with phosphate-buffered saline (PBS), and 50 μl of the substrate solution was added with a multichannel pipette. The plates were kept for 15 min at room temperature. The absorbance was then measured at 450 nm, using a microtiter plate spectrophotometer. Each sample was measured in triplicate.

4.3. Detection of the expression of TLR4 and NFκB in the aortic wall of mice by western blotting

After lysis of the aorta, the protein concentration was determined using the bicinchoninic acid (BCA) method. The samples were rinsed three times with PBS followed by lysis in an ice-cold lysis buffer for 30 min. The cells were centrifuged at 12 000 rpm for 10 min at 4 °C, and the supernatant was then collected to determine the concentration of protein by BCA assay (Pierce). A total of 20 μg of protein per cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membrane was blocked in Tris buffer solution (TBS) containing 5% non-fat milk (Wyeth) for 2 h and subsequently incubated with a primary antibody (1:1000) overnight at 4 °C, followed by incubation with the appropriate secondary antibody (1:5000) at room temperature for 1 h. Detection was performed using the chemiluminescence (ECL) method and exposure to radiographic film. β-Actin served as a loading control.

4.4. Observation of aortic sinus atherosclerotic (As) plaques stained by Oil Red O

The ApoE-/- mice were anesthetized with 3% sodium pentobarbital, and retro-orbital blood was collected. The left ventricle was perfused with 4% paraformaldehyde for 1 h. The aortic bifurcation and the heart were dissected, and the aortic sinus was frozen. The continuous sections (8 μm) were cut by a HM525 cryostat (MICROM International, GmbH). Oil Red O was used to stain fats. NIH image analysis software was applied to calculate the plaque area.

4.5. Statistical analysis

All data were processed using the SPSS 20 software package. Data are presented as the mean ± standard deviation (SD). Comparative analysis between groups was conducted using the independent-samples t test. The differences among groups were analyzed using ANOVA. A P value < 0.05 was considered to be statistically significant.

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