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# Stevioside ameliorates high-fat diet-induced insulin resistance and adipose tissue inflammation by downregulating the NF-κB pathway

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ABSTRACT

Accumulating evidence suggests that adipose tissue is the main source of pro-inflammatory molecules that predispose individuals to insulin resistance. Stevioside (SVS) is a widely used sweetener with multiple beneficial effects for diabetic patients. In this study, we investigated the effect of SVS on insulin resistance and the pro-inflammatory state of adipose tissue in mice fed with a high-fat diet (HFD). Oral administration of SVS for 1 month had no effect on body weight, but it significantly improved fasting glucose, basal insulin levels, glucose tolerance and whole body insulin sensitivity. Interestingly, these changes were accompanied with decreased expression levels of several inflammatory cytokines in adipose tissue, including TNF-α, IL6, IL10, IL1β, KC, MIP-1α, CD11b and CD14. Moreover, macrophage infiltration in adipose tissue was remarkably reduced by SVS. Finally, SVS significantly suppressed the nuclear factor-kappa b (NF-κB) signaling pathway in adipose tissue. Collectively, these results suggested that SVS may ameliorate insulin resistance in HFD-fed mice by attenuating adipose tissue inflammation and inhibiting the NF-κB pathway.

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## 1. Introduction

Insulin resistance is the fundamental defect of type 2 diabetes mellitus (T2DM). Evidence is accumulating that T2DM is a chronic low-grade inflammatory disease characterized by mild inflammation in adipose tissue, liver, skeletal muscle and pancreatic islets [1,2]. Adipose tissue is the main source of pro-inflammatory factors. Adipose tissue secretes multiple adipokines into circulation, which subsequently cause insulin resistance in the liver and skeletal muscle, thus leading to systemic insulin resistance [3,4].

Stevioside (SVS), the major component of *Stevia rebaudiana Bertoli*, is widely used as a non-caloric sweetener in many countries. SVS possesses multiple therapeutic effects for metabolic syndrome, including lowering blood pressure, reducing blood sugar, potentiating insulin secretion and improving insulin sensitivity [5,6]. Previous studies have reported that SVS decreases blood sugar and improves glucose tolerance in Goto-Kakizaki (GK) rats [7,8]. Additionally, SVS increases systemic insulin sensitivity in various types of diabetic models, including fructose-rich chow-fed rats [9], obese Zucker rats [10], alloxan-induced diabetic rats [11] and combined leptin and LDL-receptor deficiency (DKO) mice [12]. However, it remains unclear if SVS attenuates HFD-induced insulin resistance, which mimics the pathogenesis of T2DM that widely occurs in western countries resulting from high-fat food intake.

Mechanistic insights have revealed that SVS decreases phosphoenolpyruvate carboxykinase levels and slows down gluconeogenesis in rat livers [13]. Moreover, acute oral SVS administration modestly enhances insulin-stimulated glucose transport in the skeletal muscle of obese Zucker rats [10], which suggests that an important site of action of SVS is the glucose transport system of skeletal muscle. Geeraert et al. found that SVS improves insulin signaling and antioxidant defense in the adipose tissue of DKO mice [12], thus indicating that SVS may also regulate adipose tissue in insulin resistance. However, SVS modulation of the inflammatory state in adipose tissue has not been clarified.

In this study, we sought to address the effect of SVS on HFD-induced insulin resistance in mice and its role in adipose tissue inflammation.

*Abbreviations:* SVS, stevioside; HFD, high-fat diet; HEC, hyperinsulinemic-euglycemic clamp; GIR, glucose infusion rate; T2DM, type 2 diabetes mellitus; NF-κB, nuclear factor-kappa b.

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## 2. Materials and methods

### 2.1. Reagents

Stevioside (SVS; purity >98%) was purchased from Sigma–Aldrich. Unless specifically stated, all other reagents were purchased from Sigma.

