Sulfated galactofucan from Sargassum thunbergii Attenuates atherosclerosis by suppressing inflammation via TLR4/MyD88/NF-κB Signaling Pathway

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Research Article

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Abstract

Purpose: Sulfated galactofucan (SWZ-4), which was extracted from Sargassum thunbergii, has recently been reported to have anti-inflammatory and anti-cancer property. The present study aimed to evaluate if SWZ-4 could attenuate atherosclerosis in Apolipoprotein E-deficient (ApoE KO) mice by suppressing inflammatory response through TLR4/MyD88/NF-κB signaling pathway.

Methods: Male ApoE KO mice were fed with high-fat diet for 16 weeks and intraperitoneally injected with SWZ-4. RAW246.7 cells were treated with lipopolysaccharide (LPS) and SWZ-4. Atherosclerotic lesions were measured by Sudan IV and Oil Red O staining. Serum lipid profiles, inflammatory cytokines, and mRNA and protein expression levels were evaluated.

Results: SWZ-4 decreased serum TNF-α, IL-6 and IL-1 levels, but cannot improve blood lipid profiles. SWZ-4 downregulated the mRNA and protein expression of TLR4, MyD88 and phosphorylation of p65 and attenuated atherosclerosis in the ApoE KO mice ($P < 0.01$). In LPS-stimulated RAW 264.7 cells, SWZ-4 inhibited pro-inflammatory cytokines and the mRNA expression of TLR4, MyD88 and p65 and reduced the proteins expression of TLR4, MyD88 and the phosphorylation of p65 ($P < 0.01$).

Conclusion: These results suggest that SWZ-4 exerts an anti-inflammatory effect in ApoE KO atherosclerosis mice via inhibiting TLR4/MyD88/NF-κB signaling pathway in macrophages, and may potentially serve as a treatment for atherosclerosis.

Introduction

Atherosclerotic vascular disease is a major cause of morbidity and mortality worldwide [1]. Atherosclerosis has been a chronic disease characterized as inflammation and cholesterol deposition in the arterial wall [2]. Therefore, pharmacological inhibition of the vascular inflammatory state may provide to be a valuable strategy for improving therapeutic outcomes in atherosclerosis.

Many signal transduction pathways are involved in the pro-atherogenic inflammatory process. In recent years, the Toll-like receptor 4 (TLR4)/myeloid differentiation primary response protein (MyD88)/nuclear factor kappa B (NF-κB) pathway has attracted more attention because of its pivotal function in atherosclerosis. It has been reported that C3H/HeJ mice carry a missense mutation that affects the cytoplasmic portion of TLR4 and causes loss of function, leading to resistance to atherosclerosis [3]. In Apolipoprotein E-deficient (ApoE K0) mice, genetic deficiency of TLR4 or MyD88 is related with a considerable decrease of atherosclerotic plaque areas and serum levels of pro-inflammatory cytokines, despite persistent hypercholesterolemia [4]. As a primary transcription factor, NF-κB is one of the most important regulators of inflammation. It has been shown that suppressing of NF-κB signaling pathway could protect against atherosclerosis [5, 6].

Sargassum thunbergii is a brown algae and widely distributed in the seas of Japan and China. Researches on S. thunbergii has focused on its bioactive compounds, such as quinone derivatives,
phlorotannins, polyunsaturated fatty acid, and polysaccharides [7, 8]. The polysaccharides from *S. thunbergii* have been found to exhibit diverse pharmacological effects, including antioxidant, anti-inflammatory, anti-proliferative properties [9–11]. Our previous work has prepared the sulfated galactofucan (SWZ-4) isolated from *S. thunbergii* and found that SWZ-4 has anti-cancer activities by inducing cancer senescence [12]. However, few research have been reported to explore whether polysaccharides from *S. thunbergii* could attenuates atherosclerosis through suppressing inflammation. Thus, the effect and mechanism of SWZ-4 on TLR4-mediated inflammatory responses in atherosclerotic lesions is still unclear. The present study aimed to evaluate whether SWZ-4 could attenuate inflammation and atherosclerosis by inhibiting TLR4/ MyD88/NF-κB signaling pathway in ApoE KO mice. The effects were also tested in RAW246.7 cells.

