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•Original article•

Icariin attenuates vascular endothelial dysfunction by inhibiting inflammation through GPER/Sirt1/HMGB1 signaling pathway in type 1 diabetic rats

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[ABSTRACT] Icariin, a flavonoid glycoside, is extracted from *Epimedium*. This study aimed to investigate the vascular protective effects of icariin in type 1 diabetic rats by inhibiting high-mobility group box 1 (HMGB1)-related inflammation and exploring its potential mechanisms. The impact of icariin on vascular dysfunction was assessed in streptozotocin (STZ)-induced diabetic rats through vascular reactivity studies. Western blotting and immunofluorescence assays were performed to measure the expressions of target proteins. The release of HMGB1 and pro-inflammation cytokines were measured by enzyme-linked immunosorbent assay (ELISA). The results revealed that icariin administration enhanced acetylcholine-induced vasodilation in the aortas of diabetic rats. It also notably reduced the release of pro-inflammatory cytokines, including interleukin-8 (IL-8), IL-6, IL-1β, and tumor necrosis factor-alpha (TNF-α) in diabetic rats and high glucose (HG)-induced human umbilical vein endothelial cells (HUVECs). The results also unveiled that the pro-inflammatory cytokines in the culture medium of HUVECs could be increased by rHMGB1. The increased release of HMGB1 and upregulated expressions of HMGB1-related inflammatory factors, including advanced glycation end products (RAGE), Toll-like receptor 4 (TLR4), and phosphorylated p65 (p-p65) in diabetic rats and HG-induced HUVECs, were remarkably suppressed by icariin. Notably, HMGB1 translocation from the nucleus to the cytoplasm in HUVECs under HG was inhibited by icariin. Meanwhile, icariin could activate G protein-coupled estrogen receptor (GPER) and sirt1. To explore the role of GPER and Sirt1 in the inhibitory effect of icariin on HMGB1 release and HMGB-induced inflammation, GPER inhibitor and Sirt1 inhibitor were used in this study. These inhibitors diminished the effects of icariin on HMGB1 release and HMGB1-induced inflammation. Specifically, the GPER inhibitor also negated the activation of Sirt1 by icariin. These findings suggest that icariin activates GPER and increases the expression of Sirt1, which in turn reduces HMGB1 translocation and release, thereby improving vascular endothelial function in type 1 diabetic rats by inhibiting inflammation.

[KEY WORDS] Icariin; Diabetes; Inflammation; Vascular endothelial dysfunction; GPER; HMGB1
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Introduction

Diabetes, characterized by impaired glucose homeostasis, significantly elevates the risk of cardiovascular morbidity and mortality in both type 1 and type 2 forms [1]. A pivotal factor in cardiovascular disease is the alteration of endothelial function, which plays a crucial role in the pathophysiology of vascular diseases. This dysfunction manifests primarily through dysregulated vascular tone, notably impaired endothelium-dependent vasodilation [2]. Dysfunction of the vascular endothelium is also a salient feature observed in diabetes [3, 4]. The connection between vascular endothelium dysfunction and inflammation is critical in the development of cardiovascular diseases [5, 6]. Specifically, inflammation disrupts the endothelial nitric oxide synthase (eNOS) / nitric oxide (NO) signaling pathway, leading to impaired endothelium-dependent vascular relaxation [7, 8]. Inflammation is a crucial constituent in diabetes that mediates vascular endothelium dysfunction. Additionally, hypoglycemia, a common complication in diabetes, can exacerbate the risk of cardiovascular disease by further promoting vascular inflammation and endothelial dysfunction [9].

Inflammation can be triggered by high-mobility group box 1 (HMGB1) through the activation of Toll-like receptor 4 (TLR4) and receptors for advanced glycation end products (RAGE) [10]. As receptors of HMGB1, TLR4 and RAGE are instrumental in the generation of pro-inflammatory mediators and in inducing the chronic inflammatory response associated with diabetes by activating p-p65 [11]. Numerous stud-

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ies have identified HMGB1 as a pivotal mediator in various vascular diseases, including peripheral artery disease [12], cerebral artery disease [13, 14], pulmonary hypertension [15], and coronary artery disease [16, 17]. HMGB1, functioning as a cytokine, can be actively secreted or passively released from multiple cell types, including endothelial cells. The inflammation prompted by HMGB1 can impair endothelium-dependent relaxation in diabetes [18]. Notably, patients with proliferative diabetic retinopathy exhibit higher serum levels of HMGB1 compared to non-diabetic individuals [19], highlighting the critical role of HMGB1-induced inflammation in exacerbating vascular endothelium dysfunction in diabetes.

Icariin, a bioactive flavonoid compound in *Epimedium*, has shown a broad spectrum of pharmacological effects, including neuroprotection, cardiovascular protection, anti-osteoporosis, anti-inflammatory, antioxidative, antidepressant, and anti-tumor properties [20]. Recent research has explored icariin's potential in mitigating vascular endothelial dysfunction through the inhibition of inflammation. In studies involving Wistar rats with atherosclerosis, icariin administration resulted in reduced serum levels of the inflammatory mediators interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) [21]. A prior study highlighted icariin's role as a peroxisome proliferator-activated receptor α (PPARα) activator, demonstrating its ability to inhibit nuclear factor kappa-B (NF-κB) activity by activating PPARα, a critical regulator of inflammation [22]. Our previous research has confirmed icariin's efficacy in attenuating vascular endothelial dysfunction in diabetes [23]. While it is established that icariin can improve vascular endothelial function by curbing inflammation, a comprehensive investigation into the mechanisms underlying icariin's protective effects on vascular endothelial function by inhibiting inflammation in diabetes remains necessary.

A prior study revealed that icariin mitigated neuroinflammation in the hippocampus by inhibiting HMGB1 [24]. However, the impact of icariin on HMGB1-induced inflammation in vascular endothelium has yet to be established. Therefore, this study aims to elucidate whether icariin can improve vascular endothelial function in type 1 diabetes by inhibiting HMGB1-induced inflammation.

Material and Methods

Reagents and materials

Icariin (98%, HPLC; Lot: 18102102) was synthesized by the Yangtze River Pharmaceutical Group. Streptozotocin (Cat. S0130, Sigma-Aldrich, MO). The reagents and materials used in this article were as follows: RPMI1640 media (Cat. 01-100-1ACS, Bioind, Israel), fetal bovine serums (Cat. 04-001-1ACS, Bioind, Israel), D-glucose (Cat. G116307, aladdin, Nanjing, China), G1 (Cat. HY-107216, MCE, Nanjing, China), G15 (Cat. B5469, ApexBio, Houston, USA), EX527 (Cat. A10377, AdooQ, Irvine, CA), recombinant human HMGB1 (rHMGB1, Cat. HY-P70570, MCE, Nanjing, China), acetylcholine (ACh) (Cat. A800895, Macklin, Shanghai, China), sodium nitroprusside (SNP) (Cat. S817931, Macklin, Shanghai, China), NG-nitro-L-arginine methyl ester (L-NAME) (Cat. N5751, Sigma-Aldrich, St. Louis, MO), Indo (Cat. I106885, aladdin, Nanjing, China), lysis buffer (Cat. KGP702-100, KeyGEN, Nanjing, China), protease inhibitor (Cat. P1005, Beyotime, Shanghai, China), phosphatase inhibitors (Cat. KGP602, KeyGEN, Nanjing, China), Cytoplasmic and Nuclear Protein Extraction Kit (Cat. P0027, Beyotime, Shanghai, China), Pierce bicinchoninic acid (BCA) Protein Assay Kit (Cat. P0010, Beyotime, Shanghai, China), polyvinylidene difluoride (PVDF) membranes (Cat. IPVH00010, Merck millipore, Kenilworth New Jersey, USA), Fat-free milk powder (Cat. 1172GR500, Biofroxx, Germany), G protein-coupled estrogen receptor (GPER) antibody (Cat. DF2737, Affinity Biosciences, OH, USA), Sirtuin1 (Sirt1) antibody (Cat. WL02995, Wanleibio, Shenyang, China), HMGB1 antibody (Cat. A5052, Bimaker, Houston, Texas, USA), RAGE antibody (Cat. WL01514, Wanleibio, Shenyang, China), p-p65 antibody (Cat. 3033T, Cell Signaling Technology, Danvers, Massachusetts, USA), p65 antibody (Cat. 10745-1-AP, Proteintech, Chicago, USA), TLR4 antibody (Cat. WL00196, Wanleibio, Shenyang, China), eNOS antibody (Cat. WL01789, Wanleibio, Shenyang, China), p-eNOS antibody (Cat. AF3247, Affinity Biosciences, OH, USA), GAPDH antibody (Cat. 10494-1-AP, Proteintech, Chicago, USA), ELISA (Cat. YT-1264H1, MeiLian, Yancheng, China), Goat Anti-Rabbit IgG H&L (FITC) (Cat. ab6717, Abcam, Cambridge, UK), DAPI (Cat. C1002, Beyotime, Shanghai, China), Nitric Oxide assay kit (Cat. S0021S, Beyotime, Shanghai, China), Protein A + G Agarose (Fast Flow, for IP) (Cat. P2055, Beyotime, Shanghai, China), Acetyl-Lysine Mouse Monoclonal Antibody (Cat. AG0433, Beyotime, Shanghai, China), NAD + /NADH Quantification Kit (WST-8 method) (Cat. E-BC-K804-M, Elabscience, Wuhan, China), TRIzol reagent (Cat. R701-01, Invitrogen, Carlsbad, CA), HiScript III RT SuperMix for qPCR kit (Cat. R323-01, Vazyme, Nanjing, China), ChamQ SYBR qPCR Master Mix Kit (Cat. Q341-02, Vazyme, Nanjing, China).

Experimental animals

In this study, female Sprague-Dawley (SD) rats aged 4−6 weeks were provided by Qinglongshan Laboratory Animal Company (Nanjing, China). All experimental procedures complied with the National Institute of Health guidelines for the ethical use of animals and received approval from the Animal Ethics Committee of China Pharmaceutical University (Approval Number: 2019-02-010). Type 1 diabetes in rats was induced via a single intraperitoneal injection of streptozotocin (STZ) at a dosage of 55 mg·kg^{-1}. Rats in the control group were administered an equivalent volume of saline. Blood glucose levels were measured from the tail vein 72 h or one week post-STZ injection. Rats exhibiting random blood glucose levels exceeding 16.7 mmol·mL^{-1} and fasting blood glucose levels above 11.1 mmol·mL^{-1} were classified as diabetic. The rats were allocated into five groups: control, STZ, STZ + icariin-20 mg·kg^{-1}, STZ + icariin-40 mg·kg^{-1}, and STZ + icariin-80 mg·kg^{-1} (n = 8). Icariin was dissolved in carboxymethyl cellulose sodium and administered daily by

gavage to diabetic rats for nine weeks. Upon euthanasia, segments of the thoracic aorta were excised for vascular reactivity assays, while the remainder of the aortic tissue was stored at −80 °C for Western blotting analysis.