### 2.2. Animals

Male C57BL6J mice (aged 8 weeks) were purchased from the Shanghai Laboratory Animal Center at the Chinese Academy of Science (CAS). All experimental procedures were approved by the ethics committee for laboratory animals at Shanghai Jiao Tong University School of Medicine. Mice were fed in a standard animal room with 12 h dark/light cycles. After adaptive feeding for 1 week, animals were randomly divided into the following four groups with 10 mice in each group: normal chow group (CON); normal chow + SVS group (CONS); HFD group (HF); and HFD + SVS group (HFS). For the CON and HF groups, animals were fed with normal chow or HFD alone, respectively. The high fat diet consisted of 49.0% calories from fat, 15.4% calories from protein, and 35.6% calories from carbohydrates. For the CONS and HFS groups, animals were fed with normal chow or HFD, and they received SVS treatment. Animals were fed with their respective diet for 4 months and were treated daily with SVS or vehicle during the last month. SVS was dissolved in a 0.9% NaCl solution and was orally administrated twice a day at a dose of 10 mg/kg/day.

### 2.3. Glucose tolerance test and insulin tolerance test

Mice were fasted for 12 h and then injected intraperitoneally with glucose (1 g/kg) to measure glucose tolerance. Blood glucose was measured using blood samples taken from cut tail tips at baseline and at 30, 60, 90, and 120 min after the injection of glucose. The insulin tolerance test was performed by intraperitoneal injection of insulin (0.75 U/kg) in 0.9% NaCl after mice were fasted for 6 h. A tail vein blood sample was taken immediately prior to and 30, 60, 90, and 120 min after the injection for determination of blood glucose. The results were expressed as percentage of basal glucose.

### 2.4. Hyperinsulinemic-euglycemic clamp (HEC)

For the HEC studies, surgery was performed prior to the experiments. Mice were anesthetized intraperitoneally with pentobarbital sodium. A cannula was inserted into the right internal jugular vein, and mice were allowed to recover for 5 days. The cannula was flushed daily with a 0.9% NaCl solution containing 100 international units/ml heparin. For the HEC study, animals were fasted for 15 h. On the morning of the experiments, catheters were flushed with heparinized saline. After a 90-min equilibration period, mice were infused with 4 mIU/kg/min insulin and 25% glucose through a three-way connector. Blood glucose level was measured every 10 min from the tail tip, and glucose infusion rate (GIR) was adjusted to maintain blood glucose at approximately 6.7 mM. The steady state was maintained for at least 30 min, and insulin sensitivity was assessed by glucose infusion rate during the steady state as previously described [14].

### 2.5. ELISA examination

Serum was harvested from mice at the end of the experiment and stored at −80 °C. Serum insulin was measured with a mouse ELISA kit according to the manufacturer's instructions (Linco, Millipore).

### 2.6. Real-time PCR analysis

Total RNA was extracted from the epididymal adipose tissue of mice using Trizol. RNA (2 μg) was transcribed into cDNA, and mRNA expression level was determined by an ABI 7300 system with respective primers. Real-time PCR was performed with an ABI Prism 7300 instrument (Applied Biosystems, Carlsbad, California) using the SYBR Premix Ex Taq kit (Takara, Otsu, Shiga) according to the manufacturer's instructions. The cycle parameters were 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Gene expression was analyzed by relative quantification with the $2-\triangle\triangle Ct$ method. The target gene levels were normalized to GAPDH, and the results were expressed as fold changes in threshold cycle (Ct) values relative to controls.

### 2.7. Immunofluorescence

The epididymal adipose tissue was removed from anesthetized mice with pentobarbital sodium. The adipose tissue was fixed with formalin at 4 °C overnight, paraffin embedded and then cut into sections (thickness of 5 μm) before mounting on slides. The following antibodies were used for the immunofluorescence assay (overnight incubation at 4 °C): rat anti-F4/80 antibody (1:100; R&D), rat anti-CD11b antibody (1:100; R&D) and rabbit anti MIP-1α antibody (1:100; Santa Cruz). The following secondary antibodies were used for the immunofluorescence assay (2 h incubation at room temperature): Cy3-conjugated goat anti-rat IgG (1:200, Jackson Immunoresearch) and FITC-conjugated donkey anti-rabbit IgG (1:200, Jackson Immunoresearch). All slides were measured using IMAGE analysis software (Image Pro 6.0) on an Olympus microscope.

