Article

Discovery Medicine 2024; 36(186): 1408–1419
https://doi.org/10.24976/Discov.Med.202436186.131

Saikosaponin A Ameliorates Metabolic Inflammation and Intestinal Barrier Damage in DIO Mice through the Nrf2/ARE Pathway

Daoxu Huang[1,*], Zengpai Zheng[2], Yiling Huang[1], Xiang Chen[1]

[1]Department of Gastrointestinal Surgery, Ping Yang Hospital Affiliated to Wenzhou Medical University, 325400 Wenzhou, Zhejiang, China
[2]Department of Interventional Oncology, Ping Yang Hospital Affiliated to Wenzhou Medical University, 325400 Wenzhou, Zhejiang, China
*Correspondence: huangdaoxu2008@163.com (Daoxu Huang)
Published: 20 July 2024

Background: Obesity is linked to impaired intestinal barrier function and inflammation. Saikosaponin A (SSA), a triterpene saponin from *Bupleurum chinense*, has shown beneficial effects on intestinal colitis in mice. However, the mechanisms underlying SSA's protective effects against obesity are not fully understood.
Objective: To investigate the effects of SSA on body weight, metabolic disturbances, and intestinal health in diet-induced obese (DIO) mice, and to elucidate the potential mechanisms involved.
Methods: In the *in vivo* study, DIO mice were supplemented with SSA. Body weight, fasting blood glucose, and metabolic parameters were measured. Intestinal barrier function and inflammation were assessed. In the *in vitro* study, intestinal epithelial cells were treated with palmitic acid and lipopolysaccharide to induce inflammation. SSA was then administered to evaluate its effects on cell barrier integrity and inflammatory responses. The role of the nuclear factor-E2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway was investigated by silencing Nrf2.
Results: SSA supplementation significantly ($p < 0.05$) decreased body weight and fasting blood glucose levels in DIO mice, and markedly improved metabolic disturbances. This treatment also enhanced intestinal barrier function and reduced metabolic inflammation, likely through increased antioxidant capacity of intestinal epithelial cells via activation of the Nrf2/ARE signaling pathway. *In vitro*, SSA maintained cell barrier integrity and reduced inflammatory responses by activating the Nrf2/ARE signaling pathway, decreasing intracellular reactive oxygen species content, and increasing transepithelial electrical resistance. However, silencing Nrf2 abolished SSA's protective effects.
Conclusion: SSA enhances the antioxidant capacity of intestinal epithelial cells, maintains intestinal barrier integrity, and reduces intestinal inflammation in DIO mice through the activation of the Nrf2/ARE signaling pathway. These findings offer new insights into the protective role of SSA in obesity and metabolic diseases.

Keywords: saikosaponin A; obesity; intestinal barrier; Nrf2; oxidative stress; intestinal permeability; metabolic disorders

Introduction

As a common chronic metabolic disease, obesity is defined as a degree of overweight caused by excessive fat accumulation [1,2]. The global prevalence of obesity continues to increase, and by 2020, reports indicate that obese individuals will account for approximately 40% of the total population, becoming a serious public health issue [3]. Obesity and its complications include endocrine disease, cardiovascular disease, digestive disease, and many other chronic diseases [4,5]. The etiological factors contributing to obesity are diverse, encompassing genotype, diet, lifestyle, and environmental factors, and each of these elements can disrupt the energy metabolism balance, leading to the development of obesity and metabolic disorders [6]. Numerous studies demonstrate that dysfunction of the intestinal barrier and subsequent transfer of bacteria and bacterial products caused by a high-fat diet (HFD) are closely associated with obesity [7–9]. Metabolic intestinal inflammation is one of the major symptoms induced by a HFD, and impaired barrier function may lead to enhanced intestinal permeability, causing the transfer of lipopolysaccharide (LPS) endotoxin from the intestinal tract into the systemic circulation [7]. LPS amplifies the pro-inflammatory signaling cascade, resulting in insulin resistance (IR) and other metabolic disorders [8,9]. Hence, safeguarding intestinal integrity, disrupted by obesity, is paramount in intervening and treating the development and progression of metabolic inflammatory diseases and metabolic disorders.

The main components of the intestinal barrier include mechanical, chemical, immune, and biological barriers, each of which has a corresponding structural basis. The intestinal barrier is an important safeguard against the entry of harmful substances and pathogens into the intestine,

Copyright: © 2024 The Author(s). Published by Discovery Medicine. This is an open access article under the CC BY 4.0 license.
Note: *Discovery Medicine* stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

thereby contributing to the homeostasis of the body's internal environment [10,11]. Tight intercellular junctions, comprising occludin, claudin, zona occludens (ZO), and junctional adhesion molecules (JAM), are crucial components of the mechanical barrier [12]. Maintaining redox homeostasis is essential for intestinal barrier function [13]. The study has shown that a chronic HFD induces excessive radical production in intestinal epithelial cells and that reactive oxygen species (ROS) promote the peroxidation of lipids and lipoproteins [14]. Lipid peroxidation causes the accumulation of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) that exacerbate cell membrane disruption, causing further inflammation and intestinal barrier damage [15]. Therefore, it is paramount to ameliorate the intestinal oxidative stress damage and rectify the inflammatory state induced by a HFD; these actions are critical for safeguarding intestinal barrier function, and for improving the development and progression of metabolic disorders.

The Chinese herb medicine *Bupleurum chinense* contains a large number of triterpenoid saponins, for instance, saikosaponin A (SSA), with multiple pharmacological activities such as antitumour, hypoglycaemic, anti-inflammatory, and antioxidant effects [16]. Accumulated evidence shows that SSA exhibits remarkable pharmacological activities against endotoxin-induced inflammation and oxidative stress [17–19]. However, it is still unclear whether SSA administration has protective effects on metabolic inflammation and intestinal barrier damage in high-fat diet-induced obese (DIO) mice. Therefore, in the present study, the protective effects and underlying mechanisms of SSA on metabolic disorders, inflammation, intestinal barrier, and oxidative stress in the colon of DIO mice were investigated. The present findings hold promise for the use of SSA in a therapeutic regimen for the treatment of obesity and metabolic inflammation.

Materials and Methods

Materials and Chemicals

Saikosaponin A (SSA, Cat: S422516) and palmitic acid (PA, Cat: P664512) were obtained from Aladdin (Beijing, China). Normal chow diet (NCD, 10% fat, Cat: 1010001) and HFD (60% fat, Cat: XTHF60) were purchased from Xietong Pharmaceutical Co., Ltd. (Nanjing, China). Insulin (Cat: I6634) and LPS (Cat: L4516) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemical reagents used were analytical grade.

Experimental Animals

Thirty C57BL/6 male mice (2 months old, weighing 18–22 g) were purchased from Beijing Huafukang Biotechnology Co. (Certificate no. SCXK B2019-0008, Beijing, China). The mice were kept at 22 °C–24 °C and had access to food and water *ad libitum* for 7 days before the start of the experiment. The DIO mouse model was referenced from previous literature [20]. The experimental animals were divided into NCD (n = 10), HFD (n = 10), and HFD+SSA (n = 10) groups. The animals were administered saline by gavage in the NCD and HFD groups, and SSA at 10 mg/kg for the HFD+SSA group. The experiment lasted 20 weeks, based on previous studies and our preliminary data, which demonstrated effective and consistent outcomes with minimal adverse effects. The intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT) were carried out as described in a previous report [21]. The change in blood glucose concentration curve was plotted and the area under the curve (AUC) was calculated.

After 20 weeks of SSA administration, mice underwent overnight fasting and were then anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Blood samples were obtained via eyeball extirpation, centrifuged for serum preparation, and then stored at –80 °C. Euthanasia was performed through cervical dissociation, and colonic tissues were harvested and stored at –80 °C. A portion of the colonic tissue was fixed in 10% formaldehyde for hematoxylin-eosin (H&E) staining.

