doi: 10.4149/gpb_2025041

Integrated evaluation of genetic and pharmacological RAGE inhibition reveals a multidimensional therapeutic strategy for diabetic atherosclerosis

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Abstract. Atherosclerotic vascular disease is the major cause of mortality in type 2 diabetes. This study sought to examine the effects of different receptor for advanced glycation end-products (RAGE)-targeted interventions in diabetic atherosclerosis. A diabetic atherosclerosis model was constructed in ApoE$^{-/-}$ mice by high-fat diet feeding combined with streptozotocin injection. Human umbilical vein endothelial cells were treated with high glucose and oxidized low-density lipoprotein. RAGE was inhibited *via* three strategies: overexpression of soluble RAGE (sRAGE), small interfering RNA (siRNA) or short hairpin RNA (shRNA)-mediated knockdown of RAGE, and pharmacological blockade using FPS-ZM1. RAGE inhibition suppressed systemic and vascular inflammation. RAGE inhibition elevated endothelial nitric oxide synthase levels and downregulated vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 expression to improve endothelial dysfunction. RAGE blockade improved lipid metabolism, resulting in decreased total cholesterol, triglycerides, and low-density lipoprotein cholesterol levels while elevating high-density lipoprotein cholesterol concentrations. These effects were confirmed in both *in vivo* and *in vitro* models. RAGE inhibition mitigated aortic structural damage and lipid deposition in diabetic atherosclerotic mice. These protective effects were consistently observed across all three intervention strategies. Genetic and pharmacological RAGE inhibition alleviates diabetic atherosclerosis and represents a potential therapeutic target for diabetes-related macrovascular complications.

Key words: Diabetic atherosclerosis — RAGE inhibition — Inflammation — Endothelial dysfunction — Lipid metabolism

Electronic supplementary material. The online version of this article (doi: 10.4149/gpb_2025041) contains Supplementary material.
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Introduction

Diabetes mellitus and its associated complications pose a significant global health burden and remain a growing public health concern. In 2021, the worldwide incidence of diabetes among individuals aged 20 to 79 was estimated to be 10.5%, and this number is expected to increase to 12.2% by 2045 (Sun et al. 2022). Among diabetic complications, vascular abnormalities are particularly prevalent, with endothelial dysfunction and atherosclerosis being the most critical manifestations (Li et al. 2023). Compared to non-diabetic individuals, patients with diabetes have a 2–3-fold elevated risk of atherosclerosis onset, thereby initiating and promoting the development of macrovascular complications (La Sala et al. 2019; Yang et al. 2024). This heightened risk is primarily attributed to persistent hyperglycemia, dyslipidemia, systemic inflammation, and oxidative stress (Yuchen et al. 2023). Despite improvements in glycemic control and lipid-lowering strategies, the prevention and treatment of diabetic atherosclerosis remain challenging (Karakasis et al. 2025). Hence, further elucidating the underlying molecular mechanisms of diabetic atherosclerosis and identification of novel therapeutic targets are urgently needed.

The receptor for advanced glycation end-products (RAGE) is considered a crucial regulator in the development of diabetic vascular complications, including atherosclerosis (Pal et al. 2023). Functioning as a receptor capable of binding multiple ligands, RAGE localizes to the membranes of multiple cell types, such as fibroblasts, epithelial cells, and vascular endothelial cells (Ramasamy et al. 2011). Upon activation by advanced glycation end-products (AGEs) or other ligands, RAGE triggers a cascade of pro-inflammatory and pro-atherogenic signaling pathways (Mao et al. 2022; Tufail et al. 2025). Antagonism or systemic deletion of Ager, the gene encoding RAGE, has been shown to suppress the development and enhance the regression of atherosclerosis in diabetic and non-diabetic mice (Ramasamy et al. 2024). RAGE appears to play a more deleterious role in diabetes mellitus. RAGE hinders atherosclerosis regression and promotes macrophage-driven inflammation and cholesterol dysregulation *via* interferon regulatory factor 7 (IRF7) (Senatus et al. 2020). Under hyperglycemic conditions, AGEs accumulate in the plasma and bind to RAGE on the surface of vascular endothelial cells, initiating downstream signaling pathways that induce apoptosis and oxidative stress (Banarjee et al. 2018). However, the precise effects of RAGE inhibition on systemic inflammation and endothelial dysfunction in diabetic atherosclerosis remain poorly defined, and direct evaluations of different RAGE-targeted strategies are still lacking.

The objective of this study was to evaluate the efficacy of RAGE suppression in diabetic atherosclerosis. We found that RAGE inhibition, via either genetic knockdown or pharmacological blockade, attenuated vascular inflammation and improved endothelial function. These findings underscore the therapeutic potential of RAGE for managing diabetic atherosclerosis.

Materials and Methods

In vivo studies

All experimental protocols were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals. ApoE$^{-/-}$ mice (C57BL/6J background; 6–8 weeks old; 18–20 g) were obtained from SPF Biotechnology Co. (Beijing, China). Mice were maintained under specific pathogen-free (SPF) conditions, with temperature controlled at 23 ± 1°C, humidity at 60–70%, and a 12-h light/dark photoperiod, with *ad libitum* access to food and water. Mice in the model group received a high-fat diet (HFD) for 4 weeks. The composition of the HFD was as follows: basic maintenance feed (49.75%), lard (17%), sesame oil (3%), sucrose (9%), whole milk powder (8%), casein (8%), experimental animal premix (2%), calcium hydrogen phosphate (2%), and cholesterol (1.25%). To induce type 2 diabetes mellitus, mice received intraperitoneal injections of streptozotocin (STZ, 40 mg/kg), prepared in 0.05 mol/l citrate buffer (pH 4.5), administered once daily over five consecutive days after a 10-h fasting period. In the control group, mice were fed with a standard diet and received equivalent volumes of vehicle (citrate buffer) injections. Fasting blood glucose (FBG) was quantified after STZ administration with a OneTouch Ultra glucometer (LifeScan, Milpitas, CA), and mice with FBG levels exceeding 16.7 mmol/l were considered to have diabetes mellitus.