### 2.8. Western blot analysis

For western blot analysis, the epididymal adipose tissue was lysed with RIPA containing a phosphatase inhibitor cocktail. After complete homogenation, samples were centrifuged at 13000 rpm for 30 min at 4 °C. Supernatants were harvested and boiled at 99 °C for 5 min with loading buffer. Protein was separated by SDS–PAGE and transferred onto a PVDF membrane. The membrane was then blocked with 5% fat-free milk for 2 h at room temperature and then incubated with anti-IKK β and anti-IKB α antibodies. Bands were detected by Bio-Rad Quantity One software.

### 2.9. Statistical analysis

Statistical analysis was performed using SPSS 13.0 software. All data were presented as the means ± SEM unless otherwise stated. Differences among groups were analyzed by one-way ANOVA followed by a Newman-Keuls post-test. A P-value less than 0.05 was considered significant.

## 3. Results

### 3.1. SVS ameliorates glucose tolerance and systemic insulin sensitivity in HFD-fed mice

The effect of SVS on glucose metabolism was first investigated. Mice were fed with normal chow or HFD for 3 months, followed by an intervention with SVS or vehicle for 1 month Serial changes in body weight, glucose levels, fasting insulin levels, glucose tolerance and insulin sensitivity were measured. Mice belonging to

the HF group were heavier than the CON group ($P < 0.01$). However, no significant difference was observed between the HF and HFS groups (Fig. 1A). Interestingly, after the SVS treatment, fasting blood glucose levels and glucose tolerance were both reduced in the HFS group compared with the HF group (Fig. 1B and D) ($P < 0.05$), and there was no difference between the CONS and CON groups. In parallel, fasting insulin levels were also decreased in the HFS group compared with the HF group (Fig. 1C), which indicated that SVS restored insulin sensitivity. Therefore, we further determined whole body insulin sensitivity by insulin tolerance and HEC tests. After insulin injection, blood glucose decreased more significantly in the HFS group compared with the HF group (Fig. 1E). Moreover, the GIR, which represents whole body insulin sensitivity, was also significantly increased in the HFS group relative to the HF group (Fig. 1F). Taken together, these data provided compelling evidence that SVS ameliorates insulin resistance induced by HFD in C57BL6J mice.

### 3.2. SVS downregulates inflammatory cytokine mRNA levels in adipose tissue

To evaluate the effect of SVS on adipose tissue inflammation, we determined the expression levels of pro-inflammatory cytokines and macrophage-related markers using quantitative RT-PCR. The mRNA expression levels of inflammatory cytokines, including TNF-α, IL6, IL10, IL1β, MIP-1α, KC, CD11b and CD14, were significantly elevated in the adipose tissue of HFD-fed mice (Fig. 2). Increased expression of TNF-α, IL6, IL10 and IL1β indicated enhanced inflammatory response, and increased expression of CD11b, CD14, MIP-1α and KC suggested increased macrophage accumulation in the adipose tissue of HFD-fed mice. Importantly, these increases were significantly counteracted by SVS treatment in the HFS group with more than a 50% decrease in IL6, IL10, IL1β, MIP-1α, KC, and CD14 expression levels relative to the HF group, which indicated a markedly alleviated inflammatory response. No significant difference was observed in the CONS group compared with the CON group.

### 3.3. SVS reduces macrophage infiltration in adipose tissue

A hallmark of adipose tissue inflammation is immune cell infiltration. Macrophages are among the most abundant immune cells infiltrating adipose tissue after chronic high-fat feeding. Thus, macrophage relative abundance in adipose tissue from HFD-fed mice was determined using immunofluorescence. Macrophages were identified as cells positive for anti-F4/80 and anti-CD11b staining