H&E Staining

Briefly, freshly isolated colon from mice was fixed in 4% paraformaldehyde at room temperature for 24 h, dehydrated, embedded, sectioned, and stained using a H&E kit (Cat: G1120, Solarbio, Beijing, China). The colon tissues were morphologically observed and entrusted to the Affiliated Hospital of Wenzhou Medical University for pathological scoring of the colon tissues. The histopathological score was assessed as described previously [22]. Seven independent parameters were measured: the extent of inflammation (0, none; 1, mucosa; 2, mucosa and submucosa; 3, mucosa, submucosa and muscle layer; 4, transmural), infiltration by neutrophils and lympho-histiocytes (0, none; 1, focal; 2, multifocal; 3, diffuse), crypt damage (0, none; 1, basal 1/3; 2, basal 2/3; 3, entire crypt damage; 4, crypt damage and ulceration), crypt abscess (0, none; 1, focal; 2, multifocal), sub-mucosal edema (0, none; 1, focal; 2, multifocal; 3, diffuse), loss of goblet cells (0, none; 1, focal; 2, multifocal; 3, diffuse), and reactive epithelial hyperplasia (0, none; 1, focal; 2, multifocal; 3, diffuse). The histopathological scores were determined by adding all scores of the aforementioned parameters.

Biochemical Analysis

Catalase (CAT, Cat: A007-1-2), superoxide dismutase (SOD, Cat: A001-3-2), 4-hydroxynonenal (4-HNE, Cat: H268-1-2), malondialdehyde (MDA, Cat: A003-1-2), protein carbonyl content (PCC, Cat: A087-1-2), tumor necrosis factor-α (TNF-α, Cat: H052-1-1), interleukin-1β (IL-1β, Cat: H002-1-1), and interleukin-6 (IL-6, Cat: H007-1-1) were determined using biochemical kits from Jiancheng Bioengineering Institute (Nanjing, China).

Immunoblotting

Tissues or cells were lysed on ice by radioimmunoprecipitation assay (RIPA) protein lysis solution (Cat: P0013B, Beyotime, Shanghai, China). Protein content was determined by bicinchoninic acid assay (BCA). For 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, samples were transferred to a polyvinylidene fluoride (PVDF) membrane (Cat: IPVH00010, Millipore Corp, Bedford, MA, USA), followed by milk blocking, incubation of primary and secondary antibodies, and exposure development. Bands were visualized using a SuperSignal™ West Pico PLUS ECL kit (Cat: 34580, Thermo Scientific, Bedford, MA, USA) and imaged with the Chemi-Doc Touch Imaging System (170-01401, Bio-Rad, Hercules, CA, USA), and further quantified using the analysis software NIH ImageJ (version 21, Bethesda, MD, USA). Primary antibodies against nuclear factor-E2-related factor 2 (Nrf2) (Cat: ab62352, 1:1000 dilution), phospho-Nrf2 (Ser40) (Cat: ab76026, 1:1000 dilution) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Cat: ab2845, 1:2000 dilution) were obtained from Abcam (Cambridge, UK). Anti-ZO-1 (Cat: GB115686, 1:1000 dilution) and Occludin-1 (Cat: GB111401, 1:1000 dilution) antibodies were provided from Servicebio (Shanghai, China). Secondary antibodies including horseradish peroxidase (HRP)-conjugated goat anti-mouse (Cat: BA1050, 1:5000 dilution) and HRP-conjugated goat anti-rabbit (Cat: BA1054, 1:5000 dilution) were purchased from Boster Biological Technology (Wuhan, China).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Tissue (40 mg) was homogenized and RNA was extracted with TRIzol reagent (Cat: 15596026, Invitrogen, Waltham, MA, USA), and the concentration was measured by NanoDrop One (Thermo Scientific, USA), then reverse-transcribed into cDNA using a commercial kit (Cat: R302, Vazyme, Nanjing, China). qRT-PCR analysis was performed using SYBR Green fluorescent dye (Cat: Q221, Vazyme, China) to determine the expression levels of genes calculated according to the $2^{-\Delta\Delta Ct}$ method. Primers were synthesized by DynaScience (Shanghai, China), and the primer sequences are shown in Table 1.

Table 1. Designed primer sets for qRT-PCR.

Gene	Primer	5′-3′
Mus Tnf-α	sense	ACCCTCACACTCACAAACCA
	antisense	ATAGCAAATCGGCTGACGGT
Mus Il-1β	sense	TGCCACCTTTTGACAGTGATG
	antisense	AAGGTCCACGGGAAAGACAC
Mus Il-6	sense	CCCCAATTTCCAATGCTCTCC
	antisense	CGCACTAGGTTTGCCGAGTA
Mus Zo-1	sense	ACTATGACCATCGCCTACGG
	antisense	GGGGATGCTGATTCTCAAAA
Mus Occludin-1	sense	CGGTACAGCAGCAATGGTAA
	antisense	CTCCCCACCTGTCGTGTAGT
Mus Jam	sense	CAGACTGGAGTGGAAGAAGGTG
	antisense	GCTGACTTCACAGCGATACTCTC
Mus Claudin-4	sense	TGATTATGGTGCCCGTGTCC
	antisense	CGAGTAGGGCTTGTCGTTGC
Mus Ho-1	sense	CACGCATATACCCGCTACCT
	antisense	CCAGAGTGTTCATTCGAGCA
Mus Nqo1	sense	TCACCTGGGCAAGTCCATTC
	antisense	TGCCCTGAGGCTCCTAATCT
Mus β-Actin	sense	CGTGGGCCGCCCTAGGCACCA
	antisense	TTGGCCTTAGGGTTCAGGGGGG
Homo TNF-α	sense	ACTTTGGAGTGATCGGCC
	antisense	GCTTGAGGGTTTGCTACAAC
Homo IL-1β	sense	ATGCACCTGTACGATCACTG
	antisense	ACAAAGGACATGGAGAACACC
Homo IL-6	sense	CCACTCACCTCTTCAGAACG
	antisense	CATCTTTGGAAGGTTCAGGTTG
Homo ZO-1	sense	GTCCAGAATCTCGGAAAAGTGCC
	antisense	CTTTCAGCGCACCATACCAACC
Homo Occludin-1	sense	ATGGCAAAGTGAATGACAAGCGG
	antisense	CTGTAACGAGGCTGCCTGAAGT
Homo β-Actin	sense	AGACCTGTACGCCAACACAG
	antisense	TTCTGCATCCTGTCGGCAAT

qRT-PCR, quantitative reverse transcription polymerase chain reaction; *Tnf-α*, tumor necrosis factor-α; *Il-1β*, interleukin-1β; *Il-6*, interleukin-6; *Zo*, zona occludens; *Jam*, junctional adhesion molecules; *Ho-1*, heme oxygenase-1; *Nqo1*, NAD(P)H quinone dehydrogenase 1.

Cell Culture

Human epithelial colorectal adenocarcinoma (Caco-2) cells were raised in a cell culture incubator under conventional conditions (37 °C, 5% CO_2). Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) contained 10% serum and a penicillin/streptomycin mixture. The cell line was regularly authenticated using short tandem repeat (STR) profiling to confirm its identity. Additionally, it was routinely tested for the absence of mycoplasma, chlamydia, and other common contaminants to ensure the quality of the cell cultures. For cell treatment, Caco-2 cells were incubated with 200 µM PA and 10 µg/mL LPS, either in the presence or absence of 5 µM SSA (dissolved in phosphate buffered saline (PBS)), for 4 or 12 h.

Transepithelial Electrical Resistance (TEER) Measurement

TEER measurements were performed using an EVOM meter (version 2, World Precision Instruments, Sarasota, FL, USA) and chopstick-type electrodes as previously reported [23]. All TEER measurements were carried out at 37 °C. Monolayer confluence was reached and the cells were used for further experiments.