Cell culture

HUVECs from ATCC were incubated in RPMI1640 media supplemented with 11 mmol·L^{-1} glucose, 100 μg·mL^{-1} streptomycin, 100 U·mL^{-1} penicillin, and 10% (*V*/*V*) fetal bovine serum. HUVECs were cultured with 35 mmol·L^{-1} D-glucose for 48 h to mimic the high-glucose (HG) environment. In this model, the HUVECs were exposed to HG conditions and treated with various concentrations of icariin (3, 10, 30 μmol·L^{-1}) or G1 (0.1 μmol·L^{-1}) for 48 h. Additionally, HUVECs were treated with G15 (1 μmol·L^{-1}), EX527 (2 μmol·L^{-1}), or recombinant HMGB1 (rHMGB1) (100 ng·mL^{-1}) to assess the effects of these treatments on cellular responses.

Vascular reactivity study

Vascular reactivity was assessed following the methodology outlined in a previous study [23]. The vasodilatory response of the aortas was evaluated using acetylcholine (ACh) (10^{-9}–10^{-5} mol·L^{-1}) or Sodium Nitroprusside (SNP) (10^{-10}–10^{-6} mol·L^{-1}). The aortas were pre-contracted by phenylephrine (Phe) (10^{-6} mol·L^{-1}). To examine the vasodilatory effect of icariin on the aortas, they were pre-incubated with L-NAME (10^{-4} mol·L^{-1}) and indomethacin (10^{-6} mol·L^{-1}) for 30 min. Following this pre-incubation, the aortas were subjected to cumulative concentration-response curves to icariin (2.5, 5, 10, 20, and 40 g·L^{-1}).

Western blotting analysis

Frozen aortic tissue samples and cultured HUVECs from different experimental groups were lysed using an ice-cold lysis buffer containing a protease inhibitor cocktail and phosphatase inhibitors. Cytoplasmic and Nuclear Protein Extraction Kit was used to extract nuclear and cytosolic proteins in HUVECs. Protein concentration was quantified by Pierce bicinchoninic acid (BCA) Protein Assay Kit. Following separation by SDS-PAGE, protein samples were transferred onto PVDF membranes, which were subsequently blocked with 5% fat-free milk powder. The membranes were then incubated overnight with primary antibodies targeting GPER, Sirt1, HMGB1, RAGE, TLR4, p-p65, p65, p-eNOS, eNOS, and GAPDH, followed by a 2-hour exposure to anti-rabbit IgG. All immunoblots were detected by an enhanced chemiluminescence detection system and visualized in an automated imaging analysis system.

Confocal immunofluorescence microscopy

The HUVECs were cultured in laser confocal petri dishes. After the pharmaceutical intervention, the HUVECs in these dishes were fixed with 4% paraformaldehyde for 30 min and permeabilized by 0.5% (*V*/*V*) Triton-X 100 for 10 min. Subsequently, the cells were blocked with 5% (*V*/*V*) BSA for 2 h and incubated overnight at 4 °C with primary antibodies against GPER (1:200), HMGB1(1 ∶ 200), or p-eNOS (1 ∶ 100). The cells were then labeled with goat anti-rabbit IgG H&L (FITC, 1 ∶ 200) for 1 h at 37 °C and stained with DAPI for 10 min. Finally, the Zeiss LSM800 microscope was used to visualize fluorescence.

Enzyme-linked immunosorbent assay (ELISA)

According to the manufacturer's protocol, the levels of HMGB1, TNF-α, IL-6, IL-8, and IL-1β in the serum and the culture medium of HUVECs were detected by ELISA.

Determination of NO production

NO production in the serum and the culture medium of HUVECs was measured using a Nitric Oxide assay kit.

Co-immunoprecipitation (Co-IP) analysis

Protein samples were extracted from HUVECs and incubated with 1 μg of HMGB1 for 2 h at room temperature. Next, Protein A/G agarose beads were added to the samples and then incubated overnight. After centrifugation, the agarose beads were washed three times with lysis buffer and denatured with 5× loading buffer. The level of HMGB1 acetylation was assessed by Western blotting using an anti-acetylated lysine antibody.

Molecular docking

3D structures of icariin (downloaded from PubChem) and GPER protein structure (downloaded from the RCSB PDB database, https://www.pdbus.org/) were imported into AutoDockTools (v1.5.6) for pretreatment and output to PDBQT format. Then, molecular docking was performed, and the binding energy was calculated using AutoDock Vina. A binding energy below −7 kcal·mol^{-1} indicates a robust binding activity, suggesting a favorable interaction between the ligand and the receptor [25].

Small interfering RNA transfection

SiRNA-GPER and SiRNA-Sirt1 were supplied by Genomeditech. Following a six-hour incubation with siRNA-lipid complexes, HUVECs were treated with icariin for 48 h under HG conditions.

Quantitative real-time PCR

Total RNA was extracted from HUVECs using a TRIzol reagent. cDNA synthesis was performed using the HiScript III RT SuperMix for qPCR kit and amplified with the ChamQ SYBR qPCR Master Mix Kit. Primers for the PCR were purchased from GenScript Biotech Corporation (Nanjing, China), with detailed primer information provided in Table 1. The PCR was performed on the ABI StepOne PCR system (Applied Biosystems, Foster City, CA, United States), and the results were quantified by the $2^{-\Delta\Delta Ct}$ method.

Determination of NAD$^+$ levels

The concentration of NAD$^+$ was measured using the NAD$^+$/NADH Quantification Kit (WST-8 method) according to the manufacturer's instructions.

Statistical analysis

All data were presented as mean ± SD. To assess the quantitative variables between groups, the independent *t*-test was utilized for the vascular reactivity study results. A one-way analysis of variance (ANOVA) was employed for the Western blotting analysis results and other experimental outcomes. Statistical analysis of the data was conducted using IBM SPSS Statistics 19.0. $P < 0.05$ was considered statistically significant.

Table 1 mRNA primer sequences for PCR

Gene	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
Sirt1	TAGCCTTGTCAGATAAGGAAGGA	ACAGCTTCACAGTCAACTTTGT
GAPDH	CTTCTTTTGCGTCGCCAGCCGA	ACCAGGCGCCCAATACGACCAA

Results

Effect of icariin on vascular reactivity in STZ-induced diabetic female rats

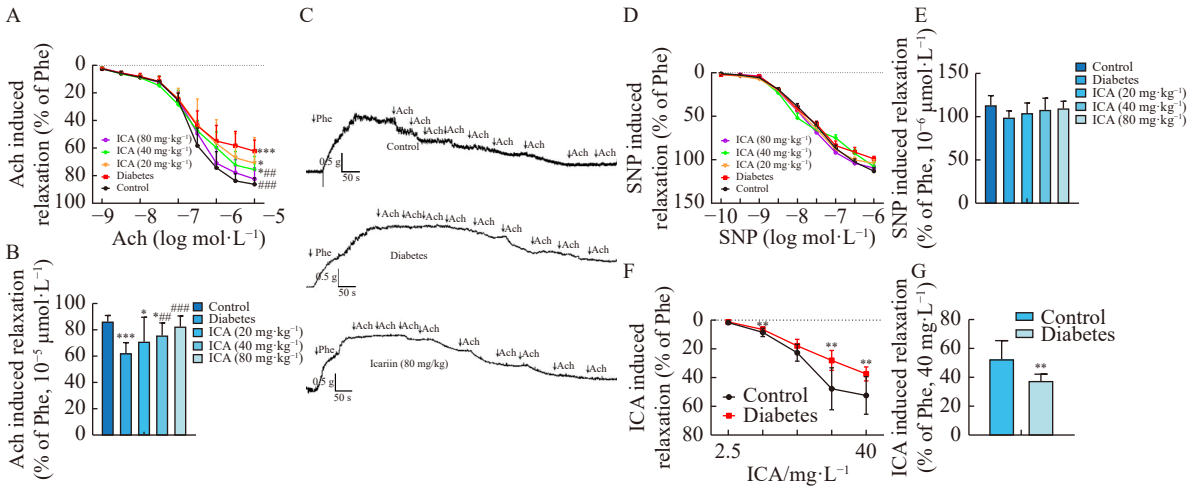
To confirm the effect of icariin on vascular reactivity in STZ-induced diabetic female rats, we assessed vascular reactivity. We observed that icariin significantly improved endothelium-dependent relaxation in response to ACh in the aortas of diabetic rats (Figs. 1A−1C). Interestingly, the response to sodium nitroprusside (SNP), which induces endothelium-independent relaxation, did not differ significantly between groups (Figs. 1D and 1E). Additionally, we evaluated the direct influence of icariin on the aortas, finding that icariin induced a dose-dependent relaxation (Figs. 1F and 1G). However, this vasorelaxant effect of icariin was negated either by damaging the aortic endothelium or by using L-NAME, an eNOS inhibitor (Figs. 1H−1K), indicating that icariin's action is mediated through endothelium-dependent activation of eNOS. Supporting this, Western blotting analyses demonstrated that icariin increased eNOS phosphorylation in the aortas of diabetic rats and in HG-induced HUVECs (Figs. 1L−1O), which were consistent with immunofluorescence results in HUVECs (Figs. 1P and S5). Moreover, icariin was shown to enhance NO production in both the serum of diabetic rats and the culture medium of HG-induced HUVECs (Figs. 1Q and 1R).

Effect of icariin on pro-inflammatory cytokines in the serum of the diabetic rats and HG-induced HUVECs culture medium

Evidence suggests that inflammation contributes to vascular endothelial dysfunction in diabetes [26]. In this context, we utilized ELISA to evaluate icariin's impact on pro-inflammatory cytokines in the serum of diabetic rats and the culture medium of HG-induced HUVECs. The concentrations of IL-8, IL-6, IL-1β, and TNF-α were notably elevated in the serum of diabetic female rats and the HG-induced HUVECs culture medium (Figs. 2A−2H). Treatment with icariin significantly reduced the levels of these pro-inflammatory cytokines (Figs. 2A−2H).