Diabetic mice were randomly categorized into the following groups (at least 6 *per* group): (1) atherosclerosis+diabetes model (HFD+STZ) group; (2) model+soluble RAGE overexpression (HFD+STZ+sRAGE) group; (3) model+short hairpin RNA (shRNA)-mediated RAGE knockdown (HFD+STZ+sh-RAGE) group; (4) model+RAGE inhibitor (FPS-ZM1) treatment (HFD+STZ+FPS-ZM1) group. Following diabetes induction, all model groups continued to receive the HFD for an additional 12 weeks. FPS-ZM1 (1 mg/kg) was administered daily (Sanajou et al. 2019) *via intraperitoneal* injection, starting from week 9 of the HFD regimen, to compensate for its short half-life and ensure sustained exposure for therapeutic efficacy. For the sRAGE overexpression and RAGE knockdown groups, lentiviral vectors (GeneChem, Shanghai, China) encoding sRAGE (LV-sRAGE) or RAGE-targeting shRNA (LV-sh-RAGE) were administered *intraperitoneally* once *per* week for 12 consecutive weeks, starting immediately after confirmation of diabetes induction. The weekly dosing schedule was designed to achieve stable and efficient transgene expres-

sion while minimizing potential immune responses. At the study endpoint, mice were anesthetized with 2% isoflurane and euthanized by cervical dislocation. Plasma and aortic tissue samples were then collected for subsequent analyses. The aorta from each mouse was carefully dissected and segmented. The aortic root was used for histological staining, and the remaining aortic arch and thoracic aorta were allocated for biochemical assays. The experimental timeline is illustrated in Figure S1 (in Supplementary material).

Hematoxylin and eosin (HE) staining

Mice were perfused with 3–5 ml of phosphate-buffered saline (PBS) after euthanasia. Whole aortas were isolated following careful dissection of surrounding connective and adipose tissues. For histological analysis, aortic root from six mice in each group were immersed in 4% paraformaldehyde for 24 h, embedded in paraffin, and sectioned at a thickness of 5 μm. Following deparaffinization, paraffin sections were stained with hematoxylin (C0105S; Beyotime, Shanghai, China) for 5 min, followed by eosin (Beyotime) for 30 s. After sealing with neutral gum, histological images were obtained using an optical microscope (Olympus, Tokyo, Japan).

Oil red O staining

The aortic root was fixed in 4% paraformaldehyde, dehydrated in a sucrose solution, and subsequently embedded in optimal cutting temperature (OCT) compound with the proximal end oriented upward. Tissue sections were cut at a thickness of approximately 6 μm using a cryostat at −20°C. Oil Red O staining solution (0.3%) was prepared by diluting a 0.5% stock solution (O0625; MilliporeSigma, Burlington, MA, USA) in isopropanol with deionized water, and then passed through a 0.2 μm filter. After infiltration with 60% isopropanol (10 min), aortic sections were incubated with Oil Red O solution for 30 min. Excess dye was removed using 60% isopropanol, and the tissue was washed with distilled water for 20 min. To assess atherosclerotic burden, lesion areas were measured from stained aortic images using ImageJ 1.8.0 (NIH, Bethesda, MD, USA), and the relative lesion area was determined by the formula: (lesion area/whole aorta area) × 100%.

Measurement of lipid profiles and inflammatory factor levels

Plasma was separated by centrifuging fresh blood at 2,000 rpm for 20 min at 4°C. Tissue homogenization of the aorta was performed using ice-cold lysis buffer. Centrifugation of the tissue homogenates was carried out at 12,000×*g* for 15 min at 4°C, after which the supernatants were carefully collected. The concentrations of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and endothelial nitric oxide synthase (eNOS) were measured in plasma, aortic homogenate supernatants, and cell culture supernatants. Lipid parameters, including total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C), were quantified in plasma and cell supernatants, while low-density lipoprotein cholesterol (LDL-C) was measured only in cell supernatants using enzyme-linked immunosorbent assay (ELISA) kits (Esebio Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's protocols. Lipid concentrations were normalized to total protein levels, which were detected employing a bicinchoninic acid (BCA) protein assay kit (P0012, Beyotime). Optical density was recorded at the designated wavelength with a microplate spectrophotometer.

Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were obtained from Icellbioscience Biotechnology Co., Ltd. (Shanghai, China) and grown in Endothelial Growth Medium-2 (EGM-2; Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 37°C containing 5% CO_2. The cells were allocated to five groups: control, High glucose (HG) and oxidized low-density lipoprotein (ox-LDL)-treated (HG+ox-LDL), HG+ox-LDL+sRAGE, HG+ox-LDL+si-RAGE, and HG+ox-LDL+FPS-ZM1. Except for the control group, cells were exposed to 33.3 mM D-glucose and 100 μg/ml ox-LDL for 48 h to mimic diabetic atherosclerotic conditions. In the HG+ox-LDL+sRAGE and HG+ox-LDL+si-RAGE groups, cells were transfected with a recombinant sRAGE overexpression plasmid (pcDNA3.1-sRAGE, 1 μg/ml; GenePharma, Shanghai, China) or small interfering RNA targeting RAGE (si-RAGE, 50 nM; GenePharma) for 24 h with Lipofectamine™ 3000 (Thermo Fisher Scientific) following the manufacturer's instructions. In the HG+ox-LDL+FPS-ZM1 group, cells were treated with the RAGE-specific inhibitor FPS-ZM1 (500 nmol/l) for 24 h.

Cell Counting Kit-8 (CCK-8) assay

The viability of cells from different groups was assessed at 0 and 24 h with the CCK-8 kit (C0038; Beyotime). A total of 1×10^5 cells were plated in 96-well plates and maintained at 37°C in a humidified incubator with 5% CO_2. At the designated time points, CCK-8 solution (10 μl) was added to each well and incubated for 2 h, after which the optical density at 450 nm was recorded using a microplate spectrophotometer.

Reverse transcription-quantitative PCR

Total RNA was extracted from arterial tissues with TRIZOL reagent (Thermo Fisher Scientific), followed by reverse transcription to synthesize cDNA. qPCR analysis was conducted using

the ABI 7500 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) with thermal cycling parameters set at 95°C for 30 s (initial denaturation), followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. GAPDH served as the internal reference, and relative gene levels were determined using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are detailed in Table S1.