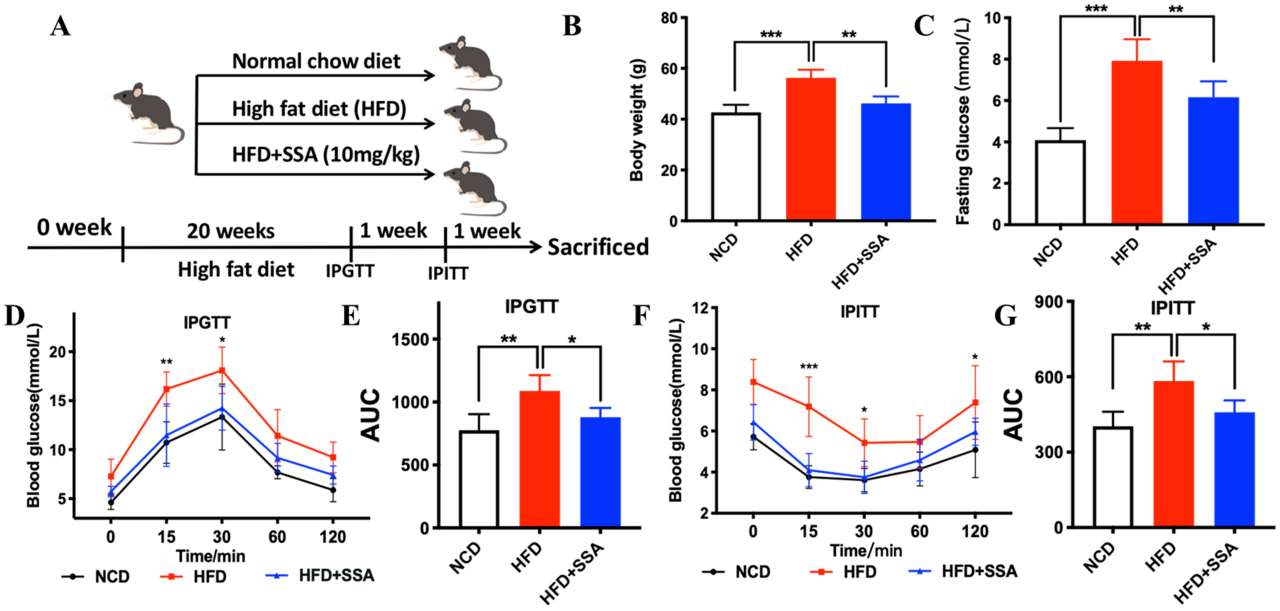

Fig. 1. Saikosaponin A (SSA) alleviates overweight, hyperglycemia and insulin resistance in diet-induced obese (DIO) mice. (A) Schematic overview for the design of the animal experiment. (B) Body weight. (C) Fasting glucose. (D) Intraperitoneal glucose tolerance test (IPGTT). (E) The area under the curve (AUC) of IPGTT. (F) Intraperitoneal insulin tolerance test (IPITT). (G) AUC of IPITT. Data are represented as mean ± standard error of the mean (SEM), n =10. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

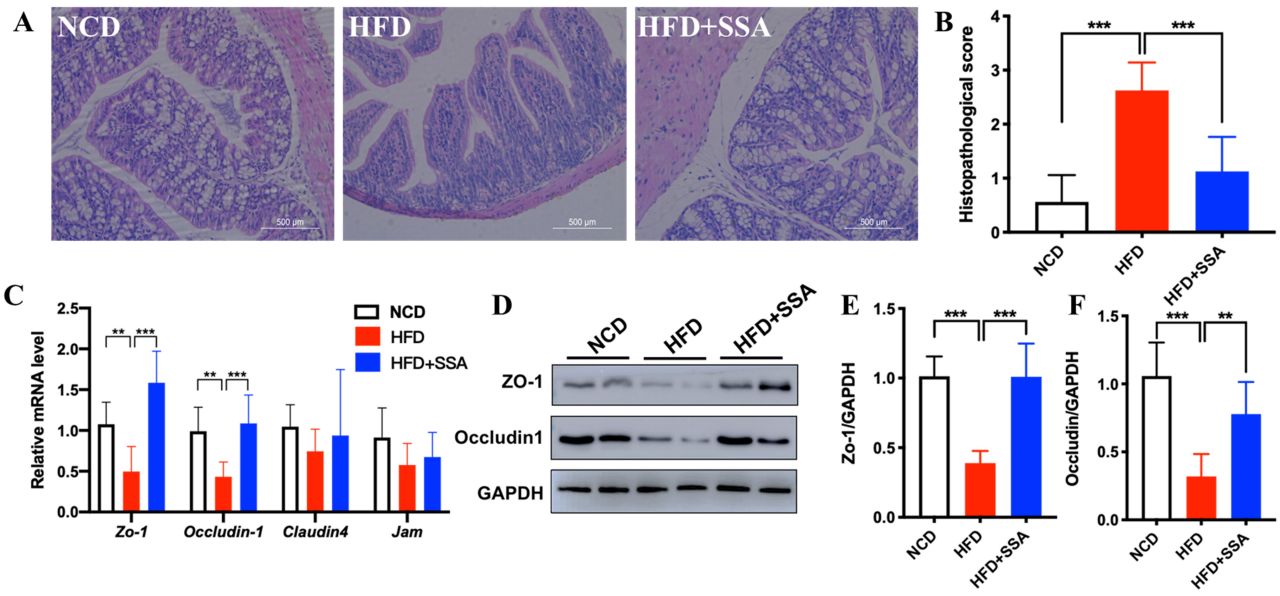

Fig. 2. SSA alleviates intestinal barrier damage in DIO mice. (A) Hematoxylin-eosin (H&E) staining of colonic tissue (400×, scale bar = 500 μm) and (B) the histopathological score; (C) the relative mRNA levels of *Zo-1*, *Occludin-1*, *Claudin-4* and *Jam*; (D) western blot and (E,F) quantification of ZO-1 and Occludin-1 in the colonic tissue. Data are expressed as mean ± SEM, n =10. $**p < 0.01$ and $***p < 0.001$.

Measurement of ROS Content

The content of ROS was measured using fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Cat: Q221, S0033S, Beyotime, China) according to the manufacturer's instructions.

siRNA Transfection

Caco-2 cells were seeded in 6-well plates and starved for 6 h after the cells grew to 70% confluence, then transfected with the above siRNA with Lipofectamine 2000 (Invitrogen, USA) for 48 h, according to the manufacturer's protocol. The specific *Nrf2* siRNA sequences synthesized:

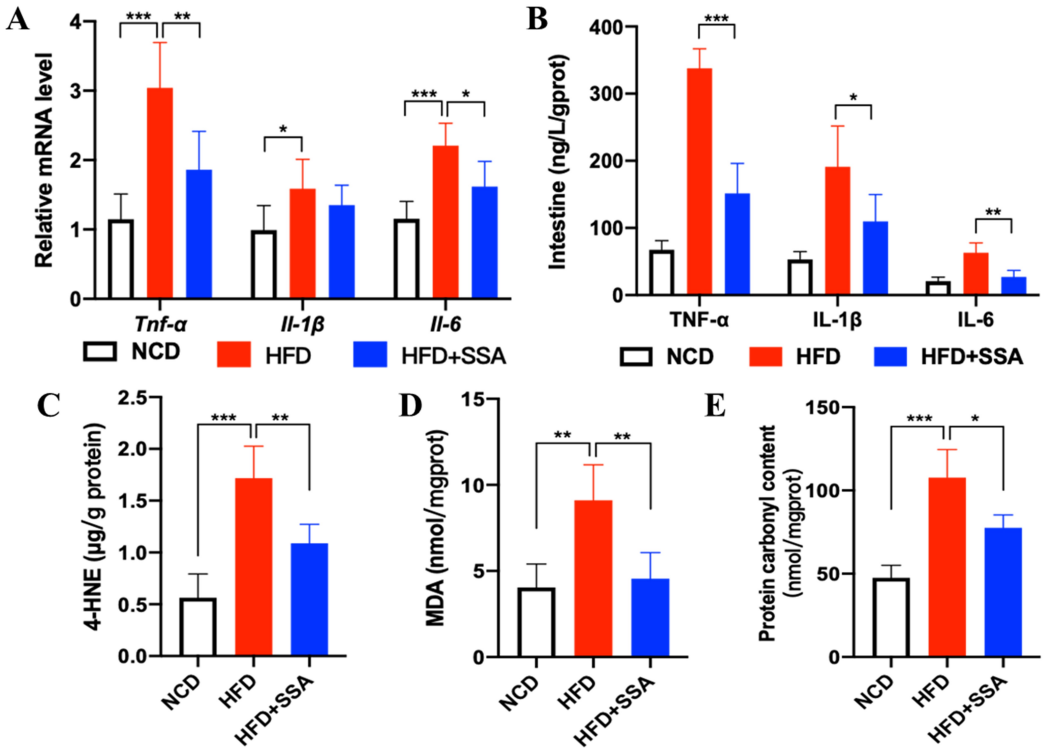

Fig. 3. SSA attenuates intestinal inflammatory responses and oxidative stress in DIO mice. (A) Relative mRNA levels and (B) content of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in colonic tissue; (C) 4-hydroxynonenal (4-HNE), (D) malondialdehyde (MDA) and (E) protein carbonyl content (PCC) in colonic tissue. Data are represented as mean ± SEM, n = 10. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

sense, 5′-GCAGCAAACAAGAGAUGGCAATT-3′; antisense, 5′-UUGCCAUCUCU UGUUUGCUGCTT-3′.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism (version 9.0, Dotmatics, Boston, MA, USA) and SPSS (version 21, IBM Corp., Chicago, IL, USA). The Shapiro–Wilk test was used to assess data distribution. For comparisons between groups, one-way analysis of variance (ANOVA) and Tukey's post hoc test were used. $p < 0.05$ was considered statistically significant.