Icariin inhibited inflammation to attenuate vascular endothelium dysfunction by inhibiting the release of HMGB1

To understand the mechanisms underlying the effects of icariin on inflammation-induced vascular endothelial dysfunction, we focused on HMGB1, a key mediator in inflammatory responses [27]. Elevated levels of HMGB1 were observed in the serum of diabetic rats and the culture medium of HG-induced HUVECs (Figs. 3A and 3B). ELISA results indicated that icariin effectively reduced the release of HMGB1 in the serum of the diabetic rats and HG-induced HUVECs culture medium (Figs. 3A and 3B). Furthermore, Western blotting and immunofluorescence analyses demonstrated that icariin inhibited the nuclear-to-cytoplasmic translocation of HMGB1 in HG-cultured HUVECs (Figs. 3C−3F). Subsequently, we examined the expression of HMGB1 receptors, specifically the RAGE and TLR4, in the aortas of diabetic rats and HG-induced HUVECs. Icariin significantly suppressed the expression of RAGE and TLR4 in these samples (Figs. 3G−3J). The phosphorylation of p65, a marker of inflammation, can be induced by HMGB1 acting through RAGE or TLR4 [28]. We observed a notable reduction in the expression of p-p65 by icariin in both the aortas of diabetic rats and HG-induced HUVECs (Figs. 3G−3J), indicating a significant decrease in HMGB1 expression and release following icariin treatment (Figs. 3G−3J). HMGB1 acetylation plays a critical role in HMGB1 nuclear-to-cytoplasmic translocation [29]. Therefore, the level of acetylated HMGB1 in HUVECs was measured by Co-IP. The results showed that HMGB1 acetylation was inhibited in HUVECs after icariin supplementation compared with the HG group (Fig. S1). EX527, as the inhibitor of Sirt1, abolished the effect of icariin on HMGB1 acetylation (Fig. S1). The functional role of



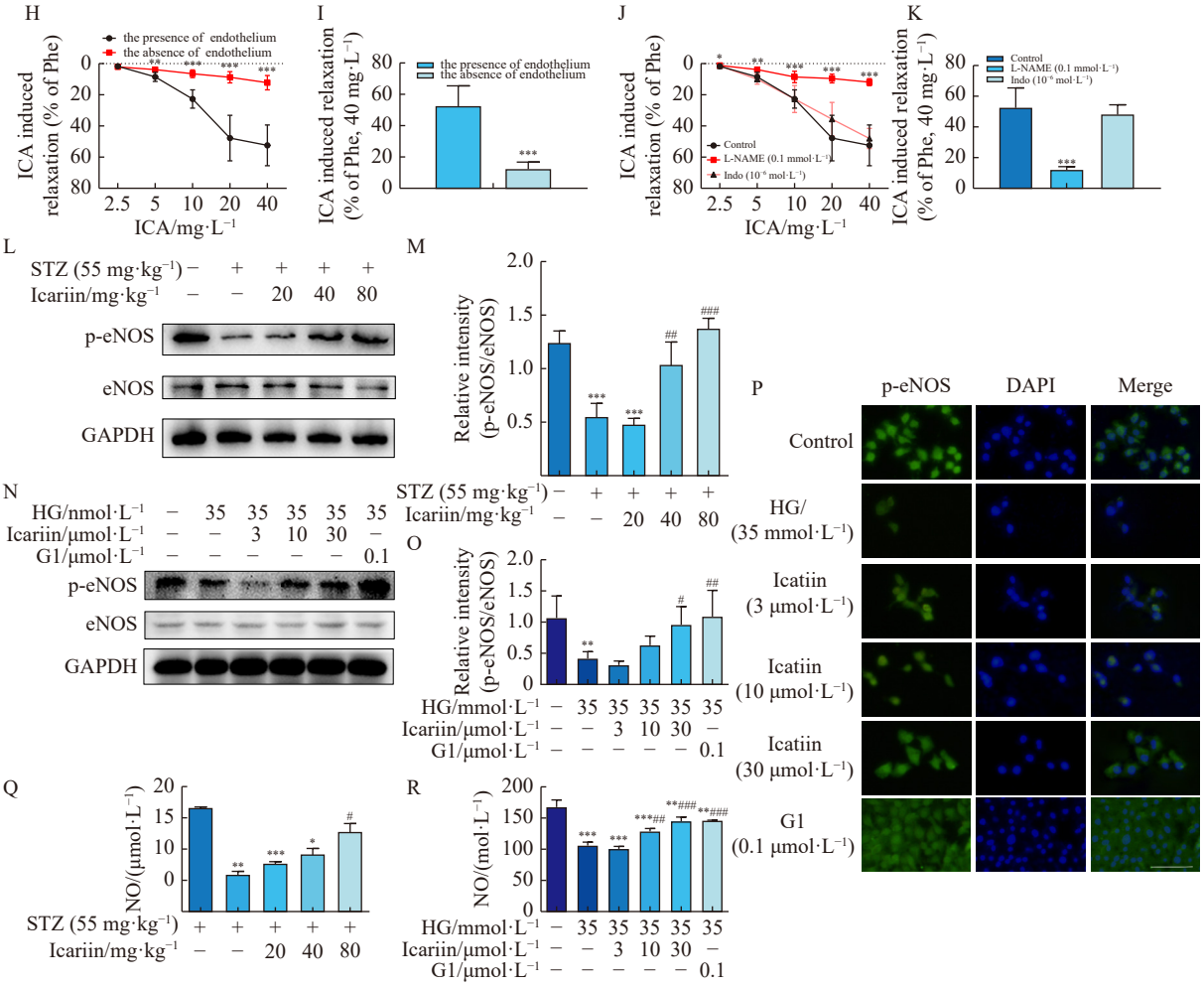


Fig. 1 Effect of icariin on vascular reactivity in STZ-induced diabetic female rats. (A−E) The diabetic rats were treated with icariin (20, 40, 80 mg·kg^{-1}). The effect of icariin on ACh or SNP induced relaxation in the aortas of diabetic rats. Data are mean ± SEM (n = 8). $^{*}P$ < 0.05, $^{**}P$ < 0.01, $^{***}P$ < 0.001 *vs* Control, $^{\#\#\#}P$ < 0.001 *vs* diabetes. (F, G) The icariin-mediated vascular response was measured. Data are mean ± SEM (n = 8). $^{**}P$ < 0.01 *vs* Control. (H, I) The influence of endothelium in icariin-induced relaxation in aortas. Data are mean ± SEM (n = 8). $^{***}P$ < 0.001 *vs* the presence of endothelium. (J, K) The effect of L-NAME and Indometacin on icariin-induced relaxation in the aortas. Data are mean ± SEM (n = 8). $^{*}P$ < 0.05, $^{**}P$ < 0.01, $^{***}P$ < 0.001 *vs* Control. (L, M) The diabetic rats were treated with icariin (20, 40, 80 mg·kg^{-1}). Western blotting assay revealed the expressions of p-eNOS and eNOS in the aortas. Data are presented as mean ± SD (n = 3). $^{***}P$ < 0.001 *vs* Control, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001 *vs* diabetes. (N, O) HUVECs were stimulated by HG (35 mmol·L^{-1}), treated with icariin (3, 10, and 30 μmol·L^{-1}) and G1 (0.1 μmol·L^{-1}) for 48 h. Western blotting assay revealed the expressions of p-eNOS and eNOS in HUVECs. Data are presented as mean ± SD (n = 3). $^{**}P$ < 0.01 *vs* Control, $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 *vs* HG.(P) The p-eNOS was detected by immunofluorescence assay in HUVECs. (Q) The NO production in the serum in different groups. (R) The NO production in the culture medium of HUVECs in different groups. Data are presented as mean ± SD (n = 3). $^{*}P$ < 0.05, $^{**}P$ < 0.01, $^{***}P$ < 0.001 *vs* Control, $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001 *vs* diabetes or HG. All images are at × 400 magnification magnification. Scale bar = 50 μm.

icariin in Sirt1 was further introduced in Results 3.6. Remarkably, the administration of rHMGB1 significantly reduced the expression of p-eNOS and increased the expressions of RAGE, TLR4, p-p65, and pro-inflammatory cytokines in HUVECs (Figs. 3K−3R). The results of rHMGB1 further demonstrated the effect of HMGB1 on vascular endothelium dysfunction.

Effect of icariin on GPER activity in STZ-induced diabetic female rats and HG-induced HUVECs

Our previous studies have shown that icariin can activate GPER in diabetes [30]. Thus, we measured the expression of GPER in the aortas of diabetic rats and HG-induced HUVECs to determine the effect of icariin on GPER. In our study, GPER was increased by icariin in the aortas of diabetic rats and HG-induced HUVECs (Figs. 4A and 4B). The results were further confirmed by immunofluorescence (Fig. 4C). The results revealed that icariin could activate GPER in STZ-induced diabetic female rats and HG-induced HUVECs. The interaction between icariin and GPER has been predicted by molecular docking (AutoDock Vina 1.5.6). The docking results, illustrated in Figure S4, showed icariin forming hydrogen bonds with ARG-155 (2.5 Å), SER-166 (2.2 Å), LEU-327 (2.4 Å), GLU-329 (2.1 Å), and THF-330 (2.6 Å), along with three hydrogen bonds with ARG-169 (2.6 Å,

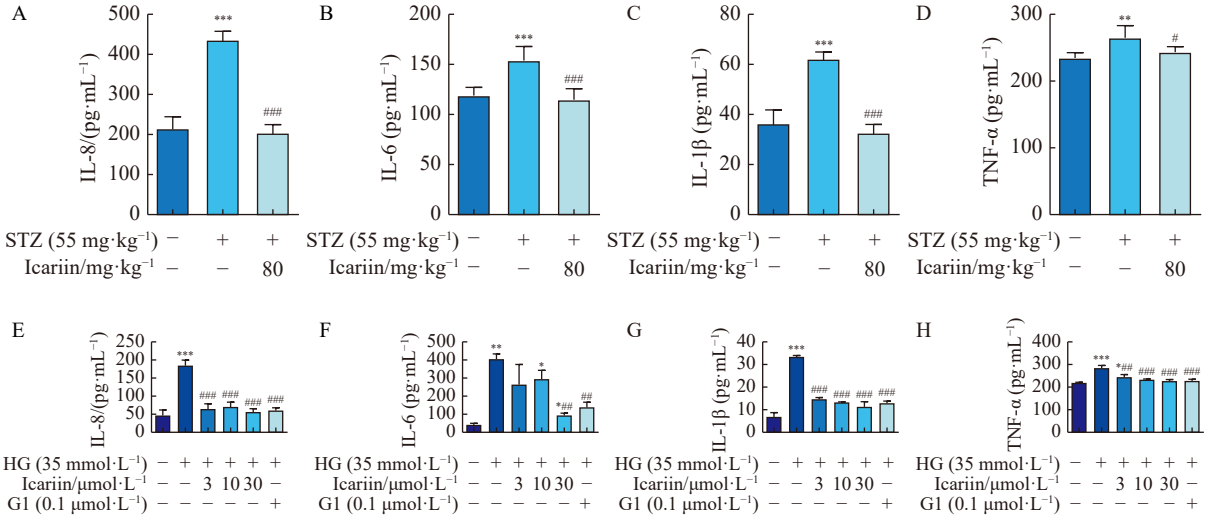


Fig. 2 Effect of icariin on pro-inflammatory cytokines in the serum of the diabetic rats and the culture medium of HG-induced HUVECs. (A−D) The diabetic rats were treated with icariin (80 mg·kg^{-1}). The pro-inflammatory cytokines in the serum of different groups were measured by ELISA (n = 6). (E−H) HUVECs were stimulated by HG (35 mmol·L^{-1}) and treated with icariin (3, 10, and 30 μmol·L^{-1}) or G1 (0.1 μmol·L^{-1}) for 48 h. The pro-inflammatory cytokines in the culture medium of HUVECs in different groups were measured by ELISA (n = 3). Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* Control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* diabetes or HG.

