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Basic Science

Shen-Yuan-Dan Capsule Inhibiting Inflammatory Reaction by Regulating Insulin Receptor Substrate 1/PI3K/Akt/NF-κB Signaling Pathway in Apoliprotein E Knockout Mice Fed with a High-Fat Diet

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Background: Shen-Yuan-Dan Capsule (SYDC), a traditional Chinese medicine, is proposed to have the capacity to prevent angina pectoris. However, the effects and the related mechanisms of SYDC on atherosclerosis (AS) are still unknown. This study was designed to investigate the effects of SYDC on AS and inflammatory reaction in the apoliprotein E-knockout ($ApoE^{-/-}$) mice fed with a high-fat diet.

Methods: Thirty eight-week-old male $ApoE^{-/-}$ mice were randomly divided into three groups (n = 10) 6 weeks after being fed with a high-fat diet: the control group, the lipitor group, and the SYDC group. The hearts were collected for hematoxylin and eosin (HE) or Van Gieson (VG) staining, and the aortas were collected for quantitative reverse transcription polymerase chain reaction (RT-PCR) and western-blotting.

Results: The data showed that the levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), atherosclerotic indexes (AI) and the corrected areas of atherosclerotic plaque of the mice on SYDC group were significantly decreased compared with those of the mice in the control group ($p < 0.01$, $p < 0.05$). SYDC can significantly increase collagen proportion in plaques as compared to the untreated mice ($p < 0.01$). In addition, the messenger ribonucleic acid (mRNA) expressions of insulin receptor substrate 1 (IRS-1), PI3K, Akt, NF-κB and tumor necrosis factor-α (TNF-α) in the mice fed with a high-fat diet were significantly reduced by SYDC ($p < 0.05$, $p < 0.01$).

Conclusions: SYDC can exert an anti-atherosclerotic effect on $ApoE^{-/-}$ mice fed with a high-fat diet. The action mechanism of SYDC was attributed to its ability to inhibit inflammatory reaction by regulating IRS-1/PI3K/Akt/NF-κB signaling pathway.

Key Words: Atherosclerosis • Inflammatory reaction • IRS-1/PI3K/Akt/NF-κB signaling pathway • Shen-Yuan-Dan Capsule

INTRODUCTION

Atherosclerosis (AS) is the common pathological mechanism of cardiovascular and cerebrovascular diseases, including angina pectoris, and has been the subject of numerous investigations over an extended period of time. In the past 100 years, the global death ratio caused by cardiovascular diseases has increased from 1/10 in the early 1900s to 1/3 in the early 2000s, becoming the lead-

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ing cause of death worldwide.[1,2] Therefore, it is essential to find therapeutic drugs to treat AS to improve the long-term prognosis of patients with angina pectoris.

Inflammatory reaction plays an important role in the pathogenesis of AS and angina pectoris.[3-5] The pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) could be involved in cardiovascular pathophysiology through their effects on endothelial function, coagulation and lipid metabolism.[6] TNF-α initiates tissue inflammation, a process mediated by the NF-κB transcription factor. In response to TNF, latent cytoplasmic NF-κB is activated, enters the nucleus, and induces inflammatory gene expression programs.[7] It has been reported that a variety of growth factors stimulate the expression of IL-6 genes via signal-transduction pathways that converge to activate the NF-κB complex of transcription factors and that PI3K/Akt were involved in the activation of NF-κB transcription factors.[8] In addition, insulin receptor substrate 1 (IRS-1) can induce the activation of PI3K/Akt signal pathway to exert biological function including inflammatory reaction.[9]

For many years, traditional Chinese medicine (TCM) has been used in the treatment of AS and angina pectoris. Shen-Yuan-Dan Capsule (SYDC), a widely used TCM prescription, consists of eight crude Chinese medicinal agents named Salvia miltiorrhiza Bge, Astragalus membranaceus Bge, root of Pilose Asiabell, Radix Scrophulariae, Hirudo nipponica (Whitman), Lumbricus, Eupolyphagasinensis (Walker), and Rhizoma Corydalis, and has been confirmed to be effective in the treatment of angina pectoris.[8,9] Our previous studies demonstrated that oral supplementation with SYDC can not only relieve symptoms of angina but also promotes recovery from cardiac dysfunction.[10,11] The related mechanisms include reduction of myocardium infarct size,[12] recovery of impaired endothelial function,[13] and inhibition of oxidative injury.[14] Additionally, a recent study showed that SYDC pharmacological post-conditioning has protective effects against myocardial ischemia/reperfusion injury in both *in vivo* and *in vitro* models, which are related to activating the PI3K/Akt pathway.[15] However, the effects and the related mechanisms of SYDC on AS are still unknown. Therefore, this study was designed to investigate the effect of SYDC on AS and the inflammatory mechanism in the ApoE-knockout ($ApoE^{-/-}$) mice fed with a high-fat diet by detecting the expressions of TNF-α, IL-6, IRS-1, PI3K, Akt, and NF-κB.