Results

SSA Alleviates Metabolic Disorders in DIO Mice

The animal experiment procedure is shown in Fig. 1A. HFD-fed mice exhibited metabolic disturbances including obesity, hyperglycemia, and glucose intolerance. Mice in the HFD+SSA group had significantly ($p < 0.05$) lower body weight and fasting glucose compared to the HFD group (Fig. 1B,C). The IPGTT test was performed on mice that had been fasted overnight. Compared to the NCD group, blood glucose (BG) continued to rise in the HFD group mice 30 minutes after oral glucose administration and remained at a higher level for the next 90 minutes (Fig. 1D). SSA treatment significantly ($p < 0.05$) inhibited the rise in BG levels in the HFD group after oral glucose administration. The AUC was significantly ($p < 0.05$) lower in the HFD+SSA mice compared to the HFD mice (Fig. 1E), suggesting that SSA improved glucose tolerance in HFD-fed mice. Similar findings were shown in the IPITT experiment; the HFD+SSA group had significantly ($p < 0.05$) lower BG levels (Fig. 1F) and AUC (Fig. 1G) than the HFD group after insulin injection, indicating that SSA increased insulin utilization and improved insulin sensitivity in HFD-fed mice. These results suggest that SSA can attenuate the metabolic disorders in DIO mice.

SSA Alleviates Intestinal Barrier Damage in DIO Mice

High energy consumption has recently been reported to induce intestinal inflammation and impaired permeability of the intestinal epithelium and is associated with the progression of several metabolic diseases [24]. Therefore, we assessed intestinal histopathology in mice by H&E staining (Fig. 2A). Compared with mice in the NCD group, mice in the HFD group had disorganized colonic tissue cells, distorted crypt structures, and reduced numbers of goblet cells, indicating that the high-fat diet severely disrupted the intestinal wall barrier structure and function.

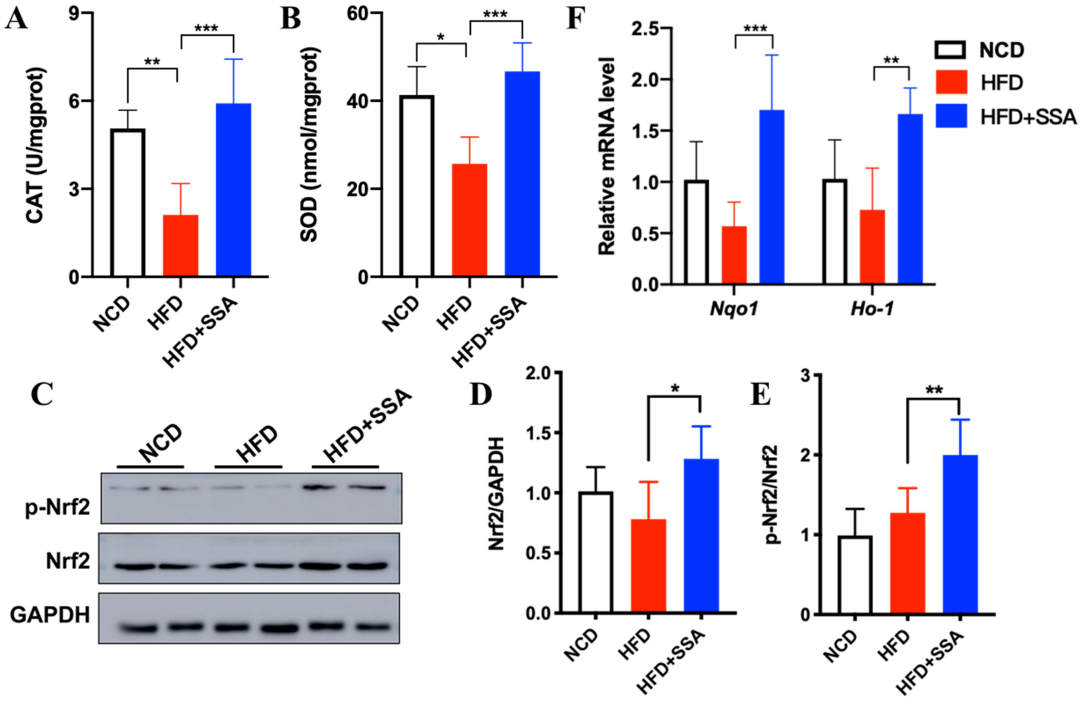

Fig. 4. SSA activates the nuclear factor-E2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway to increase antioxidant capacity in DIO mice. (A) Catalase (CAT) and (B) Superoxide dismutase (SOD) in colonic tissue. (C) The relative mRNA levels of *Nqo1* and *Ho-1*. (D) Western blot and (E,F) quantification of Nrf2 and phospho-Nrf2 (p-Nrf2) in the colon. Data are represented as mean ± SEM, n = 10. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Mice receiving SSA had tightly arranged colonic cells, intact crypt structure, increased and slightly enlarged goblet cells, and a significantly ($p < 0.05$) lower histopathological score than mice in the HFD group (Fig. 2B).

In addition, we measured the expression of molecules related to intestinal barrier function at the transcriptional level, such as epithelial tight junction molecule ZO-1, JAM, and closure proteins (Occludin-1 and Claudin-4), which are markers of epithelial cell integrity. As expected, the transcript levels of *ZO-1* and *Occludin-1* were significantly ($p < 0.05$) decreased in the colonic tissue of mice in the HFD group compared to the NCD group (Fig. 2C). Transcript levels and protein levels of *ZO-1* and *Occludin-1* were greatly increased after treatment with SSA in HFD-fed mice (Fig. 2D–F). These data suggest that SSA attenuates intestinal barrier damage in DIO mice.

SSA Attenuates Intestinal Inflammatory Responses and Oxidative Stress in DIO Mice

Inflammatory responses and oxidative stress are elevated in the gut of obese patients and progressively worsen with the disruption of the intestinal barrier [25]. As shown in Fig. 3A,B, a chronic high-fat diet increased the transcript levels of inflammatory factors (TNF-α, IL-1β, and IL-6) in the intestine of obese mice compared to the NCD group and promoted the secretion of inflammatory factors. After treatment with SSA intervention, mRNA levels of *Tnf-*α and *Il-6* were significantly ($p < 0.05$) down-regulated in the intestine of mice, and the corresponding levels of inflammatory factors were also decreased (Fig. 3A,B).

We further examined the levels of oxidative stress in the intestinal tissues of DIO mice. As noted above, 4-Hydroxynonenal (4-HNE) and malondialdehyde (MDA) are cytotoxic products of lipid peroxidation. In addition, oxidative modification of protein side chain amino acids by free radical attack results in a significant increase in protein carbonyl content (PCC). HFD dramatically increased 4-HNE levels in mouse colon tissue, whereas SSA administration significantly ($p < 0.05$) reduced this protein modification (Fig. 3C). Similarly, MDA and PCC levels in the intestine of mice in the HFD group were significantly ($p < 0.05$) increased compared to the NCD group, indicating that a chronic HFD led to intestinal oxidative stress and substantial free radical accumulation. These indices were significantly ($p < 0.05$) reduced in mice in the HFD+SSA group (Fig. 3D,E), suggesting that SSA has the effect of alleviating intestinal oxidative stress in DIO mice. In conclusion, these data suggest that SSA effectively attenuates intestinal inflammatory responses and oxidative stress in DIO mice.