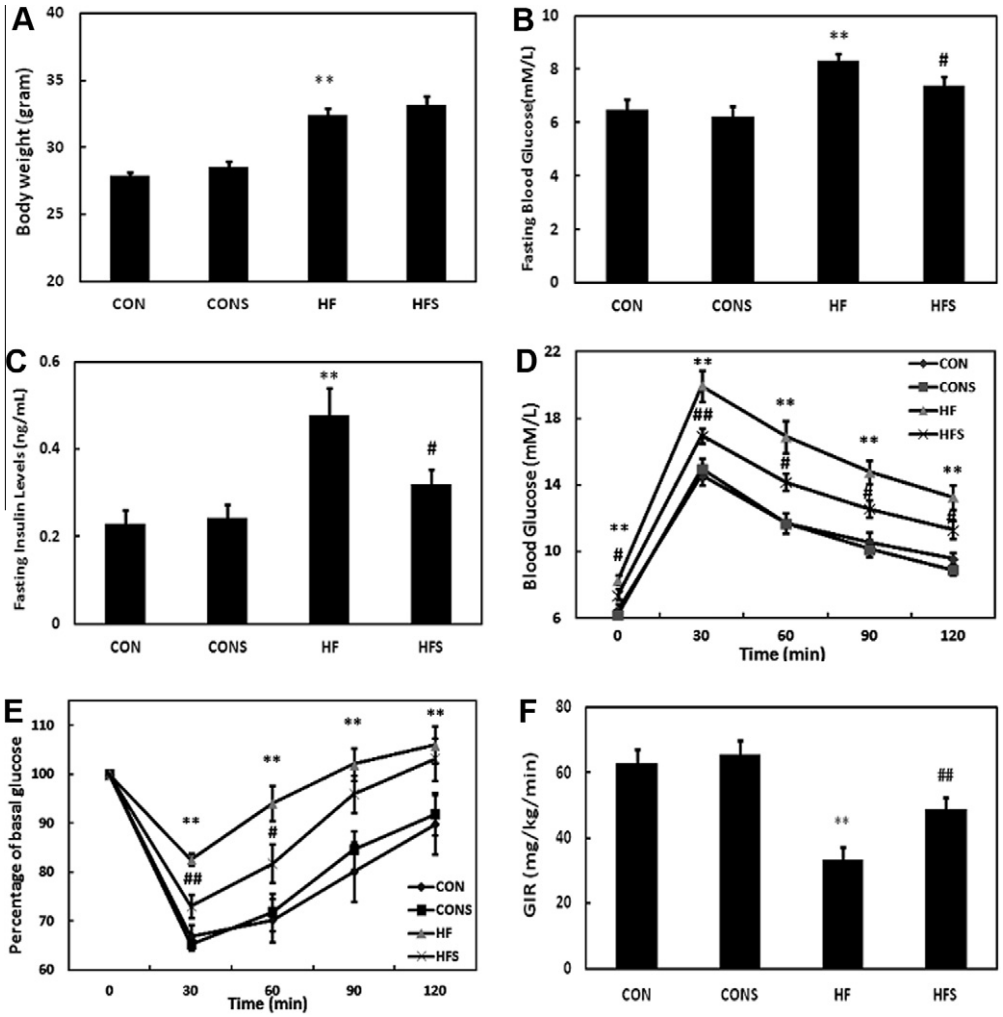


**Fig. 1.** SVS improves fasting blood glucose, fasting insulin levels, glucose tolerance and insulin sensitivity in HFD-fed mice. (A) Body weight of each group. (B) Blood glucose of each group after fasting for 12 h. (C) Fasting insulin level of each group after fasting for 12 h. (D) Blood glucose concentrations during intraperitoneal glucose tolerance test. (E) Blood glucose levels during intraperitoneal insulin tolerance test. The results were expressed as percentages of basal glucose. (F) Glucose infusion rate (GIR) during the HEC study. Data are means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. CON. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. HF group. $n = 8–10$ in each group.

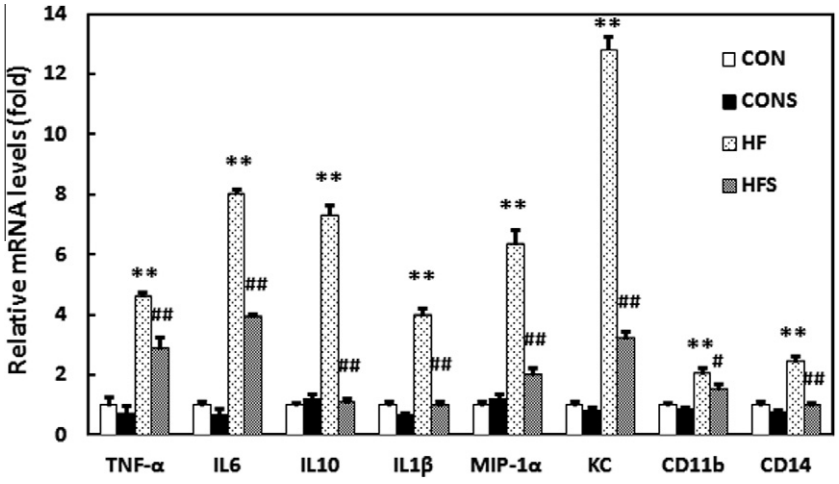


**Fig. 2.** SVS downregulates the expression of inflammatory genes in adipose tissues. The mRNA levels were detected by real-time PCR analysis, and the differences in expression levels of TNF-α, IL6, IL10, IL1β, MIP-1α, KC, CD11b and CD14 were presented as the means ± SEM ($n$ = 6–7). Data are means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. CON group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. HF group.