METHODS

Animals

Male $ApoE^{-/-}$ mice (n = 30, 8 weeks old, weighing 18-20 g) with the C57BL/6J background and 10 C57BL mice were introduced and bred by the Department of Laboratory Animal Science of Peking University Health Science Center.

Materials and reagents

The blood lipid kits were purchased from Zhongsheng Beikong Biotechnology Co., Ltd (110821, 11054, 120531, 120501, Beijing, China). Van Gieson (VG) staining kit was purchased from MAIXIN-BIO (1312178002, Fuzhou, China).

Drug sources and composition

Lipitor (Atorvastatin calcium) was purchased from Pfizer Pharmaceutical Co., Ltd (Shanghai, China, H20051408). SYDC was provided from the manufacturing laboratory of Beijing TCM Hospital (Beijing, China, Z20053327).

Establishment of atherosclerosis model

All the $ApoE^{-/-}$ mice were fed with a high-fat diet for 13 weeks, which contained 21% (wt/wt) fat from lard supplemented with 0.15% (wt/wt) cholesterol[16] from the Beijing Ke'ao Xieli Feed Co. Ltd (Beijing, China). In addition, 10 C57BL mice were fed a standard chow diet containing 4% fat.

Drug treatment

After 6 weeks on the high-fat diet, the $ApoE^{-/-}$ mice were randomized (10 mice in each group) and treated by intragastric administration with SYDC (80 mg/kg/d), lipitor (2.973 mg/kg/d, positive control group), or distilled water (control group) for 7 weeks, accompanied by high-fat diet feeding. The medical doses in mice were calculated according to the conversion coefficient (9.01) between human and mice.[17]

Histology

After 7 weeks of drug therapy, the heart, including the aortic sinus heart from each mouse, was removed

and embedded in paraffin to determine the morphology of atherosclerotic plaque by hematoxylin and eosin (HE) and Van Gieson (VG) staining. The aorta (except for the aortic root) from each mouse from all the groups were removed and stored in -80 °C.

Evaluation of atherosclerotic lesions

To quantitatively evaluate atherosclerotic lesions, a 5 μm-thick section was selected and quantified. HE staining and VG staining were used to evaluate the plaque areas and the collagen content of atherosclerotic plaque, and a morphometric analysis was performed using Image Pro Plus (Media Cybernetics, Rockville, MD, USA). The protocol for evaluating atherosclerotic lesions was conducted according to our previous method.[17]

Determination of plasma lipid concentration

Blood samples were drawn from the left ventricle of a cohort of all male ApoE$^{-/-}$ mice that had received a high-fat diet for 13 weeks. Total cholesterol (TC) and triglyceride (TG) were determined by enzyme studies in serum. Low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were determined by immunoturbidimetry. Finally, all indices were determined using the RX-2000 radiometer (Technicon Instruments Corp., Tarrytown, NY, USA). Additionally, the atherosclerosis index (AI) was calculated by the formula of AI = non-HDL-C/HDL-C.[18]

Real-time quantitative PCR

The aortic root from each mouse from all the groups were removed and stored in -80 °C to examine the messenger ribonucleic acid (mRNA) expressions of genes. The total ribonucleic acid (RNA) of the aortae was extracted using a TRIzol kit according to the manufacturer's instructions. The primers of IRS-1, PI3K, Akt, NF-κB and TNF-α are shown in Table 1. The protocol utilized for reverse transcription polymerase chain reaction (RT-PCR) was undertaken according to our previous method.[19]

Western blotting

The aorta from the other four mice per group were removed and stored in -80 °C to examine the protein expressions of PI3K and p-Akt. The protocol for western-blotting was in according with our earlier method.[20] The primary anti-bodies including PI3K (Abcam, 1:1000), p-AKT (Abcam, 1:2000) and GAPDH (Proteintech Group, 1:4000) were used in this study. Protein expression was detected with an enhanced chemiluminescence detection system (Vigorous, Beijing, China).