**Methods And Materials**

**Materials**

bovine serum albumin (BSA), oil red O, fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) culture medium were provided by Gibco (Grand Island, USA). OCT compound was from Tissue Tek (Sakura, CA). Lipopolysaccharide (LPS) was from Sigma-Aldrich (St Louis, USA).

**Preparation and compositional analysis of SWZ-4**

According to previous methods, SWZ-4 were prepared in our lab [10, 12]. Briefly, crude polysaccharides (SWZ) was extracted and underwent anion exchange chromatography. Dialysis, concentration and precipitation were then performed through ethanol. The autohydrolysis and compositional analysis of SWZ-4 was performed according to our previous study [12, 13].

**Animal experiments**

All animal studies were approved by the Ethics Committee of the Zhejiang Academy of Medical Sciences (License SYXK 2019–0011). The ApoE KO mice were randomly divided into 4 groups (n = 10): control group (NC group), high-fat diet with low-dose SWZ-4 (L-SWZ-4 group), high-fat diet with high-dose SWZ-4 (H-SWZ-4 group) and high-fat diet alone (model group). After more than a week of adaptive feeding, forty male 6-week-old ApoE KO mice were fed with high-fat diet (HFD) containing 21% fat and 0.2% cholesterol or normal chow diet for 16 weeks. All mice were kept in a SPF mouse facility and injected intraperitoneally with SWZ-4 or vehicle (saline) for a total of 16 weeks, mice in the L-SWZ-4 and H-SWZ-4 groups were received 50mg/kg and 100mg/kg SWZ-4 every two days. Body weight was measured once per 4 weeks. Before blood sampling and tissue collection, all the mice were euthanized with a lethal dose of xylazine hydrochloride (10 mg/kg).

**Cell culture**
The murine macrophage cell line RAW264.7 was obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), and cultured in DMEM supplemented with 10% FBS and 1% penicillin streptomycin at 37°C in humidified 5% CO₂ incubators. In the NC group, cells were treated only in serum-free DMEM. After pretreatment with DMSO or SWZ-4 (0.4 or 0.8mg/ml) for 1 h, the LPS group (DMSO + LPS) and SWZ-4 group (SWZ-4 + LPS) cells were stimulated with LPS (1 µg/ml) for 24 h under serum-starved culture conditions. All cell experiments were performed at least three times in triplicate.

Cell viability assay

Cell viability was measured using a Cell Counting Kit-8 (CCK-8, Tianjing Biolite Biotech, China) assay. Briefly, RAW 264.7 cells were seeded into 96-well culture plates and incubated with various concentrations of SWZ-4 (0.4, 0.8 or 1.0 mg/ml) with or without LPS (1 µg/ml). After 24h, CCK-8 reagent was added to each well and incubated for 3 h in incubator. The absorbance values were detected at a wavelength of 450 nm.

Measurement of serum lipid profile and inflammatory cytokines

Commercial kits (Nanjing Jiancheng Bioengineering Institute, China) were employed to evaluate serum lipid profile (triglycerides [TG], total cholesterol [TC], high density lipoprotein cholesterol [HDL-C] and low density lipoprotein cholesterol [LDL-C]) in ApoE KO mice. Serum inflammatory cytokine levels of TNF-α, IL-1, and IL-6 in the ApoE KO mice and RAW264.7 cells were determined by enzyme-linked immunosorbent assay (ELISA) kits (Elabscience, China). All procedures were performed strictly according to the manufacturer's instructions.

Assessment of atherosclerosis with Sudan IV and Oil Red O staining

The dissected aorta from the root to the abdominal region was embedded in paraffin or frozen in TissueTek OCT media. Serial 4 mm sections of the aortic valve were cut and stained with Oil Red O. Aortas were cut longitudinally and stained with Sudan IV solution. Two observers measured and quantified the total surface area and the total Oil Red O-positive lesion area using Image-Pro Plus 6.0. The degree of atherosclerotic lesion was evaluated by the percentage of lesion in the total area.