2.0 Å, and 2.7 Å) in GPER. The binding energy was −7.8 kcal·mol^{-1}. These findings suggest that icariin may directly bind to GPER.

Icariin inhibited HMGB1-induced inflammation to attenuate vascular endothelium dysfunction by activating GPER

To further clarify the role of GPER in the effect of icariin on vascular endothelium function, G15 (GPER antagonist) and G1 (GPER agonist) were utilized in our study. The results of Western blotting and immunofluorescence analyses indicated that G15 inhibited the icariin-induced activation of p-eNOS and NO (Figs. 5A−5D). Subsequently, we explored icariin's influence on HMGB1 release and its potential mediation by GPER. Si-GPER weakened the inhibitory effect of icariin on HMGB1 (Fig. S6). In HG-induced HUVECs, icariin and G1 reduced the levels of HMGB1 in the medium and downregulated the expression of HMGB1-associated downstream factors, including RAGE, TLR4, and p-p65 (Figs. 5A, 5B, and 5E). Conversely, G15 significantly countered the inhibitory effects of icariin on both HMGB1 expression and release (Figs. 5A, 5B, and 5E). Additionally, G15 nullified the effect of icariin on preventing HMGB1 translocation (Figs. 5F−5H) and reversed icariin's suppression of RAGE, TLR4, p-p65, and pro-inflammatory cytokines (Figs. 5A, 5B, and 5I−5L). These findings indicate that icariin mitigates vascular endothelium dysfunction in diabetes primarily by inhibiting HMGB1-induced inflammation through activating GPER.

Icariin inhibited HMGB1-induced inflammation to ameliorate vascular endothelium function by activating the GPER/Sirt1 pathway

The critical role of Sirt1 in HMGB1 deacetylation has been demonstrated in HMGB1 nuclear-to-cytoplasmic translocation [29]. Our previous study revealed that icariin critically attenuated diabetes-induced vascular endothelium dysfunction by enhancing Sirt1 activity [23]. The effect of icariin on Sirt1 and HMGB1 prompted us to examine the influence of Sirt1 in the inhibition effect of icariin on HMGB1 release in HG-induced HUVECs [31]. Prior studies have shown that 17β-estradiol (E2) upregulates Sirt1 expression *via* the GPER/EGFR/ERK/c-fos/AP-1 signaling pathway. Firstly, we investigated the potential association between GPER and Sirt1 in HG-induced HUVECs. The results demonstrated that icariin and G1 upregulated Sirt1 gene expression in HG-induced HUVECs (Fig. S2). However, icariin and G1 had no significant regulatory effect on NAD^+ (Fig. S3). Western blotting analysis revealed that G1 and icariin increased Sirt1 expression, but this effect of icariin on Sirt1 was diminished by G15 and Si-GPER, suggesting that icariin's influence on Sirt1 is partially mediated through GPER (Figs. 6A, 6B, and S6). In summary, icariin and GPER regulated Sirt1 by increasing the protein and gene expression levels. We next assessed the expression of Sirt1 in the aortas of diabetic female rats. Our results further revealed that diabetes decreased Sirt1 levels, which were subsequently increased by icariin treatment (Figs. 6C and 6D). Consistent with our prior findings, EX527, a Sirt1 inhibitor, negated icariin's effects on p-eNOS activation and NO production in HG-induced HUVECs (Figs. 6E−6H). Considering the regulatory role of Sirt1 on HMGB1 release, we employed EX527 to elucidate Sirt1's contribution to icariin's suppression of HMGB1 acetylation, as demonstrated by Co-IP analysis (Fig. S1). Si-Sirt1 abolished the inhibitory effect of icariin on HMGB1 (Fig. S7), and EX527 blocked the repression effect of icariin on HMGB1 expression and release, as well as its dampening impact on HMGB1-related inflammatory markers, including RAGE, TLR4, p-p65, and pro-inflammatory cytokines (Figs. 6E, 6F, 6I, and 6M−6P). EX527 treatment led to an unchanged ex-

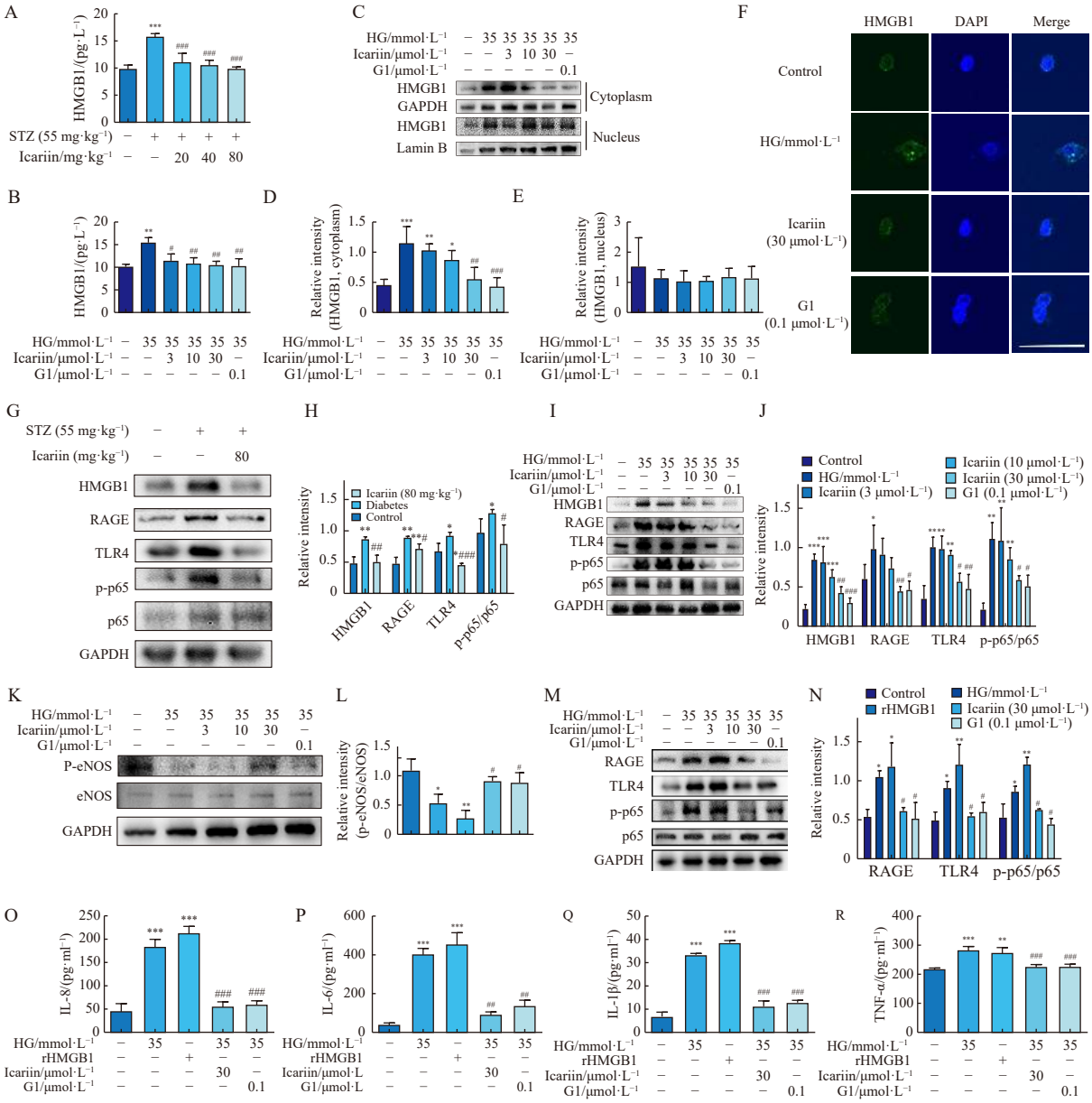


Fig. 3 Icariin inhibited inflammation to attenuate vascular endothelium dysfunction by inhibiting the release of HMGB1. (A) The diabetic rats were treated with icariin (80 mg·kg^{-1}). The HMGB1 release in the serum in different groups was measured by ELISA. (B) HUVECs were stimulated by HG (35 mmol·L^{-1}), treated with icariin (3, 10, and 30 μmol·L^{-1}) and G1 (0.1 μmol·L^{-1}) for 48 h. The HMGB1 release in the culture medium of HUVECs in different groups was measured by ELISA. (C−E) The expression of HMGB1 in the cytosol or nucleus of HUVECs was measured by Western blotting assay. (F) The distribution of HMGB1 was detected by immunofluorescence assay in HUVECs. (G, H) Western blotting assay revealed the expressions of HMGB1, RAGE, TLR4, p-p65, and p65 in the aortas. (I, J) Western blotting assay revealed the expressions of HMGB1, RAGE, TLR4, p-p65, and p65 in HUVECs. (K, L) HUVECs were stimulated by HG (35 mmol·L^{-1}), rHMGB1(100 ng·mL^{-1}), HG + icariin (30 μmol·L^{-1}), and HG + G1 (0.1 μmol·L^{-1}) for 48 h. Western blotting assay revealed the expressions of p-eNOS and eNOS in HUVECs. (M, N) Western blotting assay revealed the expressions of RAGE, TLR4, p-p65, and p65 in HUVECs. (O−R) The pro-inflammatory cytokines in the culture medium of HUVECs in different groups were measured by ELISA. Data are presented mean ± SD (n = 3). $^{*}P$ < 0.05, $^{**}P$ < 0.01, $^{***}P$ < 0.001 *vs* Control; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001 *vs* diabetes or HG. All images are at ×400 magnification. Scale bar = 50 μm.

pression of nuclear HMGB1 but increased its cytoplasmic content, significantly diminishing icariin's ability to prevent HMGB1's translocation from the nucleus to the cytoplasm (Figs. 6J−6L). Collectively, these results indicate that icariin inhibits HMGB1 release is contingent upon the activation of the GPER/Sirt1 pathway.