Statistical analysis

Mean values and standard deviations (mean ± SD) were calculated for each variable studied, and all statistical procedures were performed using SPSS 11.5 (SPSS, Chicago, IL, USA). Normally distributed data were analyzed using one-way ANOVA with a Bonferroni post hoc test to evaluate the statistical significance of intergroup differences in all the tested variables. In all cases, statistical significance was $p < 0.05$.

RESULTS

High-fat diet provokes atherosclerosis in ApoE-knockout mice

After the ApoE$^{-/-}$ mice were fed a high-fat diet for 13 weeks, typical atherosclerotic plaque scan be clearly observed in the aortic valves attachment sites of the ApoE$^{-/-}$ mice compared with C57 mice (Figure 1).

Table 1. The primers in this study

Genes	Forward	Reverse
GAPDH	5′-GCAAGTTCAACGGCACAG-3′	5′-CGCCAGTAGACTCCACGAC-3′
IRS-1	5′-TCCACACCAAGAGATGGGTA-3′	5′-AGCGTGGACAAAGAGAGGTT-3′
PI3K	5′-TCCAAATACCAGCAGGATCA-3′	5′-ATGCTTCGATAGCCGTTCTT-3′
AKT	5′-TACTCATTCCAGACCCACGA-3′	5′-GAGGTTCTCCAGCTTCAGGT-3′
NF-κB	5′-GGAGAAGGCTGGAGAAGATG-3′	5′-GCTCATACGGTTTCCCATTT-3′
IL-6	5′-GGACCAAGACCATCCAATTC-3′	5′-ACCACAGTGAGGAATGTCCA-3′
TNF-α	5′-CTAGCCAGGAGGGAGAACAG-3′	5′-GCTTTCTGTGCTCATGGTGT-3′

IL-6, interleukin-6; IRS-1, insulin receptor substrate 1; TNF-α, tumor necrosis factor-α.

The effect of SYDC on the blood lipids

Following drug treatment for 7 weeks, the levels of TG, TC, and LDL-C in serum of the ApoE$^{-/-}$ mice on the SYDC group were significantly reduced compared with those of the mice in the control group ($p = 0.04$, $p < 0.05$), but the serum levels of HDL-C were not significantly changed ($p > 0.05$) (Figure 2A).

The effect of SYDC on atherosclerotic index

The atherosclerotic indexes of the mice in the lipitor group and the SYDC group were significantly decreased compared with those of the mice in the control group ($p = 0.007$, $p < 0.01$), there was no significant difference on the two drug-treatment groups ($p > 0.05$) (Figure 2B).

The effect of SYDC on the atherosclerotic plaques and the compositions

The atherosclerotic plaque areas of the mice in the lipitor and the SYDC group were significantly decreased ($p = 0.008$, $p < 0.01$). Moreover, the proportion of collagen in the atherosclerotic plaques of the mice in the SYDC group were significantly increased compared with that of the mice in the control group ($p = 0.008$, $p < 0.01$), while the extracellular lipid composition of the mice in the SYDC group was significantly decreased ($p = 0.008$, $p < 0.01$) (Figure 3).

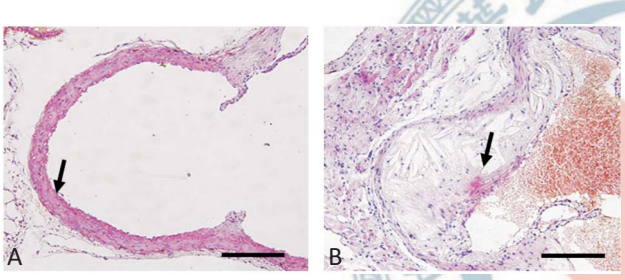

Figure 1. *Comparison of pathological morphology of the aorta of the C57BL/6J mice and the apoliprotein E-knockout (ApoE$^{-/-}$) mice under different magnification at 13 weeks after being fed with different diets. (A) The pathological morphology of the aortae of the C57BL/6J under ×200 magnification at 13 weeks after being fed with different kinds of diets (scale bars = 200 μm). (B) The pathological morphology of the aortae of the ApoE$^{-/-}$ mice under ×200 magnification at 13 weeks after being fed with different kinds of diets (scale bars = 200 μm). Hematoxylin and eosin (HE) staining, where the black arrow indicates the aorta of the mice.*