Quantitative real-time PCR analysis

mRNA expression levels were analyzed by quantitative Real-time PCR (qRT-PCR) analysis using specific primers (Table 1). Briefly, total RNA was isolated by Trizol Reagent (Invitrogen, USA) and reverse transcribed into cDNA by SuperScriptTM III Reverse Transcriptase (Invitrogen, USA). The expression of GAPDH was used as an internal control. Gene expression levels were calculated using the delta-Ct method. Each sample was assayed in triplicate.
Table 1  
Primer sequences for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Sequence(5’→3’)</th>
<th>Size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>NC_000072.7</td>
<td>F:GGGGTCCCAGCTTAGGTTCA, R:CCAATACGGGCCAATCCGTTC</td>
<td>100</td>
</tr>
<tr>
<td>TLR4</td>
<td>NC_000070.7</td>
<td>F:TCCCTGCATAGAGGTAGGTTCC, R:TCAAGGTTGGAAGCTCAGA</td>
<td>119</td>
</tr>
<tr>
<td>MyD88</td>
<td>NC_000075.7</td>
<td>F:AGGACAAACGCGGAACCTTT, R:CATGCGGCGGACACCTTCTTCT</td>
<td>196</td>
</tr>
<tr>
<td>IκBα (α)</td>
<td>NC_000078.7</td>
<td>F:GCCAGCTGACCCTGGAAAAT, R:TCATCATAGGGCAGCTCATCC</td>
<td>103</td>
</tr>
<tr>
<td>p65 (p65)</td>
<td>NC_000085.7</td>
<td>F:ACACCCACCACATCAAGATCAA, R:CTCTATAGGAACTATGGATACTGCG</td>
<td>175</td>
</tr>
<tr>
<td>IL-6</td>
<td>NC_000071.7</td>
<td>F:TTCCTCTGGTCTTCTGGAGT, R:TCTGTGACTCCAGCTTATCTTG</td>
<td>148</td>
</tr>
<tr>
<td>TNF-α</td>
<td>NC_000083.7</td>
<td>F:ACCCTCACACTCAAAACCAC, R:ACAAGGTACAACCACATCGGC</td>
<td>134</td>
</tr>
</tbody>
</table>

Western blotting analysis

Protein samples from mice aortas tissue and RAW264.7 cells were harvested and homogenized with SDS buffer. Samples were placed on ice for 30 min and centrifuged at 12,000g for 10min at 4°C, and then the supernatant was collected. Total proteins were denatured by boiling at 100°C for 10 min in 5 X loading buffer containing SDS. Protein samples were subjected to SDS-PAGE electrophoresis and then transferred onto nitrocellulose membrane. After blocking, the blots were incubated at 4°C overnight with primary antibody TLR-4 (1:1000,ab13556,abcam,UK), MyD88 (1:1000,ab219413,abcam,UK), p-IκBα (1:1000,2859S,CST,USA), IκBα (1:1000,4814S,CST,USA), p-p65 (1:1000,3033S,CST,USA), p65 (1:1000,8242S,CST,USA) and GAPDH (1:2000, Vazyme, Nanjing, Jiangsu, China). The corresponding HRP-conjugated secondary antibodies were added for 2 h. Protein bands were visualized using enhanced chemiluminescence detection reagents. Relative changes in protein expression were quantified using Image J. All western blot procedures were performed at least three times.

Statistical analysis

Data are expressed as the mean ± standard deviation (S.D.). The significant differences between groups were assessed by one-way analysis of variance followed by LSD method for multiple comparisons. All
tests were conducted using SPSS 13.0. All significance was considered at $P<0.05$.

**Results**

Mice body weight and food intake

As shown in Fig. 1a, the mice in the model, L-SWZ-4 and H-SWZ-4 groups had markedly higher body weights than those in the control group after 8 weeks. No significant differences were found in body weights among the model, L-SWZ-4 and H-SWZ-4 groups. There were no significant differences in the average daily food intake among groups (Fig. 1b).