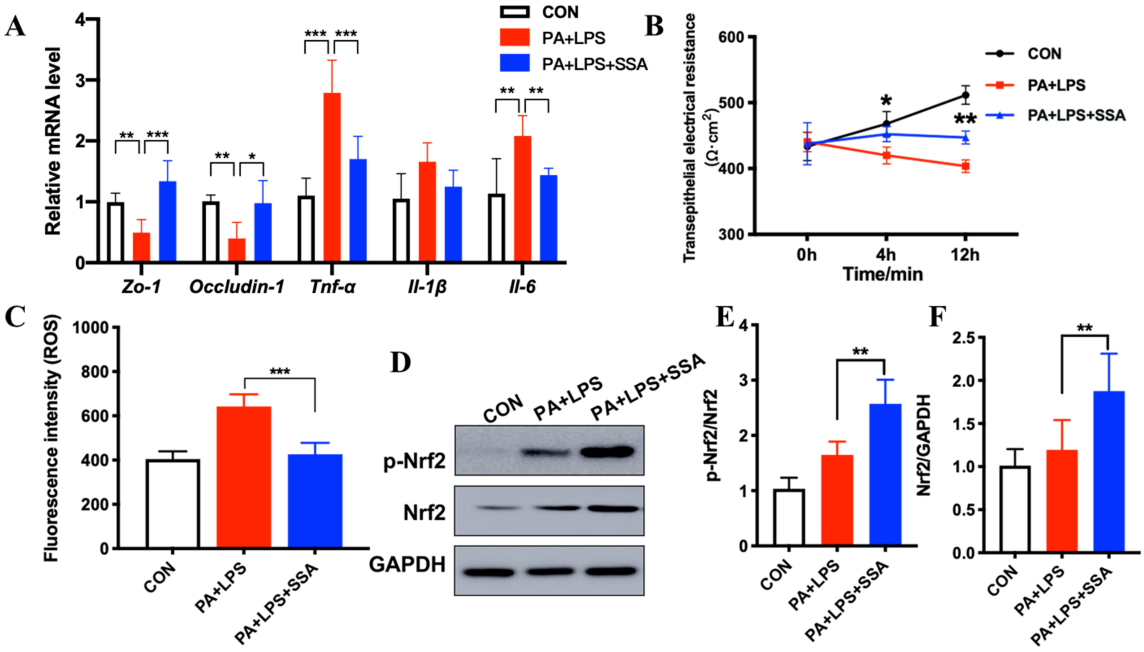

Fig. 5. SSA alleviates palmitic acid (PA) and lipopolysaccharide (LPS)-induced human epithelial colorectal adenocarcinoma (Caco-2) cell injury and oxidative stress. (A) Relative mRNA levels of pro-inflammatory cytokines (*TNF-α*, *IL-1β*, and *IL-6*) and tight junction-related proteins (*ZO-1* and *Occludin-1*). (B) The transepithelial electrical resistances (TEERs). (C) Reactive oxygen species (ROS) content. (D) Western blot of Nrf2 and p-Nrf2 and (E,F) quantification of Nrf2 and p-Nrf2 in Caco-2 cells. Data are represented as mean ± SEM, n = 6. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

SSA Activates the Nrf2/Antioxidant Response Element (ARE) Signaling Pathway to Increase Antioxidant Capacity in DIO Mice

To investigate the mechanism of SSA's role in alleviating oxidative stress in mouse intestinal tissues induced by HFD, we further measured the levels of antioxidant enzymes in the mouse intestine. Compared to the NCD group, the catalase (CAT) activity of mice in the HFD group was significantly decreased ($p < 0.01$), indicating increased levels of oxidative stress. In contrast, SSA significantly ($p < 0.001$) inhibited the decrease in CAT enzyme activity induced by HFD feeding (Fig. 4A). In addition, we also compared levels of superoxide dismutase (SOD), an important indicator of cellular antioxidant capacity. It was found that HFD feeding reduced SOD enzyme activity by approximately 50%, whereas SSA administration significantly ($p < 0.05$) inhibited this reduction (Fig. 4B).

Spontaneous or passive activation of the Nrf2/ARE pathway, an antioxidant defense system, has a preventive and reparative effect on oxidative stress damage in body tissues. Total Nrf2 and phosphorylated Nrf2, as well as its downstream antioxidant genes, including NAD(P)H quinone dehydrogenase 1 (*Nqo1*) and heme oxygenase-1 (*Ho-1*), were measured. Assessed by immunoblotting and quantitative qRT-PCR, Nrf2 protein expression was found to be significantly ($p < 0.05$) up-regulated in the intestine of mice in the HFD+SSA group compared to the HFD group (Fig. 4C–E). Furthermore, SSA promoted more Nrf2 phosphorylation, and the entry of phosphorylated Nrf2 into the nucleus continuously activated the antioxidant response and initiated the expression of antioxidant genes, as verified by the elevated transcript levels of *Nqo1* and *Ho-1* (Fig. 4F). Overall, SSA activates the Nrf2/ARE signaling pathway to increase antioxidant capacity in DIO mice.

SSA Alleviates PA and LPS-Induced Caco-2 Cell Injury and Oxidative Stress

To further investigate the mechanism by which SSA alleviates intestinal damage and oxidative stress, Caco2 cells were co-stimulated with PA (200 μM) and LPS (10 μg/mL) for 6 hours to mimic the intestinal condition of DIO mice. The co-stimulation by PA and LPS significantly ($p < 0.05$) promoted the transcription of pro-inflammatory factors (*TNF-α*, *IL-6*, and *IL-1β*) in Caco-2 cells. In contrast, transcript levels of epithelial cell tight junction molecules (*ZO-1* and *Occludin1*) were significantly ($p < 0.05$) reduced. As expected, treatment with SSA (5 μM) attenuated the increased mRNA expression of *TNF-α* and *Il-6* and decreased transcript levels of *ZO-1* and *Occludin-1*, which is similar to those observed *in vivo* (Fig. 5A). To further determine the effect of SSA on cell barrier integrity, TEER was measured at 0, 4, and 12 h in three groups of Caco-2 cells. After 4 h and 12 h of PA+LPS treatment, the TEER of the PA+LPS group was significantly ($p < 0.05$) reduced when compared to the control group, and this effect was reversed by SSA incubation (Fig. 5B), indicating that SSA treatment