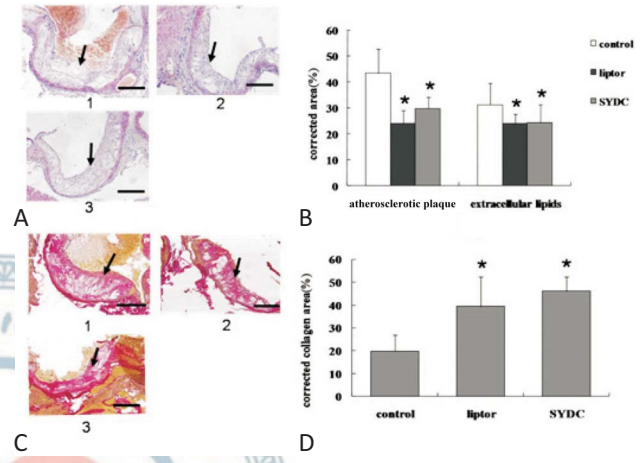

Figure 3. *Effect of SYDC on atherosclerotic plaque. (A) HE staining showing the pathological morphology change of the atherosclerotic plaque under ×200 magnification in aorta of the ApoE$^{-/-}$ mice after the treatment of SYDC. 1: control group; 2: lipitor group; 3: SYDC group. Scale bars = 200 μm, the black arrow indicates the atherosclerotic plaque in aorta. (B) The statistical analysis of the corrected area of atherosclerotic plaque and extracellular lipids content in plaque of the ApoE$^{-/-}$ mice after the treatment of SYDC. * $p < 0.01$ versus control group. (C) Van Gieson (VG) staining showing the pathological morphology change of content in atherosclerotic plaque under ×200 magnification in aorta of the ApoE$^{-/-}$ mice after the treatment of SYDC. 1: control group; 2: lipitor group; 3: SYDC group. Scale bars = 200 μm, the black arrow indicates thee collagen content in atherosclerotic plaque in aorta. (D) The statistical analysis of the corrected area of collagen in atherosclerotic plaque of the ApoE$^{-/-}$ mice after the treatment of SYDC. * $p < 0.05$ versus control group, n = 10.*

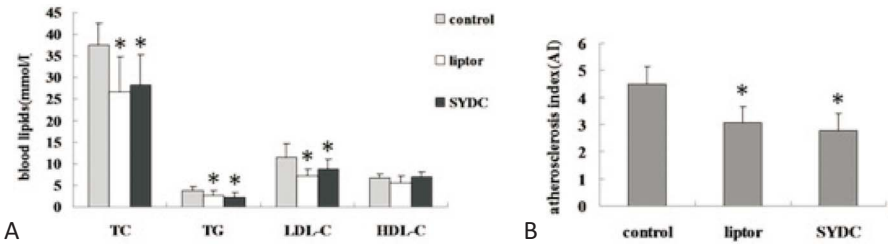

Figure 2. *Effects of Shen-Yuan-Dan Capsule (SYDC) on blood lipids in serum of ApoE$^{-/-}$ mice fed with high-fat diet. (A) Effects of SYDC on total cholesterol (CHOL), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) in serum of ApoE$^{-/-}$ mice fed with a high-fat diet. (B) Effects of SYDC on atherosclerotic index (AI, AI = non-HDL-C/HDL-C) in serum of ApoE$^{-/-}$ mice fed with a high-fat diet. * $p < 0.05$ versus control group, n = 10.*

The effect of SYDC on the mRNA expressions of NF-κB, TNF-α and IL-6

As shown in Figure 4, the mRNA expressions of NF-κB and TNF-α of the mice in the SYDC group and the lipitor group were significantly decreased in contrast with that of the control group mice ($p = 0.03$, $p = 0.006$, $p < 0.05$, $p < 0.01$), there was no significant difference in the two drug-treatment groups ($p > 0.05$). Additionally, there was no significant difference with mRNA expression of IL-6 of the mice in the SYDC group in contrast with that of the mice in the control group ($p > 0.05$).