Western blot analysis

Arterial tissue and cell samples were lysed in 1×RIPA buffer containing protease inhibitors, followed by 30 min incubation on a rotator and centrifugation at 12,000 rpm for 20 min at 4°C. The quantification of protein concentrations was performed using the BCA assay kit (P0012, Beyotime). Protein (20 μg) samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane in an ice bath. After blocking with 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 h, membranes were exposed to specific primary antibodies and incubated overnight at 4°C. Then, membranes were exposed to horseradish peroxidase (HRP)-linked secondary antibodies for 1 h, and protein signals were detected using enhanced chemiluminescence (ECL, Bio-Rad, Hercules, CA, USA) and analyzed using ImageJ software (NIH). The following primary antibodies were used in this study, all at a 1:2000 dilution: anti-nuclear factor κB (NF-κB, ab32360; Abcam, Cambridge, UK), anti-high mobility group box 1 (HMGB1, ab79823; Abcam), anti-vascular cell adhesion molecule 1 (VCAM-1, ab134047; Abcam), anti-intercellular adhesion molecule-1 (ICAM-1, ab222736; Abcam), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, ab9485; Abcam).

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 9.0 (GraphPad Software, USA). Data are expressed as mean ± standard deviation (SD). Comparisons between two groups were performed using Student's *t*-test, while one-way ANOVA followed by Tukey's *post hoc* test was applied for multiple group comparisons. A p-value < 0.05 was used to determine statistical significance.

Results

RAGE inhibition alleviates inflammation and improves endothelial function in HFD/STZ-induced diabetic atherosclerotic mice

To evaluate the impact of RAGE inhibition on systemic inflammation in diabetic atherosclerosis, we measured the levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and endothelial function-associated marker (eNOS) in plasma (Fig. 1A) and the supernatants of aortic tissue homogenates (Fig. 1B). Compared to the control group, mice receiving HFD+STZ exhibited significantly elevated levels of TNF-α, IL-1β, and IL-6, along with a marked reduction in eNOS expression. Overexpression of sRAGE, RAGE knockdown, and treatment with the RAGE inhibitor FPS-ZM1 markedly suppressed the levels of pro-inflammatory cytokines and restored eNOS expression. Collectively, these findings suggest that RAGE inhibition, *via* genetic or pharmacological strategies, alleviates systemic inflammation and endothelial dysfunction in diabetic atherosclerotic mice.

RAGE inhibition improves dyslipidemia in HFD/STZ-induced diabetic atherosclerotic mice

Subsequently, we detected TC, TG, and HDL-C levels in plasma to investigate the effects of RAGE signaling on lipid metabolism. The results showed that HFD+STZ treatment increased TC and TG levels and lowered HDL-C levels, which were notably reversed by sRAGE overexpression, RAGE knockdown, or FPS-ZM1 treatment (Fig. 2A–C). These data indicate that inhibition of RAGE signaling ameliorates lipid dysregulation in diabetic atherosclerotic mice.

RAGE inhibition attenuates aortic structural damage and lipid plaque accumulation in HFD/STZ-induced diabetic atherosclerotic mice

To evaluate the effect of RAGE inhibition on inflammatory infiltration and atherosclerotic plaque formation, HE staining and Oil Red O staining were conducted on aortic tissues from diabetic atherosclerotic mice. HE staining revealed that the aortic intima in the control group was intact and smooth, with no evident abnormalities. In contrast, mice in the HFD+STZ model group exhibited marked thickening of the aortic intima, with plaque protrusion into the lumen, numerous foam cells and lipid vacuoles beneath the intima, and infiltration of inflammatory cells. Compared to the HFD+STZ group, treatments involving sRAGE overexpression, RAGE knockdown, or administration of a RAGE inhibitor significantly reduced intimal thickness, decreased plaque formation, and led to a reduction in foam cell accumulation, lipid vacuole presence, and inflammatory cell infiltration (Fig. 3A). Moreover, Oil Red O staining revealed minimal red-stained lipid deposition beneath the aortic intima in control mice, whereas mice in the HFD+STZ group exhibited extensive red staining, indicative of substantial lipid accumulation. In comparison with the HFD+STZ group, mice treated with sRAGE overexpression, RAGE knockdown, or FPS-ZM1 showed