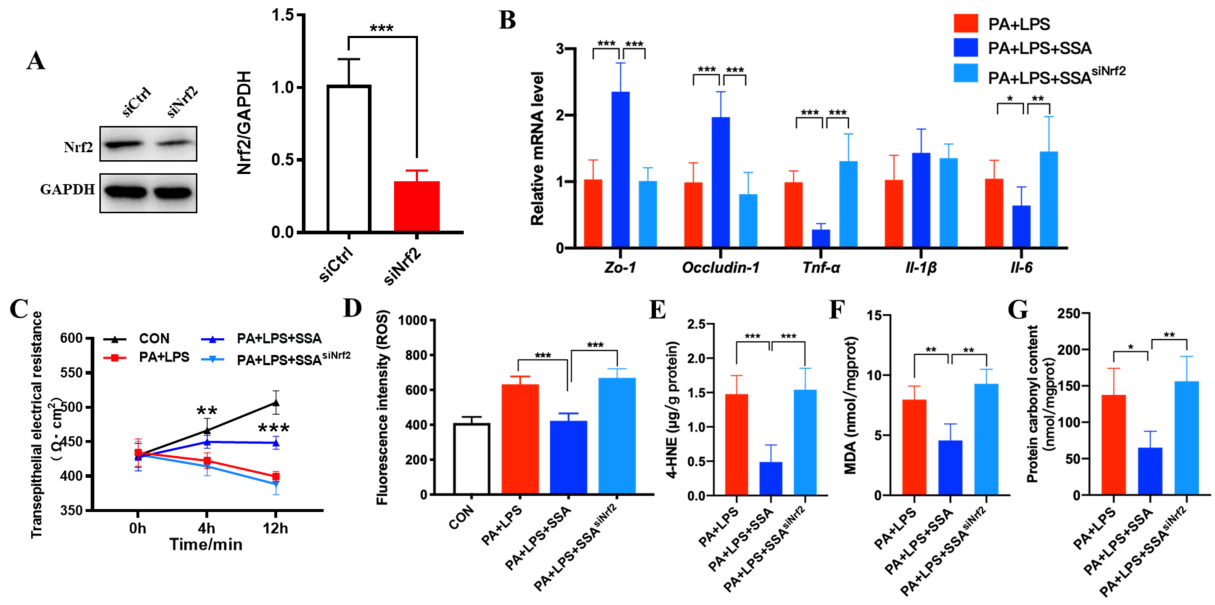

Fig. 6. The effect of SSA in reducing cellular damage and oxidative stress is mediated by the Nrf2/ARE pathway. (A) Western blot analysis demonstrating the knockdown of Nrf2 in Caco-2 cells. (B) Relative mRNA levels of pro-inflammatory cytokines (*TNF-α*, *IL-1β* and *IL-6*) and tight junction-related proteins (*ZO-1* and *Occludin-1*). (C) The TEERs. (D) ROS content. (E) 4-HNE, (F) MDA and (G) PCC in Caco-2 cells. Data are represented as mean ± SEM, n = 6. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

protects the integrity of the cell barrier. In conclusion, these findings suggest that the intestinal inflammatory response was significantly ($p < 0.05$) alleviated and cell barrier integrity increased following SSA treatment.

Intracellular ROS levels are one of the classic indicators for monitoring oxidative stress [26]. Co-culture with PA and LPS resulted in increased intracellular ROS levels in Caco-2 cells, whereas SSA intervention significantly ($p < 0.05$) reduced the accumulation of ROS in cells (Fig. 5C). Furthermore, western blot results showed that SSA significantly ($p < 0.05$) increased intracellular Nrf2 and phosphorylated Nrf2 protein levels compared to the PA+LPS group (Fig. 5D–F), suggesting that SSA activated the Nrf2/ARE pathway to attenuate PA-induced intestinal injury and oxidative stress in Caco-2 cells.

SSA Reduces Cellular Damage and Oxidative Stress via Nrf2/ARE Pathway

To further explore the Nrf2/ARE signaling pathway as a mediator of SSA action, Nrf2 was silenced by small RNA interference in Caco-2 cells (Fig. 6A). When Nrf2 was knocked down in Caco-2 cells, incubation with SSA failed to increase the transcript levels of *ZO-1* and *Occludin1*, and the repressive effect on *TNF-α* and *IL-6* transcription was lost (Fig. 6B). Also, after knock-down of Nrf2, the TEER in the SSA-treated group was much lower than in the control and PA+LPS+SSA groups, indicating that SSA was unable to maintain the integrity of the cell barrier (Fig. 6C). Furthermore, the absence of Nrf2 resulted in a dramatic increase in intracellular ROS levels, and the addition of SSA did not accelerate the clearance of ROS (Fig. 6D). Thus, SSA-only treatment effectively scavenged the accumulation of oxidized lipid substances induced by PA and LPS, whereas 4-HNE, MDA, and PCC levels remained high in the PA+LPS+SSA^{siNrf2} group (Fig. 6E–G). These data suggest that SSA works by activating the Nrf2/ARE pathway to attenuate PA- and LPS-induced Caco-2 cell damage, inflammatory responses, and oxidative stress.

Discussion

Inflammation and oxidative stress are key factors in the development of obesity and contribute to the occurrence of metabolism-related complications. Researchers believe that high calorie intake can disrupt intestinal flora and intestinal epithelial cells and cause endotoxemia, thereby promoting chronic low-grade systemic inflammation and obesity. Saikosaponins ameliorate inflammation and metabolic disease in mammals by targeting fat mass and obesity associated protein (FTO), AMP-activated protein kinase (AMPK), mitogen-activated protein kinases (MAPK), and other pathways [27,28]. However, it remains unknown whether the protective effect of SSA against obesity is linked to the modulation of intestinal barrier function and oxidative stress. In this study, we found that SSA significantly inhibited intestinal barrier damage, colonic inflammation, and oxidative stress, and increased the antioxidant capacity of epithelial cells by activating the Nrf2/ARE pathway, thereby enhancing intestinal integrity and ameliorating inflammation and metabolic disorders in DIO mice.

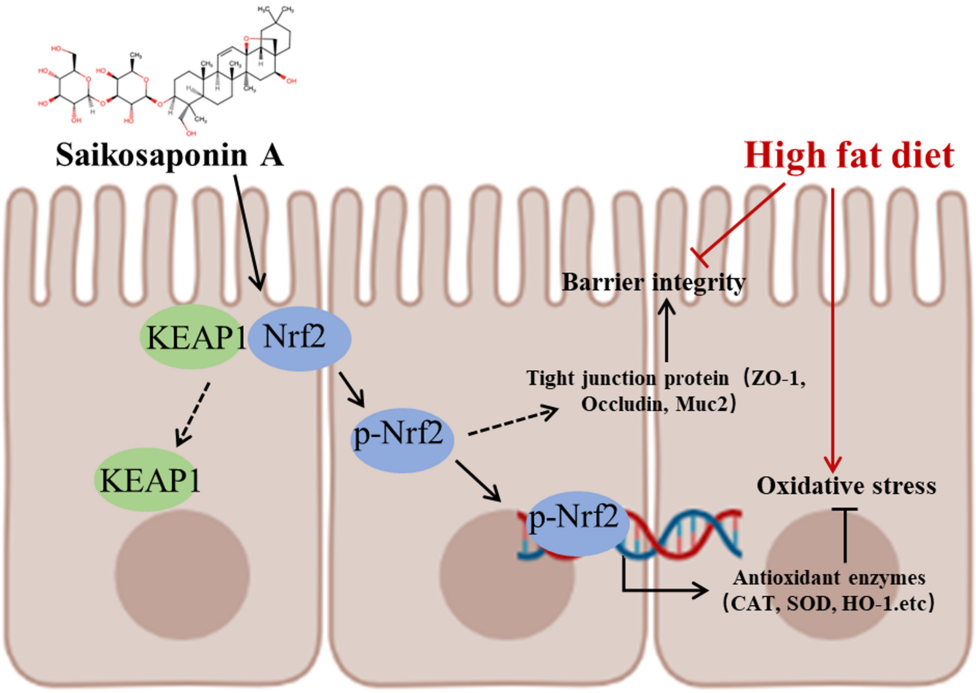

Fig. 7. Potential therapeutic mechanism of SSA to alleviate intestinal barrier damage and inflammation in DIO mice. This diagram was drawn using PowerPoint 4.0. KEAP1, Kelch-like ECH-associated protein 1.

This work provides evidence that the protection of the intestinal barrier and the inhibition of oxidative stress by SSA are mechanisms by which SSA ameliorates metabolic dysfunction and intestinal inflammation in DIO mice (Fig. 7).

A high-fat diet increases intestinal permeability, leading to the development of metabolic endotoxemia as LPS contained in food and bacterial metabolites are released into the organism [7]. Metabolic endotoxemia is an important obesogenic mechanism that triggers an inflammatory signaling cascade to activate various pro-inflammatory pathways. The binding of LPS to TLR4 increases oxidative stress, ultimately leading to systemic inflammation and IR [9,29]. Therefore, we focused on the effects of SSA on intestinal inflammation in DIO mice. Our results showed that in HFD-fed mice, SSA administration significantly reduced the levels of pro-inflammatory factors (TNF-α and IL-6) in the colon. Previous study has shown that TNF-α is closely associated with IR and that it can promote IR by activating inflammatory signaling cascades, such as the nuclear factor Kappa B (NF-κB), c-Jun NH2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) pathways, and inhibiting insulin-induced tyrosine phosphorylation and downstream signaling [30]. In addition, IL-6 has also been reported to block insulin signaling through activation of the STAT1/3 pathway, leading to metabolic disorders and reduced insulin sensitivity [31]. Thus, our results suggest that SSA can improve metabolic inflammation in the gut, which may contribute to improved metabolic disorders caused by obesity.