Discussion

In the present study, we proposed and tested a novel hypothesis that icariin can mitigate diabetes-induced vascular

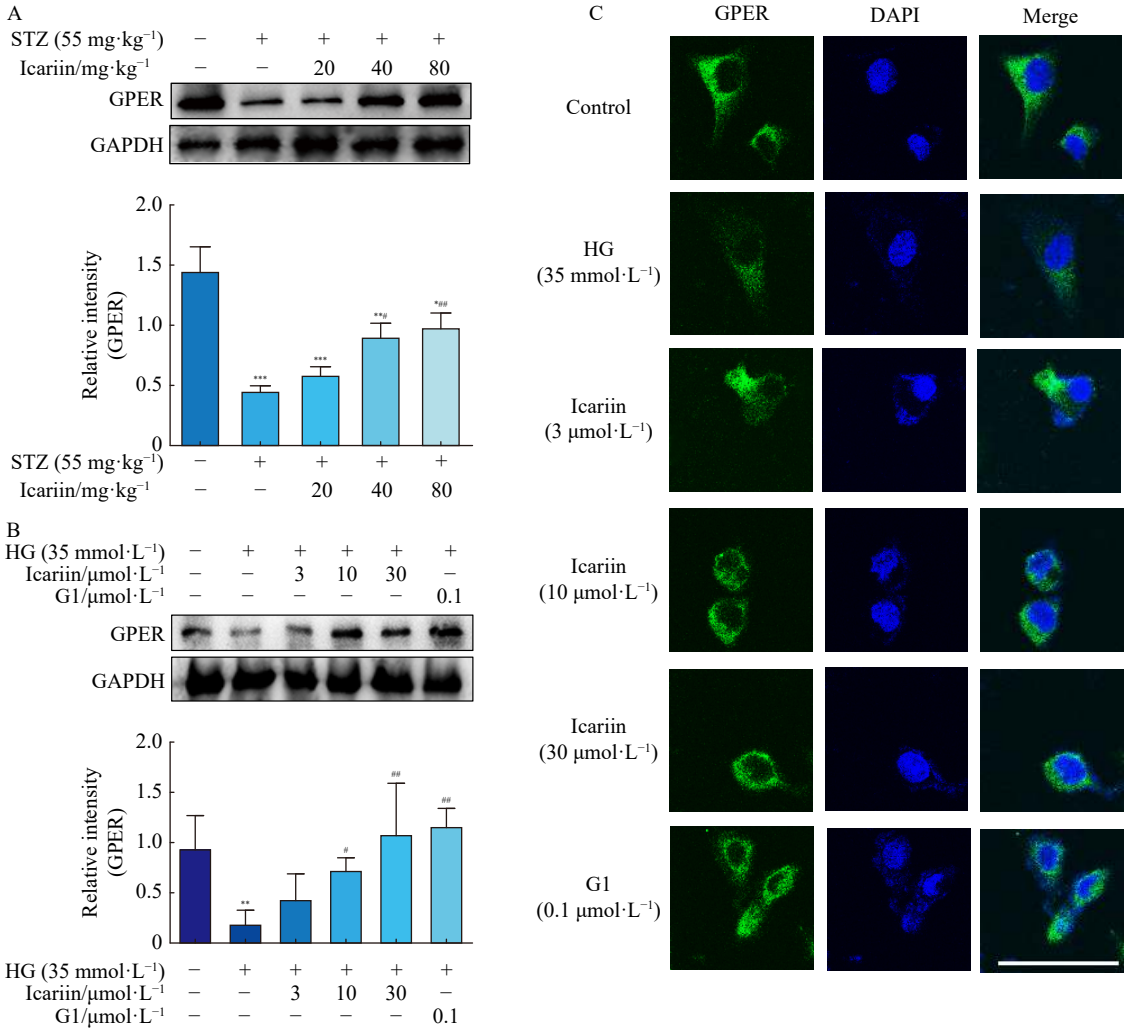


Fig. 4 Effect of icariin on GPER activity in STZ-induced diabetic female rats and HG-induced HUVECs. (A) The diabetic rats were treated with icariin (20, 40, 80 mg·kg^{-1}). Western blotting assay measured the expressions of GPER in the aortas. (B) HUVECs were stimulated by HG (35 mmol·L^{-1}), treated with icariin (3, 10, and 30 μmol·L^{-1}) and G1 (0.1 μmol·L^{-1}) for 48 h. Western blotting assay measured the expressions of GPER in HUVECs. (C) The GPER was detected by immunofluorescence assay in HUVECs. Data are presented as mean ± SD (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* Control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* diabetes or HG. All images are at ×400 magnification. Scale bar = 50 μm.

endothelial dysfunction through an anti-inflammatory mechanism. Specifically, icariin ameliorates diabetes or HG-induced inflammation by upregulating the expression of GPER and subsequently activating Sirt1, which leads to the inhibition of HMGB1 expression and its translocation from the nucleus to the cytoplasm.

Diabetes is a metabolic disorder that fosters the onset of both macrovascular and microvascular complications, chiefly characterized by progressive damage to the vascular endothelium [1]. The sensitivity of endothelium-dependent relaxation induced by ACh is significantly impaired in arteries from diabetic rats [32, 33]. Icariin, as a flavonoid isolated from *Epimedium*, has been acknowledged for its cardiovascular therapeutical effects [20]. It has been documented that icariin can facilitate endothelium-dependent relaxation of the isolated canine coronary artery by activating eNOS and the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway [34]. In our study, type 1 diabetes was induced in rats using STZ, and our findings indicated that icariin significantly enhanced vasodilation induced by ACh in the aortas of diabetic rats. Furthermore, consistent with previous research [23, 34], our data confirmed that icariin could endothelium-dependently and directly relax aortas *in vitro*, an effect that relies on the activation of eNOS. Furthermore, icariin was found to increase the expression of p-eNOS and the production of NO in type 1 diabetic rats and HG-induced HUVECs.

Vascular endothelial function is known to be compromised by inflammation in diabetes [35]. Our results also proved that pro-inflammation cytokines were increased in diabetic female rats and HG-induced HUVECs. Previous studies have demonstrated that icariin can inhibit the inflammatory response in diabetes [36, 37]. Aligning with these findings, our study demonstrated that icariin effectively reduced the levels of pro-inflammatory cytokines in both diabetic female rats

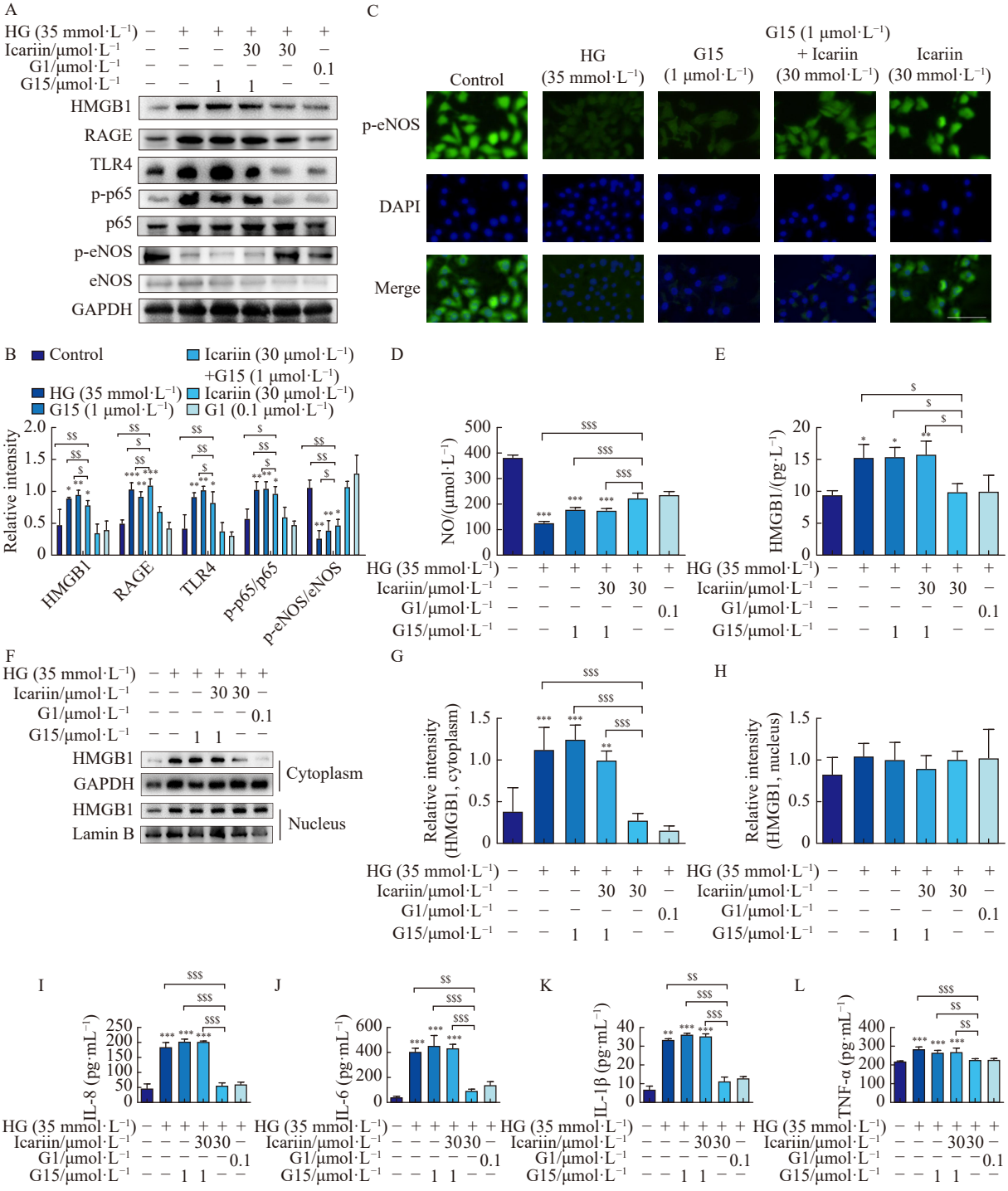


Fig. 5 Icariin inhibited HMGB1-induced inflammation to attenuate vascular endothelium dysfunction by activating GPER. (A, B) HUVECs were stimulated by HG (35 mmol·L^{-1}), treated with G15 (1 μmol·L^{-1}), G15 (1 μmol·L^{-1}) + icariin (30 μmol·L^{-1}), icariin (30 μmol·L^{-1}), G1 (0.1 μmol·L^{-1}) for 48 h. Western blotting assay measured the expressions of HMGB1, RAGE, TLR4, p-p65, and p65 in HUVECs. (C) The p-eNOS was detected by immunofluorescence assay in HUVECs. (D) The NO production in the culture medium of HUVECs in different groups was measured by ELISA. (E) The HMGB1 release in the culture medium of HUVECs in different groups was measured by ELISA. (F−H) The expression of HMGB1 in the cytosol or nucleus of HUVECs. (I-L) The pro-inflammatory cytokines in the culture medium of HUVECs in different groups were measured by ELISA. Data are presented as mean ± SD (n = 3). $^{*}P$ < 0.05, $^{**}P$< 0.01, $^{***}P$ < 0.001 *vs* Control, $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001 *vs* HG, $^{\$}P$ < 0.05, $^{\$\$}P$ < 0.01, $^{\$\$\$}P$ < 0.001 *vs* icariin. All images are at ×400 magnification. Scale bar = 50 μm.