SWZ-4-H downregulates inflammatory cytokines but cannot improve lipid profiles in the ApoE KO mice

Activating TLR4/MyD88/NF-κB signaling pathway can release different pro-inflammatory cytokines. Serum levels of TNF-α, IL-6 and IL-1 in model groups were enhanced than those in the control and SWZ-4 groups. Compared with those of controls, the expressions were higher in SWZ-4 group (Fig. 2a-c). SWZ-4 could relieve inflammatory responses in the ApoE KO mice.

To investigate the effects of SWZ-4 on lipid profiles, we measured serum lipid levels in mice. There were no significant differences in TG, TC, HDL-C and LDL-C among the model, L-SWZ-4 and H-SWZ-4 groups after 16 weeks. Compared to the controls, serum levels of TG, TC, and LDL-C were significantly higher in the model and SWZ-4 groups (Fig. 2d-g).

SWZ-4 inhibit TLR4/MyD88/NF-κB signaling pathway in the ApoE KO mice aorta

We further examined the effects of SWZ-4 on inflammation-related signaling pathways in the ApoE KO mice aorta by qRT-PCR and western blot. Compared with those in the control group, the mRNA expressions of TLR4, MyD88 and p65 were significantly upregulated in the model and SWZ-4 groups. Compared with model group, treatment with SWZ-4 could significantly suppress the expression of TLR4, MyD88 and p65. The western blot results revealed similar trends between TLR4/MyD88/NF-κB mRNA expression and protein levels (Fig. 3).

SWZ-4 attenuated the progress of atherosclerosis in the ApoE KO mice

To study the effects of SWZ-4 on atherosclerosis in vivo, the extent of atherosclerosis was measured by Sudan IV and Oil Red O staining of aorta in the apoE KO mice. According to Fig. 4, the aorta atherosclerosis plaque areas were significantly increased in the model and SWZ-4 groups than controls. Compared with those in the model group, a significant decrease was found in aorta atherosclerosis plaque areas in the L-SWZ-4 and H-SWZ-4 groups.

Cytotoxic effect of SWZ-4 and LPS

RAW264.7 cells were cultured with SWZ-4 and LPS or medium, and their viability was examined by CCK8 assay. SWZ-4 did not exhibit cytotoxicity against RAW 264.7 cells at the concentrations of 0.4 and 0.8
mg/ml with or without LPS (Fig. 5).

SWZ-4 downregulate inflammatory cytokines and TLR4/MyD88/NF-κB signaling pathway in LPS-induced RAW 264.7 cells

The comparison with the controls shown in Fig. 6, when the RAW 264.7 cells were induced by LPS, the mRNA and proteins expression levels of TNF-α and IL-6 were significantly increased. Compared with those in the LPS group, the protective effects of SWZ-4 against inflammation were significantly obvious at the 0.4 and 0.8 mg/ml concentration.

We further assessed the effects of SWZ-4 on the activation of TLR4/MyD88/NF-κB signaling pathway in LPS-induced RAW246.7 cells. Compared with the controls, both the LPS and SWZ-4 groups up-regulated TLR4, MyD88 and NF-κB mRNA and protein expressions. Compared with the levels of the LPS group, SWZ-4 suppressed the TLR4, MyD88 and NF-κB expressions of mRNA and protein (Fig. 7–8).

Discussion

The finding of the present study was that SWZ-4 treatment significantly ameliorated atherosclerosis in ApoE KO mice and reduced inflammatory cytokine expression in vivo and in vitro. These suggested that protective effects of SWZ-4 were mediated by the modulation of the TLR4/MyD88/NF-κB signaling pathway.