The effect of SYDC on the mRNA expressions of IRS-1, PI3K and Akt

The mRNA expressions of IRS-1, PI3K and Akt of the mice on the SYDC group and the lipitor group were significantly decreased in contrast with those of the mice in the control group ($p = 0.04$, $p = 0.008$, $p < 0.05$, $p < 0.01$), there was no significant difference in the two drug-treatment groups ($p > 0.05$) (Figure 5).

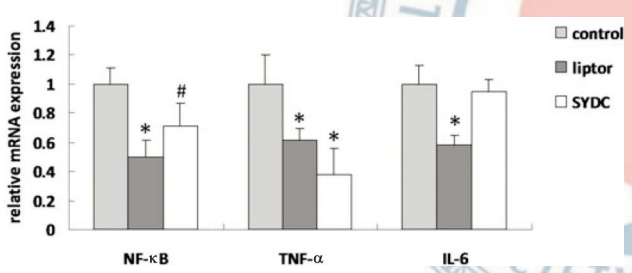

Figure 4. *Effect of SYDC on messenger ribonucleic acid (mRNA) expressions of NF-κB, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in ApoE$^{-/-}$ mice fed with a high-fat diet. # $p < 0.05$ versus control group; * $p < 0.01$ versus control group, n = 10.*

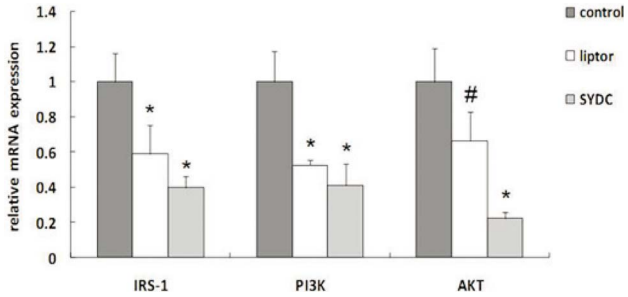

Figure 5. *Effect of SYDC on mRNA expressions of insulin receptor substrate 1 (IRS-1), PI3K and Akt in ApoE$^{-/-}$ mice fed with a high-fat diet. # $p < 0.05$ versus control group; * $p < 0.01$ versus control group, n = 10.*

The effect of SYDC on the protein expressions of PI3K and p-Akt

As shown in Figure 6, the protein expression of PI3K and p-Akt of mice in the SYDC group and the lipitor group were significantly decreased in contrast with those of the mice in the control group ($p = 0.05$, $p = 0.007$, $p < 0.05$, $p < 0.01$), there was no significant difference in the two drug-treatment groups ($p > 0.05$).

DISCUSSION

In this study, we demonstrate that SYDC can exert anti-atherosclerotic effect in ApoE$^{-/-}$ mice fed with a high-fat diet. In addition, the data showed that SYDC may inhibit the expression of TNF-α by regulating IRS-1/PI3K/Akt/NF-κB signaling pathway at the transcriptional level, which may be the main mechanism of SYDC exerting the anti-atherosclerotic effect.

SYDC, a traditional Chinese medicine compound, contains crude Chinese medicinal agents named Salvia miltiorrhiza Bge, Astragalus membranaceus Bge, root of Pilose Asiabell, Radix Scrophulariae, Hirudo nipponica (Whitman), Lumbricus, Eupolyphagasinensis (Walker), and Rhizoma Corydalis. In clinical situations, SYDC has been widely used for the treatment of angina pectoris.[10,11] It is well known that AS is the pathological basis resulting in angina pectoris. However, the effects and the related mechanisms of SYDC on AS are still unknown. In this study, the data showed that SYDC can significantly decrease the blood lipid, AI and the area of atherosclerotic plaque in the ApoE$^{-/-}$ mice fed with a high-fat diet. It suggests that SYDC can exert an inhibitive effect on the formation and progress of AS. And the inhibitive