in the stromal vascular fraction. The relative abundance of MIP-1α, a crucial chemoctactic factor in recruiting macrophage infiltration, was also determined. The immunofluorescence staining of F4/80, CD11b and MIP-1α in the HF group was comparatively more intense than in the CON group. However, the intensities of these three stains were significantly decreased in the SVS group, which indicated reduced infiltration of macrophages into the adipose tissue. Moreover, no significant difference was observed between the CONS and CON groups (Fig. 3A and B).

### 3.4. SVS suppresses the NF-κB signaling pathway in adipose tissue

To further explore the potential mechanism whereby SVS attenuates adipose tissue inflammation, we determined the effect of SVS on the NF-κB pathway by western blot analysis. Both phosphorylated IKK β and phosphorylated IKB α were significantly upregulated in the HF group compared with the CON group, which indicated activation of the NF-κB pathway (Fig. 4). As expected, this activation was markedly reversed by SVS treatment as shown by significantly decreased expression of both phosphorylated IKK β and phosphorylated IKB α in the HFS group, which provided compelling evidence that SVS interfered with the NF-κB signaling pathway. However, SVS did not affect the NF-κB pathway in the CONS group.

## 4. Discussion

Currently, obesity and T2DM have become increasingly common, and artificial sweeteners, which have low or zero calories, are widely used in the food industry to prevent excess glucose intake. However, the effect of artificial sweetener on metabolism remains controversial, and both beneficial and adverse effects have been reported [15]. In the present study, we reported for the first time the anti-inflammatory effect of the SVS sweetener in diabetes and provided compelling evidence for using SVS in food and in diabetes therapy.

The therapeutic benefits of SVS on insulin resistance have been substantially demonstrated by a number of *in vivo* studies, including studies performed in GK rats and Zucker rats, which are two types of widely used genetically inherited type 2 diabetic models [7,8]. Fructose-induced insulin resistant rats have also been used to demonstrate the therapeutic benefits of SVS *in vivo* [10]. In western countries, an excess of high-fat food intake is considered the root cause leading to insulin resistance [16,17]. However, few studies have elucidated the effect of SVS on HFD-induced insulin resistance, which mimics the pathogenesis of T2DM occurring in western countries [18]. Moreover, the underlying mechanisms for the effect of SVS on insulin resistance are only partly understood [10,12,13].

In the present study, we successfully established an insulin resistance model in C57BL6J mice via HFD feeding for 4 months. We observed the effect of long-term (1 month) SVS intervention on whole body insulin sensitivity. SVS significantly improved whole body insulin sensitivity as reflected by the enhanced glucose-lowering effect of insulin and increased GIR. Altogether, these results confirmed the protective effect of long-term SVS treatment on insulin sensitivity in HFD-fed mice.

A number of studies have described the presence of inflammation in the adipose tissue of HFD-fed mice [17,19,20], which mimics the low grade systemic inflammatory response that occurs in obesity and T2DM. Adipose tissue releases multiple pro-inflammatory factors into circulation, including TNF-α, IL6, IL8, and chemokine KC, leading to systemic insulin resistance [20,21]. Accordingly, we evaluated the inflammatory state in the HFD-fed mice and found that expression levels of pro-inflammatory factors, including TNF-α, IL6, IL10, and MIP-1α, were significantly elevated in adipose tissue, which indicated the onset of adipose tissue inflammation.