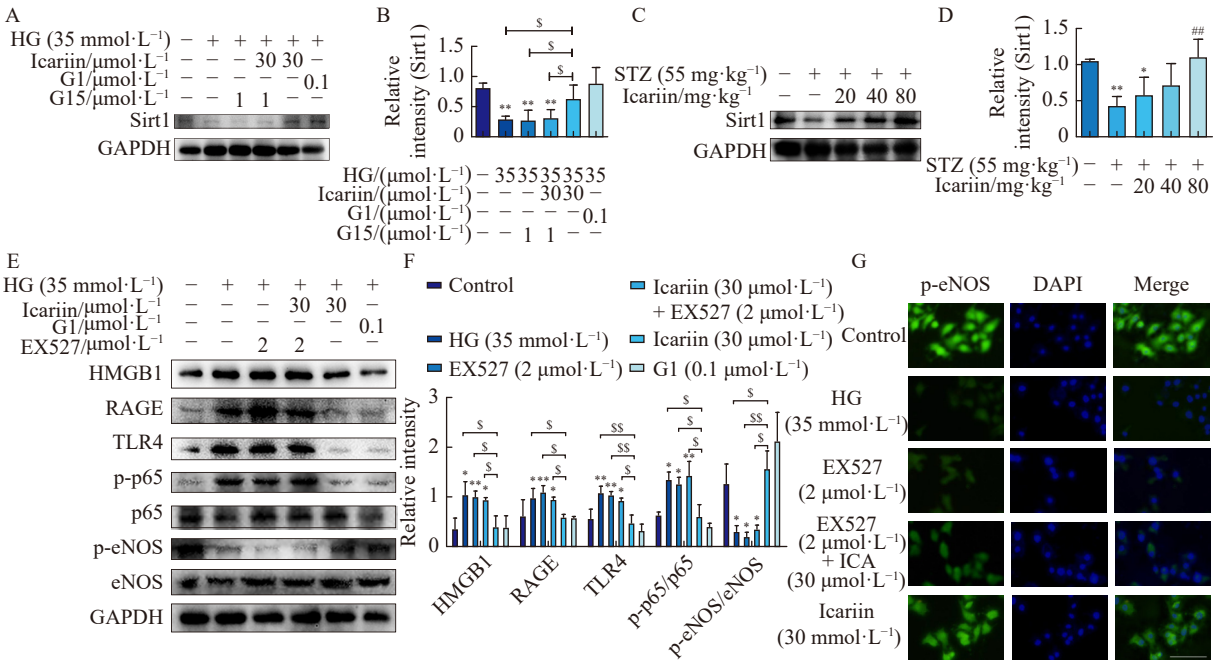
and HG-induced HUVECs.

Results from recent research implied the detrimental role of HMGB1 in cardiovascular disease by promoting inflammation [16, 38, 39]. HMGB1 and its downstream receptors, like

RAGE and TLR4, serve as suitable targets for diabetes [28]. It has been shown that icariin significantly suppresses HMGB1 activity [24], prompting us to investigate icariin's impact on HMGB1 in STZ-induced diabetic rats and HG-induced HUVECs. Therefore, we studied the effect of icariin on HMGB1 in STZ-induced diabetic rats and HG-induced HUVECs. Our findings revealed that icariin reduced the protein expression levels of HMGB1 in both STZ-induced diabetic rats and HG-induced HUVECs. HMGB1 is known to transition from the nucleus to the cytoplasm and subsequently be released into the extracellular matrix, acting as a chemotactic agent or cytokine that is actively involved in various pathologies, such as sepsis [40], ischemia-reperfusion injury [41], cardiovascular disease [16], arthritis [42], neurodegeneration [43], meningitis [44], and cancer [10, 45]. Consistent with these roles, the results of Western blotting and immunofluorescence analyses indicated that HG increased cytosolic HMGB1 levels, while icariin prevented HMGB1 translocation from the nucleus to the cytoplasm in HG-induced HUVECs. In addition, the levels of HMGB1 in the serum and cell culture medium were tested using an ELISA kit. The results demonstrated that icariin decreased HMGB1 release in type 1 diabetic rats and HG-induced HUVECs. Given the crucial role of HMGB1 acetylation in its nuclear-to-cytoplasmic translocation [29], we measured acetylated HMGB1 levels in HUVECs through Co-IP. The results revealed that icariin could inhibit HMGB1 acetylation. HMGB1 mediates inflammation *via* interactions with RAGE and TLR4, which can activate p-p65 and increase the levels of pro-inflammatory cytokines [27]. Our results showed that the expression levels of RAGE, TLR4, and p-p65 were elevated in type 1 diabetic aortas and HG-induced HUVECs, and these increases were mitigated by icariin treatment. To investigate the effect of HMGB1 on vascular endothelium inflammation, HUVECs were treated with recombinant human HMGB1, which significantly decreased p-eNOS and increased the expressions of RAGE, TLR4, p-p65, and pro-inflammatory cytokines. Overall, our findings support the notion that icariin exerts a protective effect on vascular endothelial function in diabetes by inhibiting HMGB1 release and the subsequent HMGB1-induced inflammation.

GPER, also known as GPR30, is a mediator of non-genomic estrogen signaling pathways [46]. Since its discovery, a growing body of evidence has underscored GPER's diverse physiological protective roles in diabetes, including the regulation of insulin production and action, lipid and glucose homeostasis, blood pressure, and arterial relaxation [47]. Recognized as a critical physiological regulator, GPER is extensively distributed throughout the cardiovascular system [48, 49]. According to previous results from our group, GPER could be activated by icariin [50]. In HG-induced glomerular podocytes, icariin modulated mitochondrial function and apoptosis *via* GPER [30]. In the present study, we employed STZ-induced diabetic rats and HG-induced HUVECs to determine the impact of icariin on GPER in vascular endothelium dysfunction. Our observations revealed that icariin increased the expression of GPER in diabetic aortas. Similarly, in HG-induced HUVECs, where the protein expression of GPER was found to be reduced in comparison to the control group, icariin and the GPER agonist G1 were able to increase GPER expression. Furthermore, G15, a GPER antagonist, was shown to inhibit icariin's capacity to activate eNOS in HG-induced HUVECs. These findings collectively suggest that icariin holds the potential to enhance vascular endothelial function in diabetes through the activation of GPER.

GPER has been recognized for its capacity to mitigate



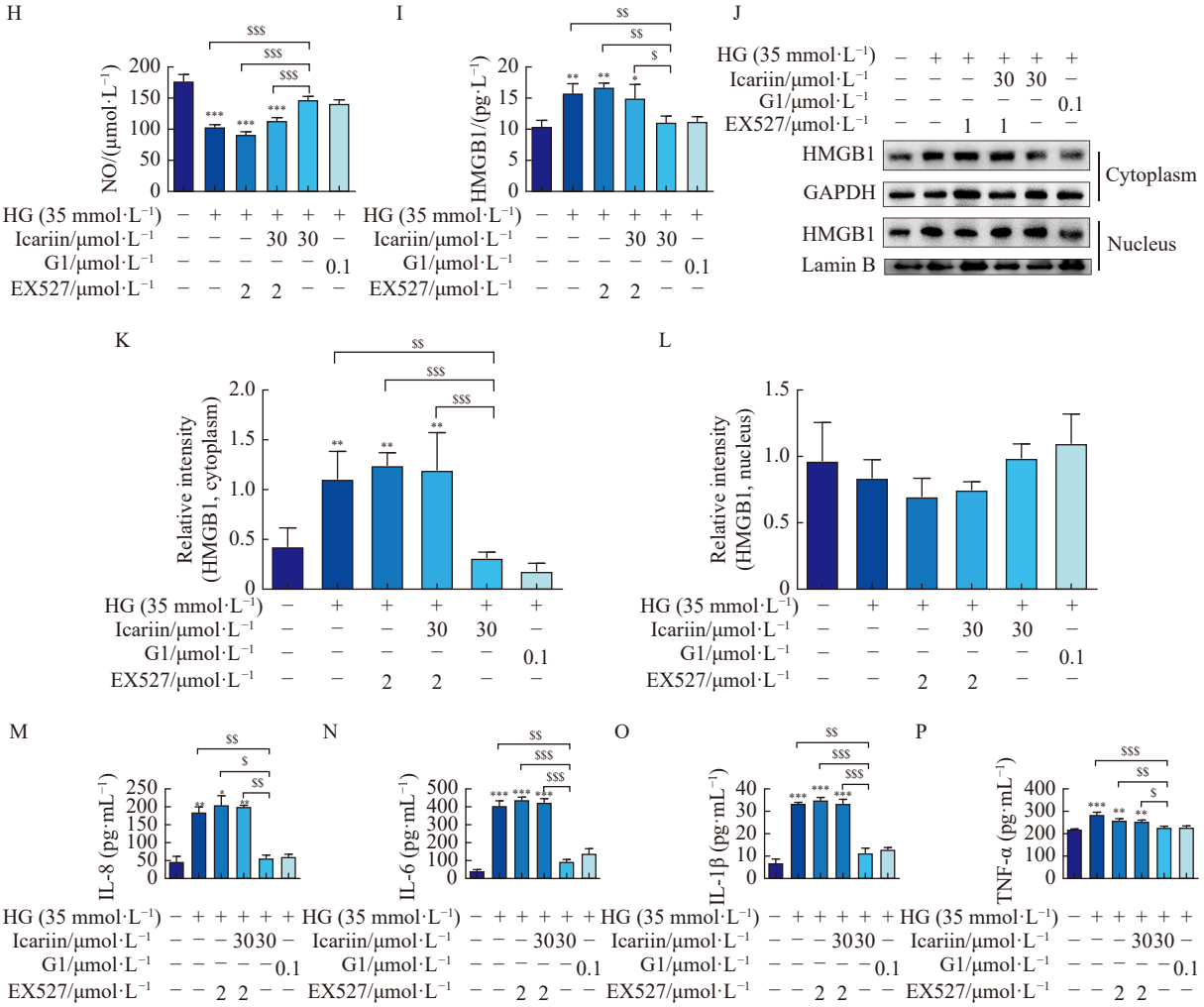


Fig. 6 Icariin inhibited HMGB1-induced inflammation to ameliorate vascular endothelium function by activating the GPER/Sirt1 pathway. (A, B) HUVECs were stimulated by HG (35 mmol·L^{-1}) and treated with G15 (1 μmol·L^{-1}), G15 (1 μmol·L^{-1}) + icariin (30 μmol·L^{-1}), icariin (30 μmol·L^{-1}), and G1 (0.1 μmol·L^{-1}) for 48 h. Western blotting assay measured the expression of Sirt1 in HUVECs. (C, D) The diabetic rats were treated with icariin (80 mg·kg^{-1}). Western blotting assay measured the expression of Sirt1 in the aortas. (E, F) HUVECs were stimulated by HG (35 mmol·L^{-1}) and treated with EX527 (2 μmol·L^{-1}), EX527 (2 μmol·L^{-1}) + icariin (30 μmol·L^{-1}), icariin (30 μmol·L^{-1}), and G1 (0.1 μmol·L^{-1}) for 48 h. Western blotting assay measured the expressions of HMGB1, RAGE, TLR4, p-p65, and p65 in HUVECs. (G) The p-eNOS was detected by immunofluorescence assay in the HUVECs. (H) The NO production in the culture medium of HUVECs in different groups was measured by ELISA. (I) The HMGB1 release in the culture medium of HUVECs in different groups was measured by ELISA. (J–L) The expression of HMGB1 in the cytosol or nucleus of HUVECs was measured by Western blotting assay. (M–P) The pro-inflammatory cytokines in the culture medium of HUVECs in different groups were measured by ELISA. Data are expressed as mean ± SD (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* Control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* HG, $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ *vs* icariin. All images are at ×400 magnification. Scale bar = 50 μm.