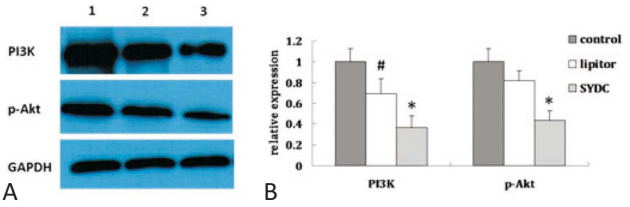

Figure 6. *Effect of SYDC on protein expressions of PI3K and p-Akt in ApoE$^{-/-}$ mice fed with a high-fat diet. (A) Western blotting (WB) results of PI3K and p-Akt levels in ApoE$^{-/-}$ mice fed with a high-fat diet. (B) Quantitative analysis (column diagram) of PI3K and p-Akt levels in mouse aorta based on WB results. # $p < 0.05$ versus control group; * $p < 0.01$ versus control group, n = 10.*

effect of SYDC on AS can mainly contribute to the entire effect from the eight crude Chinese medicinal agents with the effect of supplementing Qi or activating blood circulation.

The content in atherosclerotic plaque, especially extracellular lipids and collagen, were responsible for the stability of atherosclerotic plaque. In this study, we also revealed that SYDC can decrease the percentage of extracellular lipids and increase the percentage of collagen in atherosclerotic plaque. This suggests that SYDC may have the potential effect of promoting the stability of atherosclerotic plaque. Certainly, this conclusion needs more indexes including vulnerability index to confirm.

Inflammatory reaction plays a crucial role in the pathogenesis of AS. Chronic vascular inflammation can be induced by the pro-inflammatory cytokines including TNF-α and IL-6.[21,22] High-fat diet can also promote low-grade chronic inflammation associated with increased levels of such mediators as TNF-α and IL-6.[23] In this study, the data suggests that SYDC can inhibit inflammation reaction by down-regulating the expression of TNF-α of the ApoE$^{-/-}$ mice fed with a high-fat diet.

The NF-κB family of transcription factors regulates the induction and resolution of inflammation, and it has an essential role in inflammation.[24] Several studies showed that both IL-6 and TNF-α were regulated by NF-κB.[25-27] In this study, our results showed that SYDC can reduce the expressions of NF-κB in mice aorta after feeding with a high-fat diet. It suggests that SYDC may inhibit inflammatory reaction in the process of AS by regulating the expression of NF-κB. Our results showed that SYDC can significantly reduce the expression of NF-κB compared with the control group. It suggests that SYDC may inhibit inflammatory reaction in the process of AS by regulating the expressions of NF-κB and TNF-α, not by regulating the expression of IL-6.

It has previously been shown that the PI3K/Akt signal pathway can be an upstream activator of the NF-κB signaling cascade.[24,28] PI3Ks and their downstream target Akt are conserved family of signal transduction enzymes that are involved in regulating cellular activation, inflammatory responses, autophagy and apoptosis.[9] IRS-1 is an upstream activator of PI3K/Akt signal pathway and it can induce the activation of PI3K/Akt signal pathway to exert biological function including inflammatory reaction.[9] In addition, leptin increased interleukin-8 (IL-8) production in synovial fibroblast via the activation of IRS1/PI3K/Akt/NF-κB-dependent pathway.[29] In this study, the results showed that in transcriptional level, SYDC can significantly inhibit the expressions of IRS-1, PI3K and Akt in aortae of the ApoE$^{-/-}$ mice fed with a high-fat diet in transcriptional level. So it suggests that SYDC may suppress the expressions of IL-6 and TNF-α via regulating IRS-1/PI3K/Akt/NF-κB signaling pathway in transcriptional level. In protein level, the data show that SYDC can significantly inhibit the expression of PI3K and p-Akt in aortae of the ApoE$^{-/-}$ mice fed with a high-fat diet. It suggests that SYDC may suppress inflammatory reaction in the process of AS by activating Akt and further affecting the expression of NF-κB.

CONCLUSIONS

In summary, the results from this study show that SYDC can dramatically ameliorate AS in the ApoE$^{-/-}$ mice fed with a high-fat diet. The possible mechanism is related to inhibiting the expression of TNF-α via regulating IRS-1/PI3K/Akt/NF-κB signaling pathway in transcriptional level. The activation of Akt may be the key link of SYDC exerting anti-inflammatory effect. Therefore, it is necessary to further investigate the potential effect and mechanism of SYDC preventing the occurrence of AS.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

ACKNOWLEDGMENTS

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