Many studies reported that biological components of seaweeds have a variety of activities. As a compound of S. thunbergii, SWZ-4 have not only no apparent side-effects, but also many good biological functions. Li et al. found that polysaccharides could inhibit macrophage foam cell formation and alleviate cellular inflammation [14]. It is reported that polysaccharide have anti-angiogenesis activity against tumor migration through inhibiting VEGF/HIF-1α signaling pathway and inflammatory reaction9. In addition, the study demonstrated that polysaccharides have antioxidant effect, which is closely relates to anti-atherosclerosis [15–17]. Our previous work revealed that SWZ-4 from S. thunbergii have anti-tumour activities, which is consistent with other researches [12, 18–20]. In the present study, we found that SWS-4 have not only anti-inflammatory effect, but also anti-atherogenic effect by suppressing TLR4/MyD88/NF-κB signaling pathway.

In the past decades, atherosclerosis has been considered as an inflammatory disease characterized by immune responses that obstruct arteries. Studies showed that inflammatory reactions are pivotal in all phases of atherosclerosis and provide the relation between cardiovascular risk factors and altered arterial biology [21, 22]. Increasing numbers of studies suggested that inflammatory cytokines such as IL-6, TNF-α and hsCRP are related with atherosclerosis [23, 24]. Reduction in inflammation can lead to improve risk factors and atherosclerosis. Therefore, pharmaceutically reducing inflammatory responses appear to be a valid approach for atherosclerosis and risk factors. In the present work, HFD increased both atherosclerosis plaque area and serum levels of inflammatory markers in ApoE KO mice. And these effects were mitigated by SWZ-4 treatment in the present study.
Toll-like receptors are a family of surface molecules and important modulators of immune systems and inflammatory responses, including atherosclerosis and ischemia reperfusion [25, 26]. TLR4 is the most associated with atherosclerosis and is essential for the cell response to LPS. The expression of TLR4 has been revealed to be markedly increased in the atherosclerotic plaque macrophages. While silencing TLR4 expression in ApoE KO mice, atherosclerotic plaque size and inflammatory cytokines would decrease obviously [27]. MyD88 is not only crucial downstream key link of TLR4 but also an important adapter protein of NF-κB [28–30]. NF-κB is a widely expressed nuclear transcription factor and plays an important role in the regulation of a number of inflammatory mediators [31]. In normal conditions, NF-κB p65 is retained in the cytoplasm, and LPS stimulation could markedly elevate the phosphorylation of IκBα, and allow NF-κB p65 to translocate to the nucleus, thereby inducing transcription of inflammatory genes [32]. Several studies have been reported that the NF-κB signaling pathway functions in atherosclerosis [33, 34]. In the present study, the expressions of TLR4/MyD88/NF-κB in the aorta of ApoE KO mice and LPS-induced RAW264.7 cells were significantly elevated, the effects of SWZ-4 not only decreased the inflammatory cytokines but also reduced the expressions of TLR4/MyD88/NF-κB. The reduction levels of TLR4/MyD88/NF-κB signaling pathway and inflammatory cytokines influenced by SWZ-4 were consistent with the reduction of atherosclerotic plaque area. These results revealed that SWZ-4 could ameliorate macrophage-mediated inflammation and atherosclerosis, which was associated to TLR4/MyD88/NF-κB signaling pathway.

There are several limitations that exist in this study. First, we did not further investigate the effects of SWZ-4 on inflammation and atherosclerosis in TLR4−/− ApoE KO mice. However, previous study has proved that inhibiting TLR4 can markedly improve inflammatory responses and atherosclerosis [27]. In addition, these results do not indicate that the same effects will be seen in humans. Therefore, further clinical studies are warranted to verify efficacy and safety. Second, TLR4 in the vessel wall is expressed broadly in both endothelial and vascular smooth muscle cells, the effects of SWZ-4 on TLR4/MyD88/NF-κB signaling pathway in different cell types was insufficient in this study. Last, SWZ-4 mediated protective mechanisms other than inhibition of TLR4/MyD88/NF-κB signaling pathway may also exist. More studies are needed to further explore the relationship between SWZ-4 and atherosclerosis.

Conclusion

In conclusion, these results suggest that SWZ-4 confers anti-inflammatory and anti-atherosclerosis effects in ApoE KO mice. The protective efficacy of SWZ-4 was mediated by inhibition of TLR4/MyD88/NF-κB signaling pathway. Accordingly, SWZ-4 may be a potential therapeutic strategy for the prevention of atherosclerosis.