There is emerging evidence that SVS has anti-inflammatory properties. Initially, SVS suppresses the release of TNF-α, IL1β and NO in LPS-stimulated TH1 cells [22]. In addition, SVS modulates the TNF-α-mediated release of IL8 in T84 intestinal cells [23]. Furthermore, Geeraert et al. demonstrated that SVS improves antioxidant defenses in white visceral adipose of obese insulin resistant mice [12]. In addition, SVS potently counteracts oxidative stress by scavenging both hydroxyl and superoxide radicals [24], which may induce the inflammatory response in adipose tissue [25]. Therefore, we hypothesized that SVS may also modulate adipose tissue inflammation. To address this, we determined the mRNA expression levels of pro-inflammatory and chemotactic cytokines in adipose tissue. SVS significantly decreased the expression of TNF-α, IL6, IL1β, IL10, chemokine KC, and MIP-1α, which indicated that SVS partly reversed the deleterious effect of HFD-induced low grade inflammation. However, MCP-1, another important chemokine that is increased in diet-induced inflammation, was not affected by SVS (data not shown).

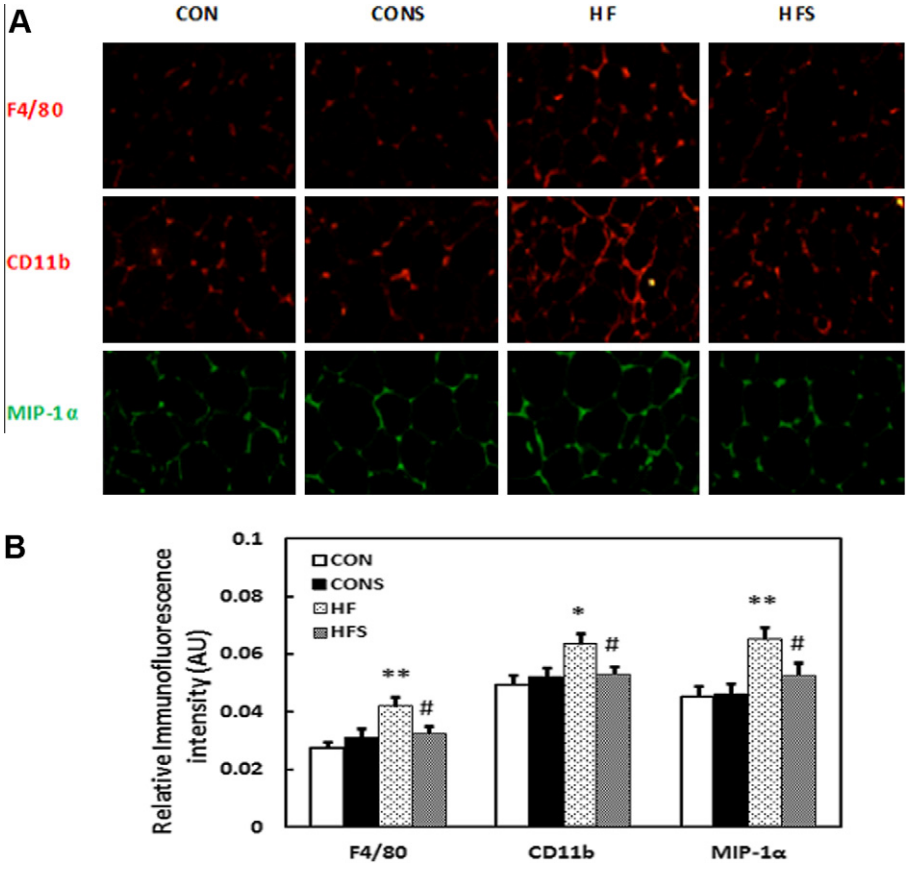


**Fig. 3.** SVS reduces macrophage infiltration into adipose tissues. Macrophages were detected by antibodies against the macrophage membrane-specific markers, F4/80 and CD11b. The chemotactic factor, MIP-1α, was also detected. (A) Representative immunofluorescence of F4/80 (red), CD11b (red) and MIP-1α (green). (B) Semiquantification of the immunofluorescence of F4/80, CD11b and MIP-1α. The immunofluorescence intensities of F4/80, CD11b and MIP-1α were higher in the HFD-fed group than in the CON group. However, the immunofluorescence intensities were remarkably lower in the SVS-treated group (HFS), which suggested that SVS also inhibited macrophage infiltration into the adipose tissue of HFD-fed mice, thus, attenuating the pro-inflammatory state. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