diabetes-mediated inflammation and thus prevent vascular endothelial dysfunction [47, 51]. Our results also showed that G1 reduced the levels of pro-inflammatory cytokines in the culture medium of HG-induced HUVECs. The anti-inflammatory effect of icariin was obstructed by a GPER inhibitor in these cells, demonstrating icariin's ability to dampen inflammation through GPER activation. Given GPER's influence on the activity of HMGB1 [52], a known inducer of inflammation through RAGE or TLR4, our study extended into the unexplored territory of GPER's effect on HMGB1 in diabetes. Our results highlighted GPER's inhibitory impact on the expression and release of HMGB1, the expression of HMGB1 receptors (including RAGE and TLR4), and the levels of pro-inflammatory cytokines in HG-induced HUVECs. Considering the inhibitory effect of icariin on HMGB1, we investigated the role of GPER icariin's suppressive effect on HMGB1. The results revealed that G15 effectively suppressed the inhibitory effect of icariin on HMGB1 release and HMGB1-induced inflammation. These insights firmly establish GPER as a critical mediator in icariin's action against HMGB1.

HMGB1 typically remains confined to the nucleus when

unmodified. Its acetylation, a form of posttranslational modification, plays a key role in its function and mobility [53, 54]. The study by Rabadi revealed that Sirt1 was involved in HMGB1 deacetylation, which inhibited HMGB1 nuclear-to-cytoplasmic translocation and systemic release [29]. Our previous study has demonstrated that Sirt1 activity is enhanced by icariin [23]. Due to the effect of icariin on Sirt1 and HMGB1, we sought to examine the influence of Sirt1 in the effect of icariin on HMGB1 in HG-induced HUVECs. Our results revealed that the expression of Sirt1 was upregulated by icariin. However, the effect of icariin on HMGB1 was reversed by Sirt1 inhibitor, indicating that icariin's suppression of HMGB1 translocation, release, and HMGB-induced inflammation depends on Sirt1 activation. Sirt1 activation has been linked to estrogen action *via* the GPER [31]. Consistent with the previous research, our results showed that G1 treatment upregulated the expression of Sirt1, while G15 treatment blocked the activation of Sirt1 by icariin in HG-induced HUVECs. Icariin and G1 also upregulated Sirt1 gene expression in HG-induced HUVECs. Consistent with the earlier findings [55], neither icariin nor G1 significantly affected NAD^+ levels, indicating that icariin and GPER regulate Sirt1 primarily by increasing its protein and gene expression levels. Thus, GPER serves as a functional intermediary linking icariin with the Sirt1/HMGB1 pathway. Previous research has established that icariin can increase the expression of sirt1 by activating PPARα [23]. However, the interplay between PPARα and GPER—both influential on Sirt1 in HUVECs but representing different receptor classes (GPER being a seven-transmembrane domain receptor and PPARα a nuclear receptor)—remains unexplored. Considering GPER and PPARα both have regulatory effects on Sirt1 in HUVECs. Therefore, the cross-talk between GPER and PPARα is under investigation by our team. Moreover, Sirt6 is another factor implicated in the regulation of HMGB1 release [56, 57], and icariin has been shown to increase Sirt6 expression, thereby attenuating inflammation [58]. Thus, icariin's inhibitory effect on HMGB1 may extend beyond its regulation of Sirt1, potentially involving Sirt6 as well. Future investigations into icariin's impact on HMGB1 could benefit from examining its interactions with Sirt6.

Conclusion

In summary, our results demonstrated that icariin protected vascular endothelium function against diabetes by inhibiting HMGB1-induced inflammation. Within this context, we found that the activated GPER by icariin increased the expression of Sirt1, thereby mitigating inflammation-induced vascular endothelium dysfunction in type 1 diabetes (Fig. 7). Our findings provide valuable insights into the intricate mechanisms by which icariin influences the vascular system in diabetes.

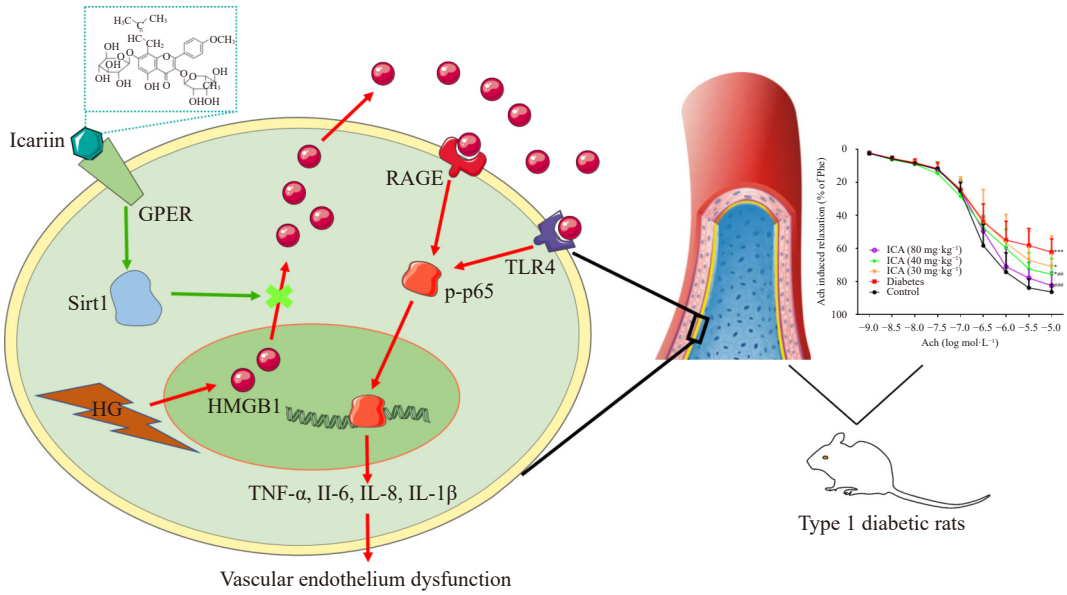


Fig. 7 Icariin attenuated vascular endothelial dysfunction by inhibiting inflammation through the GPER/Sirt1/HMGB1 signaling pathway in type 1 diabetic rats. Icariin enhanced the activity of the GPER, which in turn activated Sirt1. This activation sequence culminated in the suppression of HMGB1 release, effectively inhibiting inflammation and improving vascular endothelial function in type 1 diabetic rats.

Supplementary Materials

Supplementary data to this article can be obtained by sending an E-mail to the corresponding author.

References

[1] Rosengren A, Dikaiou P. Cardiovascular outcomes in type 1 and type 2 diabetes [J]. *Diabetologia*, 2023, **66**(3): 425-437.

[2] Hu Y, Chen M, Wang M, *et al*. Flow-mediated vasodilation through mechanosensitive G protein-coupled receptors in endothelial cells [J]. *Trends Cardiovasc Med*, 2022, **32**(2): 61-70.

[3] Yao Y, Song Q, Hu C, *et al*. Endothelial cell metabolic memory causes cardiovascular dysfunction in diabetes [J]. *Cardiovasc Res*, 2022, **118**(1): 196-211.