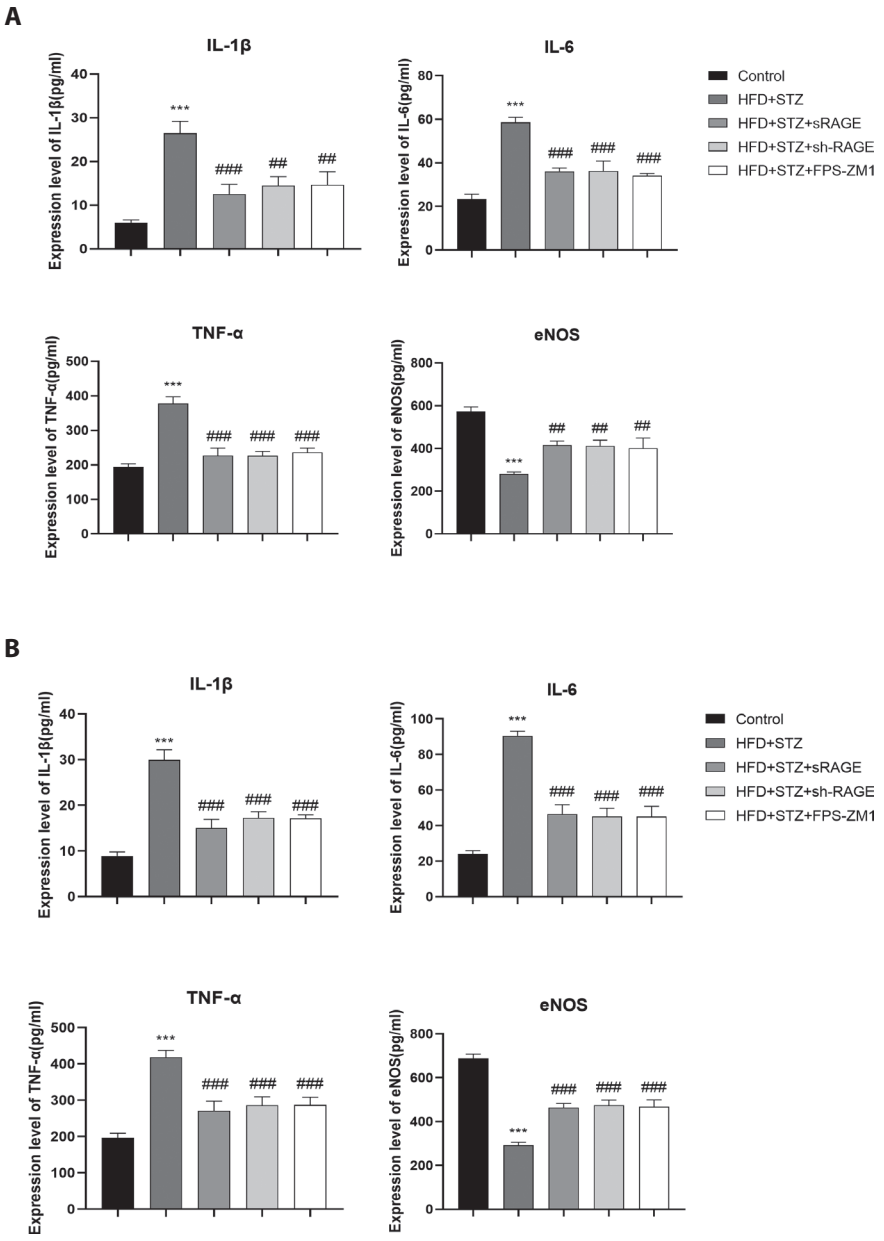


Figure 1. RAGE inhibition attenuates inflammation and restores eNOS levels in HFD/STZ-induced diabetic atherosclerosis mice. **A.** ELISA was used to quantify inflammatory cytokines IL-1β, IL-6, TNF-α, and eNOS in plasma samples from five groups: Control, HFD+STZ (high-fat diet and streptozotocin), HFD+STZ+sRAGE (soluble RAGE), HFD+STZ+sh-RAGE (RAGE knockdown), and HFD+STZ+FPS-ZM1 (RAGE-specific inhibitor). **B.** IL-1β, IL-6, TNF-α, and eNOS levels in aortic tissue supernatants collected from the same groups were detected using ELISA assays. $n \geq 6$. Data are shown as mean ± standard deviation (SD). *** $p < 0.001$ *vs.* Control group; ## $p < 0.01$, ### $p < 0.001$ *vs.* HFD+STZ group. HFD, high-fat diet; STZ, streptozotocin; sRAGE, soluble receptor for advanced glycation end-products; sh-RAGE, short hairpin RNA targeting RAGE; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor alpha; eNOS, endothelial nitric oxide synthase.

a marked reduction in Oil Red O-positive areas in the subintimal region (Fig. 3B). These findings suggest that inhibition of RAGE effectively attenuates pathological tissue changes in diabetic atherosclerotic mice.

RAGE inhibition suppresses vascular inflammation by reducing NF-κB and adhesion molecules

To determine whether RAGE inhibition alleviates inflammatory signaling associated with vascular dysfunction, we quantified the expression levels of NF-κB, HMGB1, and adhesion molecules. The findings demonstrated that the expression of NF-κB was elevated at the mRNA and protein levels in the HFD+STZ group in comparison with the control group, a trend that was significantly reversed by sRAGE, sh-RAGE, or FPS-ZM1. Similarly, expression levels of HMGB1 (a damage-associated molecular pattern protein and upstream regulator of NF-κB) and VCAM-1 and ICAM-1 (endothelial adhesion molecules) were upregulated in HFD/STZ-induced mice, which were effectively reversed by interventions targeting RAGE (Fig. 4A). Together, these findings demonstrate that targeting RAGE significantly suppresses vascular inflammatory signaling cascades in diabetic atherosclerotic mice.

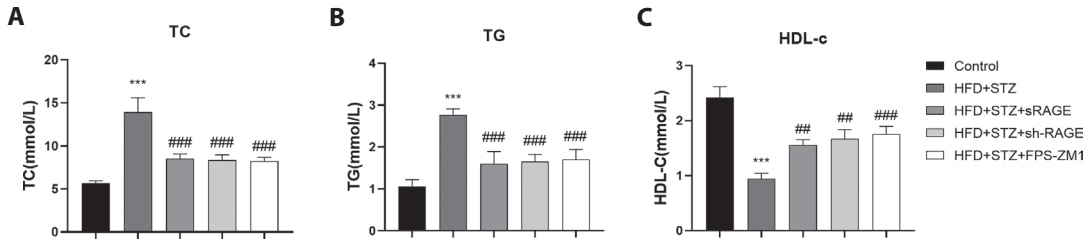


Figure 2. RAGE inhibition ameliorates dyslipidemia in HFD/STZ-induced diabetic atherosclerosis mice model. Plasma lipid profiles, including TC (**A**), TG (**B**), and HDL-C (**C**) levels, were analyzed in the Control, HFD+STZ, HFD+STZ+sRAGE, HFD+STZ+sh-RAGE, and HFD+STZ+FPS-ZM1 groups using ELISA assays. $n \geq 6$. All data are presented as mean ± standard deviation (SD). $^{***}p < 0.001$ *vs.* Control group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* HFD+STZ group. TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol. For more abbreviations, see Figure 1.

RAGE inhibition reduces cellular injury, dyslipidemia, and inflammation in HG+ox-LDL-stimulated HUVECs

Subsequently, we investigated the impact of RAGE inhibition in HUVECs exposed to hyperglycemic and pro-atherogenic stimuli. CCK-8 assays showed that cell viability was decreased in the HG+ox-LDL group relative to the control group. However, in comparison to the HG+ox-LDL group, treatment with sRAGE, si-RAGE, or FPS-ZM1 significantly restored cell viability (Fig. 5A). Lipid metabolism parameters