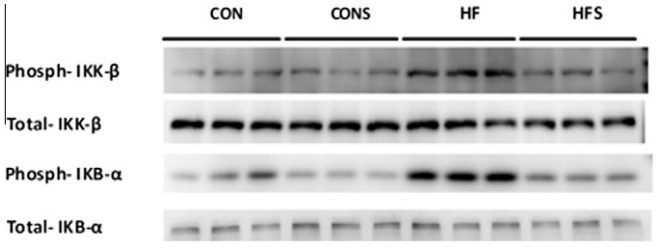


**Fig. 4.** SVS suppresses the NF-κB signaling pathway. Representative immunoblots of phosphorylated IKK β and IKB α in the adipose tissue of mice from indicated groups. Experiments were repeated independently three times.

Adipose tissue is composed of a variety of cell types, including adipocytes, preadipocytes, macrophages and lymphocytes. Macrophages are thought to be the most prominent immune cells underlying the pro-inflammatory response that leads to insulin resistance [15,26]. Moreover, the amount of infiltrated macrophages significantly increases and accounts for more than 40% of the total adipose tissue cell content from obese rodents [27]. Macrophages are responsible for the majority of TNF-α expression in adipose tissue, and they significantly contribute to the expression of IL6 and IL1β [28], which further trigger macrophage recruitment and exacerbate local inflammation [25,26,29]. There is evidence that SVS reduces macrophage infiltration in the aortic arch plaque, thus attenuating atherosclerosis [12]. Therefore, we subsequently investigated if SVS affected macrophage infiltration in adipose tissue. Using immunofluorescence, we determined the expression of two macrophage surface-specific markers, F4/80 and CD11b. The amount of F4/80- and CD11b-positive cells was significantly increased in HFD mice compared with the mice of the control group. However, SVS remarkably reversed this increase. We also determined the expression of MIP-1α, which is a crucial chemokine that mediates adipose macrophage infiltration and is increased in diet-induced obesity [28,30]. Similarly, this increase was also markedly counteracted by SVS treatment. These results indicated that SVS attenuates HFD-induced adipose tissue inflammation partly through reducing macrophage infiltration.

Together, these findings confirmed that HFD-induced mild adipose tissue inflammation was counteracted by SVS. However, in DKO mice, SVS has no obvious effects on systemic inflammation as serum TNF-α and IL6 contents are similar between placebo and SVS-treated mice [12]. SVS may not be potent enough to ameliorate the severe systemic inflammation induced by double leptin/LDL-receptor deficiency.

The NF-κB pathway is a key signaling pathway implicated in adipose tissue inflammation and insulin resistance [31]. HFD activates NF-κB both in adipocyte and adipose tissue macrophages [32], and a knockout of IKK β in myeloid cells protects mice from HFD-induced insulin resistance in adipose tissues [33]. Moreover, NF-κB-P65 transgenic mice exhibit chronic inflammation as manifested by increased macrophage infiltration and secretion of inflammatory cytokines [34]. Inhibition of NF-κB activation or

depletion of NF-κB restores insulin sensitivity in mature human adipocytes [35]. Interestingly, SVS has also been reported to interfere with the NF-κB pathway in LPS-stimulated THP-1 cells and T84 intestinal cells [22,23]. Moreover, SVS significantly lowers NF-κB1/NF-κBiα ratio in the aortic arch of DKO mice [12]. These findings suggest that SVS exerts its anti-inflammatory effect, at least in part, through modulating NF-κB activity. Accordingly, we further explored the possibility that SVS may modulate the NF-κB pathway in adipose tissue. This study demonstrated that phosphorylation of IKK β and IKB α was also significantly suppressed by SVS, thus, indicating decreased NF-κB activity. Taken together, these results strongly suggest that SVS interferes with NF-κB signaling pathway in adipose tissue, thereby contributing to its modulation of adipose tissue inflammation. Due to technical restrictions, however, we were unable to explore how SVS affects the NF-κB pathway in adipocytes or macrophages. These questions will be addressed in future work.

In conclusion, this study demonstrated for the first time that long-term SVS treatment ameliorates HFD-induced insulin resistance in mice. This insulin-sensitizing effect may partly be associated with an attenuated inflammatory state and downregulated NF-κB signaling in adipose tissue. Thus, SVS may be a promising antidiabetic drug that works through the modulation of adipose tissue inflammation and systemic insulin resistance.

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