[4] Maruhashi T, Higashi Y. Pathophysiological sssociation

between diabetes mellitus and endothelial dysfunction [J]. *Antioxidants (Basel)*, 2021, **10**(8): 1306.
[5] Stark K, Massberg S. Interplay between inflammation and thrombosis in cardiovascular pathology [J]. *Nat Rev Cardiol*, 2021, **18**(9): 666-682.
[6] Antonopoulos AS, Angelopoulos A, Papanikolaou P, *et al*. Biomarkers of vascular inflammation for cardiovascular risk prognostication: a meta-analysis [J]. *JACC Cardiovasc Imaging*, 2022, **15**(3): 460-471.
[7] Othman ZA, Zakaria Z, Suleiman JB, *et al*. Bee bread ameliorates vascular inflammation and impaired vasorelaxation in obesity-induced vascular damage rat model: the role of eNOS/NO/cGMP-signaling pathway [J]. *Int J Mol Sci*, 2021, **22**(8): 4225.
[8] De La PEñA JB, Barragan-Iglesias P, Lou TF, *et al*. Intercellular arc signaling regulates vasodilation [J]. *J Neurosci*, 2021, **41**(37): 7712-7726.
[9] Rohm TV, Meier DT, Olefsky JM, *et al*. Inflammation in obesity, diabetes, and related disorders [J]. *Immunity*, 2022, **55**(1): 31-55.
[10] Wang S, Zhang Y. HMGB1 in inflammation and cancer [J]. *J Hematol Oncol*, 2020, **13**(1): 116.
[11] Nogueira-Machado JA, Volpe CM, Veloso CA, *et al*. HMGB1, TLR and RAGE: a functional tripod that leads to diabetic inflammation [J]. *Expert Opin Ther Targets*, 2011, **15**(8): 1023-1035.
[12] Giovannini S, Tinelli G, Biscetti F, *et al*. Serum high mobility group box-1 and osteoprotegerin levels are associated with peripheral arterial disease and critical limb ischemia in type 2 diabetic subjects [J]. *Cardiovasc Diabetol*, 2017, **16**(1): 99.
[13] Wang C, Jiang J, Zhang X, *et al*. Inhibiting HMGB1 reduces cerebral ischemia reperfusion injury in diabetic mice [J]. *Inflammation*, 2016, **39**(6): 1862-1870.
[14] Xie W, Zhu T, Dong X, *et al*. HMGB1-triggered inflammation inhibition of notoginseng leaf triterpenes against cerebral ischemia and reperfusion injury *via* MAPK and NF-*κ*B signaling pathways [J]. *Biomolecules*, 2019, **9**(10): 512.
[15] Liao J, Xie S S, Deng Y, *et al*. PRDX6-mediated pulmonary artery endothelial cell ferroptosis contributes to monocrotaline-induced pulmonary hypertension [J]. *Microvasc Res*, 2023, **146**: 104471.
[16] Belmadani S, Matrougui K. Role of high mobility group box 1 in cardiovascular diseases [J]. *Inflammation*, 2022, **45**(5): 1864-1874.
[17] Ahn JG, Bae Y, Shin D, *et al*. HMGB1 gene polymorphism is associated with coronary artery lesions and intravenous immunoglobulin resistance in Kawasaki disease [J]. *Rheumatology (Oxford)*, 2019, **58**(5): 770-775.
[18] Zhu Z, Peng X, Li X, *et al*. HMGB1 impairs endothelium-dependent relaxation in diabetes through TLR4/eNOS pathway [J]. *FASEB J*, 2020, **34**(6): 8641-8652.
[19] Shen Y, Cao H, Chen F, *et al*. A cross-sectional study of vitreous and serum high mobility group box-1 levels in proliferative diabetic retinopathy [J]. *Acta Ophthalmol*, 2020, **98**(2): e212-e216.
[20] He C, Wang Z, Shi J. Pharmacological effects of icariin [J]. *Adv Pharmacol*, 2020, **87**: 179-203.
[21] Hu Y, Sun B, Liu K, *et al*. Icariin attenuates high-cholesterol diet induced atherosclerosis in rats by inhibition of inflammatory response and p38 MAPK signaling pathway [J]. *Inflammation*, 2016, **39**(1): 228-236.
[22] Dai M, Chen B, Wang X, *et al*. Icariin enhance mild hypothermia-induced neuroprotection *via* inhibiting the activation of NF-*κ*B in experimental ischemic stroke [J]. *Metab Brain Dis*, 2021, **36**(7): 1779-1790.
[23] Yao W, Wang K, Wang X, *et al*. Icariin ameliorates endothelial dysfunction in type 1 diabetic rats by suppressing ER stress *via* the PPARα/Sirt1/AMPKα pathway [J]. *J Cell Physiol*, 2021, **236**(3): 1889-1902.
[24] Liu L, Zhao Z, Lu L, *et al*. Icariin and icaritin ameliorated hippocampus neuroinflammation *via* inhibiting HMGB1-related pro-inflammatory signals in lipopolysaccharide-induced inflammation model in C57BL/6 J mice [J]. *Int Immunopharmacol*, 2019, **68**: 95-105.
[25] Liu J, Liu J, Tong X, *et al*. Network pharmacology prediction and molecular docking-based strategy to discover the potential pharmacological mechanism of Huai Hua San against ulcerative colitis [J]. *Drug Des Devel Ther*, 2021, **15**: 3255-3276.
[26] Barbu E, Popescu MR, Popescu AC, *et al*. Inflammation as a precursor of atherothrombosis, diabetes and early vascular aging [J]. *Int J Mol Sci*, 2022, **23**(2): 963.
[27] Yang H, Wang H, Andersson U. Targeting inflammation driven by HMGB1 [J]. *Front Immunol*, 2020, **11**: 484.
[28] Behl T, Sharma E, Sehgal A, *et al*. Expatiating the molecular approaches of HMGB1 in diabetes mellitus: highlighting signalling pathways *via* RAGE and TLRs [J]. *Mol Biol Rep*, 2021, **48**(2): 1869-1881.
[29] Rabadi MM, Xavier S, Vasko R, *et al*. High-mobility group box 1 is a novel deacetylation target of Sirtuin1 [J]. *Kidney Int*, 2015, **87**(1): 95-108.
[30] Qiao C, Ye W, Li S, *et al*. Icariin modulates mitochondrial function and apoptosis in high glucose-induced glomerular podocytes through G protein-coupled estrogen receptors [J]. *Mol Cell Endocrinol*, 2018, **473**: 146-155.
[31] Santolla MF, Avion S, Pellegrino M, *et al*. SIRT1 is involved in oncogenic signaling mediated by GPER in breast cancer [J]. *Cell Death Dis*, 2015, **6**(7): e1834.
[32] Juguilon C, Wang Z, Wang Y, *et al*. Mechanism of the switch from NO to H_2O_2 in endothelium-dependent vasodilation in diabetes [J]. *Basic Res Cardiol*, 2022, **117**(1): 2.
[33] He A, Guo Y, Xu Z, *et al*. Hypoglycaemia aggravates impaired endothelial-dependent vasodilation in diabetes by suppressing endothelial nitric oxide synthase activity and stimulating inducible nitric oxide synthase expression [J]. *Microvasc Res*, 2023, **146**: 104468.
[34] Xu HB, Hhuang ZQ. Vasorelaxant effects of icariin on isolated canine coronary artery [J]. *J Cardiovasc Pharmacol*, 2007, **49**(4): 207-213.
[35] Wang L, Cheng CK, Yi M, *et al*. Targeting endothelial dysfunction and inflammation [J]. *J Mol Cell Cardiol*, 2022, **168**: 58-67.
[36] Qiao C, Wang H, Song Z, *et al*. Icariin attenuates diabetic cardiomyopathy and downregulates extracellular matrix proteins in heart tissue of type 2 diabetic rats [J]. *Pharmacology*, 2020, **105**(9-10): 576-585.
[37] Singh WR, Sharma A, Devi HS, *et al*. Icariin improves cutaneous wound healing in streptozotocin-induced diabetic rats [J]. *J Tissue Viability*, 2022, **31**(1): 197-206.
[38] Yang K, Cao F, Wang W, *et al*. The relationship between HMGB1 and autophagy in the pathogenesis of diabetes and its complications [J]. *Front Endocrinol (Lausanne)*, 2023, **14**: 1141516.
[39] Liu Y, Chen L, Gao L, *et al*. LRRK2 deficiency protects the heart against myocardial infarction injury in mice *via* the P53/HMGB1 pathway [J]. *Free Radic Biol Med*, 2022, **191**: 119-127.
[40] Deng C, Zhao L, Yang Z, *et al*. Targeting HMGB1 for the treatment of sepsis and sepsis-induced organ injury [J]. *Acta Pharmacol Sin*, 2022, **43**(3): 520-528.
[41] Zhan Y, Ling Y, Deng Q, *et al*. HMGB1-mediated neutrophil

extracellular trap formation exacerbates intestinal ischemia/reperfusion-induced acute lung injury [J]. *J Immunol*, 2022, **208**(4): 968-78.
[42] Rudjito R, Agalave NM, Farinotti B, *et al*. Sex- and cell-dependent contribution of peripheral high mobility group box 1 and TLR4 in arthritis-induced pain [J]. *Pain*, 2021, **162**(2): 459-470.
[43] Ikram FZ, Arulsamy A, Retinasamy T, *et al*. The role of high mobility group box 1 (HMGB1) in neurodegeneration: a systematic review [J]. *Curr Neuropharmacol*, 2022, **20**(11): 2221-2245.
[44] Piva S, Albani F, Fagoni N, *et al*. High-mobility group box-1 protein as a novel biomarker to diagnose healthcare-associated ventriculitis and meningitis: a pilot study [J]. *Minerva Anestesiol*, 2021, **87**(1): 43-51.
[45] Wang JD, Wang YY, Lin SY, *et al*. Exosomal HMGB1 promoted cancer malignancy [J]. *Cancers*, 2021, **13**(4): 877.
[46] Arterburn JB, Prossnitz ER. G protein-coupled estrogen receptor GPER: molecular pharmacology and therapeutic applications [J]. *Annu Rev Pharmacol Toxicol*, 2023, **63**: 295-320.
[47] Barton M, Prossnitz ER. Emerging roles of GPER in diabetes and atherosclerosis [J]. *Trends Endocrinol Metab*, 2015, **26**(4): 185-192.
[48] Aryan L, Younessi D, Zargari M, *et al*. The role of estrogen receptors in cardiovascular disease [J]. *Int J Mol Sci*, 2020, **21**(12): 4314.
[49] Feidman RD, Limbird LE. GPER (GPR30): a nongenomic receptor (GPCR) for steroid hormones with implications for cardiovascular disease and cancer [J]. *Annu Rev Pharmacol Toxicol*, 2017, **57**: 567-584.
[50] Li YC, Ding XS, Li HM, *et al*. Icariin attenuates high glucose-induced type IV collagen and fibronectin accumulation in glomerular mesangial cells by inhibiting transforming growth factor-β production and signalling through G protein-coupled oestrogen receptor 1 [J]. *Clin Exp Pharmacol Physiol*, 2013, **40**(9): 635-643.
[51] Li Z, Cheng L, Liang H, *et al*. GPER inhibits diabetes-mediated RhoA activation to prevent vascular endothelial dysfunction [J]. *Eur J Cell Biol*, 2016, **95**(2): 100-113.
[52] Liu L, Liu S, Luo H, *et al*. GPR30-mediated HMGB1 upregulation in CAFs induces autophagy and tamoxifen resistance in ERα-positive breast cancer cells [J]. *Aging*, 2021, **13**(12): 16178-16197.
[53] Chen R, Kang R, Tang D. The mechanism of HMGB1 secretion and release [J]. *Exp Mol Med*, 2022, **54**(2): 91-102.
[54] Shin J, Kim YH, Lee B, *et al*. USP13 regulates HMGB1 stability and secretion through its deubiquitinase activity [J]. *Mol Med*, 2022, **28**(1): 164.
[55] Gui YL, Zhang J, Chen L, *et al*. Icariin, a flavonoid with anticancer effects, alleviated paclitaxel-induced neuropathic pain in a SIRT1-dependent manner [J]. *Mol Pain*, 2018, **14**: 1744806918768970.
[56] Wei W, Guo X, Gu L, *et al*. Bone marrow mesenchymal stem cell exosomes suppress phosphate-induced aortic calcification *via* SIRT6-HMGB1 deacetylation [J]. *Stem Cell Res Ther*, 2021, **12**(1): 235.
[57] Kong Q, Li Y, Liang Q, *et al*. SIRT6-PARP1 is involved in HMGB1 polyADP-ribosylation and acetylation and promotes chemotherapy-induced autophagy in leukemia [J]. *Cancer Biol Ther*, 2020, **21**(4): 320-331.
[58] Li L, Chen B, Aa T, *et al*. BaZiBuShen alleviates altered testicular morphology and spermatogenesis and modulates Sirt6/P53 and Sirt6/NF-κB pathways in aging mice induced by D-galactose and $NaNO_2$ [J]. *J Ethnopharmacol*, 2021, **271**: 113810.

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