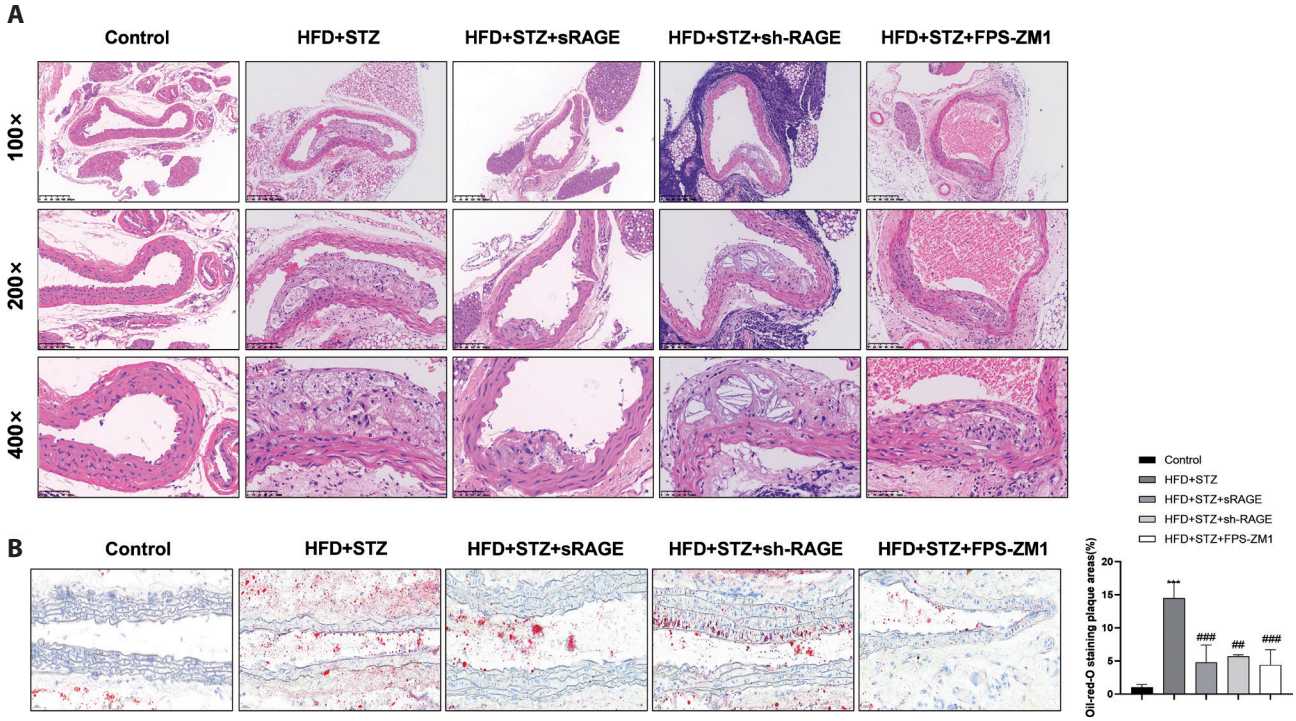


Figure 3. RAGE inhibition alleviates vascular structural damage and lipid accumulation in HFD/STZ-induced diabetic atherosclerosis mice. **A.** Representative HE-stained image of aortic tissues from five groups: Control, HFD+STZ, HFD+STZ+sRAGE, HFD+STZ+sh-RAGE, and HFD+STZ+FPS-ZM1. Samples were examined at 100× (200 μm), 200× (100 μm), and 400× (50 μm) magnifications to assess vascular wall structure, intima-media thickness, and inflammatory infiltration. **B.** Oil Red O staining of aortic tissues in the five groups was used to detect lipid deposition (red-stained areas). Representative images for each group are shown, along with quantitative analysis of plaque area (percentage of Oil Red O-positive area relative to total vascular area). $n \geq 6$. Data are expressed as mean ± standard deviation (SD). $^{***}p < 0.001$ *vs.* Control group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* HFD+STZ group. HE, hematoxylin and eosin. For more abbreviations, see Figure 1.

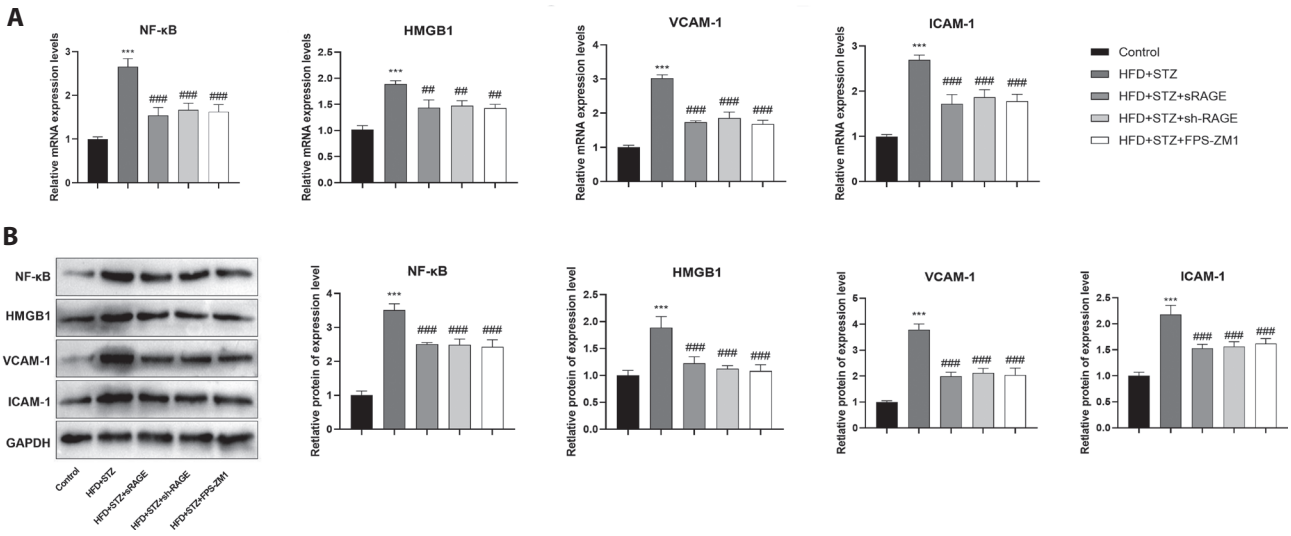


Figure 4. Inhibition of RAGE suppresses NF-κB-mediated inflammatory and endothelial activation in HFD/STZ-induced diabetic atherosclerosis mice. **A.** RT-qPCR was employed to detect mRNA expression levels of inflammatory and endothelial adhesion molecules in aortic tissues in the control, HFD+STZ, HFD+STZ+sRAGE, HFD+STZ+sh-RAGE, and HFD+STZ+FPS-ZM1 groups, including NF-κB, HMGB1, VCAM-1, and ICAM-1. **B.** Protein expression levels of NF-κB, HMGB1, VCAM-1, and ICAM-1 in five groups were assessed by western blot. GAPDH served as the loading control. $n \geq 6$. Data are expressed as mean ± standard deviation (SD). $^{***}p < 0.001$ *vs*. Control group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs*. HFD+STZ group. NF-κB, nuclear factor κB; HMGB1, high mobility group box 1; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. For more abbreviations, see Figure 1.