According to our data, DIO mice exhibited high levels of oxidative stress, as indicated by decreased CAT and SOD enzyme activity and increased levels of MDA, 4-HNE, and protein carbonylation. MDA and 4-HNE are toxic metabolites of lipid/protein peroxidation, which is widely used as a biomarker of oxidative stress during the pathogenesis of many metabolic diseases [32,33]. Similarly, excess hydroxyl radicals act on protein peptide bonds, creating carbonyl groups at the peptide bond breaks and disrupting protein function [34]. These are all indicators of elevated levels of oxidative stress in the organism. When the components of the antioxidant system are diminished, ROS cannot be adequately scavenged, leading to a redox imbalance that further triggers the development and progression of obesity and intestinal inflammation. A recent report showed that SSA exhibited significant protective effects on intestinal tissues in 5-fluorouracil-induced intestinal colitis mice by enhancing antioxidant enzymes (CAT, HO-1, SOD, NQO1, and glutathione S-transferase (GST)) while reducing MDA [35]. In SSA-treated HFD-fed mice, we observed reduced levels of MDA, PCC, and 4-HNE, and increased levels of SOD and CAT enzymes, which is also consistent with previous reports. In addition, we found that SSA administration protected the integrity of the intestinal barrier in mice, which is likely to be closely related to its effect in alleviating oxidative stress.

The NF-κB, ERK, and JNK pathways are activated by oxidative stress, and it is hypothesized that reducing oxidative stress levels is one of the important strategies for the

treatment of metabolic inflammation [23,36]. Nrf2/ARE is a classical antioxidant signaling pathway in the body, controlling the gene expression of a variety of antioxidant enzymes and phase II detoxification enzymes [37]. Due to the close relationship between oxidative stress and obesity, impaired function of the Nrf2/ARE pathway has been associated with the progression of obesity and multiple metabolic disorders. Uruno *et al*. [38] suggest that when Nrf2 is overexpressed in a mouse model, it ameliorates IR and metabolic disorders caused by a high-calorie diet. The mechanism is hypothesized to be that activation of Nrf2 significantly increases the transcript levels of genes related to antioxidant activity, energy consumption, and glucose metabolism in tissues and ultimately suppresses obesity and its complications in DIO mice. Furthermore, Jiang *et al*. [39] suggested that activation of the Nrf2/ARE signaling pathway significantly reduced the production of ROS in mitochondria and improved mitochondrial function to alleviate HFD-induced kidney injury in obese rats. Increasing evidence suggests that dietary SSA is a potential activator of Nrf2 signaling. Chen *et al*. [40] reported that SSA significantly inhibited cigarette smoke-induced MPO and MDA levels in lung tissue and suppressed lung inflammation by upregulating expression of Nrf2 and HO-1. Moreover, SSD significantly slowed H_2O_2-induced MDA and lactate dehydrogenase release, and increased SOD and CAT enzyme activity, thereby reducing apoptosis. Lin *et al*. [41] also showed that another saikosaponin compound could inhibit H_2O_2-induced apoptosis in PC12 cells by scavenging ROS, and the mechanism may be related to blocking MAPK-dependent oxidative damage. A recent study also found that SSA attenuated acute pancreatic injury induced by sodium taurocholate injection, and the mechanism may be related to the Nrf2-mediated antioxidant pathway [42]. Our results show that SSA activates the Nrf2/ARE antioxidant signaling pathway and enhances the enzymatic activity of SOD and CAT to scavenge excess oxygen radicals and lipid peroxidation products.

While our study demonstrates the beneficial effects of SSA on metabolic disorders and intestinal inflammation in DIO mice, there are several limitations that must be acknowledged. First, our research relied solely on a single animal model, the DIO mouse, which, although widely used, may not fully represent the complexity of human obesity and metabolic diseases. The physiological and metabolic responses in mice can differ significantly from those in humans, which may limit the translatability of our findings. Additionally, we did not use a variety of dosages and treatment durations for SSA, which means that the optimal dose and treatment regimen for potential clinical applications remain undetermined. Furthermore, our study focused on the Nrf2/ARE signaling pathway to explain the protective mechanisms of SSA, but other pathways and mechanisms might also play significant roles. Future studies should explore these additional pathways to provide a more comprehensive understanding of SSA's effects. Lastly, the study did not consider potential long-term effects or toxicity of SSA administration, which are critical for evaluating its safety and efficacy for clinical use. Addressing these limitations in future research will be essential for translating these promising findings into practical therapeutic strategies for obesity and related metabolic disorders.

Conclusion

In this study, we demonstrated that SSA effectively alleviates metabolic disorders, intestinal inflammation, and oxidative stress in DIO mice. The protective effect of SSA is likely due to the increased antioxidant capacity of the intestinal epithelium through activating the Nrf2/ARE signaling pathway, inhibiting diet-induced intestinal inflammation and oxidative stress damage. The resulting low levels of ROS benefit intestinal barrier function and reduce the possibility of LPS leakage into the circulation. These findings provide new insights into the protective role of SSA in obesity and metabolic diseases, suggesting its potential as a therapeutic agent for improving intestinal health and mitigating metabolic disturbances associated with obesity.

Availability of Data and Materials

The data are available from the corresponding author on reasonable request.

Author Contributions

DH: Conceptualization, Methodology, Resources, Investigation, Formal analysis, Visualization, Data curation, Project administration, and Funding acquisition, Writing - original draft, manuscript revision. ZZ: Experimental, Data curation, Manuscript editing, Formatting, Manuscript revision. YH: Software, Data curation, Project administration, Manuscript editing, Review. XC: Visualization, Data curation, Manuscript revision, Project administration. DH had primary responsibility for the final content. All authors have read and approved the final manuscript. All authors have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

All animal experiments were approved by the Wenzhou Medical University Animal Care and Use Committee (Approval No.: 2023102).

Acknowledgment

We acknowledge Dr. Yan Zhang from Wenzhou Medical University for their valuable suggestions for this research.

Funding

This work was financially supported by the Science and Technology Program of Wenzhou (Y2023449).

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Piché ME, Tchernof A, Després JP. Obesity Phenotypes, Diabetes, and Cardiovascular Diseases. Circulation Research. 2020; 126: 1477–1500.

[2] Blüher M. Obesity: global epidemiology and pathogenesis. Nature Reviews Endocrinology. 2019; 15: 288–298.

[3] Young KL, Graff M, Fernandez-Rhodes L, North KE. Genetics of Obesity in Diverse Populations. Current Diabetes Reports. 2018; 18: 145.

[4] Kachur S, Lavie CJ, de Schutter A, Milani RV, Ventura HO. Obesity and cardiovascular diseases. Minerva Medica. 2017; 108: 212–228.

[5] Chen L, Li L, Cui D, Huang Y, Tong H, Zabihi H, *et al*. Extracellular macrophage migration inhibitory factor (MIF) downregulates adipose hormone-sensitive lipase (HSL) and contributes to obesity. Molecular Metabolism. 2024; 79: 101834.

[6] Howell KR, Powell TL. Effects of maternal obesity on placental function and fetal development. Reproduction. 2017; 153: R97–R108.

[7] Gérard P. Gut microbiota and obesity. Cellular and Molecular Life Sciences. 2016; 73: 147–162.

[8] Cuevas-Sierra A, Ramos-Lopez O, Riezu-Boj JI, Milagro FI, Martinez JA. Diet, Gut Microbiota, and Obesity: Links with Host Genetics and Epigenetics and Potential Applications. Advances in Nutrition. 2019; 10: S17–S30.

[9] Wu S, Zuo J, Cheng Y, Zhang Y, Zhang Z, Wu M, *et al*. Ethanol extract of Sargarsum fusiforme alleviates HFD/STZ-induced hyperglycemia in association with modulation of gut microbiota and intestinal metabolites in type 2 diabetic mice. Food Research International. 2021; 147: 110550.