Declarations

Contributors: KF Zhu and XH Wang contribute equally to this article. All authors contributed to the design and interpretation of the study and to further drafts.
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**Data Availability:** All the data in this study are available upon reasonable request from the corresponding author.

**Competing interest:** No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

**Ethical approval:** The study was approved by the Institutional Animal Care and Use Committee (IACUC) at Animal Lab Center of Zhejiang Academy of Medical Sciences.

**Consent to publish:** All authors read and approved the final manuscript and submitted it for consideration for publication.

**References**


**Figures**

![Figure 1](image)

**Figure 1**

Body weight and food intake in the ApoE KO mice. (a) Body weight was measured once 4 weeks; (b) Food intake during the experiment. Values are expressed as mean ± SD. (n = 10). #P < 0.01 versus control group.
Figure 2

Serum levels of inflammatory cytokine and lipid profiles in the ApoE KO mice. (a) TNF-α, (b) IL-6, (c) IL-1, (d) TG, (e) TC, (f) HDL-C, (g) LDL-C. Values are expressed as mean ± SD. (n = 10). #P < 0.01 versus control group, *P < 0.01 versus model group.

Figure 3

Effects of SWZ-4 on TLR4/MyD88/NF-κB signaling pathway in the ApoE KO mice aorta. (a-d) The mRNA expression of TLR4, MyD88, IκBα and p65 determined by qRT-PCR. (e-i) The protein expression of TLR4, MyD88, p-IκBα, IκBα, p-p65 and p65 determined by western blot. Values are expressed as mean ± SD. (n = 3). #P < 0.01 versus control group, *P < 0.01 versus model group.
SWZ-4 attenuates atherosclerotic lesion in the ApoE KO mice aorta. (a) Representative morphological images (ORO) of aortic sinus sections. The scale bar is 500 μm. (b) Representative images of Sudan IV stained en face aorta. (c) Quantification of the percentage of lesion area stained by Sudan IV in the total aorta intima area. Values are expressed as mean ± SD. (n = 5). #P < 0.01 versus control group, *P < 0.01 versus model group.

Figure 5

Effect of SWZ-4 and LPS on the viability of RAW26.47 cells. Cells were pretreated with 0.4, 0.8 and 1 mg/ml of SWZ-4 for 1 h, followed by LPS (1 μg/ml) stimulation for 24 h. Cell viability was determined by CCK8 assay. Values are expressed as mean ± SD. (n = 3). #P < 0.01 versus control group, *P < 0.01 versus control.
Figure 6

Effect of SWZ-4 on mRNA expression and release of TNF-α and IL-6 in LPS-induced RAW264.7 cells. (a-b) For mRNA expression analysis, cells were pretreated with 0.4 and 0.8 mg/ml of SWZ-4 and LPS (1 μg/ml) stimulation for 6 h. The mRNA levels of TNF-α and IL-6 were measured by qRT-PCR. (c-d) After cells were treated with SWZ-4 and LPS for 24 h, the culture supernatants were collected and TNF-α and IL-6 were measured by ELISA. Values are expressed as mean ± SD. (n = 3). #P < 0.01 versus control group, *P < 0.01 versus LPS group.

Figure 7

Effect of SWZ-4 on LPS-induced TLR4/MyD88/NF-κB mRNA expression in RAW264.7 cells. (a) TLR4 mRNA expression. (b) MyD88 mRNA expression. (c) IκBα mRNA expression. (d) p65 mRNA expression. Values are expressed as mean ± SD. (n = 3). #P < 0.01 versus control group, *P < 0.01 versus LPS group.

Figure 8

Effect of SWZ-4 on LPS-induced TLR4/MyD88/NF-κB protein expression in RAW264.7 cells. (a-e) The protein expression of TLR4, MyD88, p-IκBα, IκBα, p-p65 and p65 determined by western blot. Values are expressed as mean ± SD. (n = 3). #P < 0.01 versus control group, *P < 0.01 versus LPS group.