were then assessed. HG+ox-LDL stimulation increased TC, TG, and LDL-C, while reducing HDL-C levels in the cell supernatants, and intervention with sRAGE, si-RAGE, or FPS-ZM1 significantly normalized these lipid profiles (Fig. 5B). The levels of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) were upregulated after HG+ox-LDL exposure, while RAGE blockade significantly suppressed their expression levels in the cell supernatants (Fig. 6A). HG+ox-LDL markedly downregulated eNOS levels in comparison with the control group, while sRAGE, si-RAGE, or FPS-ZM1 treatment markedly restored eNOS levels in the cell culture supernatants (Fig. 6B). Furthermore, Western blot analysis revealed that HG+ox-LDL treatment resulted in markedly elevated expression of NF-κB, HMGB1, VCAM-1, and ICAM-1 compared to the control group. Treatment with sRAGE, si-RAGE, or the RAGE-specific inhibitor FPS-ZM1 significantly reduced the protein levels of NF-κB, HMGB1, VCAM-1, and ICAM-1 in HG+ox-LDL-stimulated HUVECs (Fig. 7). Collectively, these findings demonstrate that RAGE inhibition attenuates cell injury, inflammation, lipid dysregulation, and endothelial dysfunction in HG+ox-LDL-stimulated HUVECs.

Discussion

Atherosclerotic cardiovascular disease constitutes a major macrovascular complication of diabetes and continues to be the primary contributor to disease burden and death among individuals with diabetes (Steen Carlsson et al. 2023; Shah et al. 2024). Diabetic atherosclerosis is typified by persistent vascular inflammation, endothelial dysfunction, and accelerated atheromatous plaque formation (Pirri et al. 2020; Gao et al. 2023). In this study, we found that inhibition ofRAGE through sRAGE overexpression, siRNA-mediated or shRNA-mediated knockdown, or pharmacological inhibition *via* FPS-ZM1, effectively attenuated inflammation and improved endothelial function in *in vivo* and *in vitro* models of diabetic atherosclerosis. RAGE is minimally expressed under physiological conditions; however, in metabolic inflammatory disorders such as diabetes, its expression is markedly elevated in vascular and immune cells (Chen et al. 2024). Ligand engagement with RAGE initiates downstream signaling cascades that promote inflammation and endothelial dysfunction, representing an essential pathway driving the progression of diabetic atherosclerosis (Dong et al. 2022). Through its binding to ligands AGEs, HMGB1, and S100 family proteins, RAGE activates several intracellular signaling cascades, including NF-κB, mitogen-activated protein kinases (MAPKs), and phosphatidylinositol 3-kinase (PI3K), subsequently promoting the production of pro-inflammatory cytokines and cell adhesion molecules such as interleukin-1alpha (IL-1α), IL-6, TNF-α, and VCAM-1 (Zeng et al. 2019; Taguchi et al. 2023). Consistent with these mechanisms, our data revealed

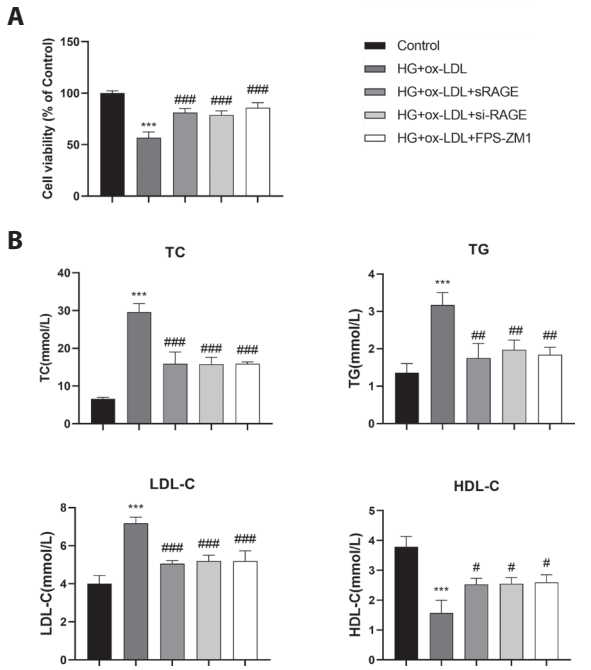


Figure 5. RAGE inhibition enhanced endothelial HUVEC viability and lipid metabolism under HG and ox-LDL conditions. **A.** HUVEC viability was evaluated using the CCK-8 assay following 24 h exposure to HG (33.3 mM) and ox-LDL (100 μg/ml), with subsequent co-treatment using sRAGE, sh-RAGE, or the RAGE inhibitor FPS-ZM1. **B.** The levels of lipids in the cell supernatants, including TC, TG, LDL-C, and HDL-C, were measured using ELISA assays in the control, HG+ox-LDL, HG+ox-LDL+sRAGE, HG+ox-LDL+sh-RAGE, and HG+ox-LDL+FPS-ZM1 groups. Data are presented as mean ± standard deviation (SD) from at least three independent experiments. $^{***}p < 0.001$ *vs.* control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* HG+ox-LDL group. HG, high glucose; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; CCK-8, Cell Counting Kit-8. For more abbreviations, see Figure 1.

that RAGE inhibition reduced HMGB1 and NF-κB levels in both plasma and arterial tissues, confirming that these molecules are key downstream effectors in RAGE-mediated inflammation. Endothelial dysfunction represents an early hallmark of vasculopathy and serves as a critical prognostic marker for diabetic vascular complications (An et al. 2023). Through upregulation of VCAM-1, ICAM-1, and various chemokines, RAGE activation facilitates monocyte adhesion, transendothelial migration, and the formation of early lipid streaks in atherosclerosis (Prasad et al. 2022). In our study, the expression levels of VCAM-1 and ICAM-1 were markedly increased in diabetic mice. RAGE inhibition suppressed their expression, which may consequently interfere with early plaque formation. In addition to inflammatory regulation, our study also revealed that RAGE inhibition alleviated dyslipidemia in diabetic atherosclerosis, with reductions in TC, TG, and LDL-C, accompanied by a significant elevation in HDL-C levels. A recent study has shown that through stimulation of the RAGE/NF-κB/Caveolin-1 axis, AGEs facilitate increased transcytosis of LDL in endothelial cells, thereby accelerating the progression of atherosclerosis in diabetes (Shu et al. 2023). Our findings suggest that blocking RAGE dampens inflammation and restores lipid metabolic homeostasis, thereby exerting protective effects against diabetic vascular complications.