[10] Di Tommaso N, Gasbarrini A, Ponziani FR. Intestinal Barrier in Human Health and Disease. International Journal of Environmental Research and Public Health. 2021; 18: 12836.

[11] Luissint AC, Parkos CA, Nusrat A. Inflammation and the Intestinal Barrier: Leukocyte-Epithelial Cell Interactions, Cell Junction Remodeling, and Mucosal Repair. Gastroenterology. 2016; 151: 616–632.

[12] Farré R, Fiorani M, Abdu Rahiman S, Matteoli G. Intestinal Permeability, Inflammation and the Role of Nutrients. Nutrients. 2020; 12: 1185.

[13] Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. Physiological Reviews. 2014; 94: 329–354.

[14] Lee JY, Bae E, Kim HY, Lee KM, Yoon SS, Lee DC. High-Fat-Diet-Induced Oxidative Stress Linked to the Increased Colonization of Lactobacillus sakei in an Obese Population. Microbiology Spectrum. 2021; 9: e0007421.

[15] Diaz de Barboza G, Guizzardi S, Moine L, Tolosa de Talamoni N. Oxidative stress, antioxidants and intestinal calcium absorption. World Journal of Gastroenterology. 2017; 23: 2841–2853.

[16] Liu M, Zhang G, Naqvi S, Zhang F, Kang T, Duan Q, *et al*. Cytotoxicity of Saikosaponin A targets HEKa cell through apoptosis induction by ROS accumulation and inflammation suppression via NF-κB pathway. International Immunopharmacology. 2020; 86: 106751.

[17] Du ZA, Sun MN, Hu ZS. Saikosaponin a Ameliorates LPS-Induced Acute Lung Injury in Mice. Inflammation. 2018; 41: 193–198.

[18] Zhou F, Wang N, Yang L, Zhang LC, Meng LJ, Xia YC. Saikosaponin A protects against dextran sulfate sodium-induced colitis in mice. International Immunopharmacology. 2019; 72: 454–458.

[19] Fu Y, Hu X, Cao Y, Zhang Z, Zhang N. Saikosaponin a inhibits lipopolysaccharide-oxidative stress and inflammation in Human umbilical vein endothelial cells via preventing TLR4 translocation into lipid rafts. Free Radical Biology & Medicine. 2015; 89: 777–785.

[20] Zhang Y, Cheng Y, Liu J, Zuo J, Yan L, Thring RW, *et al*. Tauroursodeoxycholic acid functions as a critical effector mediating insulin sensitization of metformin in obese mice. Redox Biology. 2022; 57: 102481.

[21] Zhang Y, Zuo J, Yan L, Cheng Y, Li Q, Wu S, *et al*. *Sargassum fusiforme* Fucoidan Alleviates High-Fat Diet-Induced Obesity and Insulin Resistance Associated with the Improvement of Hepatic Oxidative Stress and Gut Microbiota Profile. Journal of Agricultural and Food Chemistry. 2020; 68: 10626–10638.

[22] Zhuge X, Jin X, Ji T, Li R, Xue L, Yu W, *et al*. Geniposide ameliorates dextran sulfate sodium-induced ulcerative colitis via KEAP1-Nrf2 signaling pathway. Journal of Ethnopharmacology. 2023; 314: 116626.

[23] Wang W, Zhao J, Gui W, Sun D, Dai H, Xiao L, *et al*. Tauroursodeoxycholic acid inhibits intestinal inflammation and barrier disruption in mice with non-alcoholic fatty liver disease. British Journal of Pharmacology. 2018; 175: 469–484.

[24] Tilg H, Zmora N, Adolph TE, Elinav E. The intestinal microbiota fuelling metabolic inflammation. Nature Reviews Endocrinology. 2020; 20: 40–54.

[25] Malesza IJ, Malesza M, Walkowiak J, Mussin N, Walkowiak D, Aringazina R, *et al*. High-Fat, Western-Style Diet, Systemic Inflammation, and Gut Microbiota: A Narrative Review. Cells. 2021; 10: 3164.

[26] Nathan C, Cunningham-Bussel A. Beyond oxidative stress: an immunologist's guide to reactive oxygen species. Nature Reviews Endocrinology. 2013; 13: 349–361.

[27] Li X, Li X, Huang N, Liu R, Sun R. A comprehensive review and perspectives on pharmacology and toxicology of saikosaponins. Phytomedicine. 2018; 50: 73–87.

[28] Sun K, Du Y, Hou Y, Zhao M, Li J, Du Y, *et al*. Saikosaponin D exhibits anti-leukemic activity by targeting FTO/m^6A signaling. Theranostics. 2021; 11: 5831–5846.

[29] Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. The Journal of Clinical Investigation. 2006; 116: 3015–3025.

[30] Nieto-Vazquez I, Fernández-Veledo S, Krämer DK, Vila-Bedmar R, Garcia-Guerra L, Lorenzo M. Insulin resistance associated to obesity: the link TNF-alpha. Archives of Physiology and Biochemistry. 2008; 114: 183–194.

[31] Akbari M, Hassan-Zadeh V. IL-6 signalling pathways and the development of type 2 diabetes. Inflammopharmacology. 2018; 26: 685–698.

[32] Zhou Q, Zhu L, Zhang D, Li N, Li Q, Dai P, *et al*. Oxidative Stress-Related Biomarkers in Postmenopausal Osteoporosis: A Systematic Review and Meta-Analyses. Disease Markers. 2016; 2016: 7067984.

[33] Liu G, Ji W, Huang J, Liu L, Wang Y. 4-HNE expression in diabetic rat kidneys and the protective effects of probucol. Journal of Endocrinological Investigation. 2016; 39: 865–873.

[34] Hauck AK, Huang Y, Hertzel AV, Bernlohr DA. Adipose oxidative stress and protein carbonylation. The Journal of Biological Chemistry. 2019; 294: 1083–1088.

[35] Ali J, Khan AU, Shah FA, Ali H, Islam SU, Kim YS, *et al*. Mucoprotective effects of Saikosaponin-A in 5-fluorouracil-induced intestinal mucositis in mice model. Life Sciences. 2019; 239: 116888.

[36] Yang H, Xie Y, Yang D, Ren D. Oxidative stress-induced apoptosis in granulosa cells involves JNK, p53 and Puma. Oncotarget. 2017; 8: 25310–25322.

[37] Uruno A, Yagishita Y, Yamamoto M. The Keap1-Nrf2 system and diabetes mellitus. Archives of Biochemistry and Biophysics. 2015; 566: 76–84.

[38] Uruno A, Matsumaru D, Ryoke R, Saito R, Kadoguchi S, Saigusa D, *et al*. Nrf2 Suppresses Oxidative Stress and Inflammation in *App* Knock-In Alzheimer's Disease Model Mice. Molecular and Cellular Biology. 2020; 40: e00467–19.

[39] Jiang XS, Cai MY, Li XJ, Zhong Q, Li ML, Xia YF, *et al*. Activation of the Nrf2/ARE signaling pathway protects against palmitic acid-induced renal tubular epithelial cell injury by ameliorating mitochondrial reactive oxygen species-mediated mitochondrial dysfunction. Frontiers in Medicine. 2022; 9: 939149.

[40] Chen RJ, Guo XY, Cheng BH, Gong YQ, Ying BY, Lin MX. Saikosaponin a Inhibits Cigarette Smoke-Induced Oxidant Stress and Inflammatory Responses by Activation of Nrf2. Inflammation. 2018; 41: 1297–1303.

[41] Lin X, Wu S, Wang Q, Shi Y, Liu G, Zhi J, *et al*. Saikosaponin-D Reduces H_2O_2-Induced PC12 Cell Apoptosis by Removing ROS and Blocking MAPK-Dependent Oxidative Damage. Cellular and Molecular Neurobiology. 2016; 36: 1365–1375.

[42] Li J, Han J, Lv J, Wang S, Qu L, Jiang Y. Saikosaponin A-Induced Gut Microbiota Changes Attenuate Severe Acute Pancreatitis through the Activation of Keap1/Nrf2-ARE Antioxidant Signaling. Oxidative Medicine and Cellular Longevity. 2020; 2020: 9217219.