Importantly, inhibiting the AGE-RAGE axis has been identified as a promising therapeutic target for diabetic atherosclerosis. Stachybotrys microspora triprenyl phenol-44D (SMTP-44D) attenuates atherosclerotic plaque development in ApoE$^{-/-}$ mice by partially downregulating the AGEs-RAGE axis (Terasaki et al. 2023). Pharmacological agents such as gliclazide can suppress AGE-induced M1 polarization in macrophages by interfering with the RAGE/TLR4/ROS/NF-κB signaling cascade (Jahan et al. 2021). In our study, siRNA-mediated knockdown, sRAGE, and FPS-ZM1 showed comparable efficacy in reducing vascular inflammation, highlighting the potential of targeting RAGE with decoy receptors or small-molecule inhibitors. sRAGE functions as a decoy receptor that competitively engages RAGE ligands to block RAGE-mediated signaling. As an endogenous inhibitor of RAGE-driven inflammatory processes, a higher AGE/sRAGE ratio may serve as an insightful and feasible surrogate or universal biomarker in AGE-RAGE axis–related diseases, regardless of absolute serum sRAGE levels (Steenbeke et al. 2021). Moreover, in type 1 diabetes, treatment with lipid-lowering drugs has been associated with increased sRAGE levels and decreased RAGE end products (Melin et al. 2020). Additionally, it is reported that repeated intravenous administration of miR-21-3p significantly reduced atherosclerotic lesion size and increased circulating sRAGE levels in diabetic mice with atherosclerosis (Shao et al. 2020). In addition to endogenous inhibitors, small molecule-based RAGE antagonists such as FPS-ZM1 have shown promise in preclinical studies and entered clinical trials (Bongarzone et al. 2017; Singh et al. 2022). FPS-ZM1 has been shown to reduce methylglyoxal (MGO)-AGE-mediated neurotoxicity and inflammation (Liu et al. 2025). Interaction between α-synuclein fibrils and RAGE activates neuroinflammatory responses, which can be attenuated by genetic ablation of RAGE or pharmacological inhibition using FPS-ZM1 (Long et al. 2022). Deletion of RAGE attenuates oxidative stress and inflammatory responses triggered by elevated uric acid, and treatment with the RAGE inhibitor FPS-ZM1 effectively lowers circulating uric acid, mitigates pancreatic inflammation, and ameliorates insulin resistance (Zhao et al. 2025). However, most prior studies focused either

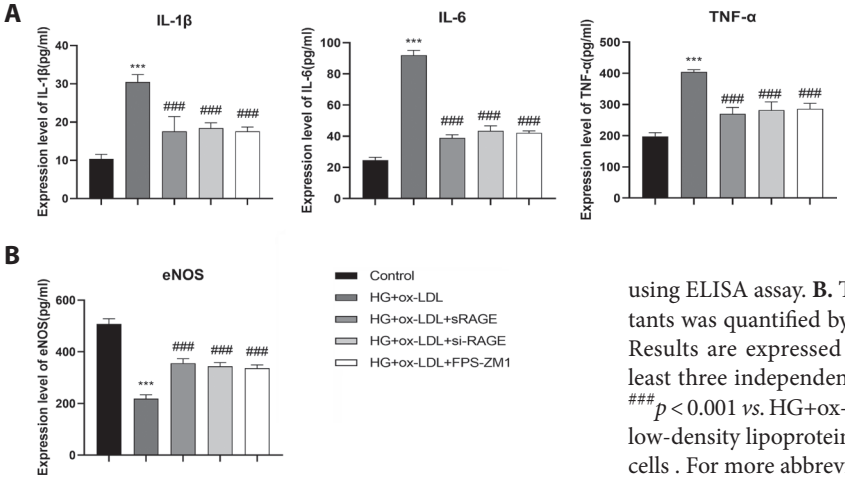


Figure 6. RAGE inhibition lowers inflammatory cytokine production and increases eNOS levels in HG+ox-LDL-induced endothelial injury. **A.** Pro-inflammatory cytokine levels of IL-1β, IL-6, and TNF-α in the cell supernatants were quantified in HUVEC supernatants after 24 h exposure to HG (33.3 mM) and ox-LDL (100 μg/ml), followed by co-treatment with sRAGE, sh-RAGE, or FPS-ZM1 (1 μM), using ELISA assay. **B.** The concentration of eNOS in the cell supernatants was quantified by ELISA under the same treatment conditions. Results are expressed as mean ± standard deviation (SD) from at least three independent experiments. $^{***}p < 0.001$ *vs.* Control group; $^{###}p < 0.001$ *vs.* HG+ox-LDL group. HG, high glucose; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells . For more abbreviations, see Figure 1.

on systemic outcomes or on isolated RAGE antagonism, lacking comprehensive comparisons between genetic and pharmacological RAGE inhibition approaches. By integrating both *in vivo* (ApoE$^{-/-}$ diabetic mice) and *in vitro* (HG+ox-LDL-stimulated HUVECs) models, our study provides a more translationally relevant evaluation of the therapeutic effect of RAGE inhibition. Taken together, our findings extend current knowledge by directly comparing multiple RAGE-targeted approaches in both systemic and vascular compartments. These data suggest that RAGE blockade, either genetically or pharmacologically, could serve as a viable adjunctive strategy to current anti-diabetic and lipid-lowering therapies in the clinical management of diabetic atherosclerosis.

Conclusion

Our study demonstrates that inhibition of RAGE, through sRAGE overexpression, shRNA-mediated knockdown,

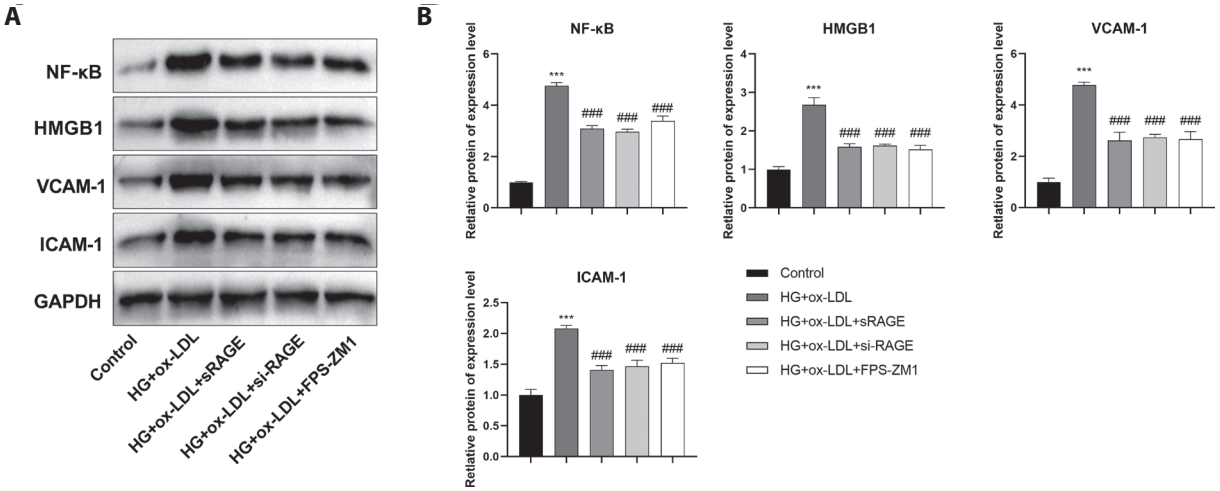


Figure 7. RAGE inhibition reduces NF-κB-mediated inflammatory signaling and endothelial adhesion molecule expression in HG+ox-LDL-treated HUVEC. **A.** Western blot analysis was used to measure inflammatory and endothelial adhesion-related proteins, including NF-κB, HMGB1, VCAM-1, and ICAM-1, in the control, HG+ox-LDL, HG+ox-LDL+sRAGE, HG+ox-LDL+sh-RAGE, and HG+ox-LDL+FPS-ZM1 groups. GAPDH was used as an internal loading control. **B.** Quantitative analysis of protein expression levels for NF-κB, HMGB1, VCAM-1, and ICAM-1 was performed by densitometry and normalized to GAPDH. Data are shown as mean ± standard deviation (SD) from at least three independent experiments. $^{***}p < 0.001$ *vs.* Control group; $^{###}p < 0.001$ *vs.* HG+ox-LDL group. HG, high glucose; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; NF-κB, nuclear factor κB; HMGB1, high mobility group box 1; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. For more abbreviations, see Figure 1.

or pharmacological blockade using FPS-ZM1, effectively attenuates systemic and vascular inflammation, improves endothelial function, and ameliorates dyslipidemia. These effects may be associated with the suppression of NF-κB and HMGB1 signaling, as well as downregulation of vascular adhesion molecules such as VCAM-1 and ICAM-1. These findings underscore the central role of RAGE in the pathogenesis of diabetic atherosclerosis and highlight its potential as a promising therapeutic target for the prevention and treatment of diabetes-associated macrovascular complications.

Declarations. Ethics approval and consent to participate: The experiments conformed to the Guide for the Care and Use of Laboratory Animals. Animal study has been approved by the Animal Ethics Committee of Guangdong Provincial People's Hospital. All methods are reported in accordance with ARRIVE guidelines.

Availability of data and materials. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interests. The author declares that they have no competing interests.

Funding. This work was supported by grants from the Science and Technology Program of Guangzhou, China (No. 202002030088) and Talents' plan Foundation of Guangdong Second Provincial General Hospital (No.2024B004)

Author contributions: YH: conceptualization, formal analysis, methodology, writing – original draft, validation, resources; FW and JL: formal analysis, methodology, validation, editing; ZY and JL: data curation, investigation, software, review and editing. All authors have read and approved the manuscript.

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Received: June 24, 2025
Final version accepted: September 29, 2025

doi: 10.4149/gpb_2025041

Supplementary Material

Integrated evaluation of genetic and pharmacological RAGE inhibition reveals a multidimensional therapeutic strategy for diabetic atherosclerosis

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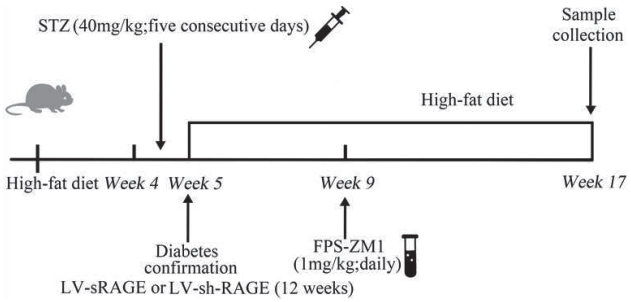
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Supplementary Table

Table S1. Primer sequences used in RT-qPCR

Primer name	Sequence (5'-3')
GAPDH-F	GAGTCAACGGATTTGGTCGT
GAPDH-R	TTGATTTTGGAGGGATCTCG
NF-κB-F	GGACCTATGAGACCTTCAAGAG
NF-κB-R	CAGAAGTTGAGTTTCGGGTAGG
HMGB1-F	AGGGAACCAGCTTCCAAAAT
HMGB1-R	AAGACCAACCTCGCTTTTCA
VCAM-1-F	ATTTTCTGGGGCAGGAAGTT
VCAM-1-R	ACGTCAGAACAACCGAATCC
ICAM-1-F	AGCACCTCCCCACCTACTTT
ICAM-1-R	AGCTTGCACGACCCTTCTAA

Supplementary Figure



Supplementary Figure 1. Schematic timeline of the experimental design for the diabetic atherosclerosis mouse model and intervention treatments.